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Drugs in the Pipeline Series:

Orally Active Epoxyeicosatrienoic Acid Analogs

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Abstract

Biologically active epoxyeicosatrienoic acid regioisomers (EETs) are synthesized from arachidonic acid by cytochrome P450 epoxygenases of endothelial, myocardial and renal tubular cells. EETs relax vascular smooth muscle and decrease inflammatory cell adhesion and cytokine release. Renal EETs promote sodium excretion and vasodilation to decrease hypertension. Cardiac EETs reduce infarct size following ischemia-reperfusion injury and decrease fibrosis and inflammation in heart failure. In diabetes, EETs improve insulin sensitivity, increase glucose tolerance and reduce the renal injury. These actions of EETs emphasize their therapeutic potential. To minimize metabolic inactivation, 14,15-EET agonist analogs with stable epoxide bioisosteres and carboxyl surrogates were developed. In pre-clinical rat models, a subset of agonist analogs, termed EET-A, EET-B and EET-C22, are orally active with good pharmacokinetic properties. These orally active EET agonists lower blood pressure and reduce cardiac and renal injury in spontaneous and angiotensin hypertension. Other beneficial cardiovascular actions include improved endothelial function and cardiac anti-remodeling actions. In rats, EET analogs effectively combat acute and chronic kidney disease including drug- and radiation-induced kidney damage, hypertension and cardiorenal syndrome kidney damage, and metabolic syndrome and diabetes nephropathy. The compelling pre-clinical efficacy supports the prospect of advancing EET analogs to human clinical trials for kidney and cardiovascular diseases.

Keywords

epoxyeicosatrienoic acid; hypertension; diabetes; epoxygenase; inflammation; heart failure; kidney injury

Introduction

Epoxyeicosatrienoic acids (EETs) are cytochrome P450 (CYP) metabolites of arachidonic acid (1–3). Four regioisomeric EETs are biosynthesized. They differ in the placement of the

epoxide at the *cis*-double bonds of arachidonic acid resulting in 14,15-, 11,12-, 8,9- and 5,6-EET. A limited number of isozymes of the CYP gene family catalyze EET biosynthesis, and these isozymes vary in their metabolic repertoire, i.e., relative abundance and stereochemistry, of the EET regioisomers produced (4–7). Additionally, CYP isozyme expression varies with gender, cell type, metabolic state, starvation, exposure to xenobiotics and other factors. The biological significance of these differences in EET synthesis is not completely understood. As with other eicosanoids, activation of phospholipases and the release of arachidonic acid from membrane phospholipids is the step regulating cellular EET synthesis and release (8)(Figure 1). Cell injury, shear stress and hormones such as bradykinin, acetylcholine, angiotensin II and adenosine are among the phospholipase activators that initiate EET synthesis (9–14). CYP epoxygenases are expressed and EETs are synthesized in cells and tissues of cardiovascular importance. Some of these cells include endothelial cells of arteries and veins, cardiac myocytes, renal tubular epithelial cells, macrophages and adrenal zona glomerulosa cells (15–19).

EETs function as local, autocrine or paracrine mediators (13, 20). They are synthesized by one cell type and exert their biological activity on the same or a different adjacent cell type. For example, in arteries, endothelial cells synthesize EETs that may stimulate endothelial cell growth and migration or relax adjacent smooth muscle cells (9, 21, 22). In some cases, EET regioisomers and stereoisomers have differing biological activities (21–24). For many activities, only a single racemic EET regioisomer has been tested. Thus, the pharmacology of the EETs is incomplete.

Several pathways terminate the biological activity of the EETs (8) (Figure 1). The EET regioisomers and stereoisomers vary in their inactivation by these pathways (25–28). EETs are fatty acids with a free carboxyl group at carbon-1 so are substrates for β -oxidation and esterification. β -Oxidation results in shortening of the 20 carbon backbone to 18-, 16 or 14-carbon metabolites and loss of biological activity (8, 23, 25). This process is relatively slow requiring hours for complete metabolism in cultured cells. EETs are rapidly esterified into cellular phospholipids (8, 25, 26). Free EETs are biologically active, so esterification diminishes the concentration of free EETs and terminates activity. Esterified EETs may make subtle changes in the lipid environment of cell membranes and affect cell function. Activation of phospholipases will release the esterified EETs and restore the active form. Soluble epoxide hydrolase (sEH) hydrates the epoxide group of the EETs to produce the corresponding vicinal diols or dihydroxyeicosatrienoic acids (DHETs) (27, 29–31). This metabolic pathway is also rapid. In most instances, the *cis*-epoxide group is required for full biological activity, and the DHETs are inactive or less active. There are activities described for the EETs where the corresponding DHETs have not been tested so caution should be used in generalizing about EETs being active and DHETs not. ω -Oxidation of the EETs and EET conjugation to glutathione are documented but have not been extensively studied (32, 33).

Summary of Cardiovascular Actions of the EETs

The cardiovascular actions of the EETs are briefly summarized. However, more detailed reviews are available (8, 20, 31, 34–37).

Regulation of vascular tone

EETs function as endothelium-derived hyperpolarizing factors (EDHFs) in arteries of humans and experimental animals including cows and pigs (9, 13, 18, 38). EETs are synthesized by endothelial cells, but not smooth muscle cells, and are released in response to bradykinin, acetylcholine and shear stress (9, 10, 13, 18, 39). Endothelial cells contain CYP 2C8, 2C9 and 2J2 and release predominately 14,15- and 11,12-EET. These EETs are transferred to the smooth muscle where they activate the large conductance, calcium-activated potassium (BK_{Ca}) channel through a guanine nucleotide binding (G) protein-mediated mechanism (14, 40, 41). This increases K efflux and hyperpolarizes the smooth muscle cell membrane resulting in relaxation. The four EET regioisomers are equally active in vasorelaxation (9). The relaxations to bradykinin and acetylcholine are endothelium-dependent whereas relaxations to the EETs are endothelium-independent (42, 43). These relaxations are inhibited by the BK_{Ca} blockers iberiotoxin and charybdotoxin, the CYP inhibitors miconazole, MS-PPOH and CYP2C antisense oligonucleotides and the EET antagonist, 14,15-epoxyeicosa-5Z-enoic acid (14,15-EE5ZE) (9, 18, 38, 43, 44). In normal and hypertensive subjects treated with aspirin and N^G -monomethyl-L-arginine to block vascular prostaglandin and nitric oxide synthesis, bradykinin increases forearm blood flow that is inhibited by the CYP inhibitor sulfaphenazole (45). Other studies document the dilator role of EETs in humans and human arteries (38, 43, 46).

Renal blood flow and sodium excretion

In rats, 11,12-EET dilates renal arteries whereas 14,15-EET is less active (47). CYP is expressed in the thick ascending limb and collecting duct and converts arachidonic acid to EETs (48–51). Endogenous and exogenous EETs inhibit the epithelial sodium channel (ENaC) resulting in sodium excretion (48, 50, 52, 53). Deletion of CYP2c44 in mice increases ENaC activity, sodium retention and blood pressure (50, 52, 54). The blood pressure is lowered by the ENaC inhibitor amiloride. Thus, renal tubular EETs may also regulate blood pressure.

Hypertension

Since EETs cause vasodilation and increase renal sodium excretion, an anti-hypertension action is predictable. Inhibition of sEH lowers blood pressure in spontaneously hypertensive (SH) rats as well as rats with angiotensin II- and deoxycorticosterone acetate/salt-hypertension (55–59). Similarly, overexpression of CYPs reduces blood pressure in SH rats and hypertensive mice (56, 60).

Cardioprotection

EETs reduce infarct size and improve functional recovery of the myocardium after ischemia in mice, rats, rabbits and dogs (61–65). In perfused mouse hearts, global ischemia followed by reperfusion produces contractile dysfunction (62, 63). Increasing endogenous EETs by increased CYP expression or reducing the activity of sEH diminishes the contractile dysfunction. This is blocked by CYP inhibition or the EET antagonist 14,15-EE5ZE. Without treatment, thirty minutes of ischemia and 2 hours of reperfusion produce a 55% infarct (65). Both 11,12- and 14,15-EET are equipotent in reducing the size of the infarct to

31% in anesthetized rats. Similar results were obtained in anesthetized dogs (64). Importantly, exogenous EETs are effective when given before ischemia or, most notably, at reperfusion. Inhibition of ATP-sensitive K (K_{ATP}) channels with glibenclamide blocks the beneficial effects of EETs in mice, rats and dogs (63–65). Other studies implicate the glycogen synthase kinase-3 β , phosphatidylinositol-3 (PI-3)-kinase, mitogen-activated protein (MAP) kinase and reactive oxygen species in the cardioprotection afforded by EETs (61, 63, 65).

Heart failure

sEH was identified as a heart failure susceptibility gene in SH heart failure rats (66). In mice, inhibition of sEH increases endogenous EETs, improves pressure overload heart failure and reduces the incidence of arrhythmias (67). Similarly, cardiac overexpression of CYP2J2 reduces mortality and arrhythmias in transverse aortic constriction and isoproterenol infusion models of heart failure compared to wild type mice (68). Angiotensin-induced cardiac fibrosis, inflammation and hypertrophy are also reduced in CYP2J2 mice (69, 70). Thus, endogenous EETs are beneficial in models of both systolic and diastolic heart failure.

Platelet adhesion and fibrinolysis

EETs inhibit expression of the adhesion molecule P-selectin on human platelets under basal conditions and with ADP-stimulation (71). Platelet adhesion to human endothelial cells is decreased. This is mediated by K_{Ca} channel activation and hyperpolarization of the platelet membrane. 11,12-EET is more active than 8,9- or 14,15-EET. The concentrations required for activity are μ M. In human and bovine endothelial cells, the four EET regioisomers in nM concentrations increase the expression and release of tissue plasminogen activator (tPA); however, 11,12-EET is the most active (72). EET release of tPA requires a guanine nucleotide binding protein, G α s, and an increase in cyclic AMP. Thus, EETs inhibit platelet adhesion and stimulate thrombolysis.

Inhibition of inflammation

EETs decrease inflammation by a variety of mechanisms (73, 74). Tumor necrosis factor- α (TNF α) increases the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin in human and bovine endothelial cells (74, 75). Interleukin-1 α and lipopolysaccharide act similarly. 11,12-EET inhibits the expression of these adhesion molecules by all three inflammatory stimuli in nM concentrations. 8,9-EET and 5,6-EET are less active than 11,12-EET. 14,15-EET is without activity. The structural features of 11,12-EET required for this activity were defined using a series of analogs (24). TNF α also increases the adhesion of mononuclear cells to the endothelium in mice *in vivo* (75). It is also blocked by 11,12-EET, but not 14,15-EET. In endothelial cells, TNF α increases the nuclear accumulation of NF κ B by increasing the phosphorylation I κ B by I κ B kinase (IKK) and its degradation. 11,12-EET inhibits IKK preventing NF κ B translocation to the nucleus and transcription of adhesion molecules. Participation of peroxisome proliferator-activated receptor- γ (PPAR γ) in EET inhibition of NF κ B was suggested; however, several discrepancies question this mechanism (76). EETs bind and activate PPAR γ in μ M concentrations but inhibit NF κ B in nM concentrations (75,

76). Also, 14,15-EET activates PPAR γ but does not inhibit NF κ B in endothelial cells. NF κ B regulates numerous pro-inflammatory pathways including adhesion molecule expression, cytokine release and cyclooxygenase expression (77). Thus, it represents a key mechanism in the anti-inflammatory action of the EETs. Increasing endogenous EET production by increased expression of CYPs or inhibition of sEH increases I κ B accumulation and inhibits the cytokine release and accumulation of inflammatory cells associated with renal, lung, hepatic and cardiac injury (57, 70, 78, 79).

Nuclear erythroid-related factor 2 (Nrf2) contributes to the resolution of inflammation by increasing the expression of antioxidant proteins and enzymes (80). 14,15-EET activates Nrf2 accumulation and nuclear translocation in lung epithelial cells (81). Also, 11,12-EET, 14,15-EET and CYP2C8 overexpression increases Nrf2 expression and reduces TNF α -induced endothelial cell reactive oxygen species production and apoptosis (82). These effects are blocked by transfection with a Nrf2 small interfering RNA. While less studied than NF κ B, Nrf2 may represent an important pathway for the anti-inflammatory actions of EETs.

Angiogenesis and wound healing

Endogenous and exogenous EETs promote angiogenesis, thus increasing endothelial cells growth, migration and tube formation (21, 22, 83–85). These *in vitro* activities require μ M concentrations of the EETs. The regioisomer(s) causing these activities vary with the vascular source and species of endothelial cells. 11,12-EET is the most extensively studied. Angiogenesis is an important mechanism contributing to EET accelerating wound healing and organ regeneration (86, 87).

Diabetes

Endogenous EETs contribute to glucose tolerance, insulin sensitivity and glucose uptake as well as reduce the kidney and liver damage and improve the wound healing in diabetes. Increasing endogenous EETs by increasing CYP or decreasing sEH reduces blood pressure, reverses insulin resistance and increases glucose tolerance in fructose-treated rats and db/db mice (88–90). Renal injury and inflammation are reduced in streptozotocin-induced diabetic mice and FVB mice on a high fat diet (91, 92). In contrast, glucose tolerance, insulin sensitivity and glucose uptake are decreased in CYP2c44 deleted mice compared to wild type mice (93). Plasma EET concentrations positively correlates with insulin sensitivity in human subjects (93). 11,12-EET also improves wound healing in ob/ob mice by decreasing accumulation of inflammatory cells, reducing cytokine production and increasing angiogenesis (87). Similarly, increases in CYP expression in db/db mice decrease hepatic inflammation by the same mechanisms (89).

EET Receptors and Signaling Mechanisms

As indicated above, a number of cell signaling pathways are implicated in the actions of the EETs (8, 37). For example, K $_{Ca}$ channel activation is involved in relaxation of vascular smooth muscle and inhibition of platelet adhesion to the endothelium (41, 71). EETs activate BK $_{Ca}$ channels and adenylyl cyclase/cyclic AMP accumulation through G α s (21, 41, 72).

Endothelial tPA release, Trp channels and K_{Ca} channel activation as well as inhibition of smooth muscle cell migration use cyclic AMP as a second messenger (21, 72, 94, 95). $NF\kappa B$ inhibition, possibly Nrf2 activation, mediates the anti-inflammatory activity of the EETs (75, 81, 82). EETs also increase the ERK1/2 and Akt kinase pathways to promote cell growth (83). Other signaling pathways have been described (37, 61, 63, 65).

Characterization of EET receptors has lagged behind the discovery of signaling mechanisms. Clearly, defining the EET receptors is important to the therapeutic development of EET agonist analogs. A high affinity binding site for 3H -14,15-EET was described in mononuclear cells and membranes (96–98). The binding is specific, saturable and reversible. We designed 20- ^{125}I -14,15-EE8ZE and –EE5ZE as chemically stable, high specific activity agonist and antagonist radioligands, respectively (99–101). In cell membranes, specific, saturable, reversible and high affinity ($K_d=8.5$ nM) binding occurs with 20- ^{125}I -14,15-EE8ZE (101). 14,15- and 11,12-EET and 14,15-EE5ZE displaced the radioligand ($IC_{50}=12$ –40 nM) whereas 14,15-DHET and other inactive 14,15-EET analogs do not. Similar results were obtained for antagonist binding with 20- ^{125}I -14,15-EE5ZE ($K_d=1.1$ nM) (99).

The role of G-proteins in EET action has been investigated. The specific binding of 20- ^{125}I -14,15-EE8ZE is inhibited by GTP γ S implicating G protein coupling to the binding (101). Using cell-attached patch clamp measurements of BK_{Ca} channels in smooth muscle cells, 14,15- and 11,12-EET increase K channel activity at 1–100 nM concentrations (9, 41). In contrast, the EETs are without activity in inside-out pulled off patches; however, GTP, but not ATP, restores EET-induced K channel activity. The EET activity was inhibited by an anti-G α_s , but not anti-G α_i or anti-G $\beta\gamma$, specific antibodies. Similarly, in endothelial cells, an anti-G α_s antibody and small interfering RNAs inhibit EET activities (21, 72). Others have also confirmed that some EET actions are GTP-dependent and G α_s mediated (102, 103). These studies indicate that a high affinity 14,15-EET receptor exists. It is a GPCR coupled to G α_s in smooth muscle and endothelial cells. The identity of this 14,15-EET receptor is not known. There are no studies documenting high affinity EET receptors for 11,12- or 8,9-EET. However, PPAR γ , GPR40, E prostanoid type 2 (EP2) and thromboxane prostanoid (TP) receptors have been described as possible receptors for EETs (76, 104–107). Their order of EET potency and requirement for μ M EET concentrations for activation exclude them as the high affinity EET receptors.

Initially, differences in the rank order of potency of prostaglandins provided compelling evidence for multiple prostaglandin receptors (108). This may be the case for EETs as well, but the data comparing EET regioisomers are incomplete. For some biological activities, one regioisomer is more active than others; however, the regioisomers are equally active in other cases (9, 21, 22, 72, 95). This suggests that multiple EET receptors exist. Additionally, regioisomer-specific EET antagonists have been developed and implicate multiple EET receptors (44, 109–111). In coronary arteries, 14,15-EE5ZE inhibits the relaxations to all four EET regioisomers. In contrast, 14,15-dihydroxy-E5ZE inhibits relaxations to 14,15-EET without affecting 11,12- or 8,9-EET relaxations (109). 11,12,20-Trihydroxy-E8ZE inhibits relaxations to 11,12-EET, but not 14,15- or 8,9-EET (110). These data suggest there are separate EET receptors for 14,15-, 11,12- and 8,9-EET and indicate the need to investigate and characterize possible 11,12- and 8,9-EET receptors.

Therapeutic Considerations

The EET pathway may be enhanced by direct and indirect strategies that include increasing EET synthesis by induction of cellular CYP epoxygenase(s), decreasing EET degradation to DHETs by sEH inhibition and EET agonists. Each of these strategies has been used (31, 55, 112, 113). Increasing epoxygenases and inhibition of sEH increase the accumulation of all of the EETs. EET regioisomers differ in their actions so untoward or off-target effects may accompany the therapeutic benefit. Furthermore, inhibitors of sEH depend on EET biosynthesis to increase endogenous levels of EETs. Endogenous EET synthesis may vary with disease or concomitant drug ingestion and blunt or enhance the therapeutic effect of the sEH inhibitor. Orally active, metabolically stable EET agonist analogs represent an analog of a single regioisomer and are therefore expected to be more selective in their action. Also, the dose of the agonist analog is directly related to the therapeutic effect.

EET Agonist Analogs and Structure Activity Relationships (SARs)

We initiated a long-term, inter-laboratory program of EET analog development. Our goals included: (i) elucidation of SARs, (ii) improve potency, (iii) obviate autooxidation, (iv) extend *in vivo* half-life, (v) increase water solubility, and (vi) streamline chemical syntheses/reduce production costs. The earliest effort to develop more robust analogs involved simple modification of the carboxylic acid of 14,15-EET (**1**) (114). (The numbers in **bold** refer to the specific structures provided in the text.) The *N*-methylsulfonylamides of 14,15-EET (**2**) and 11,12-EET (**3**) were introduced to reduce β -oxidation and esterification of the parent EETs during *in vitro* studies of mitogenesis and tyrosine phosphorylation stimulation in renal epithelial cells (115). Analog **3** was subsequently utilized by Imig et al to demonstrate the activation of protein kinase A during afferent arteriolar vasodilation and again to evaluate renal microvascular reactivity during angiotensin II-induced hypertension (115, 116). However, **2** and **3** are rapidly hydrolyzed in plasma and other biological milieu and should be considered as primarily pro-drugs when used for *in vivo* applications.

Following the proposal by Campbell *et al.* that EETs were EDHFs in some species, the first generation of structural analogs was evaluated using a bovine coronary artery isometric tension assay and the results were compared with equimolar **1** (9, 23). These studies identified the carboxylic acid (cf., analog **4**) and an intact epoxide (cf., analog **5**) as essential to vascular relaxation. Systematic removal of the olefins beginning one at a time, then two at a time led to analog **6** which retained full vasorelaxation activity with respect to **1** whereas the ^{5,6}-regioisomer **7** and fully saturated analog **8** proved to be antagonists. Analog **6**, with its simplified structure and stability towards autooxidation, was selected as the lead scaffold for the next generation of analogs. Analog **7**, due to its greater potency as a competitive antagonist compared with **8**, has found wide utility as a pharmacological tool (44).

The impact of 14,15-EET stereochemistry upon vasodilation was also tested (23, 117). The 14(*S*),15(*R*)-enantiomer **9** was more potent than its antipode **10** which in turn was stronger than *trans*-14,15-EET **11**. Other studies have also reported enantioselective-dependent EET responses and metabolism (27, 97).

Structural Requirements for 14,15-EET-induced Vasorelaxation

- ◆ carboxylic acid or acidic proton at C(1)
- ◆ *cis*-8,9-olefin
- ◆ *cis*-epoxide
- ◆ 20-carbon chain

A parallel study of 11,12-EET looking at the structural determinants for inhibition of TNF- α -induced VCAM-1 expression allowed us to propose a recognition or binding domain map (Figure 2) (24). Again, a *cis*-8,9-olefin was critical for maximum activity. An ionizable (acidic) group at C(1) that participates in ionic bonding and hydrophobic regions flanking the epoxide/olefinic moieties were likewise important. Introduction of heteroatoms or bulky functional groups in these regions as well as at the ω -end proved detrimental.

Substitution of the epoxide with an acyclic ether led to a series of analogs, e.g., **12** (NUDSA) that essentially maintained the atom sequence without the rigidity and susceptibility of the three-membered epoxide to sEH cleavage (118). This was counterbalanced by a partial loss of potency compared with 11,12-EET.

The second generation of analogs focused primarily on the search for more efficacious, sEH-resistant epoxide bioisosteres or surrogates (119). Candidates were evaluated for both vascular relaxation and inhibition of sEH (Table 1). The simple 1,3-disubstituted urea **13** was about equal to the acyclic ether **12**; however, truncated versions such as **14** performed poorly so this approach was terminated. Mono-*N*-alkylation of the urea gave mixed results depending upon the location of introduction (15 vs 16); vasorelaxation of the di-*N*-alkylation version **17** seemed to share the negative and positive contributions in equal proportions. Notably, as the level of substitution increased (13 vs 17), the ability to inhibit sEH declined progressively and was essentially independent of the vasorelaxant effects. Regrettably, the impressive activity of thiourea analog **18** was dampened by its rapid metabolism in

microsomal fractions. Since sulfur is considered a weaker hydrogen bond acceptor than oxygen, the improvement in ED₅₀ for **18** compared with **13** would be consistent with coordination to a metal center in the epoxide binding region. Various urethanes were prepared as represented by **19**, but none rose above the activity of urea **13**. A series of amides were instructive: progressing from secondary amide **20** to the tertiary amides **21** or **22** saw a loss of more than two orders of magnitude in sEH inhibition. Importantly, increasing the steric size of the nitrogen substituent from *N*-methyl **21** to *N*-isopropyl **22** significantly boosted the vasorelaxation efficacy. On the other hand, reversing the placement order of the amide, **20** vs **23**, almost abolished the analogs ability to relax the vessels (121). Oxamide **24** stands out as a good choice for a reagent that displays EET-like EDHF effects, yet has little sEH inhibitory activity. Unlike urea **17**, *N,N'*-dimethyloxamide **25** was not as effective in either assay as its unsubstituted parent. A variety of oxygen-containing heterocycles, e.g., **26**, were also evaluated, but with no promising results. The reader will appreciate that all of the epoxide bioisosteres in Table 1 are achiral, i.e., do not contain an asymmetric center. This architectural simplification, we anticipate, will lead to agonists capable of binding universally to receptors or recognition sites irrespective of the sites' enantiomeric preferences.

The remaining major modification addressed replacement of the carboxylic acid and drew extensively from the large pool of established carboxylic acid bioisosteres (120). Collectively, they comprise the third generation of 14,15-EET analogs and were mainly targeted at minimizing or preventing β -oxidation and esterification while simultaneously promoting better water solubility, improving oral bioavailability, and extending *in vivo* half-life (121). Some typical examples are shown in Table 2. Small molecular weight amides such as aspartate **27** were easily prepared from the parent carboxylic acid and reasonably functional while conversely phosphonate **28** was less effective despite its inherent greater acidity versus a carboxylic acid. Many small heterocyclic bioisosteres satisfied the principle goals of improved potency, water solubility, and oral bioavailability, e.g., 5-sulfonyl-1*H*, 1,2,3-triazole **29**, tetrazole **30**, and oxathiadiazole-2-oxides **32** and **33**. The latter two had virtually identical vasorelaxation effects, but starkly different behavior on sEH since the former contained a urea and the latter an *N*-isopropyl amide as their respective epoxide surrogates. Notwithstanding the wide use 2,4-thiazolidinedione as a bioisostere in the glitazone class of antidiabetic drugs and elsewhere, **31** was disappointing (122). In a significant departure from the basic carbon backbone utilized in the majority of examples, it is clear from the fused bicyclic example **34** than there is wide latitude in the design of EET agonists. With only a few exceptions, the reiterative approach ending in the third generation of analogs has delivered a cornucopia of drug candidates that are comparable or surpass the activity of 14,15-EET either with or without significant sEH inhibition. From these examples, a potential therapeutic should emerge as they are further vetted through advanced absorption, distribution, metabolism, excretion screens.

Therapeutic Application of Orally Active EET Agonists in Pre-Clinical Rat Models

The therapeutic potential for manipulating EETs has been well recognized for the past decade with the development of sEH inhibitors that increase the EET to DHET ratio (31, 123). The promising biological actions of EETs in a number of pre-clinical disease models has led to interest in developing EET-based therapeutic strategies and inspired the development of sEH inhibitors (Figure 3). As a result, there have been human clinical trials for hypertension, diabetes, and chronic obstructive pulmonary disease with two sEH inhibitors (31, 124). Specifically, sEH inhibition improves endothelial function in obese smokers with chronic obstructive pulmonary disease (124). Despite this positive cardiovascular outcome, sEH inhibitors have not yet garnered approval for use in humans. Drawbacks to this approach include: (i) indirect increases in endogenous EETs and epoxides of other fatty acids, (ii) requirement for a functioning CYP epoxygenase for EET generation and (iii) chemical and metabolic lability of endogenous EETs. Our group developed EET agonist analogs with key features important for stability and bioavailability. Third generation of orally active EET agonist analogs demonstrate great potential as therapy for cardiovascular and kidney diseases in pre-clinical rat models (121). There have been no clinical studies with these EET agonist analogs.

The precise molecular and protein target for the EETs has yet to be identified which does not allow for target-based screening drug design approaches. As indicated in the EET receptor section above, identification of EET receptors is important and active research is ongoing in this area. Our group has taken a structure-activity approach to define the pharmacophore for EET agonist analogs. Cell-signaling mechanisms are extensively described for EETs. We have determined that EET analogs utilize these cell-signaling mechanisms and that EET antagonists block these actions (37, 121). The pharmacophore for EET agonists was used to develop second and third generation orally active EET agonist drug candidates through a phenotypic screening process (121). Interestingly, when comparing FDA drugs approved between 1999–2008, phenotypic screening was the most successful approach for first-in-class drugs whereas target-based screening was most successful for follower drugs (125). The phenotypic screening data for EET analogs in cardiovascular and kidney diseases by our group and others provide clear and convincing evidence that EET analogs are viable drug candidates.

Cardiovascular Diseases

Numerous drugs on the market exist for the treatment of cardiovascular diseases like hypertension and heart failure and include renin-angiotensin system blockers, diuretics, and

beta-adrenergic blockers. Even with this being the case, there is significant need for better cardiovascular therapeutic agents and none of the drugs used for heart failure with reduced systolic function are effective in diastolic dysfunction. With an aging population and increased prevalence of metabolic syndrome, therapies for diastolic heart failure are needed. A major shortfall with current therapeutics is that co-morbid events, end organ damage, and cardiovascular-related mortality remain problematic (126–128). Endothelial dysfunction is predictive for cardiovascular events in pre-clinical animal models and humans (128). EET agonist analogs provide a novel therapeutic approach that improves endothelial function and decreases end organ damage in cardiovascular disease (37).

Initial studies with EET agonist analogs focused on hypertension. A second generation EET analog, NUDSA **12**, was successfully used *in vivo* in rodents (129, 130). NUDSA was developed by evaluating the second generation of EET analogs for dilation of the afferent arteriole and mesenteric artery via activation of vascular smooth muscle cell K_{Ca} channels (118, 131). Modification of EET analogs was based on the SAR for afferent arteriolar dilation and pharmacological properties necessary for *in vivo* administration (129). A single intraperitoneal (i.p.) injection identified EET analogs that dramatically decrease blood pressure to normal values over 4 to 12 hours in rats with established angiotensin hypertension (129). Importantly, EET analogs with no or weak afferent arteriolar dilatory actions fail to lower blood pressure in angiotensin hypertensive rats (129). EET agonist analogs that lower blood pressure in angiotensin hypertension were then tested in SH rats. EET analogs demonstrate variability in lowering blood pressure in SH rats and require at least three days for a maximum blood pressure lowering effect (129). After discontinuing the EET analog, blood pressure rises to hypertensive levels over 4 to 5 days. These findings raise the possibility that mechanisms other than vasodilation are necessary for EET analogs to be antihypertensive. EETs and EET analogs inhibit ENaC and promote natriuresis and may serve as a longer-term mechanism to decrease blood pressure (113). The discovery that the injection of NUDSA achieves adequate therapeutic levels to lower blood pressure to normal in two hypertensive animal models was a major breakthrough (129). Additionally, in heme oxygenase 2 null mice that develop metabolic syndrome, NUDSA dramatically lowers blood pressure, lowers blood glucose, decreases body weight, and improves vascular function in these metabolic syndrome mice (130). Although NUDSA demonstrated great therapeutic potential, the major drawback is that NUDSA requires i.p. administration to be effective.

The design, synthesis, and screening of a series of approximately 50 EET analogs resulted in three novel orally active EET analogs (EET-A **27**, EET-B **34**, and EET-C22 **30**) (112, 121). These EET analogs were evaluated for their ability to relax coronary arteries and sEH inhibition. Potential EET analogs with therapeutic anti-hypertensive activity were determined in a similar manner in angiotensin hypertensive and SH rats (113). EET-A and EET-B demonstrate anti-hypertensive activity with i.p. injection (112, 113). In addition to vasodilator activity, EET-A inhibits sEH with an IC_{50} of 392 nM and EET-B has no sEH inhibitory activity below 30 μ M (121). Oral administration of the EET analogs, EET-A or EET-B to SH and angiotensin II hypertensive rats for two weeks lowers blood pressure to normal levels and decreases urinary albumin excretion, an indicator for decreased renal

injury (112, 113). Importantly, LC-MS-MS methods to measure plasma and tissue EET analog levels show that orally administered EET analogs achieve plasma levels that are sufficient for biologically activity (112, 132, 133). EET-A administered orally for two weeks prevents the development of angiotensin-mediated malignant hypertension in Cyp1a1-Ren-2 transgenic rats (134). However, when EET-A is administered for one week during the late stage of established malignant hypertension where significant renal damage is present, EET-A fails to lower blood pressure (134). A longer duration of EET analog treatment may be necessary to lower blood pressure at a late stage of malignant hypertension. In a thorough evaluation of EET-A, it vasodilates arterioles, inhibits ENaC activity in cultured collecting ducts, reduces ENaC expression in nephrons, and lowers blood pressure in *Cyp2c44*^{-/-} mice with salt-sensitive hypertension (112, 113, 134). Thus, the blood pressure lowering actions for EET analogs depends on vasodilator and natriuretic actions. Additionally, these findings are consistent with EET analog antihypertensive actions being multi-factorial and not purely due to vasodilation and as such, requires days to weeks for maximal blood pressure lowering to occur in hypertensive pre-clinical animal models (112, 113). Another consistent finding in hypertension models treated with EET analogs is a marked decrease in renal macrophage infiltration and diminished monocyte chemoattractant protein-1 (MCP-1) levels (112, 113, 133). Although renal inflammation contributes to hypertension, the contribution of the renal anti-inflammatory actions of EET analogs to lowering blood pressure remains to be determined. More importantly, the biological actions of EET analogs have the potential to lower co-morbid events by decreasing end organ damage, improving endothelial function, and significantly reducing cardiovascular related mortality.

Cardioprotective actions for EETs and EET analogs depend on their multi-functional actions (135–137). Endogenous and exogenous EETs and sEH inhibitors are beneficial in cardiac ischemia reperfusion injury. Pathophysiological responses to myocardial ischemia reperfusion injury such as myocardial infarction and reduced left ventricular function are improved by EET treatment (136, 137). Importantly, EETs given after reperfusion improve left ventricular heart function (64, 65). Likewise, NUDSA improves cardiac function when given to mice after myocardial infarction (138). Mice were subjected to left anterior descending artery ligation, and five days later NUDSA treatment was started and continued for four weeks. By echocardiography and cardiac histological analysis, NUDSA treatment returns left ventricular end diastolic area and fractional area to normal. More importantly, cardiac fibrosis and measures of diastolic dysfunction significantly decreased at four weeks following myocardial infarction with NUDSA (138). EET agonist analog treatment attenuates the progression of congestive heart failure and cardiac fibrosis following myocardial infarction induced by a 30 minute left coronary artery occlusion in SH rats (139). EET-B was administered for seven weeks following a myocardial infarction. It diminishes congestive heart failure-induced lung edema and dramatically reduces myocardial fibrosis at the ischemic zone (139). Cardioprotection by EETs and EET analogs depends on activation of pro-survival mechanisms, opposing apoptosis, and mitochondrial protection. EET analogs increase OPA1 oligomers and mitochondrial cristae density, activate mitochondrial K_{ATP} channels, and activate MAP kinase pro-survival signaling mechanisms to prevent cardiac cell apoptosis following an ischemic stress (136). EET mediated ischemia reperfusion protection of the heart also involves sarcK_{ATP} channel activation and PI-3-kinase

signaling mechanisms. EET analogs decrease left ventricular hypertrophy and cardiac fibrosis in hypertension (137). Importantly, EET-based therapies consistently prevent cardiac fibrosis in hearts after myocardial infarction, in hearts subjected to pressure overload, and in animals with insulin resistance (37, 137). Taken together, these findings strongly support the idea that EET analog treatment will provide beneficial anti-remodeling in the injured myocardium and may provide a novel approach to treating both systolic and diastolic cardiac dysfunction.

Kidney Diseases

EET agonist analogs combat kidney damage and improve renal function in acute and chronic kidney disease models (132, 133). EET analogs have actions that are ideally suited to treat kidney diseases including vasodilation, inhibition of platelet adhesion, blood pressure lowering action that takes days to weeks, and long-term anti-inflammatory, anti-apoptotic, and anti-fibrotic actions over the course of weeks to months. More importantly, the combined renal and cardiovascular actions make EET analogs an outstanding therapeutic candidate for kidney diseases associated with drug-induced organ toxicity, hypertension, cardiorenal syndrome, and diabetes.

Orally active EET agonist analogs were initially evaluated in a drug-induced acute kidney injury animal model. EET analogs prevent cisplatin-induced increases in blood urea nitrogen (BUN), plasma creatinine, albuminuria, renal tubular cast formation as well as urinary N-acetyl-(*D*)-glucosaminidase activity (NAG) and kidney injury molecule-1 (KIM-1) excretion (132). Renal injury markers are diminished 40–80% by EET analogs in cisplatin-induced nephrotoxicity. EET analogs also reduce renal oxidative stress, inflammation, apoptosis, and endoplasmic reticulum stress. In fact, these findings are consistent with other reports that EETs reduce critical apoptotic events including Bcl2 activated proapoptotic signaling and caspase-3 activity (140, 141). Likewise, EET analogs protect against the progression of chronic kidney injury in hypertension (112, 113, 133, 134). EET analog treatment of Dahl salt sensitive hypertensive, SH, or angiotensin hypertensive rats markedly reduces urinary albumin along with significant reductions in glomerular injury, intra-tubular cast formation, and kidney fibrosis (112, 113, 133, 134). Renal inflammation, oxidative stress, and endoplasmic reticulum stress dramatically decreases with EET analog treatment in hypertensive rats. Inflammatory MCP-1 levels and macrophage infiltration are consistently lowered by 60–80% with EET analogs in hypertensive animal models. EET analogs also protect the glomerular barrier by preserving nephrin expression and decrease renal fibrosis associated with hypertensive kidney injury (133). In Cyp11a1-Ren-2 transgenic malignant hypertensive rats administration of EET analogs for two weeks decreased oxidative stress, improved renal hemodynamics, and diminished renal injury (134). These data indicate that EET analogs prevent and treat kidney disease if given during the progression of acute or chronic kidney disease. The effectiveness of EET analogs in late stages of kidney diseases needs further study.

Orally active EET analogs protect the kidney from chronic injury through combined anti-inflammatory, anti-apoptotic, and anti-fibrotic mechanisms (142–144). The ability for EET-A to mitigate radiation nephropathy caused by total body irradiation (TBI) indicates that

EET analog intervention prevents progression of chronic kidney disease (142). EET-A treatment started two days after TBI and continuing for three months results in reduced BUN, albuminuria, and improved renal histopathological changes. Renal inflammation and oxidative stress do not contribute to TBI mediated renal injury. Therefore, TBI evaluates the ability of EET analog to directly combat renal endothelial dysfunction and epithelial apoptosis. Renal parenchymal apoptosis in TBI rats is reduced by EET-A actions on the p53/Fas/FasL (Fas ligand) apoptotic pathway (142). Renal afferent arteriolar endothelial dysfunction following TBI is dramatically decreased by EET analog treatment (142). These findings clearly show that EET analogs would be an effective treatment in kidney diseases where renal endothelial dysfunction and epithelial apoptosis are prominent.

EET analogs have significant therapeutic potential for organ transplantation. Not only is acute and chronic rejection of implanted organs a problem, but the major immunosuppressive treatment to prevent organ rejection, calcineurin inhibitors, causes hypertension and renal injury. Late rejection is the principal cause of graft failure. Consequently, the ability of an EET analog to oppose cyclosporine-mediated hypertension and renal injury was determined in rats. Oral treatment with an EET agonist analog decreases blood pressure and renal injury induced by four weeks of cyclosporine exposure (143). EET analog treatment also decreases cyclosporine-induced renal oxidative stress, inflammation, fibrosis, and apoptosis by 50–80%. Additionally, the EET analog eliminates cyclosporine-induced apoptosis in cultured NRK-52E renal epithelial cells (143). EET analog treatment decreases endoplasmic reticulum stress and activation of apoptotic pathways by decreasing GRP78 levels in renal epithelial cells (132, 133, 143). More recently, experimental studies in the unilateral ureter obstruction (UUO) mouse kidney fibrosis model have determined the ability of EET analogs to decrease fibrosis, independent of other confounding pathologies causing kidney damage. The anti-fibrotic mechanism by which EET-A diminishes chronic kidney disease was evaluated. EET-A treatment for 10 days produces clear anti-fibrotic actions by decreasing renal collagen positive area, kidney hydroxyproline content, and renal α -smooth muscle actin levels by 50–70% (144). A critical factor that contributes to kidney fibrosis is renal epithelial to mesenchymal transition (EMT) and involves renal epithelial cells that undergo phenotypic and functional changes to myofibroblasts (145). In UUO mice, the anti-fibrotic actions of EET analog treatment are due to a marked decline in E-cadherin transcription factors and renal EMT signaling pathways (144). Taken together, these findings demonstrate that EET analogs prevent and treat kidney diseases through direct renal epithelial cellular protective actions.

Summary of EET Agonist Analog Therapeutic Considerations

EET agonist analogs have exceptional therapeutic potential to combat cardiovascular and kidney diseases (Figure 3). They are effective for short-term or long-term therapy depending on the cardiovascular or renal disease being treated. These EET analogs possess biological activities ideally suited to treat these diseases. Novel, orally active EET analogs have been tested in a range of pre-clinical disease models and demonstrated clear benefit. Importantly, EET analogs not only prevent disease progression but also effectively intervene after the start of the disease. Cardiovascular diseases amenable to treatment by EET analogs include hypertension, drug-induced cardiotoxicity, myocardial infarction, left ventricular fibrosis,

chronic left heart disease, and acute coronary artery syndrome. EET analogs also have tremendous potential to treat drug-induced nephrotoxicity, acute kidney injury, hypertensive chronic kidney disease, nephritis, and diabetic nephropathy and ischemia reperfusion injury. Organ transplantation is an intriguing potential therapeutic use for EET analogs. EET analogs could protect the organ being implanted as well as protect the kidney from the deleterious actions of calcineurin inhibitors like cyclosporine. Defining and identifying the EET receptors will be an important step in developing EET agonists for human therapeutics. Next steps will be to determine and prioritize the clinical circumstances that are best suited for EET agonist treatment and make significant advances towards human clinical trials.

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Figure 1.
EET synthesis and metabolism pathways

Figure 2.
Proposed recognition/binding domain map of 11,12-EET

Cardiovascular Diseases

Hypertension
Vascular Hypertrophy
Diastolic Heart Failure
Systolic Heart Failure

Inflammation and Injury

Organ Inflammation
Transplant Organ Protection
Wound Healing

EET Analogs Broad Therapeutic Potential

Metabolic Disorders

**Prevention of Obesity
Related
Metabolic Disease**
Insulin Sensitizing

*Supported by preliminary studies
Deduced from literature*

Kidney Diseases

**Acute Kidney Injury
Chronic Kidney Disease**
Anti-apoptotic

Figure 3.
Therapeutic uses of orally active EET agonist analogs

Table 1Selected examples of second generation 14,15-EET analogs.^a

Compd	Analog	Vascular % (10 μ M)	Relax. ED ₅₀ (μ M)	sEHi IC ₅₀ (nM)
13		63	7.5	46
14		16	>10	1355
15		59	7.6	71.5
16		88	3.2	1451
17		83	3.2	8484
18		99	1.5	770
19		53	9.3	3480
20		64.5	6.1	79
21		64.5	4.3	11194
22		100	1.7	10712
23		12	>10	13877

Compd	Analog	Vascular	Relax.	sEHi
		% (10 μ M)	ED ₅₀ (μ M)	IC ₅₀ (nM)
24		89	1.7	58712
25		70	4.4	17622
26		11	>10	7147

^aAt 10 μ M, 14,15-EET induces 85% of the maximum vasorelaxation and its ED₅₀ is 2.2 μ M. Recombinant human sEH was utilized.

Table 2Representative third generation 14,15-EET analogs.^a

Compd	Analog	Vascular % (10 μ M)	Relax. ED ₅₀ (μ M)	sEHi IC ₅₀ (nM)
27		91	1.6	392
28		71	>10	>500
29		92	3.1	23
30		110	1.1	32
31		47	>10	57
32		109	0.34	10
33		109	0.32	>500
34		96	1.3	>500

^a At 10 μ M, 14,15-EET induces 85% of the maximum vasorelaxation and its ED₅₀ is 2.2 μ M. Recombinant human sEH was utilized.