The Soluble Epoxide Hydrolase as a Pharmaceutical Target for Hypertension

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Abstract: The soluble epoxide hydrolase appears to be a promising target for the development of antihypertensive therapies based on a previously unexplored mechanism of action. Epoxide hydrolases are enzymes that add water to three membered cyclic ethers known as epoxides. The soluble epoxide hydrolase in mammalian systems (sEH) is a member of the α/β-hydrolase fold family of enzymes and it shows a high degree of selectivity for epoxides of fatty acids. The regioisomeric epoxides of arachidonic acid or epoxyeicosanoids (EETs) are particularly good substrates. These EETs appear to be major components of the endothelium-derived hyperpolarizing factors (EDHFs). As such, EETs cause vasodilation and reduce blood pressure. The EETs also are strongly anti-inflammatory and analgesic. By inhibiting sEH, the increase in circulating EETs leads to a reduction in blood pressure in a number of animal models. Potent transition state mimic inhibitors have been developed for the sEH. Some of these sEH inhibitors (sEHIs) show nanomolar to picomolar potency and good pharmacokinetic properties. Because of their unique mode of action they show promise in treating hypertension while reducing problems with end organ failure, vascular inflammation and diabetes. Indeed, the anti-inflammatory properties of the sEHI may make them particularly suitable for treating hypertension in patients with other concomitant metabolic syndromes. They are more potent on a molar basis than most nonsteroidal anti-inflammatory drugs (NSAIDs) in reducing PGE_2 in inflammation models, they strongly synergize with NSAIDs, and appear to ameliorate apparently unfavorable eicosanoid profiles associated with some cyclo-oxygenase-2 inhibitors.

Key Words: soluble epoxide hydrolase, epoxide hydrolase, epoxide hydrolase inhibitor, inflammation, hypertension, analgesia, epoxyeicatrienoic acids, arachidonic acid, cyclooxygenase

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NEED FOR NEW ANTIHYPERTENSIVE DRUGS

A large number of classes of antihypertensive agents are available in the market for the treatment of hypertension [diuretics, β-blockers, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers, calcium channel blockers, and α-blockers], and they have all been shown to be relatively safe and effective in lowering blood pressure.[1,2] The recent approval of a renin inhibitor, aliskiren (Tekturna) adds another tool to the antihypertensive arsenal. There are several critical issues which need to be carefully and fully considered in deciding the best antihypertensive agents to use. A large proportion of patients with hypertension also have other concomitant diseases, for example, diabetes, coronary artery disease, hyperlipidemia, and metabolic syndrome. Indeed, a large number of clinical trials in recent years have been conducted to address whether there are important differences in clinical outcomes among different antihypertensive agents.[2] Of these trials, the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) study represents one of the largest studies.[3] Even though there were no significant differences in the primary outcome [cardiovascular-related mortality or nonfatal myocardial infarction (MI)] among the following antihypertensive agents including a diuretics (chlorthalidone), calcium channel blocker (amlodipine), and ACE inhibitor (lisinopril), it was found that thiazide-type diuretics are superior in preventing one or more major forms of cardiovascular disease and are less expensive.[3] Moreover, there was an increase in cardiovascular events in β-blocker (doxazosin) arm.[3] Recent clinical trials comparing treatments with similar blood pressure-lowering effects also suggest that interruption of hypertrophic signaling pathways may confer differing degrees of clinical benefit independent of its blood pressure-lowering effects.[4–6] In contrast, persistence of cardiac hypertrophy (despite similar blood pressure changes) predicted an adverse outcome.

From the above discussion, it seems clear that antihypertensive treatment may need to be individualized, and the treatment goals should not be limited to merely lowering blood pressure. Each antihypertensive agent possesses very different mechanisms of action and adverse event profiles. It is very likely that certain groups of patients—such as those with diabetes, left ventricular dysfunction, angina pectoris, migraine, prostatism, or lipid disorders—will derive different advantages from different antihypertensive drug groups.[7] Initial antihypertensive drug choices need to take into account the comorbid conditions of the patients being treated (Table 1) as well as consideration of target-organ damage

TABLE 1. Cardiovascular Risk Factors or Concomitant Disorders That May Affect Prognosis and Guide the Anti-Hypertensive Treatment

Cigarette smoking
Metabolic syndrome including
Obesity (BMI ≥30)
Dyslipidemia
Diabetes mellitus
Physical inactivity
Microalbuminuria or estimated GFR <60 mL/min
Age (>55 years for men, >65 years for women)
Family history of premature cardiovascular disease (men <55 years or women <65 years)

BMI indicates body mass index calculated as weight in kilograms divided by the square of height in meters; GFR, glomercular filtration rate.[7,110–113]

(left ventricular hypertrophy, coronary artery disease, chronic kidney disease, peripheral arterial disease, cerebrovascular disease). In addition, other key features which need further consideration include compliance issues. In a proportion of patients with elevated blood pressure, multiple drugs are often needed to achieve an adequate level of blood pressure reduction. Therefore, from a public health perspective, it seems clear that the focus for the antihypertensive therapy should be directed towards an overall decrease in the risk factors for cardiovascular diseases using antihypertensive agents which provide salutary effects and without exacerbating other comorbid factors. Thus, a diversity of pharmaceuticals with different mechanisms of action is attractive and will greatly enhance the likelihood of achieving the above treatment goals.

THE SOLUBLE EPOXIDE HYDROLASE AS A NOVEL TARGET TO TREAT HYPERTENSION

Among the many possible targets evaluated to find new treatments for hypertension, the soluble epoxide hydrolase (sEH) appears to be among the most overlooked. There are probably many reasons for this oversight, including the fact that most pharmacologists have the mistaken impression that epoxide hydrolases are only involved in xenobiotic metabolism. Epoxides of arachidonic acid or epoxyeicosatrienoic acids (EETs, Fig. 1) are excellent substrates for the sEH, but only recently have these compounds been recognized as likely endothelium-derived hyperpolarizing factors (EDHFs)[8] involved in reducing hypertension and inflammation and even yielding analgesia. The epoxide of EETs (unlike the polyunsaturated fatty acid backbone) is relatively stable chemically, it is not a potent alkylating agent, and it does not intercalate with DNA. However, biologically the EETs are turned over so quickly that it was difficult to show biological effects in vivo.

In fact, the entire cytochrome P450 branch of the arachidonic acid cascade has received scant attention in spite of the cyclooxygenase (Cox) and lipoxygenase (Lox) branches of the cascade being the target for roughly 15% of the world's pharmaceuticals. It also is commonly thought that the action of chemical mediators largely can be modulated by altering biosynthesis or developing agonists and antagonists. The concept that the efficacy of chemical mediators can be radically altered by inhibiting their degradation is not commonly used in pharmacology although there are a few dramatic examples of success using this approach. Regardless of the reason, only a handful of laboratories have evaluated the action of inhibitors of the soluble epoxide hydrolase (sEHIs) as novel antihypertensive agents, yet the information to date suggests that this enzyme may be a very valuable pharmaceutical target.[9]

The sEHIs offer the first radically unique mechanism for the treatment of blood pressure in many years. Since there is a trend in the treatment of hypertension toward the use of drug combinations, a first-in-class series of compounds offering a new mechanism may prove very useful for combination therapy. Moreover, it appears that the epoxyeicosanoids that are stabilized by sEHIs have at least three independent actions. Not discussed here are direct analgesic effects.[10–12] Certainly of direct interest with hypertensive patients are the dramatic anti-inflammatory effects of the sEHIs. In a murine sepsis model, the compounds not only are more potent than common nonsteroidal anti-inflammatory drugs (NSAIDs) in reducing predominantly inflammatory eicosanoids like prostaglandin PGE_2 but they synergize with NSAIDs in reducing inflammation.[11–13] These effects can be anticipated from the mechanism of action where the NSAIDs inhibit Cox 1 and/or 2 and shift the arachidonate acid substrate toward other pathways including the P450 pathway, while the EETs stabilized by sEHIs transcriptionally reduce the Cox 2 (and other enzymes and proteins) induced by inflammation.[12] Thus, one can anticipate that inhibitors of Cox and other enzymes in the arachidonate cascade will synergize both the antihypertensive and anti-inflammatory roles of sEHIs and that the sEHIs by stabilizing EETs may transcriptionally regulate other pathways in the arachidonate cascade. Thus the sEHI should reduce the dose needed of some Cox and Lox inhibitors for a targeted therapy.

Among the many possible targets evaluated to find new treatments for hypertension, the soluble epoxide hydrolase (sEH) appears to be among the most overlooked.

The thrombic events associated with the use of rofecoxib and other NSAIDs have been attributed to high dose but also to destabilizing platelets due to the increased ratio of TXA_2 to PGI_2.[14] In addition, some NSAIDs increase blood pressure. sEHIs coadministered with indomethacin, celecoxib, and rofecoxib reduce the amount of NSAID needed for reduction in inflammatory PGE_2, they decrease the TXA_2 to PGI_2 ratio and reduce blood pressure. Thus, the sEHIs could be considered synergizers for NSAIDs and may help specifically to control hypertension in individuals on high doses of NSAIDs and related modulator of the arachidonate cascade for inflammatory and other diseases.[11] Certainly, end organ

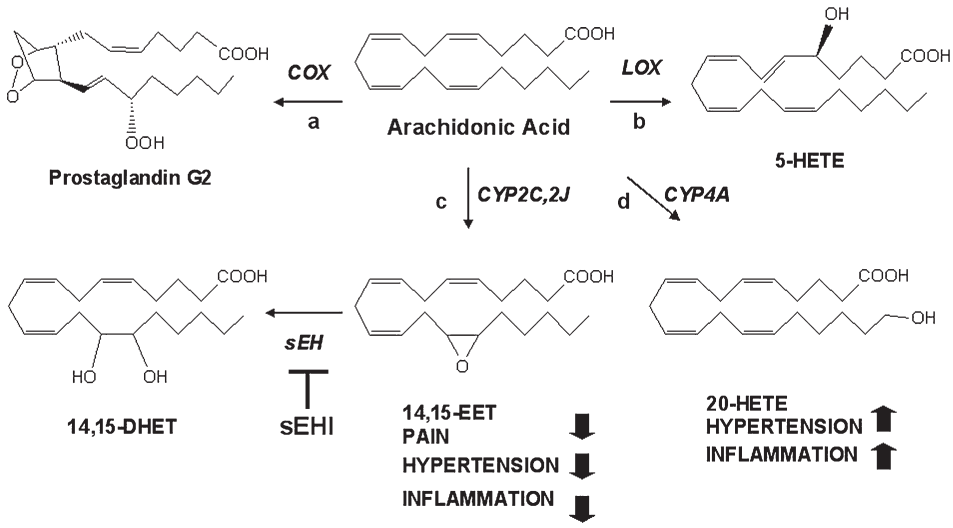

FIGURE 1. Simplified view of the arachidonic acid cascade. Arachidonic acid can be metabolized to (A) prostaglandin G2 by cyclooxygenase, (B) 5-HETE by lipoxygenase, (C) 14,15-EET and other regioisomers by cytochrome P450 2J/2C, or (D) 20-HETE by cytochrome P450 4A. EETs are then converting to less biologically active DHETs by sEH. This figure also represents the major biological effects of 14,15-EET and 20-HETE. The target sEH converts a variety of lipid epoxides to their corresponding diols. The sEHI act by blocking this enzyme, increasing the EETs which have both direct actions on hypertension, pain, and inflammation as well as transcriptional regulation of cytokines and other key enzymes in the arachidonate cascade. DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; sEHI, soluble epoxide hydrolase inhibitor.

damage can be considered a major mortality factor associated with hypertension. In several models, sEHIs have protected the kidney from hypertension-induced damage.[15–17] As mentioned above, sEHIs are promising in preventing ischemia-reperfusion injury in the heart[18] and also the brain.[19]

PHYSIOLOGY OF THE ACTION OF SEHIS

sEHIs Alter the Arachidonic Acid Cascade

The arachidonic acid cascade is arguably one of the most elaborate signaling systems in biology. Arachidonic acid is an important constituent of cellular membranes that is esterified to the *sn*-2 position of glycerophospholipids. Arachidonic acid is derived directly from linolenic acid or is ingested as a dietary constituent. Increasingly, it is a "value added" additive of food. Free arachidonic acid is taken up by cells and stored rapidly by esterification into membrane phospholipids. As a result, only trace amount of free arachidonate may be found in resting cells. Such tight control is necessary due to its potential toxicity and the wide range of signaling pathways that may ensue. Arachidonic acid is released in response to neuromodulators and various stimuli such as histamine and platelet-derived growth factor. At least three distinct phospholipases are thought to generate free arachidonic acid, either directly or indirectly: phospholipase A_2 (PLA_2), phospholipase C (PLC), and phospholipase D (PLD).[20]

Arachidonic acid is metabolized to eicosanoid mediators involving three major pathways via cyclooxygenase (Cox), lipoxygenase (Lox), and cytochrome *P*-450 (CYP) monooxygenase. Each branch produces numerous bioactive metabolites whose actions are often not easily summarized. Increasingly amide derivatives of compounds in each of the above three pathways are being found to be biologically active. The Cox pathway results in the formation of prostaglandin G_2 (PGG_2) from arachidonic acid by a cyclooxygenase reaction (Fig. 1A). PGG_2 serves as a substrate for cell-specific isomerases and synthases, producing other eicosanoids such as prostacyclin (PGI_2) and thromboxane A_2 (TXA_2). Proinflammatory mediators such as PGE_2 arise from the Cox branch as well. The Lox pathway forms hydroperoxyeicosatetraenoic acids (HPETEs) by lipoxygenase and subsequently converts these to hydroxyeicosatetraenoic acids (HETEs, Fig. 1B). The leukotriene family also comes from lipogenase enzymes. The CYP pathway produces two types of eicosanoid products: the EETs, formed by CYP epoxygenases, which are primarily members of the CYP 2C and 2J classes. (Fig. 1C), and the hydroxyeicosatetraenoic acids such as 20-HETE, formed by CYP ω-oxidases (Fig. 1D). Very simplistically, one can think of these as antagonistic pathways where 20-HETE is proinflammatory and hypertensive and the EET pathway is antiinflammatory, antihypertensive, and analgesic. Thus, one can increase EETs by inhibiting their major route of degradation through the sEH enzyme[9,21,22] or conceptually by increasing their production or substrate availability.

Metabolites of Epoxyeicosatrienoic Acids

The main EET catabolic pathway is catalyzed by sEH to form the corresponding dihydroxyeicosatrienoic acids (DHETs) (Fig. 2). This enzyme effectively utilizes 8,9-, 11,12-, and 14,15-EET while the hydrolytically unstable 5,6-EET is a poor substrate. Fig. 2 shows other metabolic pathways for EETs including Lox, Cox, glutathione-*S*-transferase (GSH), CYP ω-oxidase, β-oxidation, and chain-elongation. These are generally thought to yield eicosanoids of dramatically different or reduced biological activity, but human coronary endothelial cells chain shorten 14,15-EET to a biologically active metabolite,[21] for example,10,11-epoxyhexadecadienoic acid (16:2). Possibly the lack of toxicity so far observed with the sEHIs results because it is difficult to obtain total inhibition of an enzyme in vivo and even with total inhibition of sEH, there are alternate pathways for metabolism of EETs and other bioactive lipids.

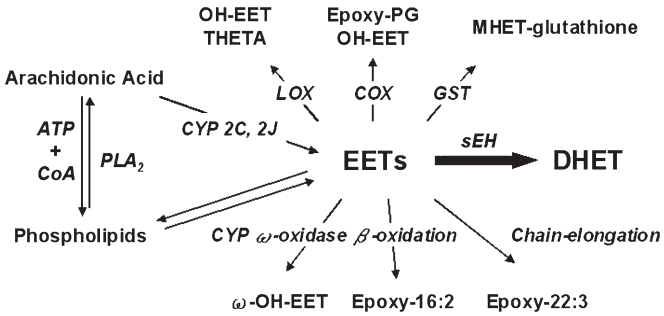

FIGURE 2. Major metabolites of EETs. This figure shows the overview of EET metabolism. According to different locations and corresponding enzyme expression, EETs have different metabolic pathways. The EETs arise from arachidonic acid released from phospholipids by hydrolysis by several enzymes including phosphlipase A2. Following synthesis by several cytochromes P450, they are largely metabolized to corresponding diols by sEH. When sEH is reduced as by treatment with sEHI, EETs are metabolized by other pathways such as chain-elongation and β-oxidation. They can be converted into other biologically active materials as by synthesis of their corresponding amides. DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; THETA, hydroxyepoxyeicosatrienoic acid; MHET, monohydroxyeicosatrienoic acid.

Biological Activities of EETs

EETs function primarily as autocrine and paracrine effectors in the cardiovascular system and kidney[23–25] and have been shown to modulate ion transport and gene expression, producing vasorelaxation as well as anti-inflammatory and profibrinolytic effects. EETs act at vascular, renal, and cardiac levels in blood pressure regulation.[26] EETs are formed subsequent to activation of cells leading to the formation of arachidonic acid, and the released arachidonic acid is converted to EETs by the CYP epoxygenases. Alternatively, activated phospholipase may release the preformed EETs stored in the phospholipids. The initial action of EETs remains uncertain. EETs may bind to a putative plasma membrane receptor linked to an intracellular signaling pathway to initiate the functional responses (membrane receptor mechanism), or EETs may directly interact with and activate K^+ channels,[27] signal transduction components, or transcription factors (intracellular mechanism). The intracellular signaling pathways are active in different tissues under unique conditions. It is likely that actions of EETs may be mediated by both mechanisms above. Activation of large-conductance Ca^{2+}-activated K^+ channels (BK channels) by EETs occurs via a $G_{\alpha s}$, protein coupled to the putative membrane receptor[28] and leads to vasodilation and blood pressure lowering effects. All EET regioisomers show distinct vasodilatory actions and function as endogenous hypotensive agents.[29–31] EETs are further metabolized by the sEH to form the corresponding diols (DHETs).[32–34] In the renal microcirculation, the vasoconstriction stimulated by 20-HETE (Ca^{2+} activated K^+ channel antagonist) is attenuated by EETs (Ca^{2+}-activated K^+ channel agonists).[26]

A number of other signaling cascades have been shown to be activated by EETs under various conditions including tyrosine kinase, Src kinase, mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI-3K)/Akt.[22,26,35–41]

Certainly of direct interest with hypertensive patients are the dramatic anti-inflammatory effects of the sEHIs.

Previous studies have shown that EETs inhibit apoptosis by activation of a PI-3K/Akt signaling pathway.[35,36]

The anti-inflammatory actions of EETs appear to be mediated by interfering with the activation of the proinflammatory transcription factor, NF-κB.[42–44] Activated NF-κB is a critical cell-signaling molecule for the induction of numerous inflammatory mediators in the vascular wall. EETs appear to inhibit NF-κB activity by blocking the activation of IκB kinase.[44] In addition, inflammatory cytokines have been demonstrated to decrease CYP2 gene expression.[45–47]

Efficacy of Epoxide Hydrolase Inhibitors

The cardiovascular protective actions of sEHIs have been documented in animal models of hypertension (Table 2).[9,32,48,49] Although one might anticipate a large reduction in blood pressure following targeted disruption of the sEH gene,[50] recent studies suggest that there is little if any reduction possibly due to adaptation and an increase in 20-HETE.[51] There is accumulating evidence to demonstrate that sEHIs lower blood pressure in several animal models of

TABLE 2. Possible Unique Advantages of sEHI as an Anti-Hypertensive Agent

Function	References
Unique mechanism of action in reducing blood pressure	9,16,48,49,114,115
Possible value in combination therapies with other anti-hypertensive agents	9,16,114
Possible value in treating recalcitrant hypertension	9,16,48,49
Reduction in end organ damage	15,114
Reduction in vascular inflammation	13,44
Amelioration of co-morbidities (cardiac and cerebral ischemia—reperfusion, diabetes, cardiac hypertrophy)	18,40,116
Avoidance of side effects of some existing therapies (edema, erectile dysfunction)	117
Synergism with aspirin and NSAIDs	11
Reduction of hypertension in patients treated for inflammatory disease	9,44

hypertension, for example, spontaneously hypertensive rats (SHR) as well as angiotensin-induced hypertensive models.[15,32,48,49] However, the mechanisms of action of sEHIs in blood pressure lowering are not fully understood. These data demonstrate that sEHIs lower blood pressure and ameliorate renal damage in angiotensin-dependent, salt-sensitive hypertension.[49] More recent studies have demonstrated the effectiveness of orally administered sEHIs in blood pressure lowering.[49,52] In addition, it was shown that sEHIs could influence the generation of Cox metabolites.[49] These changes do not relate to the blood pressure lowering actions of sEH inhibitors but may be due to the anti-inflammatory actions of sEHIs.

Apart from their antihypertensive action, sEHIs offer protective effects from cardiovascular disease-related end organ damage. Renal vascular and glomerular injury induced by angiotensin-mediated hypertension is decreased following chronic administration of sEHIs.[15,49] In addition, the renal protection appears to be independent of blood pressure lowering (Dr. Imig's personal communication), and is likely the result of the anti-inflammatory actions of sEHIs. Indeed, using rodent models, investigators have demonstrated that sEHIs are effective in the treatment of inflammatory diseases.[10,12,13] Using lipopolysaccharide-induced systematic inflammation in mice, treatment with sEHIs reduces the production of nitric oxide, cytokines, and proinflammatory lipid mediators and significantly improves survival.[13] These findings are consistent with the notion that EETs prevent amplification of an inflammatory event by inhibiting NF-κB translocation to the nucleus.[43,44]

sEHIs have also been shown to have protective effects in myocardial ischemia and reperfusion. Overexpression of epoxygenase enzymes (CYP2J2) in mice resulted in the improvement myocardial function following ischemia and reperfusion.[40] CYP2J2 transgenic mice exhibit increased expression of phospho-p42/p44 mitogen-activated protein kinase (MAPK) after ischemia, and addition of the p42/p44 MAPK kinase (MEK) inhibitor PD98059 during reperfusion abolishes the cardioprotective effects of CYP2J2 overexpression. These data suggest that CYP2J2-derived metabolites are cardioprotective after ischemia, and the mechanism for this cardioprotection involves activation of mitoK(ATP) and p42/p44 MAPK.[40] More recent data have shown that mice with targeted disruption of sEH have increased availability of cardioprotective EETs and improvement in cardiac function after ischemia/reperfusion injury compared to the wild-type animals.[18]

Apart from their antihypertensive action, sEHIs offer protective effects from cardiovascular disease-related end organ damage.

Because inhibitors of the soluble epoxide hydrolase (sEHIs) stabilize EETs as endogenous chemical mediators, they have unique advantages. The sEHI will not create EETs where the body is not producing them. They are likely to change the regioisomeric ratios of the EETs slightly due to substrate preference and other factors, but the ratios are likely to be dominated by biosynthesis which sEHIs should not influence. The sEHIs are known to increase the absolute amounts of epoxylipids in the blood and the epoxide to diol ratios. This effect will presumably be dose dependent. However, once the sEH enzyme is largely inhibited there will not be additional increases in epoxides or decreases in diols with increasing dose of sEHI. The EET buildup will be limited by other pathways of degradation (Fig. 2). Thus, bearing off target toxicity, it is likely that sEHI will have a massive therapeutic index; it will be very difficult to overdose with the drug because there will be a maximum effect on increased EET concentration regardless of the sEHI dose.

There is of course a downside to the above mechanism of action of sEHI: one cannot stabilize EETs that are not there. In spite of the very high potency of some sEHI, one is likely to quickly reach a maximum effective dose of sEHI leading to a maximum increase in lipid epoxides. No additional clinical benefit will be gained by increasing the sEHI dose. Thus one may not see the efficacy at high doses that one sees with an ARB for example. Similarly if there are disorders associated with a decrease in EET production, the sEHI will offer limited unless EET production can be supplemented or EETs administered exogenously.

Potential Additive and Synergistic Effects

Combination therapy has become common and often unavoidable in many patients with hypertension.[3,5] Because sEHIs act via a unique target in their antihypertensive actions, it is anticipated that these actions will be additive with other antihypertensive agents currently available in the market; for example, diuretics, ACE inhibitors, renin receptor antagonists, β-blockers, and Ca^{2+} channel blockers. Inhibition of the Cox pathways by NSAIDs will be expected to shift arachidonic acid towards the P450 pathway, thereby increasing the level of EETs. Hence, the effects may be predicted to be synergistic. Finally, the increase in the endogenous level of EETs by sEHIs is expected to exert an anti-inflammatory action,[11–13] which may provide synergistic effects in combination with other cardiovascular drugs; for example, statins. A caution of course is that our understanding of cardiovascular and renal physiology is far from complete. It is critical to test the potential interactions of sEHI with other pharmaceuticals.

Polymorphisms of sEH

Previous studies have documented that sEH enzyme activity in humans shows high interindividual variation. Consistent with these findings, a variety of polymorphisms in the human sEH gene (*EPHX2*) has been found, some of which result in amino acid substitutions. Moreover, using the crystal structure of the murine sEH, predictions were made on some of the amino acid variants, which may lead to changes in enzymatic activities.[53] Indeed, more recent findings in patients enrolled in Atherosclerosis Risk in Communities (ARIC) study suggest that genetic variation in *EPHX2*, particularly the presence of the *K55R* polymorphism variant allele, may be an important risk factor for the development of congenital heart defect clinical events in whites.[54] Interestingly, this single nucleotide polymorphism (SNP) increases sEH activity

resulting in a decrease in the fatty acid epoxide to diol ratio, which one would assume would be associated with a decrease in antihypertensive and anti-inflammatory effects. Association studies in different populations will undoubtedly be required to validate these findings as well as studies to evaluate the roles of sEH, endothelial function, and cardiovascular disease risk. These SNPs as well as changes in the plasma levels of regulatory lipids may make it possible to phenotype clinical trials and aid in individualizing treatment for hypertension.

BACKGROUND ON THE SOLUBLE EPOXIDE HYDROLASE

Discovery of the Soluble Epoxide Hydrolase

Research on biological insecticides based on insect endocrinology led to two important advances relevant to the sEH as a target to treat hypertension. The first advance was the concept that one could obtain profound biological effects by blocking the degradation of a chemical mediator.[55] These effects are particularly dramatic in short-lived insects where developmental events occur over very short periods. The second key advance was the discovery of the mammalian sEH while studying the murine metabolism of insect hormones and their commercial analogs.[56,57] Initially the enzyme was thought to be a variant of the more commonly studied microsomal epoxide hydrolase, but this hypothesis was soon dispelled.[58,59] Early workers assumed that the enzyme was overlooked because it was selective for terpene epoxides, but fatty acid epoxides were soon found to be turned over very rapidly.[58,60,61] and shortly thereafter epoxides of arachidonic acid were found to be good substrates.[62,63] The enzyme was later shown to have both regio and stereoselectivity for the EET substrates.[64] Of course, in parallel to work on the enzyme and the realization that it was a key component of the arachidonic acid cascade was work demonstrating that cytochrome P450-dependent metabolites of arachidonic acid and other fatty acids were key chemical mediators.[8,9,16,22,65] Several reviews cover early work on the sEH.[66,67]

Biochemistry and Properties of the Soluble Epoxide Hydrolase

The sEH in mammals is a homodimer composed of two 60-kDa monomers. Each monomer has an N-terminal domain shown to have lipid phosphatase activity but to be of uncertain biological role[68–71] and a larger C-terminal domain, which is a classical α/β-hydrolase fold and which has epoxide hydrolase activity. The domains are joined by a proline rich bridge.[68] Such divalent proteins are quite rare in biology and often appear joined for a biological function. The domains are separate proteins, messages and genes in plants and early animals.[72,73] The liver and kidney are the richest sources of the enzyme; however, an early clue to its role in chemical mediation rather than xenobiotic metabolism was its unique distribution. The distribution is being studied more carefully with modern tools, but relative to hypertension it appears to occur commonly in the vascular endothelium and likely in the vascular smooth muscle where it often is rapidly lost in culture. The finding of the enzyme in astrocytes and other brain regions may relate to the central effects of epoxidized lipids and the reduction of ischemic damage by sEHIs following experimentally induced stroke[19] and/or the analgesic effects.[10] The fine points of the subcellular distribution of the enzyme are confusing; however, the greatest mass of the enzyme is in the cytosol of mammals, although the highest concentration sometimes appears in the peroxisomes.[74] The enzyme in mammals has two incomplete peroxisome targeting sequences. Small amounts of the enzyme are tightly associated with the endoplasmic reticulum. The enzyme is induced by PPARα ligands in rodents, but response elements for the PPAR receptor have not been found in the upstream region of the human gene. The enzyme is coded by the *EPXH2* gene and, with the exception of murine ovary to date,[75] it appears to yield a single transcript and a single protein. The enzyme has been cloned from a number of species and its evolutionary pathway traced back to prokaryotes for both the N and C-terminal domains of the enzyme. Due to space limitations the biochemistry of the N-terminal domain of sEH will not be discussed. However, the domain has phosphatase activity, and the fatty acid alcohol and diol substrates along with some terpene phosphates appear to be good substrates.[76,77]

Assays for Epoxide Hydrolase

Analytical methods for an enzyme are of course critical first to its purification, cloning and study, to determine its catalytic mechanism, and finally for high throughput screens to develop potent inhibitors. The early assays for the enzyme were laborious radiochemical assays based on separation of substrate and product by thin layer chromatography, but the observation that the massive difference in logP (polarity) between many epoxides and their diols permitted rapid partition assays that revolutionized the field. These radiochemical partition assays remain the best methods for evaluating large numbers of tissue samples since interfering activities can be eliminated by removing cofactors. Since fatty acid epoxides are thought to be endogenous substrates, they also are useful tools for assay based on gas liquid chromatography (GLC), liquid chromatography (LC), and radiochemical procedures. These partition and chromatographic methods have been reviewed.[67,78] Fortunately both *trans*- and *cis*-stilbene oxides are available radiolabeled and with care they provide diagnostic assays for the soluble and microsomal enzyme, respectively.[79] There are more sensitive radiochemical assays but so far they are not commercially available.[80,81]

Spectral assays offer numerous advantages in avoiding radioactivity and of course the ability to multiplex the assays and increase throughput. For most epoxides and diols, there are only small spectral differences between the diols and epoxides. There are exceptions[82] with a number of possible assays based on internal cyclization to yield a large change in a chromophore due to hydrogen bonding.[67,83] Many of these assays have the disadvantage of being in the ultraviolet (UV) or near UV region. An extension of this approach is to couple the opening of the epoxide to a release of a chromophore,[84] and it was this colorimetric assay that dramatically reduced the laborious point assays used for screening for inhibitors and led to the general classes of sEHIs being evaluated for treating hypertension. However, these assays have several limitations.

By 1999 in the laboratory, it had become clear that it was rather straightforward to design potent inhibitors of the sEH; the key was, of course, to develop materials with properties yielding good absorption, distribution, metabolism, and excretion (ADME).

One limitation was that the substrates were relatively unstable in buffer resulting in short assays that were somewhat difficult to automate in true high throughput mode. The other disadvantage was that the kinetic properties did not allow one to distinguish among the potencies of compounds if their IC_{50}s were in the low nanomolar to picomolar range. The discovery of a new class of chromophores which had high molar absorptivity, intense fluorescence, very large Stokes' shift, high hydrolytic stability, and synthetic simplicity presented a way around these problems.[85] These chromophores are aliphatic rather than aromatic esters, which lead to vastly improved hydrolytic stability and thus signal to noise. It was the availability of these substrates that permitted the high throughput screening for alternate inhibitory pharmacophores and the fine tuning of existing inhibitory structures, since these assays distinguish among inhibitors with picomolar IC_{50}s.[86] It must be cautioned that esterases, glutathione transferases, monooxygenases, reductases, and other enzymes can interfere with the assay. Of course once a recombinant enzyme and a potent, stable inhibitor are available, numerous competitive binding assays for screening molecules which bind to the sEH can be devised. A caution is that all surrogate substrate-based assays should be compared with the putative endogenous substrate of interest.

Catalytic Mechanism and Structure

Determining the catalytic mechanism of an enzyme gives one considerable power in designing inhibitors based on mimicking transition states or developing suicide inhibitors. Epoxide hydration is of course energetically favorable with water as the only cofactor. Probably most chemists who first hear of an epoxide hydrolase think in terms of a carbocation based mechanism, since many epoxides are labile to acid catalyzed hydrolysis. This mechanism is used in some epoxide hydrolases, but not the mammalian microsomal or sEH or any of the enzymes in the α/β-hydrolase fold family. Evidence for a general base catalyzed mechanism for the sEH was generated quite early.[87] However, key to the elucidation of details of the mechanism was the generation of an affinity purification system for the enzyme, which provided sufficient pure protein to study.[88,89] Some insight was gained from kinetic and proteomic studies, but it was the cloning of the cDNAs coding for the murine and human enzymes that had a revolutionary impact.[72,90,91] These sequences made it clear that the sEH was an α/β-hydrolase fold enzyme and thus related to the haloalkane dehalogenase from *Pseudomonas*. This dehalogenase had an X-ray structure, which in turn made a number of experiments obvious to several research groups to test a two step hypothetical mechanism for both the microsomal and the sEH.[24] The later elucidation of the X-ray structure of both the murine and human sEHs with a series of inhibitors bound in the catalytic site supported the two step mechanism and suggested that two tyrosines polarize the epoxide (Fig. 3).[69,71,92]

The overall structure of the C-terminal domain of the sEH is that of the α/β-hydrolase fold family defined as having a

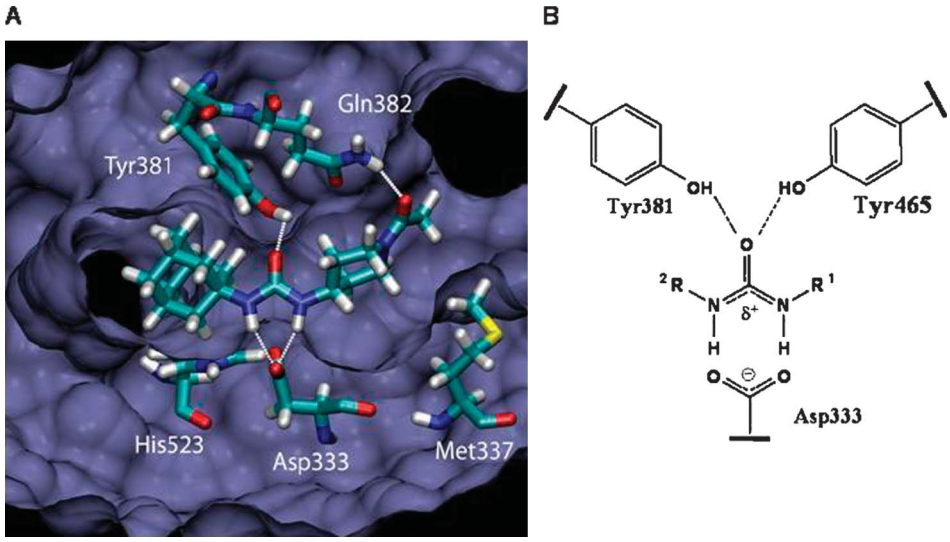

FIGURE 3. Binding of sEHI in the human sEH catalytic site. (A) The model of the catalytic site of the human sEH is based on a series of structures from the Christianson laboratory.[93] The view is from the inside of the enzyme looking out toward black solvent water on either side of the catalytic site and viewing a sEHI in a cutaway view of the catalytic tunnel. The sEHI, N-(1-acetylpiperdin-4-yl)-N′-(adamant-1-yl)urea (APAU,1153)[109] was modeled into the site by Dr. Paul Jones. This shows the interaction between the central pharmacophore of the sEHI and Tyr381/Asp333 in sEH active site. His 523 is part of a ''catalytic triad'' common to α/β-hydrolase fold enzymes which orients and activates the nucleophilic aspartic acid. Hydrogen bonds are indicated by yellow lines. Tyr465 which also polarizes the urea is removed the clarity. The first linker in the sEHI is a piperidine and the secondary pharmacophore or Y is an acetamide hydrogen bonding to Gln 382. (B) Simplified structure of sEH active site bound to a urea central pharmacophore. The sEHI mimics the transition state of epoxide hydration. This partial salt bridge is stabilized by two hydrogen bonds to Asp 333 and the carbonyl is polarized by Tyr381 and 465.

"floor" of 5–8 beta sheets connected by α-helices and existing in a globular state. The catalytic site is at a right angle of the catalytic tunnel deep within the protein. Figure 3 shows a potent inhibitor modeled into the enzyme toward the center of a 14-Å long hydrophobic tunnel. There is some question if the tunnel really exists or if the enzyme opens in a "Pac-man"-like way, and if one end of the tunnel is truly solvent accessible. However, the majority of the evidence points to a tunnel structure with two openings to solvent as shown in Figure 3. Like all α/β-hydrolase proteins, the sEH works by a two step mechanism with the first step being a nucleophilic attack by the aspartic acid 333 of the enzyme on an epoxide carbon yielding a covalent hydroxyl-alkyl enzyme intermediate.[93] In the case of the human sEH, the nucleophile is Asp^{333}, which is oriented and activated by a His^{523}-Asp^{495} pair. A nucleophilic acid may seem extraordinary; however, this acid is located in an exceptionally hydrophobic environment. Following formation of a covalent ester intermediate with the substrate, the enzyme changes confirmation, and water is then activated by the His-Glu pair. The water attacks not the substrate molecule but the enzyme aspartic ester to cleave the ester, release the substrate and regenerate the enzyme. The epoxide is polarized by Tyr^{381} and Tyr^{465}.[68,94] On either side of the catalytic site, the hydrophobic tunnel extends toward solvent. On the "right" side of the catalytic site as seen in Figure 3, the tunnel opens up as it approaches the solvent, allowing for the presence of polar groups in an inhibitor. There are several possible hydrogen bonding sites on the right side of the catalytic site roughly 8Å from the carbonyl of the central pharmacophore of the urea as illustrated by the inhibitors shown in Figures 3A, 4, and 5. This group hydrogen bonding group in the inhibitor is referred to as the secondary pharmacophore Y.

Early Work on Inhibitors

Several ions and alternate substrates were found to be moderate inhibitors of the sEH but chalcones[95] and later glycidols[96] were the first inhibitors sufficiently potent to be of any value in elucidating the biological role of the enzyme. The chalcone oxides and glycidols probably act as substrates for the sEH, which are turned over with a very low k_{CAT} analogous to the turn over of carbamate insecticides by another α/β-hydrolase fold enzyme, acetylcholinesterase.[97] These compounds have been used to evaluate the biological role of the sEH, but their poor pharmacokinetic properties, rapid reaction with glutathione, and their slow turn over by the sEH itself have limited their utility. However, they have been the basis of an affinity purification system for the enzyme that allows several hundred–fold purification to homogeneity in a single step.[88,89] Several other approaches of the inhibitor design relied on the presence of reactive groups in the active site or upon the formation of an incipient carbocation in the reaction.[98,99]

DEVELOPMENT OF SEHIS AS PHARMACEUTICALS

Transition State Inhibitors of sEH

Attempts to make alternate substrates and suicide substrates for the sEH led to useful laboratory reagents, but they are probes of only marginal utility in vivo. However, determination of the catalytic mechanism of the enzyme facilitated the design of transition state mimic enzyme inhibitors.[24] Linus Pauling advanced the concept that one way catalysts work is by stabilizing transition states along the reaction coordinated by binding far more tightly to the transition state than either the substrate or the product. He further reasoned that the rate acceleration of enzymatic reactions suggests that this binding may be very tight not only for a transition state but for even a rather crude mimic of a transition state or transient intermediate.[100] Based on this idea, ureas, amides and carbamates were found to be powerful, competitive, tight binding inhibitors of the she (Figs. 3A, 3B, and 4A).[101] Interestingly, dicyclohexylurea (DCU, a common synthetic byproduct, Fig. 4A) is in fact a very potent inhibitor of the enzyme. Multiple X-ray structures and several lines of biochemical evidence support a transition state like inhibition possibly with a partial salt bridge stabilized by hydrogen bonds approximating one of several binding forms (Fig. 3B). Note that the X-ray structure shows the nucleophilic aspartic acid oriented 90 degrees from the plane of the urea in Figure 3A rather than the coplanar structure depicted in Figure 3B. The potency of some amide inhibitors suggests that a single NH alpha to a carbonyl is sufficient for activity.[24] The general structure shows that one needs a carbonyl with at least one H bonding group alpha to it. Thus one needs a NH at position A or B (amides and carbamates) or in ureas, both positions. For significant activity on the enzyme one needs a lipophilic group on either side of the central A-C(O)-B pharmacophore. In Figure 3A, an adamantine on the left side is seen to fill the hydrophobic pocket on the left side of the catalytic site.

The key, of course, to optimize these structures were high throughput assays based on the recombinant and affinity purified murine and human sEH enzymes and a rapid colorimetric assay. These tools led quickly to development of structure-activity relationships resulting in inhibitors with IC_{50}s in the low nanomolar region[102,103] and differential inhibition of the microsomal and soluble EHs (Fig. 4A).[104] Figure 4A illustrates an improvement in potency of the inhibitors on the human sEH enzyme using classical SAR aided by computer docking into models. As expected of high melting lipophilic amides and ureas, these early compounds had poor physical properties. A compound like DCU of course had little if any *in vivo* biological activity unless it was administered in true solution. However, as discussed above, with careful formulation these high melting lipophilic solids could be used to demonstrate that inhibition of the sEH could reduce blood pressure in spontaneously hypertensive rats.[32]

Improving Physical Properties

Lipinski's rules and similar approaches to medicinal chemistry suggest that most small molecule drugs have a fairly narrow range of physical properties.[105] Certainly a molecule that will be sufficiently simple for routine biological use to test hypotheses of the endogenous role of the sEH required a molecule that was more water soluble, lower melting, and reasonably soluble in the organic cosolvents used to deliver molecules to cell culture and animal systems. Thus, a number of physical parameters were followed to drive synthesis of

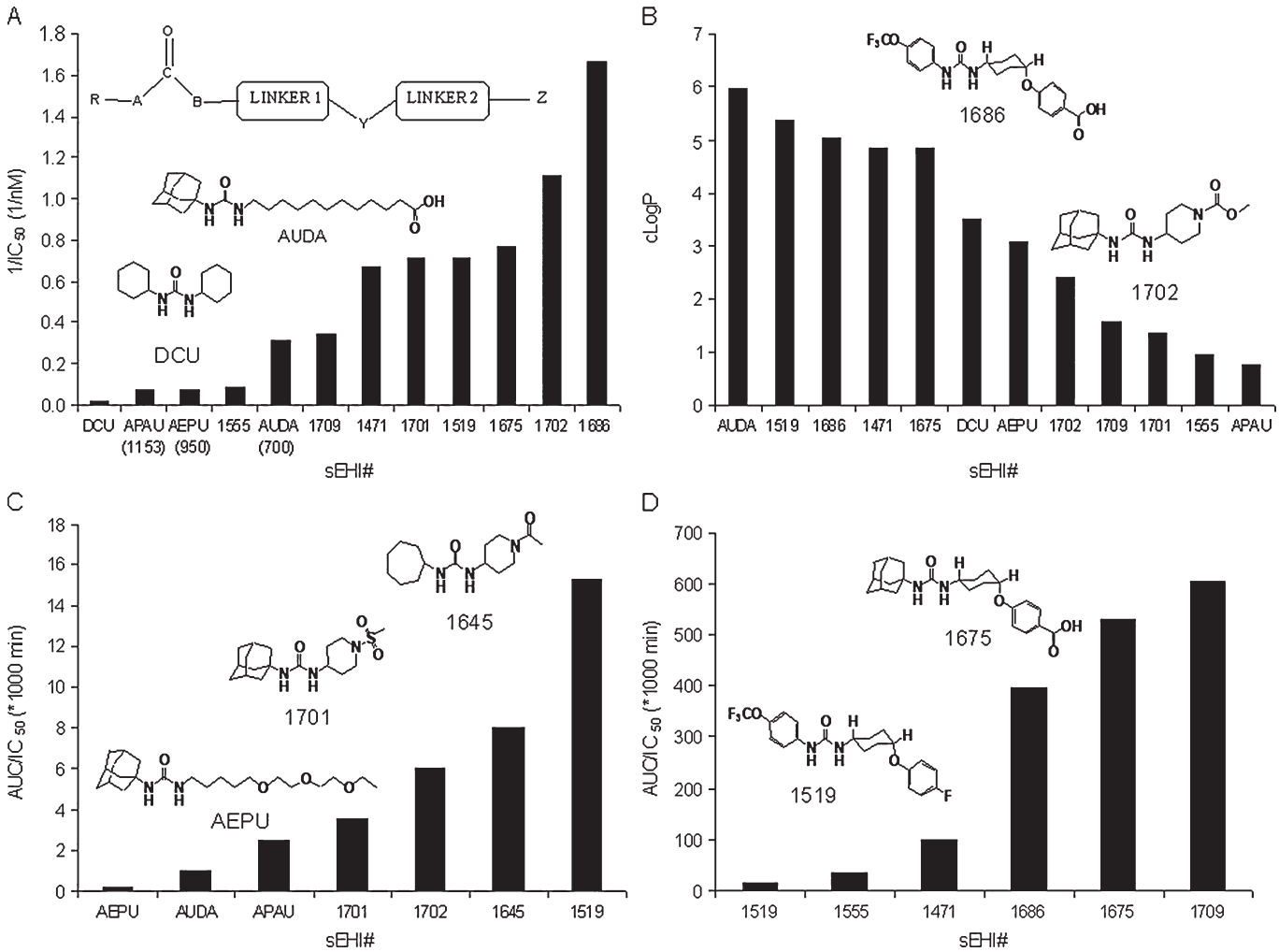

FIGURE 4. Structural improvements of urea sEHI. (A) Classical SAR approaches led to dramatic increases in potency of sEHI from the DCU initial lead as measured by IC_{50} on the human sEH. IC_{50}s as low as 200 pmolar have been achieved. The general structure shows a central pharmacophore A–C(=O)–B, a left R group (adamantane or trifluoromethoxyphenyl here), a first linker, a polar secondary pharmacophore Y, sometimes a second linker, and a terminal or tertiary pharmacophore Z. (B) The clogP demonstrates that the developed sEHI are less lipophilic and are more water soluble. There are a number of current, highly potent inhibitors which fall within Lipinski's rules for drug design. (C and D) Some potent inhibitors of the human sEH have good ADME (note different scales) in a canine model. The bars indicate a function of exposure or area under the blood curve and potency on the human sEH with compounds like 1709 having both a large area under the curve and very low IC_{50}.

potent compounds with drug like physical properties. This approach is illustrated for clogP in Figure 4B. Highly lipophilic compounds (high logP) are associated with a lack of selectivity for a single target and problems with pharmacokinetics and formulations. Several structural modifications led to a compound which has been commonly used by biologists: AUDA (700) (Fig. 4A-C). Surprisingly, it was found that adamantyl ureas were lower melting and more soluble than cyclohexyl ureas. Also a terminal carboxylic acid group in the Z position was used as a mimic of the hypothetical endogenous fatty acid epoxide substrates.[106] AUDA if solubilized carefully in 3-hydroxypropyl-β-cyclodextran can reach effective doses if given in drinking water; its butyl and other esters are effective following administration in the food or by subcutaneous or intraperitoneal injection. AUDA and its esters and salts have been widely used to evaluate the biological role of the enzyme. However, its rapid metabolism, moderate oral availability, and poor physical properties require careful use. A compound like AUDA that is both high melting and has low water solubility is very difficult to formulate. This coupled with rapid beta oxidation limited its utility. However, even a small increase in polarity, a lower melting point, or a 10-fold increase in potency would make it a very valuable compound. AUDA is a highly potent material that may find unique therapeutic nitches.

AUDA was followed by several compounds which incorporated groups anticipated to bind to hydrogen bonding sites within the catalytic site. One of these compounds, termed AEPU (950), (Fig. 4C for structure) was dramatically more water soluble, had good oral bioavailability in some species,

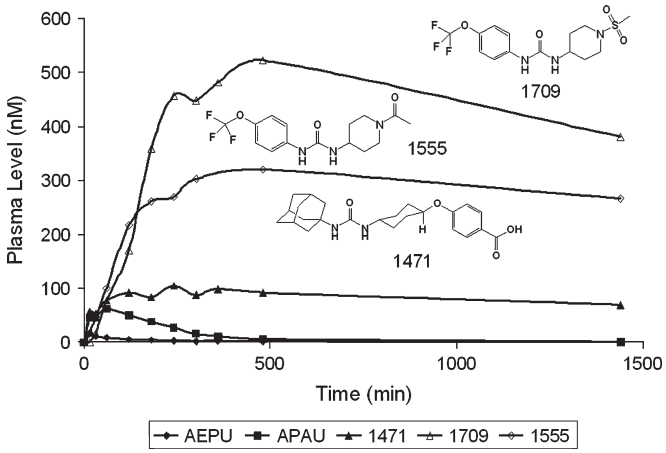

FIGURE 5. Typical blood profiles of sEHI in a canine model following oral administration at 0.3 mg/kg body weight. AEPU (950) and AUDA (700) have been used successfully following oral administration in a variety of biological models of hypertension and comorbidities. However, the plasma levels and half-lives are very short compared to materials such as APAU (1153), 1471, 1555, and 1709. In general, there is a good correlation between dose and blood level for the compounds tested. Unpublished compounds were synthesized by Dr. Sung Hee Huang and Dr. Paul Jones.

and had a lower melting point facilitating formulation.[107] This and other studies demonstrated the concept that one could place a polar group approximately 8Å from the central carbonyl in a position to bind to the limited polar residues lining an otherwise hydrophobic catalytic tunnel of sEH (position Y, Fig. 3A). Many structures with improved physical properties have resulted from placing hydroxyl, carbonyl, sulfonyl, ether, ester, carbamate, and other functionalities at the so-called secondary pharmacophore indicated in the generalized structure in Figure 4A as Y. Esters at the secondary pharmacophore represent soft drug approaches in that they are very active but quickly metabolized into biologically inactive materials by esterases.

One compound in this series, AEPU, has been used in a number of studies and offers the advantage of probably not being an effective EET mimic and not being a PPAR ligand like AUDA. Although very potent in vivo, its rapid metabolism leads to low blood concentrations of AEPU as shown by the canine ADME in Figure 5. However, it passes freely through cell membranes and shows efficacy in vivo that are better than those predicted from blood levels. It is easy to formulate and it gives similar blood levels whether administered orally in triglyceride, water, or as a dry powder. It is a unique compound where its in vivo efficacy is far greater than predicted from its concentration in the blood. Like AUDA the unique properties of the polyethylene glycol tail may provide specialty applications, potentially as a soft drug.

Thus the general structures of sEHIs include a urea, carbamate, or amide central pharmacophore at A–C(=O)–B with relatively hydrophobic R, linker 1, and linker 2 groups. Either A or B needs to have a hydrogen bond donor in either a linear or cyclic structure. One can have a polar residue such as an ester, sulfonamide, ether, or carbamate at Y and similarly a polar group at Z as in AUDA. Usually the inclusion of polar residues in a potent scaffold like DCU do not dramatically increase potency, but they dramatically alter physical and pharmacokinetic properties and dramatically increase selectivity for the target if these polar groups, such as Y and Z, are in the proper position to hydrogen bond in the catalytic pocket.

By 1999 in the laboratory, it had become clear that it was rather straightforward to design potent inhibitors of the sEH; the key was, of course, to develop materials with properties yielding good absorption, distribution, metabolism, and excretion (ADME). This resulted in the synthesis of compounds driven by in vitro stability in microsomes and in vivo pharmacokinetics (Fig. 5). This work was made possible in an academic setting by advances in mass spectrometry allowing the rapid determination of sEHIs into the picomolar range with little work up in only a few microliters of blood. Subsequently our SAR work has been driven largely by an understanding of physical properties of the inhibitors and by very rapid feed back of ADME information (Fig. 4C and D). This approach has resulted in several series of compounds that are both potent inhibitors of the human and other sEHs and give good ADME, as indicated by Figure 4 C and D (note different scales), using IC_{50} and area under the curve (AUC) following oral administration of 0.3 mg/kg to dogs as an indication of potency and exposure, respectively. For example compounds 1686, 1675, and 1709 show both very high potency on the human sEH and good oral ADME in canines. In 1686, R is a trifluoromethoxyphenyl that fits tightly in the left hand hydrophobic pocket. The first linker is a cyclohexyl, Y, or the secondary pharmacophore is an ether, the second linker is a phenyl, and Z is a carboxylic acid. In APAU (1153), shown in Figure 3 as well as 1701 and 1702 (AUPC), R is adamantine, there is a urea central pharmacophore, a piperidine first linker, and the secondary pharmacophore is an acetamide, sulfonamide, and carbamate, respectively. Typical blood profiles are shown in Figure 5 for several compounds. Many of the resulting compounds will hopefully be leads for the pharmaceutical industry and at least offer biologists sEHIs that are far easier to use in a variety of animal models. Rapid solid phase and solution phase synthesis methods have been developed which should facilitate fine tuning of the structures by industry,[108] and high throughput assays should facilitate finding alternate pharmacophores for the target. Figure 4 illustrates that there are highly potent compounds available with a variety of physical properties that demonstrate a great range in their speed of clearance and in their oral availability. Compound 1519 (TCUB) for example, is quite lipophilic and suitable for subcutaneous administration, whereas compounds like 1555 (TPAU), 1153 (APAU), 1675 (ACUB) and 1709 (TUPS) are suitable for oral and other routes of administration.

Thus, almost 40 years of fundamental research has yielded very potent tools to investigate the roles of the sEH in eicosanoid metabolism. The sEHIs along with agonists and antagonists for the natural ligands and P450 inhibitors allow one to dissect the biology of the P450 branch of the arachidonate cascade. Possibly, the lead structures and tools developed here will also be valuable leads for the pharmaceutical industry in developing human and veterinary

therapeutics because these structures are particularly attractive in their high selectivity and apparent safety profile as well as the picomolar potency and good ADME of some structures.

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