Published in final edited form as:

J Med Chem. 2014 June 12; 57(11): 4454–4465. doi:10.1021/jm401431x.

Prostanoid Receptor EP2 as a Therapeutic Target

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Abstract

Cyclooxygenase-2 (COX-2) induction is prevalent in a variety of (brain and peripheral) injury models where COX-2 levels correlate with disease progression. Thus, COX-2 has been widely explored for anti-inflammatory therapy with COX-2 inhibitors, which proved to be effective in reducing the pain and inflammation in patients with arthritis and menstrual cramps, but they have not provided any benefit to patients with chronic inflammatory neurodegenerative disease. Recently, two COX-2 drugs: rofecoxib and valdecoxib were withdrawn from the United States market due to cardiovascular side effects. Thus, future anti-inflammatory therapy could be targeted through a specific prostanoid receptor downstream of COX-2. The PGE₂ receptor EP2 is emerging as a pro-inflammatory target in a variety of CNS and peripheral diseases. Here we highlight the latest developments on the role of EP2 in diseases, mechanism of activation and small molecule discovery targeted either to enhance or to block the function of this receptor.

Keywords

Inflammation; EP2 receptor; COX-2; agonist; antagonist; epilepsy; arthritis; Alzheimer's disease

INTRODUCTION

Inflammation plays a vital role in the pathogenesis of many chronic neurodegenerative^{1, 2} and peripheral diseases.³⁻⁶ An acute injury to the brain or to the body induces inflammatory pathways as an immune response. Common features of inflammation include upregulation of cytokines and chemokines, activation of microglia (in the central nervous system) or macrophages (in the periphery), induction of cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) and oxidative-stress.^{1, 2} All of these features are manifested in patients as well as animal models of chronic neurodegenerative diseases such as Alzheimer's disease (AD),^{7, 8} Parkinson's disease (PD),^{9, 10} epilepsy,¹¹⁻¹⁴ amyotrophic lateral sclerosis (ALS),¹⁵ and traumatic brain injury (TBI).^{16, 17} The inflammatory features are also evident in patients with other diseases such as rheumatoid arthritis (RA),⁴ chronic obstructive pulmonary disease (COPD)⁵ and inflammatory bowel diseases.³ Prolonged activation of these inflammatory pathways results in a secondary damage to the neighboring neurons in the brain, and to the cells and tissues in the body exacerbating the disease pathology. Thus, inhibition of inflammatory pathways with small molecule inhibitors should provide benefit

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to patients by reducing the severity of the diseases. It is important to note that the inflammatory responses are critical for survival and homeostasis. However, more than one inflammatory pathway or mediator is activated simultaneously during and after an injury to maintain homeostasis and aid in repair. There is also cross-talk among some of these inflammatory mediators (cytokines, iNOS and COX-2). An important issue that is often difficult to address is whether these inflammatory mediators act independently or in concert to fight against potential pathogens or clearing the debris from the tissue. As a result, it will be ambitious to comprehend an anti-inflammatory therapeutic agent that blocks a single inflammatory target or pathway resulting in alleviation of an entire inflammatory cascade without interfering with compensatory mechanisms. However, such a therapeutic agent might mitigate the severity of the inflammation and disease progression to some extent. Nonetheless, small molecular agents that block a single biological target such as COX-2, iNOS, cytokines and glia are currently being pursued by investigators for anti-inflammatory therapy.

The cyclooxygenase enzymes (COX-1 and COX-2) have been widely explored for anti-inflammatory therapy.¹⁸ COX-1 is constitutively expressed throughout the body and is involved in a range of physiological functions including cellular homeostasis and protection of gastrointestinal integrity.^{19, 20} COX-2 is rapidly induced or upregulated after a variety of stimuli or injuries and remains upregulated in chronic inflammatory diseases. Both COX-1 and COX-2 isoforms activate prostaglandin signaling (Figure 1). Several COX-1 selective inhibitors (a.k.a. non steroidal anti-inflammatory drugs (NSAIDs)) have been shown to produce on-target mediated side-effects, mainly gastrointestinal tract and renal toxicity.²¹ It has been hypothesized that selective inhibition of COX-2 by small molecules could potentially eliminate COX-1 mediated toxicities, because COX-2 is induced only upon an insult or injury in inflamed joints and tissues.^{22, 23} Thus, several selective COX-2 inhibitors were discovered.^{24, 25} For example, celecoxib (Celebrex), rofecoxib (Vioxx) and valdecoxib (Bextra) have been clinically proven as anti-inflammatory and pain relievers for chronic indications such as osteoarthritis and rheumatoid arthritis.^{26, 27} Surprisingly, although elevated levels of COX-2 were observed in AD patients and COX-2 levels were correlated with progression of AD,²⁸ COX-2 inhibitors and NSAIDs have not shown any cognitive benefits to patients with AD. The data suggest that COX-2 inhibitors could have an adverse effect on AD pathogenesis.^{29, 30} Moreover, chronic use of COX-2 drugs like rofecoxib and valdecoxib for arthritis and other diseases also resulted in adverse cardiovascular side effects including myocardial infarction and stroke in a significant number of patients.³¹ Consequently, rofecoxib and valdecoxib were withdrawn from the USA market. These side effects could impose severe restrictions on the future use of COX-2 drugs, particularly for elderly patients with AD, who are already at increased risk for heart disease. It was demonstrated that inhibition of the prostanoid receptor IP, which is activated by COX-2 derived prostaglandin PGI₂ (see Figure 1), is responsible for myocardial infarction, stroke and atherosclerosis.^{32, 33} Thus, future generations of anti-inflammatory therapy should be directed to target specific prostaglandin synthases or prostanoid receptors downstream of COX-2 rather than the generic block of the entire COX-2 signaling cascade (Figure 1).

COX/PROSTANOID SIGNALING CASCADE

Both COX-1 and COX-2 catalyze the conversion of arachidonic acid into endoperoxide intermediate prostaglandin- H_2 (PGH₂), which is then transformed by a variety of cell specific prostaglandin synthases to produce five prostaglandins (PGD₂, PGE₂, PGF₂, PGI₂ and TXA₂). These natural ligands activate eleven different G protein-coupled prostanoid receptors (DP1, DP2, EP1, EP2, EP3, EP4, FP α , β , IP and TP α , β).³⁴ These prostanoid receptors are differentially expressed in various cells and tissues and show a physiological and pathological role in a variety of conditions.³⁵ Based on structural and functional properties, these prostanoid receptors are classified into three major groups. The first group called relaxant receptors, consists of DP1, EP2, EP4 and IP receptors, because they mediate smooth muscle relaxation through cyclic AMP (cAMP) production. The second group known as contractile receptors, consists of EP1, FP and TP receptors, because they induce smooth muscle contraction by intracellular Ca²⁺ mobilization. The third group known as inhibitory receptors, has only two members (EP3 and DP2), that inhibit the production of cAMP. Activation of the DP2 receptor has been shown to increase intracellular Ca²⁺ levels as well (Figure 1). Additional articles regarding the detailed description of prostanoid receptor properties and function have been published recently.^{34, 36-38} Thus, in this perspective article, we focus specifically on the EP2 receptor and the binding of the natural ligand prostaglandin-E₂ (PGE₂)³⁹ (**1**) (Figure 4) and synthetic analogs. Both a beneficial and deleterious role of EP2 has been suggested in central nervous system (CNS) and peripheral diseases by making use of EP2 knockout mouse models and selective small molecular agonists and antagonists developed for EP2 (see below).

EP2 RECEPTOR IS A TARGET FOR A VARIETY OF CNS AND PHERIPHERAL DISEASES-AN OVERVIEW

The EP2 receptor is widely distributed in the body.⁴⁰ In the brain, EP2 is expressed in the cerebral cortex, thalamus, hypothalamus, spinal cord, hippocampus and striatum.^{41, 42} At the cellular level, EP2 is expressed in neurons, astrocytes and microglia.^{41, 42} A significant body of evidence shows that the EP2 receptor plays both protective and deleterious role in brain injury models. EP2 activation by butaprost (a.k.a. TR4979⁴³) (**2**) (Figure 4) (a relatively selective EP2 agonist) protects neurons from glutamate-, and NMDA-induced excitotoxicity in rat hippocampal cultures *in vitro*.^{42, 44} EP2 activation by **2** reduces 6-hydroxydopamine (6-OHDA)-mediated neurotoxicity in primary neuronal cultures prepared from rat midbrain.⁴⁵ An EP2 selective allosteric potentiator TG3-95-1⁴⁶ (**10**) (Figure 4), also displays a neuroprotective effect *in vitro* against NMDA-induced excitotoxicity in rat cultures⁴⁶ indicating that EP2 is involved in neuroprotection (see next section for neuroprotection in EP2 knockout models).

In contrast to the above results, several studies indicate that EP2 activation promotes inflammation and neurotoxicity in chronic neurodegenerative disease models. For example, deletion of EP2 receptors reduces the oxidative-damage and amyloid burden in a model of AD,⁴⁷ attenuates neurotoxicity by α -synuclein aggregation in a mouse model of PD,⁴⁸ and improves motor strength while extending the survival of ALS mice (G93ASOD model).⁴¹ Mice lacking the EP2 receptor are less prone to cerebral oxidative damage produced by the

activation of innate immunity.⁴⁹ Microglia cultures from mice lacking EP2 are less sensitive to neurotoxicity indicating that microglial EP2 is involved in paracrine neurotoxicity.⁵⁰ Microglia lacking EP2 also show enhanced phagocytosis of amyloid- β (A β) peptides, which are hallmarks of AD, and initiate AD progression. Microglia lacking EP2 are also less sensitive to neurotoxicity when activated by A β -peptides⁵¹ suggesting that EP2 plays a key role in AD pathology. PGE₂ (**1**) acting on EP2 and EP4 receptors mediates the production of A β -peptides as EP2 knockout mice show reduced amounts of A β -peptides in the brain.⁵² Taken together, these data suggest that EP2 exacerbates AD by maintaining high levels of A β -peptides. Furthermore, EP2 receptor activation increases expression of iNOS, an enzyme that produces nitric oxide (NO), which is converted into reactive nitrates and nitrites, in cytokine activated astrocytes,⁵³ creating an environment for oxidative-stress that has been shown to damage the brain tissue.

EP2 receptors are also widely distributed in the periphery. For example, EP2 is highly expressed in retinal tissue, dendritic cells, endometriotic cells, skin epidermis and in a variety of cancer cells.⁵⁴⁻⁵⁶ Studies also indicate that EP2 plays protective and deleterious roles in the peripheral disease models. EP2 activation by a selective agonist ONO-AE1-259-01⁵⁷ (**5**) (Figure 4) in a rat model of retinal degeneration, increases the retinal arteriolar diameter and fundus blood flow and also prevents NMDA induced excitotoxic injuries to rat retina, suggesting that EP2 plays a role in the regulation of ocular hemodynamics.^{58, 59} An EP2 agonist CP-533536⁶⁰ (**7**) (Figure 4) has been shown to accelerate bone-healing in a preclinical canine model of bone fracture.⁶⁰⁻⁶² EP2 activation by **1** also improves renal function in an HgCl₂ model of renal failure,⁶³ and survival of epithelial cells after radiation injury.⁶⁴

EP2 activation exacerbates symptoms of experimental inflammatory bowel disease (colitis) by increasing IL-23 expression and reducing both IL-12 and IL-27, causing T-cells to differentiate to Th17 effectors (Figure 2).^{55, 65} Activation of EP2 also mediates joint inflammation in a mouse model of collagen induced arthritis.⁶⁶ EP2 has been suggested to function in UV-induced skin inflammation, where loss of EP2 significantly reduces inflammatory cell infiltration into the local skin, and local blood flow after UV exposure.⁶⁷ EP2 has also been shown to promote tumor angiogenesis by enhancing endothelial cell motility and opposing apoptosis,^{68, 69} and mediating epidermal growth and tumor formation in response to UV radiation⁷⁰ or chemical carcinogenesis.⁵⁶ PGE₂ promotes survival of human endometriotic cells through EP2 and EP4 receptors by activating multiple cell survival signaling pathways. Selective or combined inhibition of EP2 and EP4 receptors impairs these survival pathways and activates intrinsic apoptotic pathways, thereby promoting apoptosis of human endometriotic cells, indicating the potential of non-estrogenic therapy for the treatment of endometriosis in women using an EP2 inhibition strategy.⁵⁴

EP2 AGONISM AND ANTAGONISM FOR DRUG DISCOVERY

The above overview of results indicates the pleiotropic nature of the EP2 receptor in the CNS and periphery, thus rendering it as a potential target for medicinal chemistry approaches to discover treatments for a variety of disease indications (Figure 3). The results also suggest that EP2 receptors play a context dependent protective and deleterious role.

Thus, both EP2 agonistic and antagonistic strategies may be applied for therapeutic intervention, depending upon the disease and the role played by EP2 in that setting. So far, there is no small molecule agent that either activates or inhibits this G protein-coupled receptor that is approved for clinical use in patients. However, recent preclinical data using a selective EP2 agonist in ischemic stroke and glaucoma models, an EP2 antagonist in seizure and status epilepticus models, suggests that EP2 modulators (agonists and antagonists) should be explored aggressively in the clinical setting.

EP2 Agonism for stroke

Stroke affects about 800,000 Americans each year. Treatment and disability for ischemic cerebral injury is estimated to cost about 73 billion/year (American Heart Association, Heart Disease and Stroke Statistics, 2013 Update). So far, a significant number of neuroprotective agents, that have been shown to be effective in preclinical models, failed to show efficacy in patients in clinical studies.⁷¹ Thus activation of the EP2 receptor may provide neuroprotective benefits to stroke patients. McCullough et al.,⁴² and Liu et al.,⁷² have demonstrated that mice lacking EP2 show a significantly larger infarct size and larger stroke volumes compared to wild-type control mice in models of both transient and permanent focal ischemia, with middle cerebral artery occlusion (MCAO). However, both EP2 knockout and wild type mice show similar physiological parameters and vascular anatomy.^{42, 72} Independently, Ahmad et al.⁷³ also demonstrated that mice lacking EP2 show higher neurologic deficit scores and larger infarct sizes than those of wild-type controls after MCAO (transient- and permanent-middle cerebral artery occlusion; *t*MCAO and *p*MCAO). Importantly, an injection of a selective EP2 agonist **5** (Figure 4) (1-2 nM) into the right lateral ventricle of mice prior to *t*MCAO displayed fewer neurologic deficits and smaller hemispheric infarct volumes in the brain compared to mice injected with only vehicle.⁷³ Moreover, Serrano et al.,⁷⁴ have shown that a single injection of butaprost free acid⁷⁵(**3**) (Figure 4) immediately after termination of status epilepticus shows about 50% neuroprotection in the hippocampal CA1. These groups have chosen to administer EP2 agonists, either **3** or **5**, directly into the lateral ventricle. Although this is not the method of choice for the dosing of a drug to patients, given the poor brain penetration properties of these carboxylic acids, this method of administration may be justified. Thus, it is also possible that bolus dosing of an EP2 agonist early on could potentially saturate and desensitize the EP2 receptors by internalization, as described before for other receptors⁷⁶⁻⁷⁸ and negatively regulate the deleterious functions of the receptor, leading to a net neuroprotective effect. However, Desai et al.,⁷⁹ showed that 1 μ M **1** (about 500-fold higher concentration than its EC₅₀ for EP2) for 0-60 minutes does not internalize EP2 receptors expressed on 293-EBNA cell line, but in comparison, the same dose of **1** internalizes EP4 receptors by 40%. In the study involving an ischemic stroke model,⁷³ the EP2 agonist **5** was dosed to produce cerebrospinal fluid (CSF) concentrations of only 1-2 nM, a concentration at its EC₅₀ for the EP2 receptor (see Table 1). It is less likely that EP2 receptors are desensitized in this situation. However, in a mouse model of status epilepticus, Serrano et al., used butaprost free acid **3** at concentration about 3-fold higher than its EC₅₀ for EP2.⁷⁴ In this case, there is a possibility of receptor desensitization. Overall, the very weak internalization tendency of EP2 receptors *in vitro*,^{77, 79} in conjunction with results from mice lacking EP2 receptors⁴² rule out the possibility of receptor desensitization is the cause

of beneficial effects in the stroke models^{73, 74} and provide a rationale for using EP2-activation as an attractive strategy for a neuroprotection therapy in ischemic stroke patients, by using a highly selective, orally available, brain penetrant EP2 agonist. Whether the animal models of *r*MCAO and *p*MCAO, as well as pilocarpine induced status epilepticus discussed here^{42, 72-74} are relevant to the human disease, or whether they will translate into the clinical setting is beyond the scope of this review.

EP2 Agonism for ocular disease

Treatments for lowering intraocular pressure with hypotensive agents that antagonize β 2-adrenergic receptors, or agonize α 2-adrenergic receptors and prostanoid receptor FP are currently marketed.^{80, 81} Recent studies from Pfizer, using an EP2 agonist taprenepag isopropyl (PF-04217329)⁸² (**9**) (Figure 4) suggest EP2 as an attractive target for open-angle glaucoma and ocular hypertension therapy. Compound **9** (a prodrug of CP-544326⁸² (**8**)) has been shown to control intraocular pressure in preclinical models of rabbit, dog and monkey.⁸² Moreover, this compound has been investigated in a phase 2 clinical trial of 28 days of monotherapy, where it significantly reduced intraocular pressure in patients with open-angle glaucoma and ocular hypertension.^{83, 84} Due to the poor aqueous solubility of **9**, it was formulated with a surface active ingredient, (1% tween-80, 5% Cremophor-L and 0.02% benzalkonium chloride in 100 mM phosphate buffer pH 5.5) as an ophthalmic solution. This compound, at a concentration of 0.0025% to 0.03%, produced benefits comparable to a 0.005% latanoprost solution (a current standard of care) in reducing intraocular pressure in patients.^{83, 84} A point to note is that topical administration of an EP2 agonist, like **9**, is unlikely to be efficacious in treating other CNS and peripheral diseases. Although it is unknown whether systemic administration of an EP2 agonist will cause hypotension, EP2 knockout mice and mice injected with an EP2 agonist (by intracerebroventricular route), after MCAO have shown no differences in mean arterial cerebral blood flow in comparison to control mice.^{42, 73} Nonetheless, long-term oral treatment with an EP2 agonist may cause hypotension as a side effect.

EP2 Antagonism for neurodegenerative disease

Chronic inflammatory neurodegenerative diseases such as epilepsy, AD, PD and ALS contribute to the mortality and morbidity of a significant fraction of the population in the USA. AD and PD are particularly associated with dementia symptoms in the elderly. However, all of these diseases share a common feature of overactivated inflammatory pathways.^{1, 2, 7} Interestingly, PGE₂ is a major prostaglandin produced during the progression of these diseases and has been shown to mediate proinflammatory functions via EP2 (and to a lesser extent by EP4) receptors. Recently, Jiang et al.,⁸⁵ have shown that administration of a potent and selective EP2 antagonist, TG4-155⁸⁵ (**12**) (Figure 4), substantially blocks neuronal damage in the hippocampus (> 60% in the hilus, 80% in the CA3 and > 90% in the CA1 region) in a pilocarpine model of status epilepticus. Jiang et al.,⁸⁶ also showed that another potent EP2 antagonist, TG6-10-1⁸⁶ (**13**) (Figure 4), suppresses neurodegeneration, upregulation of inflammatory cytokines and chemokines and activation of glia in mice following status epilepticus, suggesting a potential therapeutic benefit for an EP2 antagonist to block epileptogenesis. Based on beneficial effects observed in EP2-knockout mouse models of AD, PD and ALS (vide supra),^{41, 47, 48} a potent and

highly selective EP2 receptor antagonist may show utility as an adjunct therapeutic agent for chronic inflammatory neurodegenerative diseases including epilepsy, AD, PD and ALS.

EFFECTS OF EP2 DELETION IN ANIMAL MODELS

Creation and use of EP2 genetic knockout models provided a wealth of information regarding the function of EP2 receptor in physiological and pathophysiological conditions. However, it is important to note that EP2 knockout mice exhibited significant cognitive and social memory deficits, impaired spatial learning and prepulse inhibition, and heightened anxiety, suggesting an association between the EP2 receptor and long term hippocampal depression and learning and memory.^{87, 88} The loss of EP2 also has functional ramifications for reproduction, as EP2-lacking mice have decreased ovulation number and fertilization rate compared to wild type mice, and EP2 null females produce lower than normal litter sizes.⁸⁹ Other functional roles of EP2 identified by genetic manipulation studies in pregnant EP2 mice include arterial dilatation, salt-sensitive hypertension during pregnancy, and reduced female fertility.⁹⁰ Reports such as these may have contributed to the paucity in antagonistic drug discovery approaches for this receptor. However, it is unlikely that these developmental compromises will be observed with small molecule inhibitors. Thus, use of a highly specific EP2 receptor antagonist for longer periods of time may provide a definitive answer to whether EP2 antagonism has a therapeutic advantage in comparison to genetically manipulated mice.

EP2-DOWNSTREAM MECHANISMS OF ACTION

A significant body of evidence suggests that EP2 activates downstream signaling either by a G protein-coupled or by G protein-independent mechanism.⁹¹⁻⁹⁵ As shown briefly in Figure 5, activation of EP2 by PGE₂ stimulates adenylate cyclase (*via* Gas) resulting in cAMP, which mediates events either through protein kinase A (PKA),⁹⁵ or exchange protein activated (Epac).^{96, 97} PKA activates transcription factors such as cAMP-responsive element binding protein (CREB), which is involved in neuronal survival and neurogenesis.^{98, 99} Epac initiates downstream effectors Rap1/2 to regulate neuronal differentiation, neuronal excitability, learning, memory and social interactions.¹⁰⁰⁻¹⁰² It has been shown that there is cross-talk between PKA and Epac and that PKA and Epac work synergistically to activate CREB. However, more often they work antagonistically. Generally, it is believed that PKA activation is associated with neuronal survival, whereas Epac activation leads to neuronal injury. EP2 also regulates other downstream effectors independent of G protein-coupling *via* β -arrestin to promote or exacerbate disease conditions including cancer proliferation and metastasis (Figure 3 and Figure 5). Thus, EP2 is a complex target displaying pleiotrophic roles depending on the circumstances. For simplicity, only a few downstream pathways are described here. However, it is important to remember that some of the downstream effectors shown in Figure 5 are also influenced by other G protein-coupled receptors, like EP4.⁹¹⁻⁹⁵

EP2 STRUCTURE AND BINDING SITE

The EP2 receptor belongs to the seven-transmembrane G protein-coupled receptor family. Human EP2 encodes a protein of 488 amino acids with a molecular mass of 53 kDa. The amino acid sequence identity between human and mouse EP2 is 88%. The difference

between these receptors is the presence of an extra 25 amino acids at the NH₂-terminus of the mouse EP2 protein, and a difference of six consecutive amino acids in the third extracellular loop. Despite these differences, the pharmacological properties of human EP2 receptors are similar to those of mouse EP2 receptors.⁴⁰ Within the family of prostanoid receptors, the amino acid identity is limited. The identity of EP2 amino acids to EP1, EP3 and EP4 is in the range of 20-30%. However, EP2 is more homologous to DP1 with a structural identity of 44%, and then to the IP receptor with 40% identity.¹⁰³ No 3D structure of the EP2 receptor or any other prostanoid receptor has yet been solved which might provide details for the binding of small molecule (natural and synthetic) EP2 modulators. However, the second extracellular loop region has shown a highly conserved amino acid sequence and it is presumed that the ligand-recognition site is located in this region.³⁴ So far, small molecule discovery (*vide infra*) has been carried out only on the basis of intuitive ligand-based design strategy.

IN VITRO BIOACTIVITIES OF EP2 AGONISTS AND ANTAGONISTS

In comparison to other prostanoid receptors, relatively fewer efforts have appeared towards discovery and development of small molecule modulators of EP2.^{34, 38} There are only two classes of agonists currently available. Representative members of these classes that are widely used and chemically optimized are shown in Figure 4. The first class of agonists is composed of molecules that are structurally related to the endogenous ligand **1**, a non selective EP2 agonist with EP2 $K_i = 38$ nM determined by a competitive binding assay.⁷⁵ The synthetic agonist **2** (butaprost) is about 63-fold less potent than **1** with an EP2 $K_i = 2400$ nM. The agonist **2** is selective for EP2 over other prostanoid receptors in receptor binding assays, but was equally effective in activating IP receptors in a functional assay (see Table 1).⁷⁵ In a separate study, **2** was shown to be only 3 to 4-fold selective for EP2 over EP3 and EP4 receptors, but very selective against the IP receptor in binding assays.¹⁰⁴ The ester group of **2** is unstable and is expected to be cleaved metabolically to produce acid **3** (Figure 4). The acid **3** is more potent than **2** with an EP2 K_i of 73 nM. By comparison, **3** is only 2-fold less potent than **1**, however, the free acid **3** has lost selectivity to EP2 binding over IP receptor binding (IP $K_i = 870$ nM). In a potency assay, **3** showed nearly the same activity for EP2 and IP receptors with an EC₅₀ of 32 nM and 25 nM, respectively (Table 1).⁷⁵ The other agonist in the same class represented as compound **4**⁵⁷ (Figure 4) appears very selective for the EP2 receptor over other prostanoid receptors. The affinity of **4** to EP2 is nearly 10-fold higher than that of **1** and 20-fold higher than that of **3** (Table 1). However, in functional assays, it has nearly the same potency as **1**, and roughly a 10-fold higher potency than **3**.⁵⁷ Compound **4** is found to be unstable as the free acid, but its stability is markedly improved when it is converted to the lysine salt.⁵⁷ Compound **5** shows a higher binding affinity for EP2 ($K_i = 1.7$ nM), a 22-fold higher affinity than **1** and 42-fold higher affinity than **3**. Compound **5** also shows ~700 to 1500-fold selectivity for EP2 over other prostanoid receptors such as EP1, EP3, EP4 and IP.⁵⁷ In a functional assay, it has about an 18-fold higher potency than **2** and **3** (Table 1). The non-selective EP2 agonist AH-13205^{105, 106} (**6**) (Figure 4) is also a member of this class, but because of its low potency it has not received much attention.

The second class of EP2-agonists are pyridyl-sulfonamides. Compound **7** is the initial lead compound in the class, and has high binding affinity to EP2 ($K_i = 50$ nM) with high selectivity for EP2 over other prostanoid receptors (16-fold selectivity against DP1 and 50 to 60-fold selectivity against EP1, EP3, EP4 and IP receptors).⁶⁰ A related compound in this class, **8** also displays high binding affinity to EP2 and at least a 270-fold selectivity against EP1, EP3 and EP4 receptors. The potency of **8** is equal to **1** in a cAMP determination assay (Table 1). Importantly, a prodrug of **8**, taprenepag isopropyl (**9**) has been developed as a clinical candidate (*vide supra*).⁸³ The only class of EP2-allosteric potentiators reported, represented by **10** (Figure 4) was developed recently at Emory University. Compound **10** potentiates EP2 receptors with $EC_{50} = 7.8$ μ M in a cAMP assay and also displays selective potentiation of EP2 receptors versus EP4 and β 2-AR receptors.⁴⁶ Allosteric potentiators are designed to function in the presence of high PGE₂ concentrations that are expected to be present during tissue injury. Two drawbacks of these potentiators are that they are weakly active and poorly water soluble for dosing into animal models. However, **10** may be useful for *in vitro* studies.⁴⁶

The only EP2 antagonist widely used for probing the roles of EP2 is AH-6809¹⁰⁷ (**11**) (Figure 4). **11** is commercially available, but it shows weak antagonistic activity (EP2 $K_i = 1150$ nM) and very poor selectivity against other prostanoid receptors such as EP1, EP3 and DP1 (Table 2).¹⁰⁴ Recently, Pfizer revealed the azetidine-carboxylic acid derivative PF-04418948¹⁰⁸ (**15**) (Figure 4) as the first of a new class of selective EP2 antagonists with an IC_{50} of 16 nM and Schild K_B of 1.8 nM (a concentration required to cause a twofold increase of the EC_{50} of PGE₂).¹⁰⁸ This antagonist exhibits >10,000-fold selectivity for the EP2 receptor over other prostanoid receptors (i.e. EP1, EP3, EP4, DP1 and IP) (Table 2) and does not show significant effects on a diverse panel of other GPCRs.¹⁰⁸ However, brain penetration of this antagonist has not been disclosed and thus it is unclear whether this compound could be useful for *in vivo* evaluation in mouse models of CNS disease. Nonetheless, **15** has been shown to attenuate butaprost-induced vasodilatory blood flow in rats, demonstrating that it has the requisite plasma pharmacokinetics and tissue penetration to be useful as an *in vivo* probe for a variety of peripheral diseases.¹⁰⁸

Independently from Pfizer, Emory University identified a novel cinnamic acid amide class of EP2 antagonists, and has investigated them for proof of concept in mouse models of status epilepticus.^{85, 86} These compounds are unique in structure and do not belong to any other class of agonists or antagonists previously reported. Importantly, the Emory EP2 antagonists do not possess a carboxylic acid, which is intrinsic to the majority of the EP2-agonists and -antagonists discovered thus far (Figure 4). Two representatives in this class of EP2 antagonists are **12** and **13** whose structures are shown in Figure 4.⁸⁵ The prototype compound **12** has an EP2 Schild K_B of 2.4 nM and displays 550-4750-fold selectivity for EP2 over EP1, EP3, EP4 and IP, but only 14-fold selectivity against the DP1 receptor (Table 2).⁸⁵ Another lead compound in this class of EP2 receptor antagonists is **13**, which has an EP2 Schild K_B of 17.8 nM, and displays greater than 300-fold selectivity against EP3, EP4 and IP receptors. Compound **13** shows a 100-fold selectivity against EP1, FP and TP receptors, but only 10-fold selectivity against the DP1 receptor.⁸⁶ Moreover, **13** shows a plasma half-life ($t_{1/2}$) of 1.6 h and brain to plasma ratio of 1.6 in mice. These two Emory

compounds (**12** and **13**) did not show significant off-target activity against a diverse panel of 47 enzymes and receptors including COX, NOS, PDE, adrenergic receptors, serotonin receptors and cytochrome P450 enzymes. So far **12** and **13** have only shown significant off-target activity at the serotonin 5-HT_{2B} receptor with an IC₅₀ 2.6 μ M and 7.5 μ M respectively.^{86, 109} These IC₅₀ values far exceed their respective EP2 Schild K_B values (1080-fold higher for **12**, 420-fold higher for **13**). The data obtained thus far on these novel EP2 receptor antagonists suggest that **13** may serve as a valuable probe for proof of concept studies in CNS and other neurodegenerative animal models where EP2 receptors may mediate deleterious effects. Very recently, Emory university group discovered a second class of antagonists that are carbamothioylacrylamides.¹¹⁰ A lead compound, TG6-129¹¹⁰ (**14**) (Figure 4) displayed EP2 potency at low nanomolar concentrations (EP2 Schild K_B = 8.8 nM). Unlike **12** and **13**, compound **14** has shown high selectivity against DP1 (1660-fold) and EP4 (440-fold) receptors, but only 22-fold selectivity against the IP receptor. This compound did not cross the blood brain barrier and thus it might be useful as a probe for a variety of peripheral disease models shown in Figure 3.

EP2 ANTAGONISTS REPORTED IN PATENT LITERATURE

Although very little bioactivity or biology is included in the patent literature, a brief summary on patented EP2 antagonists is presented due to the relatively limited number of chemical scaffolds available so far. Two pharmaceutical companies; Pfizer and Bayer Schering Pharma have identified small molecule antagonists for the EP2 receptor. Representative structures **16-22** are illustrated in Figure 6. Pfizer has created 3-azetidine carboxylic acids and 3-azetidine carboxamides (**16-17**) with potent EP2 inhibition activity.¹¹¹ Compound **15** (Figure 4; Table 2) emerged as a lead compound from the general structure **16**. Bayer Schering Pharma has also developed four distinct classes of antagonists represented by **18-21**,¹¹²⁻¹¹⁵ but *in vivo* data is not available for any lead structure from these scaffolds **18-22**. Emory University also created a class of compounds represented by **22**, which display potent EP2 antagonist activity,¹¹⁶ from which **13** (Figure 4) has emerged as a lead compound. Several compounds from each class (**16-22**) show EP2 potency in the range of low nanomolar to low micromolar level (Figure 6), and are available as backups for development into therapeutically useful compounds. Due to the large number of EP2 agonists published in the patent literature and scholarly articles, they are not highlighted here.¹²²⁻¹²⁵

CONCLUSIONS AND PROSPECTS

PGE₂ is a major product of increased COX-2 activity in the brain of rodents and human patients following various insults. It activates four G protein-coupled receptors (EP1, EP2, EP3 and EP4). EP2 receptor is expressed in various parts of the body and brain and plays context-dependent, beneficial, and deleterious roles. Thus, concerns are inevitable about whether EP2 receptor is druggable with small molecules, and if so, whether activation or inhibition is a better therapeutic strategy. Recent studies with mouse models of ischemic stroke and inflammatory neurodegenerative disease model of status epilepticus (*vide supra*) suggest both agonism and antagonism strategies may be explored for therapeutic development. Studies also suggest that the cell type from which EP2 receptors originate may

affect the role of the receptor. For example, in the brain, EP2 receptors are expressed in neurons and glia. Thus, it is also possible that EP2 of neuronal origin may act, mainly through PKA activation, in an autocrine fashion to protect neurons from acute brain injury (as in stroke, or just after status epilepticus). The signaling from dying neurons, in conjunction with several other proinflammatory mediators produced during acute brain injury may activate EP2 receptors in microglia and astrocytes, which, predominantly *via* the Epac activation pathway, may exacerbate paracrine toxicity leading to neurodegeneration. Thus, as illustrated in Figure 7, an appropriate therapeutic approach may be to use an EP2 agonist or an allosteric potentiator immediately after brain injury to maximize the beneficial effects. An alternative therapeutic strategy would be to use an EP2 antagonist, starting several hours to days after brain insult to break the glia-induced vicious inflammatory cycle, as a first line or adjunct therapeutic agent in patients with neurodegenerative diseases such as epilepsy, AD, PD, TBI and ALS.

As recently described by Weiss et al.,¹¹⁷ it is not always true that the phenotype of small molecular inhibitors of a target mimics the phenotype of genetic knockout animals lacking the same target. There are several receptors and enzymes including a few kinases (e.g. Aurora B) known to show divergence. EP2 knockout mice were created many years ago and have been used extensively to explore the role of EP2 in a variety of disease models. However, EP2 receptor antagonists have been reported recently and have been explored in limited neurodegenerative disease models. Thus they should be examined for on-target mediated effects on healthy mice during chronic dosing (2-3 months) in order to characterize memory deficits, fertility complications, formation and loss of synapses in the hippocampus (*vide supra*). This experiment would yield valuable information to rule in or out whether an antagonism of EP2 is therapeutically beneficial.

There are several highly characterized EP2 specific agonists now available (Table 1), but a limited number of selective antagonists (Table 2) that are useful for CNS and other disease models. Other than steroids, NSAIDs, and COX-2 inhibitors, currently, there is no other FDA approved anti-inflammatory therapeutic agent available which works through a specific inflammatory target. However, selective inhibitors of microsomal prostaglandin-E-synthase (mPGES)¹¹⁸ and antagonists of prostanoid receptor EP4¹¹⁹ are currently under preclinical evaluation. These agents (mPGSE-1 and EP4 inhibitors) may also alleviate some of the adverse side effects caused by COX-2 inhibitors. However, experiments designed to reduce inflammation or neurodegeneration (or both) in chronic inflammatory or neurodegenerative disease models (examples; arthritis, AD, epilepsy) using COX-2, and EP2 or EP4 or mPEGS-1 selective inhibitors head-to-head, is worth pursuing.

Although the available EP2 agonists and antagonists are selective for EP2 in binding and *in vitro* functional assays (Table 1, 2), *in vivo* target-engagement studies^{120, 121} are lacking to ascertain whether the observed efficacy is mediated through the binding of EP2 modulators to the receptor *in vivo*. Demonstration of target engagement is a formidable task for EP2 receptor, and is complicated by the fact that there is no specific biomarker produced solely by the EP2 receptor, and lack of available radio-labeled agonists or antagonists that specifically bind to EP2 *in vivo*. Nonetheless, this study is very important, and if target

engagement can be demonstrated, it will strengthen the published results and advance the EP2 receptor as a target for drug discovery.

Acknowledgments

TG thanks lab members, Drs. Asheebo Rojas, Jianxiong Jiang, Ray Dingledine and Ms. Nadia Lelutiu for reading the proof of the manuscript and comments.

Funding Sources Dr. Ganesh is supported by NIH/NINDS grant U01NS058158 (P.I. Ray Dingledine)

Dedication: To Dr. Raymond J. Dingledine for his outstanding contributions in epilepsy research, and in celebration of his 65th birthday.

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Dr. Thota Ganesh obtained his M. Sc. and Ph. D degrees from Osmania University, Hyderabad, India. After his postdoctoral studies at IIT-Bombay (India), University of Durham (UK) and Virginia Tech (USA), he began working as an Assistant Professor at Department of Pharmacology, Emory University. Dr. Ganesh's current research focuses on developing small molecule agents to mitigate the inflammatory pathologies in neurodegenerative diseases, including epilepsy and Alzheimer's disease. His past research includes, total synthesis, medicinal chemistry of Taxol, epothilones and other bioactive molecules, and development of small molecules inhibitors for HSP90 and histone methyltransferase enzymes. Dr. Ganesh is grateful to his past mentors Drs. Sambasivarao Kotha (IIT-Bombay, India), Patrick G. Steel (University Durham, UK), David G.I. Kingston (Virginia Tech, USA) and Dennis Liotta (Emory University).

ABBREVIATIONS

PGE₂	Prostaglandin-E ₂
cAMP	cyclic adenosine monophosphate
AD	Alzheimer's disease
PD	Parkinson's disease
ALS	amyotrophic lateral sclerosis
SE	status epilepticus
RA	rheumatoid arthritis
IBD	inflammatory bowel disease
DC	dendritic cells
NMDA	N-Methyl-D-aspartate
CNS	central nervous system
OGD	oxygen-glucose deprivation
MCAO	middle cerebral artery occlusion
PDE	phosphodiesterase

GPCR	G protein-coupled receptor
MMP	matrix metalloproteinase

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Figure 1.

COX/prostaglandin signaling cascade. Arachidonic acid is converted to PGG_2 and then to PGH_2 by both COX-1 and COX-2 enzymes. Cell specific synthases convert the intermediate PGH_2 into prostaglandin ligands, which activate one or more G coupled-prostanoid receptors. There are four receptors DP1, EP2, EP4 and IP which promote cAMP production. cAMP then activates PKA-, and Epac-signaling. COX-2 inhibitor (red) would block every prostanoid receptor type, but an EP2 antagonist (blue) could block only one out of eleven prostanoid receptors and may avoid COX-2 inhibition mediated side-effects.

Figure 2.

PGE₂ via EP2-receptor stimulates the differentiation of Th0 to Th17, which exacerbates the disease progression and severity in peripheral diseases such as arthritis and IBD. Additional EP2 signaling is shown in Figure 5

Figure 3.

EP2 receptors are involved in a variety of CNS and peripheral diseases. EP2 receptors shown to play a protective role in models of stroke, bone-fracture and glaucoma. But, mouse models of cancer, endometriosis, AD, PD, ALS, arthritis and IBD suggest EP2 promotes inflammatory cascade or exacerbates disease pathology. Thus EP2 may be targeted for discovery of therapeutics for these diseases.

EP2 Agonists/potentiators

EP2 Antagonists

Figure 4.
Structures of EP2 agonists, EP2 allosteric potentiator and EP2 antagonists

Figure 5.

EP2 mediates both beneficial and deleterious effects by G protein-coupled and G protein-independent downstream mechanisms. Upon PGE₂ binding, EP2 activates and dissociates G_s protein. Then G_s regulates activities of various effectors. Abbreviations used; PKA = protein kinase A, CREB = cAMP responsive binding element protein, PI3K = phosphatidylinositol-3-kinase, ERK = extracellular signal regulated kinase, JNK = c-Jun N-terminal kinase, EGFR = epidermal growth factor receptor.

Figure 6.

General structural scaffolds of EP2 antagonists reported in the patent literature. EP2 inhibition activity range from nanomolar to micromolar among the members in each class, is shown here for comparison purposes.

Figure 7.

Two potential therapeutic strategies that may be useful for EP2 receptor mediated effects in the brain for neuroprotection and for blocking the neurodegeneration

Table 1

Pharmacological properties of selected EP2 agonists

Entry	Compound name/ID	<u>EC₅₀ / IC₅₀ (nM)</u>		<u>Binding K_i (nM)</u>				
		EP2	IP	EP2	EP1	EP3	EP4	IP
1	PGE ₂	2.1	260	38	18	5	3.1	>10,000
2	Butaprost	33	37	2400	>10,000	>10,000	>10,000	>10,000
3	Butaprost free acid ^π	32	25	73	>10,000	>10,000	>10,000	870
4	Compound 4	3.8	NA	3.3	>10,000	>10,000	>10,000	10,000
5	ONO-AE1-259-01	1.8	NA	1.7	1300	2700	2300	10,000
6	AH-13205	NA	NA	240	NA	82	NA	NA
7	CP-533536	5	NA	50	>2800	>2800	>3200	>3200
8	CP-544326	2.8	NA	10	>3200	>3200	>3200	NA

^π An independent study indicates butaprost free acid (**3**) is only 17-fold selective to EP2 over EP3 receptors.¹⁰⁴ NA = data not available

Table 2Pharmacological properties of EP2 antagonists^a

Entry	Compound ID	K_i EP2 (nM)	Functional cAMP assays; Schild K_B (nM)					
			EP2	EP1	EP3	EP4	DP1	IP
11	AH-6809	1150	1150	1200	1600	>100,000	1400	>100,000
12	TG4-155	9.9	2.4	2100	10,000	>11,400	34.5	1320
13	TG6-10-1	210	17.8	1700	5300	17,200	167	8120
14	TG6-129	ND	8.8	ND	ND	3,900	14,600	199
15	PF-04418948	16	1.8	>100,000	>100,000	>10,000	>10,000	>10,000

^a K_i values are taken from binding assay. Schild K_B s are derived from a cAMP functional assay. For compound AH-6809 Schild K_B values are not available; so, K_i values are presented in the table to show the non-selectivity and poor EP2 activity