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SOLUBLE EPOXIDE HYDROLASE: A NOVEL THERAPEUTIC TARGET FOR METABOLIC SYNDROME

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Abstract

Metabolic syndrome is a pathology that has exponentially increased its worldwide prevalence in the last few decades. However, its treatment is still not optimal, hence there is a big necessity to find better therapies. Soluble Epoxide Hydrolase, an enzyme involved in the arachidonic acid cascade, has recently arisen as one of the potential targets to treat metabolic syndrome. It is believed that the selective inhibition of this enzyme could lead to positive effects in several disorders that conform the disease. Many compounds have been synthesized and tested, several of them presenting a high inhibitory potency. Nevertheless, none has yet reached the market due to their poor pharmacokinetic profiles. Over the years, many strategies have been followed to overcome this issue. In this dissertation, the enzyme structure and function, the physiologic role of its metabolic substrates, and the design of the different classes of inhibitors are reviewed.

Keywords: metabolic syndrome, soluble Epoxide Hydrolase, lead optimisation, pharmacokinetic profile

Resumen

El síndrome metabólico es una patología que ha incrementado exponencialmente su prevalencia mundial en las últimas décadas. No obstante, su tratamiento aún no es óptimo, por lo que resulta necesario encontrar terapias más eficaces. La epóxido hidrolasa soluble, una enzima involucrada en la cascada del ácido araquidónico, se ha revelado recientemente como una de las posibles dianas para tratar esta enfermedad. Se cree que la inhibición selectiva de esta enzima podría conllevar efectos positivos en muchas de las enfermedades que conforman el síndrome metabólico. Se han sintetizado y probado un gran número de compuestos, y muchos de ellos presentan una elevada potencia inhibitoria. A pesar de ello, ninguno ha llegado a ser comercializado debido a su pobre perfil farmacocinético. A lo largo de los años se han seguido muchas estrategias para afrontar este problema. En este trabajo se describe la estructura y la función de la enzima, el papel fisiológico de sus sustratos y el diseño de las diferentes clases de inhibidores.

Palabras clave: síndrome metabólico, epóxido hidrolasa soluble, optimización de compuestos, perfil farmacocinético.

Resum

La síndrome metabòlica és una patologia que ha incrementat exponencialment la seva prevalença mundial en les últimes dècades. No obstant això, el seu tractament encara no és òptim i, per tant, hi ha una gran necessitat de trobar teràpies millors. L'epòxid hidrolasa soluble, un enzim involucrat en la cascada de l'àcid araquidònic, s'ha erigit recentment com una de les possibles dianes per tractar aquesta malaltia. Es creu que la inhibició selectiva d'aquest enzim podria comportar efectes positius en moltes de les malalties que conformen la síndrome metabòlica. Un gran nombre de compostos s'han sintetitzat i provat, i molts 'presenten una elevada potència inhibidora. Tot i això, cap ha arribat a ser comercialitzat a causa del seu pobre perfil farmacocinètic. Al llarg dels anys s'han seguit moltes estratègies per superar aquest problema. En

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aquest treball es descriu l'estructura i la funció de l'enzim, el paper fisiològic dels seus substrats i el disseny de les diferents classes d'inhibidors.

Paraules clau: síndrome metabòlica, epòxid hidrolasa soluble, optimització de compostos, perfil farmacocinètic.

1. Introduction

Metabolic syndrome is a combination of physiologic and metabolic disorders that have the potential to compromise people's health, essentially by increasing the probability of suffering from cardiovascular episodes (Grundy, 2006) "ISBN" : "1558-3597 (Electronic. The typical signs include body weight gain, excess visceral adipose deposition, hyperglycaemia, glucose and insulin intolerances, hypertension, dyslipidaemia, endothelial damage, cardiovascular hypertrophy, inflammation, ventricular contractile dysfunction, fibrosis and fatty liver disease (Iyer et al., 2012). All these diseases can lead, eventually, to death.

Nowadays, metabolic syndrome is believed to be extremely prevalent in developed countries, affecting 25-30% of the total population, especially elderly people (Ford; Earl, S. & Giles, W.H., 2002), therefore representing a major challenge to health worldwide (Iyer et al., 2010). Of note, its prevalence is also alarmingly increasing in developing countries such as China and India (Wu et al., 2008). This fact highlights the importance of understanding the root causes of the disease and the need to develop better drugs to prevent its effects, which would eventually lead to a better quality of life for patients (Kahn, 2008). Furthermore, it would also decrease the expense of national health systems, since it is calculated that the comorbidity of metabolic syndrome together with other side diseases affect their treatment.

The treatment that metabolic syndrome patients follow is based on two strategies, one related to their life-style and the other based on pharmacological treatment (Grundy & Vigersky, 2008). In the first case, recommendations focus on following a hypocaloric and balanced diet while increasing physical activity (Grundy & Vigersky, 2008). It is also advisable to avoid factors with the potential to increase inflammation and oxidative stress, such as alcohol and tobacco (Grundy & Vigersky, 2008). All these basic recommendations for a healthy life might prevent the development of the disease. However, this is normally not enough, which results in the necessity of initiating pharmacological treatment. Thus, the therapy aims at treating each factor separately, focusing especially on blood hypertension, hyperglycaemia and cholesterol. Consequently, antihypertensive drugs (for instance, diuretics, β -blockers and angiotensin converting enzyme inhibitors), antidiabetic drugs (such as metformin, sulfonylureas, and others) and lipid-lowering drugs (statins or fibrates), are widely used in the treatment of this pathologic condition.

To sum up, metabolic syndrome patients are exposed to polypharmacy, which is neither optimal therapy nor economically sustainable for national health systems. Consequently, finding a common target to treat all these disorders is of major importance. Recent studies (Iyer et al., 2012), both in vitro and in vivo, show that this potential target could be the enzyme soluble Epoxide Hydrolase (sEH). This review highlights current knowledge in the field in order to provide an overview of the topic.

2. Objectives

The main objectives of the present dissertation are to:

- Study the physiological roles known so far for the different metabolites involved in the sEH pathway
- Analyse the structure of sEH and its catalytic mechanism
- Explain and understand the rationale followed to design the different classes of inhibitors tested up to the present time
- Review the different classes of inhibitors discovered in chronological order, highlighting their binding mode, potency, and main advantages and drawbacks
- Give an overview of the newest therapeutic strategies followed using sEH as a target

3. Materials and methods

This work is based on bibliographic research of scientific databases, especially *Sci-Finder* (<http://scifinder.cas.org>) and *PubMed* (<http://www.ncbi.nlm.nih.gov/pubmed>). Organic chemistry and biochemistry textbooks have been a useful point of support for the acknowledgement of basic concepts and notions cited in the publications and documents used.

In order to show the structure of sEH and the binding mode of its inhibitors, some selected X-ray crystal structures have been used. The files have been searched and downloaded from the Protein Data Bank webpage (<http://www.rcsb.org/pdb/home/home.do>), and the file code has been added each time in the figure legend. To open and work with these files, the program PyMOL (property of Schrödinger, Inc.) for Windows® has been used, specifically the open access software available to the University of Barcelona, which is a limited version of the commercial one. The style and colour of the (macro)molecules shown is specified in each case, except for the heteroatom colour code, which is always as follows: nitrogen in blue, oxygen in red, and sulphur in gold.

4. Results

4.1. The Arachidonic Acid cascade

The enzyme sEH is involved in the Arachidonic Acid (ARA) cascade. The ARA cascade is a group of metabolic pathways in which ARA, an all-*cis*-5,8,11,14-eicosatetraenoic acid, is the central molecule (Morisseau & Hammock, 2013). ARA is a ω -6 polyunsaturated fatty acid which is a major component of cell membranes, and it resides attached to membrane phospholipids, usually at position C2 of the glycerol molecule (Imig, 2012; Spector et al., 2004; Zeldin, 2001). Once liberated from cell membrane phospholipids, a cleavage mediated by Phospholipase A2 (PLA2), ARA is converted by a series of different enzymes to numerous biological active metabolites termed eicosanoids (Zeldin, 2001; Spector et al., 2004; Imig, 2012; Imig & Hammock, 2009). This pathway produces a wide range of endogenous signalling molecules that regulate multiple biological processes, inflammation being the most relevant (Imig, 2012; Imig & Hammock, 2009; Spector et al., 2004; Zeldin, 2001) (Figure 1).

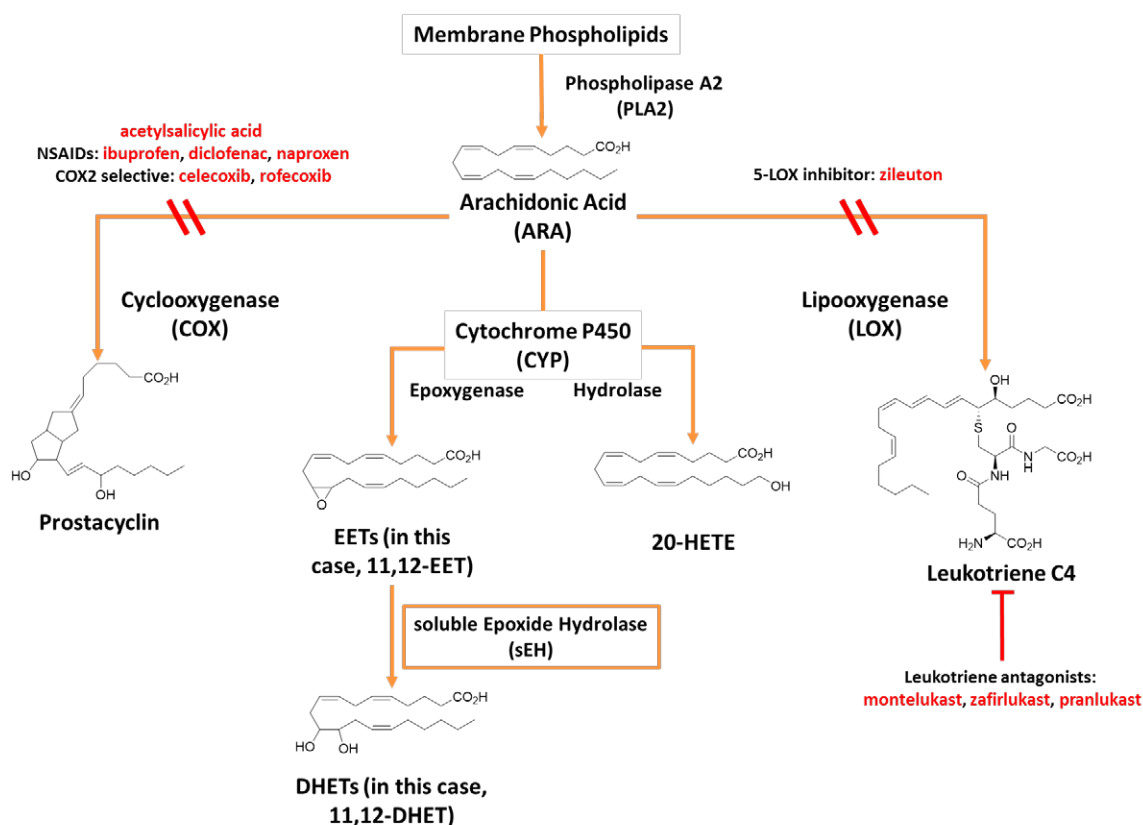


Figure 1. The three key pathways that can metabolize arachidonic acid: cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) pathways, with their intermediates. Highlighted in red, the marketed drugs that target these routes. Adapted from Imig & Hammock, 2009.

A large number of pharmaceutical drugs target these lipid mediators. The first pathway to be targeted was the cyclooxygenase (COX) pathway, which produces prostaglandins (Fitzgerald, 2004; Grosser et al., 2006). Indeed, acetylsalicylic acid and non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, naproxen or diclofenac, including specific COX2 inhibitors, such as celecoxib or etoricoxib, are effective drugs to treat pain, inflammation and in preventing cardiovascular diseases (Fitzgerald, 2004; Grosser et al., 2006).

The second eicosanoid and inflammatory pathway that was therapeutically targeted was the generation of leukotrienes by lipoxygenase (LOX) (Imig & Hammock, 2009). ARA 5-LOX inhibitors, such as zileuton, and leukotriene receptor antagonists like montelukast, zafirlukast and pranlukast, antileukotrienes in general, have been developed for treatment of asthma and seasonal allergies (Savari et al., 2014; Ribeiro et al., 2006).

Table 1. Biological effects and marketed drugs targeting the main metabolites of ARA, and their mechanism of action. DHETs (dihydroxieicosatrienoic acids) are discussed in forthcoming sections.

	Biological effects	Marketed drugs
Prostacyclins	Pain Inflammation Blood clotting Pulmonary hypertension	Acetylsalicylic acid NSAIDs: ibuprofen, diclofenac, naproxen COXIBs: celecoxib, etoricoxib
Leukotriene C4	Asthma Allergies	5-LOX inhibitors: zileuton Leukotriene antagonists: montelukast, zafirlukast, pranlukast
DHETs	Cardiovascular disease Pain Inflammation Diabetes Kidney disease	Potential modes of action: • EET agonism • sEH inhibition • combining sEH inhibition and EET agonism

Finally, a third eicosanoid pathway, the cytochrome P450 (CYP) pathway, was described in 1980 (Imig & Hammock, 2009). It is formed by two different enzymatic routes, one catalysed by the hydroxylases and the other one by the epoxygenases (Ballou et al., 1987; Oliw et al., 1981). CYP enzymes belong to a complex superfamily of genes with a common evolutionary origin, a conserved peptide that provides them with a cysteine heme ligand and the capacity to take an active form of atomic oxygen to ground state carbons (Capdevila et al., 2007; Imig, 2012).

On the one hand, CYP hydroxylase enzymes (mostly CYP4A and 4F) convert ARA into hydroxyeicosatetraenoic acids (HETEs) (Duflot et al., 2014; Imig, 2016). 20-HETE, the main metabolite of this pathway, is a pro-inflammatory molecule that plays an important role in regulating vascular function. This pathway, and specifically this metabolite, are currently being targeted for the treatment of cardiovascular diseases like hypertension and stroke (Imig & Hammock, 2009; Imig, 2016).

On the other hand, the CYP epoxygenase enzymes, mostly CYP2C and CYP2J (Iyer et al., 2012), generate epoxyeicosatrienoic acids (EETs). This function is achieved by catalysing the epoxidation of ARA olefin bonds, resulting in the production of four regioisomeric EETs: 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET, depending on the reacted double bond (Harris & Hammock, 2013; Imig, 2012). Importantly, epoxygenase enzymes generate *cis*-epoxides with a high degree of enantiofacial selectivity consisting of a mixture of (*R,S*) and (*S,R*) enantiomers (Imig, 2012). However, both *cis*-EETs and *trans*-EETs have been found in plasma (Capdevila et al., 2007) (Figure 2), and hence the biochemical pathway that generates *trans*-EETs remains to be determined (Imig, 2012).

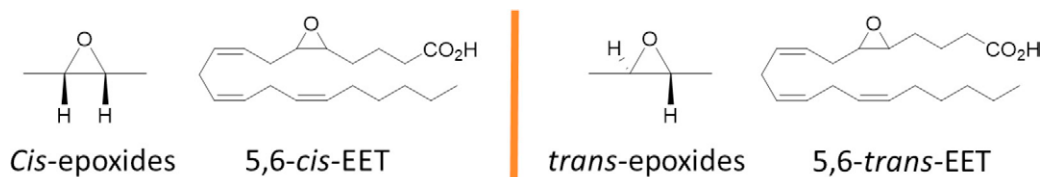


Figure 2: CYP epoxygenases generate *cis*-epoxides, whereas both *cis*- and *trans*- exist in plasma. The mechanism that forms *trans*-EETs remains unknown but could occur through radical-driven reactions. In the figure, both sorts of isomeric epoxides and the corresponding EET, in this case 5,6-EET. Adapted from Imig, 2012.

As with other CYP enzymes, CYP epoxygenase enzymes are also expressed in a gender-, age- and organ-specific manner, and even hormonal/paracrine factors as well as environmental factors and diseases can alter CYP expression and activity (Spector et al., 2004). The CYP epoxygenases that play a role in the ARA cascade are primarily members of the CYP2C and CYP2J classes, which are located in the endoplasmic reticulum (Capdevila et al., 2007; Zeldin, 2001).

4.2. Biological effects of EETs

EETs metabolism and cellular localisation are important considerations due to the fact that these can influence their biological actions (Imig & Hammock, 2009). Since the first descriptions of these physiological effects, which include increases in epithelial transport in the kidney and dilatation of small mesenteric resistance arteries, interest in EETs has grown exponentially. Especially since 1996, after the identification of EETs as endothelium-derived hyperpolarising factors (EDHFs) (Campbell et al., 1996), which means they act effectively as vasodilator molecules. Over the past decade, it has become increasingly apparent that EETs have many cardioprotective effects, accomplished mostly by their antihypertensive activity, through decreasing the vascular tone in small resistance arteries and also by enhancing natriuresis in the kidney (Morisseau, 2013). Apart from that, inhibition of EETs metabolising has proved to cause anti-inflammatory, neuro-protective, antidiabetic and analgesic effects (Morisseau, 2013).

4.3. EETs are metabolised to DHET by sEH

Once EETs are formed, their main catabolic pathway is conversion to the corresponding diols by the sEH enzyme (Ingraham et al., 2011; Duflot et al., 2014; Harris & Hammock, 2013) (Figure 1 and 3). Other secondary metabolic pathways include: 1) ω -oxidation, that adds a methyl-terminal hydroxyl group to 8,9-EET, 11,12-EET and 14,15-EET; 2) β -oxidation, to form 16-carbon long epoxy fatty acids, and 3) chain elongation to obtain a 22-carbon product (Kodani & Hammock, 2015; Duflot et al., 2014; Imig, 2012).

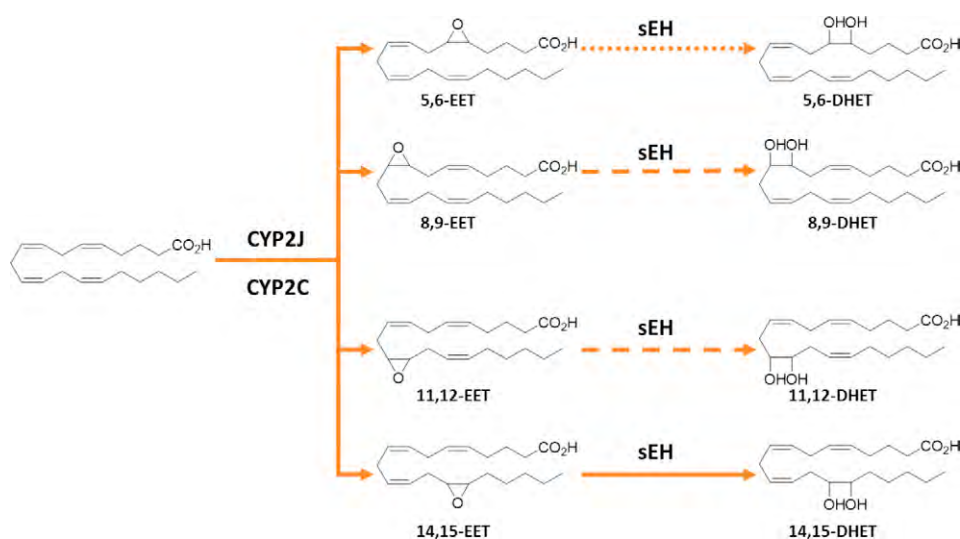


Figure 3. Metabolism of ARA in the CYP epoxygenase pathway. CYP2J and CYP2C are the main CYP subfamilies that metabolise ARA to EETs. Once EETs are formed, they are metabolised by sEH in variable affinities depending on the epoxigenated double bond. The preferred substrate is 14,15-EET (straight arrow), followed by 11,12 and 8,9-EET (dashed arrow), while 5,6-EET is a poor substrate (dotted arrow). Adapted from Imig, 2012.

Conversion of EETs to their corresponding DHETs by sEH is responsible for decreasing EET levels and thereby diminishing their beneficial properties (Imig & Hammock, 2009). Consequently, and considering the biological effects of EETs, inhibition of this enzyme is a promising therapeutic strategy for treating cardiovascular diseases, including the ones originated in metabolic syndrome. Several attempts to achieve a sEH inhibitor (sEHI) that could be used as a successful drug have been made, but to date none has reached the market. Interestingly, however, several studies have been conducted with sEHIs as proof-of-concept. They have shown cardio protective effects in hypertension, cerebral ischaemia, cardiac ischaemia, cardiac hypertrophy and atherosclerosis (Imig & Hammock, 2009; Shen, 2010), resulting in broad potential for the treatment of many cardiovascular diseases and associated morbidity. In addition, some sEHIs have shown protective activity from acute renal injury induced by the chemotherapeutic agent cisplatin (Parrish et al., 2009), as well as antidiabetic (Luria et al., 2011; Luo et al., 2010), analgesic (Wagner et al., 2011) and even antidepressant effects (Ren et al., 2016).

4.3. Structural features of sEH

sEH is one of the two well-studied α/β -hydrolase fold epoxide hydrolase (EH) enzymes, the other one being the microsomal epoxide hydrolase (mEH), both ubiquitously expressed (Morisseau & Hammock, 2005; Imig & Hammock, 2009; Ingraham et al., 2011). They differ in their subcellular localization and also in their substrate selectivity (Morisseau & Hammock, 2005). Whereas mEH is found primarily attached to the smooth endoplasmic reticulum and associated with the nuclear and cytoplasmic membranes, sEH is found predominantly in the cytosol, except in peroxisome-rich tissue, where it is also localised in peroxisomes (Luo et al., 2008) the only known mammalian protein contain-

ing a terminal methionine PTS1 (SKM. Furthermore, in terms of substrate selectivity, mEH is described to be a key enzyme in the metabolism of environmental contaminants, specifically the ones that contain cyclic and arene epoxides (Morisseau & Hammock, 2013). However, as previously mentioned, sEH is related to the metabolism of EETs and other epoxides involved in the regulation of the cardiovascular function (Imig, 2012).

sEH is encoded in the *EPHX2* gene, cloned and characterised in 1993, which is a 60 kbp gene formed by 19 exons and located in the chromosomal region 8p21-p12 in humans (Duflot et al., 2014). Transcribed and translated, that results in sEH, a 555 amino acid long enzyme with a molecular weight of 62 kDa (Duflot et al., 2014). Its structure has been thoroughly studied over the years. The structure of the murine sEH was solved for the first time in 1999, but it was not until 2004 that the first X-ray crystal structure of the human protein was released. Since then, 96 more X-ray crystal structures complexed with different molecules have been published (information acquired from the Protein Data Bank webpage). The existing X-ray crystal structure of full length sEH with the best resolution acquired so far (at 1.7 Å) is shown in Figure 4.

This data has allowed its structural features to be almost completely disclosed. sEH is a highly conserved homodimer, with its monomers arranged in an anti-parallel fashion (Duflot et al., 2014; Morisseau & Hammock, 2013; Shen & Hammock, 2012). Importantly, each monomer is composed of two domains: the N-terminal domain, which contains phosphatase activity and presents major similarities with the bacterial haloacid dehalogenase enzyme; and the C-terminal domain, which has EH activity and presents a high degree of homology with the bacterial haloalkane dehalogenase enzyme (Duflot et al., 2014). There is also a proline-rich linker region in between both domains (Duflot et al., 2014).

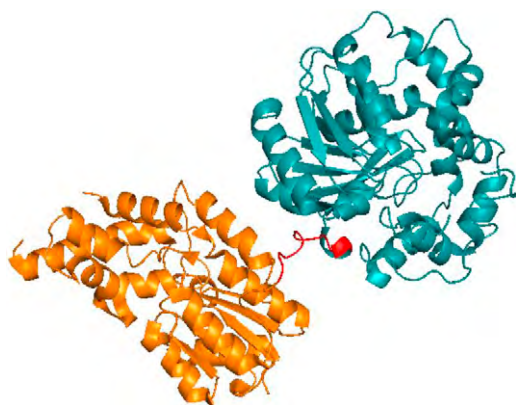
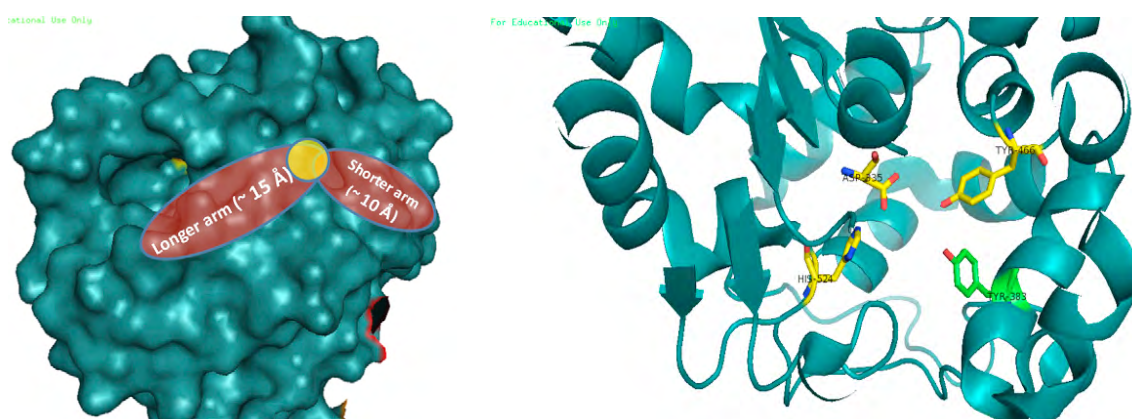


Figure 4. X-ray crystal structure of sEH (PDB file: 5AM2) represented in cartoon. In orange, the N-terminal domain (from amino acid 1 to 224); in red, the proline-rich linker region (from amino acid 225 to 234); in blue, the C-terminal domain (from amino acid 235 to 555) that contains the EH activity.

The function of the N-terminal domain is poorly understood and it still remains unclear. It is a 25 kDa fragment, from amino acid 1 to 224 (Duflot et al., 2014). It has been postulated that one of its functions might be the stabilization of the EH activity of the other fragment, since the expression of the C-terminal domain on its own shows a reduced EH activity (Morisseau et al., 2012). In addition, it has been observed that some cholesterol precursors and lysophosphatidic acid are dephosphorylated by this domain in vitro (Morisseau et al., 2012). It is also important in terms of promoting dimerization of the enzyme, since one of the key interactions for the dimerization to happen occurs between Arg287 from one monomer and Glu254 in the other monomer through an ionic bond (Duflot et al., 2014). Finally, it is also complexed with a metal cofactor, Mg^{+2} , through Asp9, Asp11 and Asp185 (Duflot et al., 2014).

Alternatively, the function of the C-terminal domain is well-known and it provides the EH activity (Ingraham et al., 2011). It comprises from amino acid 235 to 555, forming a 35 kDa fragment (Duflot et al., 2014). A number of lipid epoxides have been identified as physiological substrates, but the best characterised are the four regioisomeric EETs (Ingraham et al., 2011). Apart from them, two linoleic acid derived epoxides, 9,10- and 12,13, and other minor lipid epoxides appear to be also physiological substrates (Ingraham et al., 2011). As a result, the active site of the enzyme is found in this domain. It is formed by an L-shaped cavity within the enzyme (Figure 5), with its longer arm measuring ca. 15 Å and the shorter one ca. 10 Å (Shen & Hammock, 2012). The central vertex is where the catalytic triad is found, formed by Asp335 (nucleophile), Tyr466 (proton donor) and His524 (proton acceptor), with Tyr383 acting as the key binding site of the substrate (Shen & Hammock, 2012) (Figure 6).



Figures 5 and 6. X-ray crystal structure of the C-terminal domain of human sEH (PDB file: 1S8O), crystallised with polyethylene glycol 400 (not shown).

Figure 5 (left). C-terminal domain of the enzyme, represented in blue surface mode. Shaded in red, the L-shaped tunnel where its substrates locate while being metabolised. Shaded in yellow, the vertex where the epoxides locate to react with the catalytic triad. Adapted from H. Shen and B. Hammock (Shen & Hammock, 2012).

Figure 6 (right). Catalytic cavity of the enzyme, represented in blue cartoon. Labelled in yellow sticks, the catalytic triad of the enzyme: Asp335, Tyr466 and His524. Moreover, labelled and in green sticks, Tyr383, key for the binding of the substrate epoxides.

4.5. In pursuit of a successful sEHI

4.5.1. sEH catalytic mechanism

As has been previously highlighted, taking into account the many potential therapeutic targets for a sEHI it is not surprising that many laboratories in industry and academia have pursued their discovery in recent decades. To understand the success in identifying the best sEHIs, it is important to take a closer look at the sEH active site and its catalytic mechanism, discovered initially with X-ray crystal structure from murine sEH (Figure 7) (Ingraham et al., 2011), together with theoretical calculations using Molecular Dynamics (Hopmann & Himo, 2006; Schiott & Bruice, 2002) and quantum mechanics/molecular mechanics modelling (Lonsdale et al., 2012).

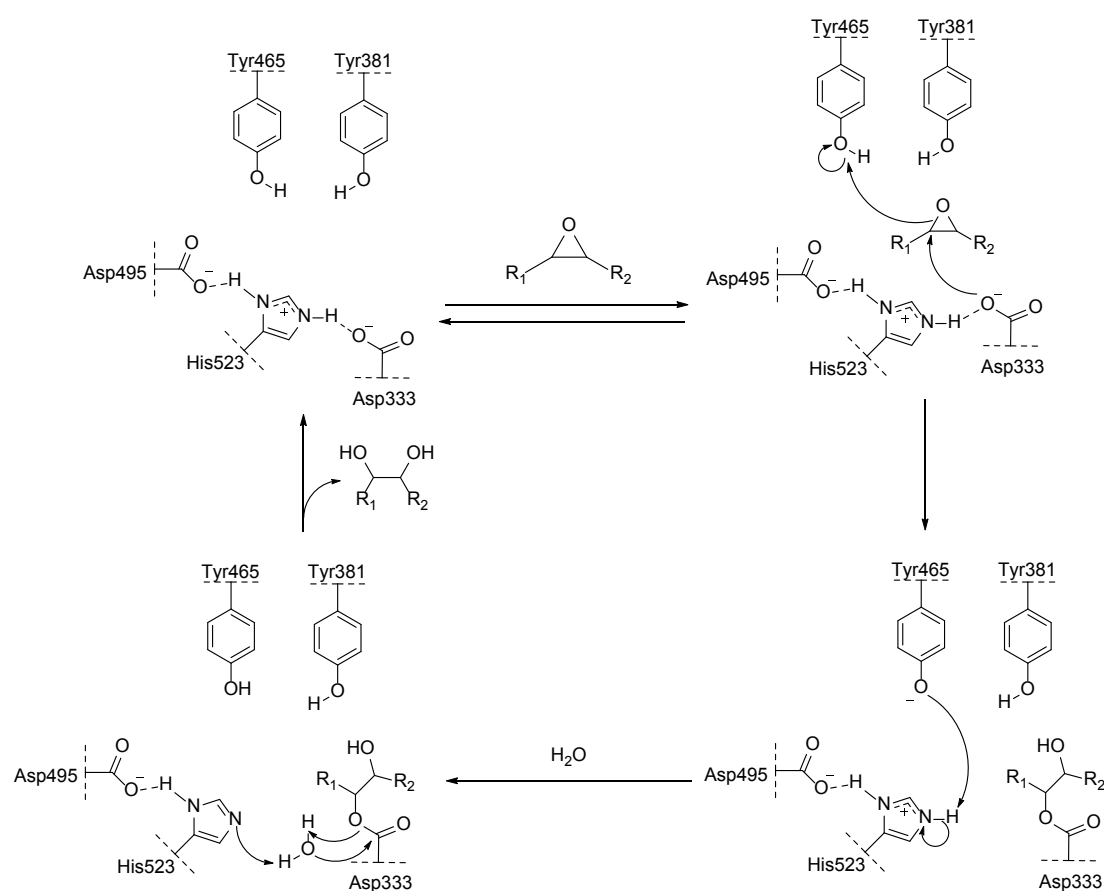
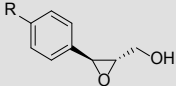
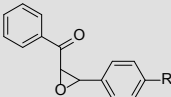
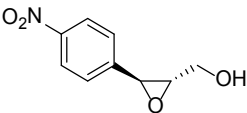
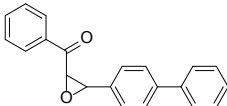
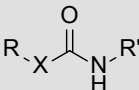
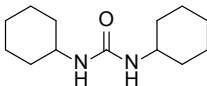
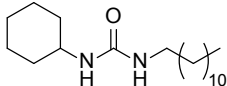
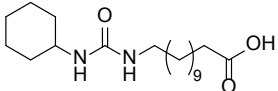
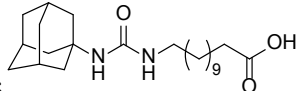
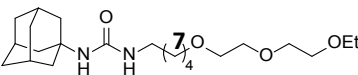
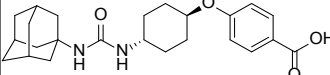
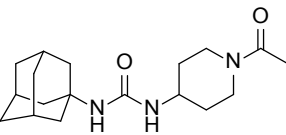
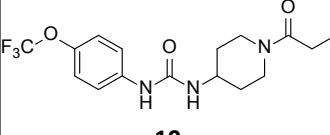
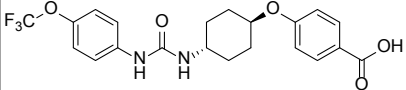
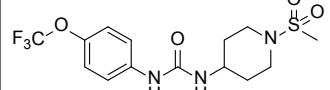
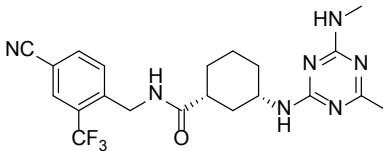
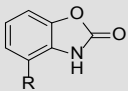
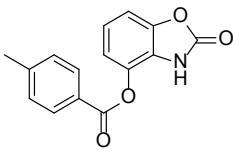
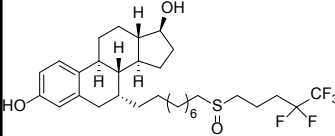


Figure 7. Catalytic mechanism of murine sEH, in which each amino acid of the catalytic triad (plus Asp495 and Tyr381) plays a role in the hydrolysis of the epoxide of the EET to the corresponding diol to form DHET using one molecule of water. The numeration of the residues corresponds to the murine sEH. Adapted from R. H. Ingraham et al., 2011.

As has been previously shown, the active site is located within an L-shaped lipophilic tunnel on the surface of the C-terminal domain of the enzyme (Shen & Hammock, 2012). In the mechanism proposed for murine sEH, the epoxide of the EET molecule binds within the tunnel and is orientated and polarised for nucleophilic attack via the hydrogen bonds formed with the two tyrosine residues, Tyr381 and Tyr465. One of the epoxide's carbon is then attacked by the nucleophilic carboxylate Asp333, activated by His523, which forms an ester bond with the substrate as the epoxide bond is subsequently broken. Simultaneously, the alkoxide formed from the epoxide opening captures a proton from the more acidic phenol of Tyr465, forming a phenoxide. As a consequence, the phenoxide then captures a proton from His523, which in turn is affected by Asp495. Next, the covalent intermediate previously formed, the ester on the Asp333, is attacked by a water molecule that is activated by the His523-Asp495 interaction, resulting in the release of a diol product and the reconstitution of the catalytic triad (Morisseau & Hammock, 2013; Argiriadi et al., 2000; Ingraham et al., 2011).

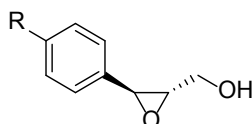
Table 2. Main classes of sEHs, main examples that appear in section 4.7 and their inhibitory potency. The sources of the IC_{50} values shown are indicated in each case, and they refer to human sEH. Of note, IC_{50} values were obtained using different assays, therefore they are not entirely comparable.

Trans-phenylglycidols 		Chalcone oxides 	
(2S,3S)-3-(4-nitrophenyl) glycidol $IC_{50} = 1.6 \mu M$	1 	4-PCO $IC_{50} = 200 \text{ nM}$	2 
Urea derivatives 			
DCU $IC_{50} = 63 \text{ nM}$	3 	CDU $IC_{50} = 85 \text{ nM}$	4 
CUDA $IC_{50} = 112 \text{ nM}$	5 	AUDA $IC_{50} = 69 \text{ nM}$	6 
AEPU $IC_{50} = 14 \text{ nM}$		t-AUCB $IC_{50} = 1.3 \text{ nM}$	8 
APAU (or AR9281) $IC_{50} = 8 \text{ nM}$	9 	TPPU $IC_{50} = 3.7 \text{ nM}$	10 
t-TUCB $IC_{50} = 1 \text{ nM}$	11 	TUPS $IC_{50} = 3 \text{ nM}$	12 
GSK2256294 $IC_{50} = 0.66 \text{ nM}$	13 		
4-substituted benzoxazolone 		Sulfoxides 	

Benzoxazolinoyl <i>p</i> -methylbenzoate $IC_{50} = 1.07 \mu M$	 14	Fulvestrant $IC_{50} = 6 \text{ nM}$	 15
IUPAC names: 4-PCO: 4-phenylchalcone oxide; DCU: N,N'-dicyclohexylurea; CDU: N-cyclohexyl-N'-dodecylurea; CUDA: N-cyclohexyl-N'-dodecanoic acid urea; AUDA: N-adamantanyln'-dodecanoic acid urea; AEPu: 1-adamantanyln-3-(5-(2-(2-ethoxyethoxy)ethoxy)pentyl)urea; t-AUCB: <i>Trans</i> -4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid; APAU: 1-(1-acetypiperidin-4-yl)-3-adamantanylurea; TPPU: 1-(trifluoromethoxyphenyl)-3-(1-propionylpiperidin-4-yl)urea; t-TUCB: <i>trans</i> -4-[3-(4-(trifluoromethoxy-phenyl)-ureido)-cyclohexyloxy]benzoic acid TUPS: 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)urea; GSK2256294: (1 <i>R</i> ,3 <i>S</i>)-N-(4-cyano-2-(trifluoromethyl)benzyl)-3-((4-methyl-6-(methylamino)-1,3,5-triazin-2-yl)amino)cyclohexanecarboxamide.			

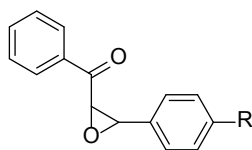
4.6. Families of sEHIs

4.6.1. *Trans*-3-phenylglycidols



These appear as the first class of sEHIs explored, being enantioselective slow-binding inhibitors of sEH (Dietze et al., 1991). Some examples show potency in the low micromolar range, except the ones with a blocked hydroxyl group or without it, that present clearly decreased potency (Dietze et al., 1991). For instance, compound **1**, the most potent inhibitor tested of this class of inhibitors, presents an IC_{50} of 1.6 μM , whereas its enantiomer, (2*R*,3*R*), only has an IC_{50} of 1200 μM (Dietze et al., 1991). Due to the lack of excellent potency and physiological liability (they react with glutathione), research on this series was discontinued.

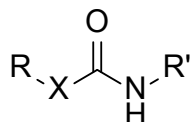
4.6.2. Chalcone oxides



Substituted chalcone oxides are more potent inhibitors than the previous ones, mostly because their mechanism of action involves a stable covalent enzyme-inhibitor intermediate (Morisseau et al., 1998). Their mechanism of inhibition involves two steps, the first one being the attack of Asp335 to the oxirane ring, leading to ring opening and formation of a hydroxyalkylated enzyme (Morisseau et al., 1998). In the second step, the covalent intermediate is hydrolysed by a water molecule activated by the His524-Asp496 pair (Morisseau et al., 1998). The time-dependent inhibition with slow recovery observed with chalcone oxide inhibition of sEH is consistent with this mechanism, which liberates a non-active product. However, these compounds are also attacked by glutathione, which means they are unsuitable when used in physiological conditions (Morisseau et al., 1998). It was observed that hydrophobic substituents in position 4, for instance 4-PCO (compound **2**), strengthened inhibition (Morisseau et al., 1998).

Nevertheless, its use has recently been evaluated for patients with pulmonary pathologies, such as restrictive airway diseases or asthma, since its reactivity with glutathione would not be such a major drawback (Shen, 2010).

4.6.3. Urea derivatives



Significantly more potent inhibitors were identified when ureas and their derivatives, especially amides and carbamates, were tested. Interestingly, in an attempt to cover potential chemical space of their own property, companies like Arête Therapeutics Inc. explored alternative primary pharmacophores, such as sulphonamide, thiourea, sulfonylurea, aminomethylene, hydroxyamide and ketoamide groups (Shen & Hammock, 2012). Unfortunately, they found that almost all replacements led to substantial loss of sEH inhibitory potency.

This class of inhibitors act as transition state inhibitors of sEH or, in other words, they resemble the transition complex formed in the catalytic cycle when the enzyme has its maximum free energy (Imig & Hammock, 2009; Ingraham et al., 2011; Kodani & Hammock, 2015; Wagner et al., 2011). These transition state mimics were designed based on the knowledge generated from the mechanism previously shown. Early examples include the industrial byproduct DCU (compound **3**) (Kodani & Hammock, 2015; Duflot et al., 2014; Shen, 2010; Shen & Hammock, 2012), which was the first urea used in vivo to demonstrate potential beneficial cardiovascular effects. However, it did not have the physicochemical properties necessary for pharmaceutical formulation, since it is too hydrophobic to become a useful drug. Shortly afterwards, variations in the cyclohexyl ring indicated that one of the cycloalkyl groups could potentially be replaced by a long, flexible chain, which is the case of CDU (compound **4**) (Kodani & Hammock, 2015; Imig & Hammock, 2009). However, its use was limited due to a rapid decomposition in hepatic microsomes. One of the metabolic reactions was a Phase I metabolic multi-step pathway, which resulted in a more water soluble carboxylic derivative (Duflot et al., 2014). In fact, this metabolite, CUDA (compound **5**), also maintains sEH inhibitory activity (Duflot et al., 2014).

Subsequent efforts to improve solubility and decrease metabolic liability of this family of inhibitors, and Structure-Activity Relationships (SAR) studies in order to find a proper drug candidate, led to the proposal of the so-called *Pharmacophore Model*, proposed by Prof. Bruce Hammock and his co-workers (Ingraham et al., 2011; Shen & Hammock, 2012) (Figure 8). In the model, the primary pharmacophore (P_1) may be a carbamate, amide or preferably urea group, bearing a bulky, hydrophobic substituent. The main group would have a second polar group, the secondary pharmacophore (P_2), located at a distance of ca. 7.5 Å (around five or six carbons away from the carbamate, amide or urea carbonyl group). Finally, an optional tertiary pharmacophore (P_3) can be located at least 12 Å away from the carbonyl group of P_1 . All three polar pharmacophore moieties should be connected by hydrophobic linkers L_1 and L_2 . Importantly, L_2 and P_3 are to some extent optional and tolerate a wide range of variation (Ingraham et al., 2011; Shen & Hammock, 2012).

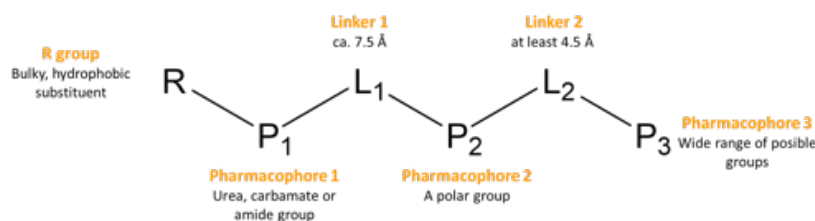


Figure 8. Schematic representation of the *Pharmacophore Model* proposed by Prof. Hammock and co-workers.

At least one free NH is needed, since hydrogen bonds are formed between this group and residues of the C-terminal domain of sEH, specially Asp335 (Ingraham et al., 2011; Pillarisetti & Khanna, 2012). This supports the hypothesis that ureas and their derivatives mimic features that are present in transient intermediates or transition states that occur during the epoxide ring opening of the EETs by sEH.

In that sense, a significant improvement in terms of solubility and melting point of the compounds in order to achieve more drug-like properties happened when a polar carboxylic functionality in a long aliphatic chain was introduced, the case of AUDA (compound **6**) (Pillarisetti & Khanna, 2012; Duflot et al., 2014; Shen, 2010; Imig & Hammock, 2009). This compound was prepared as a mimic of 14,15-EET, since within the molecule a dodecanoic acid was added to resemble the α end of ARA and, on the other nitrogen of the urea group, an adamantane was installed to mimic the ω hydrophobic tail (Morisseau et al., 2002). Interestingly, the adamantane was a good choice, as it provided high sensitivity for detection on liquid chromatography/mass spectrometry (LC/MS), facilitating the in vivo determination of the compound's concentration for pharmacokinetic (PK) studies (Watanabe et al., 2006). AEPU, (compound **7**) was a very similar compound that also presented high potency and improved solubility. Both of AUDA and AEPU's disadvantage was that they were rapidly metabolised due to their long alkyl chain. In addition, their activity both as a moderate peroxisome proliferator-activated receptor- α (PPAR α) agonist (Fang et al., 2005) and as a reasonable potent mimic of EETs (Olearczyk et al., 2006) have made them therapeutically unfavourable.

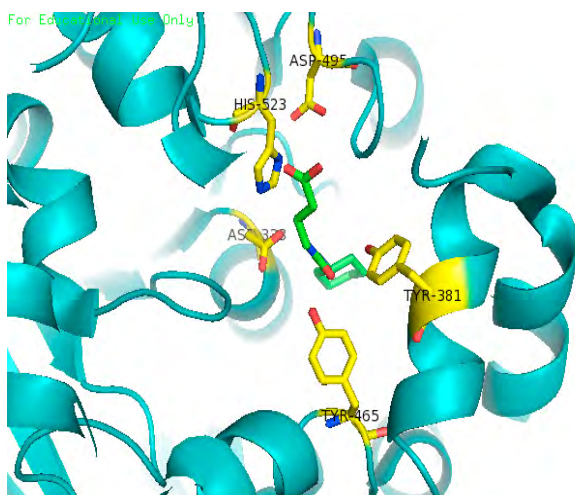


Figure 9: X-ray crystal structure of murine sEH C-terminal domain (blue cartoon) co-crystallised with a urea class of inhibitor: 4-(3-cyclohexylureido)-butyric acid (green sticks). Relevant residues for binding are represented in yellow sticks and labelled. Of note, the relevant interactions are:

- Free -NH of the urea group with Asp333 (Asp335 in human sEH).
- Carbonyl of the urea group with Tyr381 and Tyr465 (Tyr383 and Tyr466 in human sEH).

Subsequent work in this series has led to compounds of improved physical properties and PK profile. Normally, the urea pharmacophore is retained, but carbamate or amide groups have been also tested. In general, increasing water solubility, decreasing melting point and lowering the oxidation of the compounds while maintaining high potency have been the important goals in the development of this series.

Therefore, to eliminate the problem of β -oxidation, the alkyl chain present in AUDA and AEPU was replaced by a conformationally restricted scaffold, which was achieved by using linkers between P_1 and P_2 like saturated or unsaturated rings (Shen & Hammock, 2012). In that sense, *c*-AUCB and *t*-AUCB (compound **8**), with a cyclohexyl followed by a phenyl group, improved substantially the metabolic profile of sEHIs, allowing them to be used experimentally in diet-induced metabolic syndrome in rats (Iyer et al., 2012). Moreover, Arête Therapeutics Inc. synthesised APAU (or AR9281, compound **9**), with a piperidine heterocycle as the substituent of the urea, which was the first sEHI to be used in clinical trials, reaching phase IIa (Shen & Hammock, 2012; Imig & Hammock, 2009).

Finally, further modifications of the left side of the scaffold – the bulky hydrophobic substituent of the primary pharmacophore – resulted in the replacement of the adamantane ring for aryl groups, producing analogues with subnanomolar IC₅₀ against sEH. In addition, some of these compounds also showed improved PK in rat models (Rose et al., 2010). That is the case, for instance, of TPPU (compound **10**), which has even shown good PK profile in primate species and has been used for many laboratories as a model sEHI (Ulu et al., 2012). Interestingly, another example originated from these types of modifications, *t*-TUCB (compound **11**) is currently in trials for the neuropathic pain of equine laminitis (Guedes et al., 2013) and is being evaluated for canine arthritis and feline joint pain (Kodani & Hammock, 2015).

To sum up, it can be considered that urea-based sEHIs have undergone a clear development in the past decade, directed towards increasing or retaining potency, improving their PK profile and lowering hydrophobicity, evolving to a more suitable scaffold to become a successful marketed drug (Figure 10).

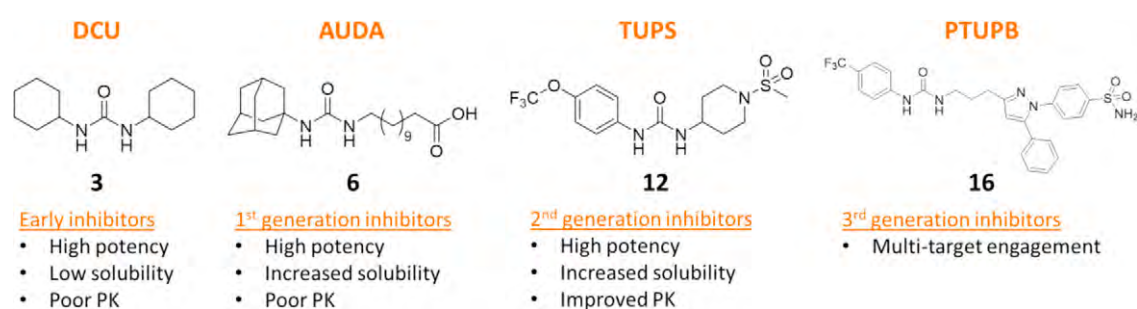
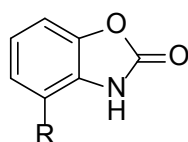


Figure 10: Evolution of sEHIs over the past decade, from the early inhibitors that presented solubility issues to the last generation of inhibitors, which present activity against more targets than just sEH (this last type of compound will be discussed in section 4.8). Adapted from S. Kodani and B. Hammock, 2015.

Urea is by far the most explored chemical group in this series due to the fact that the most promising compounds presented the group in their structure. However, the only compound that is currently in clinical trials does not contain a urea in its structure, but an amide group. This is the case of GSK2256294 (Lazaar et al., 2015; Podolin et al., 2013) (compound **13**), a compound discovered and developed by the pharmaceutical

company GSK. The compound was initially described in 2013 (Thalji et al., 2013), and the pre-clinical results showed target selectivity, concentration-dependent inhibition of conversion of EETs to DHETs, decent physicochemical properties (including good aqueous solubility at lower pH) and a very good PK profile, exhibiting high oral bioavailability in the species that were examined and a decent half-life (Podolin et al., 2013). Eventually, the compound was investigated in clinical trials, in a study named “first-time-in-human” (FTIH, identifier NCT01762774), where it showed positive results in a study with 42 obese smokers and 14 healthy subjects (Lazaar et al., 2015). The results obtained suggest that this compound is safe and could be used for further investigation in patients with endothelial dysfunction or abnormal tissue repair, such as in diabetes, wound healing or chronic obstructive pulmonary disease (Lazaar et al., 2015). Clinical trials are ongoing.

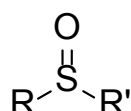
4.6.4. Substituted benzoxazolones



This family of molecules are promising sEHs that can be dosed orally (Tang et al., 2013). Several substituents have been evaluated to improve water solubility and bioavailability compared to the other families of sEHs (Dufлот et al., 2014).

Initially, benzoate and besilate substituents at 4-position were introduced, but the inhibitory activity of the compounds was not high enough (Tang et al., 2013). A step forward came when glycosyl and amino acid substituents allowed the formation of bioactive compounds, and another when using phenyl, pyrrolidine or sulfhydryl as the amino acid substituents further increased the inhibitory activity of the compounds against sEH (Tang et al., 2013). Furthermore, substitution with a meta-electron withdrawing group or a para-electron donor increases the inhibitory effect of the 4-substituted benzoxazolone in the low micromolar range, such as compound **14** (Tang et al., 2013). Importantly, the development of this new class of inhibitors is preferred due to the use of similar compounds as antibacterial and anti-inflammatory drugs (Shankaran et al., 1997), which means they are to some extent tolerated, have a good PK profile and present almost no toxicity, lowering the risk of clinical failure (Tang et al., 2013).

4.6.5. Sulphoxides



This new class of competitive sEHs has been recently discovered, and its lead compound is fulvestrant (compound **15**), which presents a low nanomolar potency ($IC_{50} = 6$ nM) for human sEH (Morisseau et al., 2013). Fulvestrant (Faslodex; AstraZeneca), which is a marketed drug for hormone receptor-positive metastatic breast cancer for postmenopausal women that acts as an oestrogen receptor antagonist (AstraZeneca n.d.),

also binds to the hydrolase catalytic pocket of sEH (Morisseau et al., 2013). To do so, its sulphur atom of the sulfoxide interacts with Asp335, an interaction quite similar to the one reported between the nitrogen atoms of the urea group and the same Asp335 residue in the urea derivative sEHIs (Morisseau et al., 2013; Schmelzer et al., 2005). In the case of fulvestrant, the oxygen atom of the sulfoxide pharmacophore forms hydrogen bonds with two more key catalytic residues (Tyr383 and Tyr466), the same as the oxygen atom of the carbonyl in the urea group (Morisseau et al., 2013; Schmelzer et al., 2005) (Figures 11 and 12).

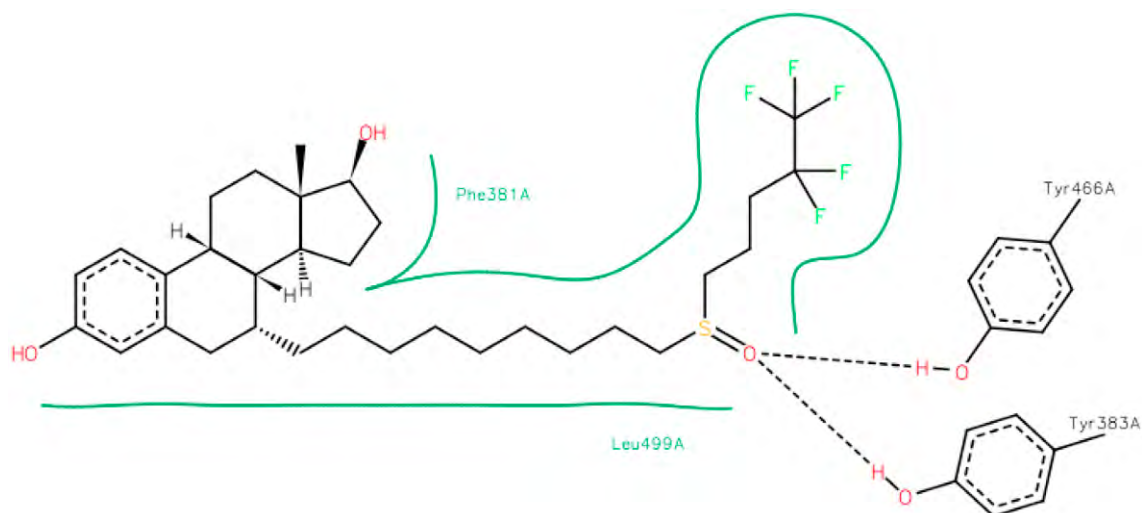


Figure 11 (above): Schematic representation of fulvestrant's binding mode, with hydrogen bonds represented in black dashed lines and hydrophobic interactions represented by a straight green line. Image obtained in the supplementary information of the PDB file 4J03.

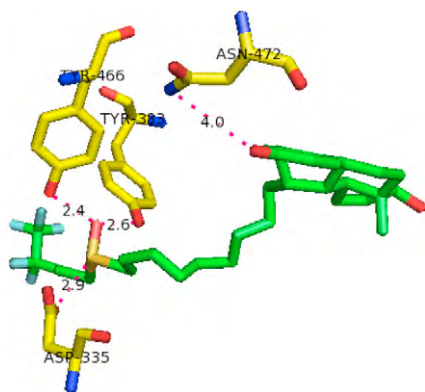
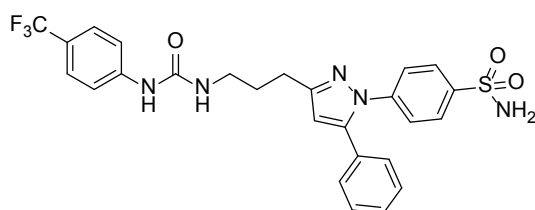


Figure 12 (below): X-ray crystal structure of sEH with fulvestrant (PDB file: 4J03). The key residues necessary for the interaction are represented in yellow sticks and labelled, and fulvestrant is represented in green sticks. The measurements of the distance between the polar interactions are shown in pink dashed lines.

4.7. New strategies – Dual inhibitors

4.7.1. sEH and COX inhibition

Hammock and co-workers have also studied the effect of the combination of sEHIs with COX and LOX pathway inhibitors in order to enhance the anti-inflammatory effect of such compounds. Therefore, a dual COX/sEH inhibitor named PTUPB (compound **16**, Figure 10) was generated by combining pharmacophores of celecoxib and the urea class sEHIs through a 3-carbon linker chain (Hwang et al., 2011). The resulting compound, which was even orally bioavailable, showed excellent sEH inhibition potency ($IC_{50} = 0.9$ nM) and a minor but not negligible COX inhibition ($IC_{50} = 1.3$ μ M) (Hwang et al., 2011).

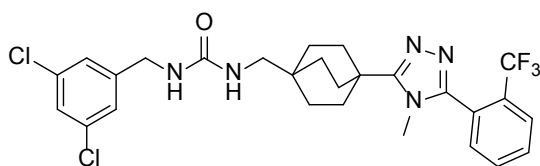


16: Dual COX/sEH inhibitor that mimics celecoxib's pharmacophore and the urea sEH scaffold.

PTUPB: 4-(5-Phenyl-3-{3-[3-(4-trifluoromethyl-phenyl)-ureido]-propyl}-pyrazol-1-yl)-benzenesulfonamide

4.7.2. sEH and 11 β -HSD1 inhibition

Hammock and co-workers were not the only ones exploring the dual inhibitors field. GSK disclosed a series of inhibitors targeting both sEH and 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1), an enzyme that has the biological function of reducing cortisone to the active hormone cortisol (Shen, 2010). There have been major attempts to target this enzyme in order to treat type II diabetes and cardiovascular disease, so far unsuccessfully. Interestingly, this series of inhibitors were generated by combining a known pharmacophore for 11 β -HSD1, a triazole-substituted [2.2.2] bridged system, with the sEH pharmacophore, using a substituted urea group (compound **17**).



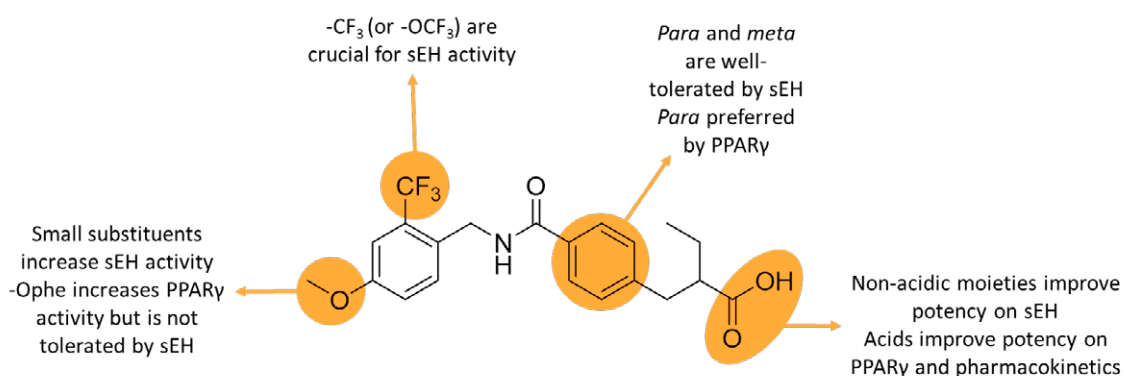
17: Dual 11 β -HSD1/sEH inhibitor mimicking known pharmacophores for both enzymes.

IUPAC Name: 1-(3,5-dichlorobenzyl)-3-((4-(4-methyl-5-(2-(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-yl)bicyclo[2.2.2]octan-1-yl)methyl)urea

4.7.3. sEH and PPAR γ inhibition

Finally, a new series of dual inhibitors, in this case modulating sEH and PPAR γ , has been recently published (Blöcher et al., 2016). PPAR γ is a member of the PPAR nuclear receptor family, and it is widely known it plays a key role in adipogenesis, regulation of lipid metabolism and glucose homeostasis, as well as in anti-inflammatory processes. Therefore, it is targeted in the treatment, for example, of type II diabetes (Blöcher et al., 2016).

The dual inhibition strategy was originated from the study of known inhibitors for these two enzymes, and it was observed that several previously reported series shared a common pharmacophore: the N-benzilamide group (Blöcher et al., 2016). Using it as a starting point, many derivatives were synthesized, until a potent (sEH IC₅₀ = 0.3 μ M / PPAR γ EC₅₀ = 0.3 μ M) and orally available compound was tested (Blöcher et al., 2016). Even the SAR was explored (compound 18), resulting in a promising series to be explored and improved to become a successful metabolic syndrome treatment.



18: Essential SAR of sEH/PPAR γ dual modulator. Adapted from Blöcher et al. (2016).

IUPAC Name: 2-Ethyl-3-[4-(N-(4-methoxy(2-trifluoromethyl)benzyl)- benzamide)]propionic acid

5. Conclusions

The main conclusions that can be extracted from this dissertation are:

- The pharmacological treatment of metabolic syndrome needs to be improved since it relies mostly on polypharmacy.
- Much evidence supports the hypothesis that inhibition of sEH could be a suitable treatment for metabolic syndrome.
- The structure and catalytic mechanism of the enzyme has proved useful to the rational design of successful sEHIs.
- Optimisation of sEHIs has led to potent and bioavailable drug-like compounds, some of which have reached clinical trials.
- The newest strategies to treat metabolic syndrome are directed towards dual inhibition of sEH together with another enzyme.

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