Selectively targeting prostanoid E (EP) receptor-mediated cell signalling pathways: implications for lung health and disease

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Running title: Targeting $EP_{1\text{-}4}$ receptors in respiratory disease

Abstract

Arachidonic acid is metabolized by cyclooxygenases (COX-1 and COX-2) into various prostanoids which exert different functions in mammalian physiology. One of these prostanoids, prostaglandin E_2 (PGE_2), interacts with four different G protein-coupled receptors, named EP_1, EP_2, EP_3 and EP_4, to initiate different downstream signaling pathways. Prostanoid receptors are diversely expressed throughout different tissues all over the body and PGE_2 is responsible for a large variety of beneficial and disadvantageous effects. We have recently achieved a greater understanding of the biology of prostanoid E receptors and the potential for specific drug targeting with the advent of potent and selective EP receptor agonists and antagonists. This has important implications for lung health and disease as PGE_2-mediated EP receptor activation impacts upon migration of airway smooth muscle cells, airway microvascular leak, tone regulation of pulmonary blood vessels, mast cell degranulation, bronchodilatation, cough, angiogenesis and airway inflammation, to name a few.

In this review, we overview the EP receptor family and the related signalling pathways, summarize a variety of $EP_{1\text{-}4}$ receptor agonists and antagonists, provide an overview of pharmacological tools used to implicate EP receptor function in the context of respiratory health and disease and finally highlight some of the more selective pharmacological reagents that have recently been developed. The availability of selective pharmacological agonists and antagonists for the distinct EP receptors, as well as the development of specific prostanoid receptor knock-out mice, offer hitherto unattainable opportunities for achieving an in depth understanding of the role and function of PGE_2 in respiratory disease and the exciting potential of targeting EP receptors more broadly.

Non-standard abbreviations: ASM, airway smooth muscle; BAL, bronchoalveolar; CHO, Chinese hamster ovary; COX, cyclooxygenase; EP, prostanoid E; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptors, GSK-3, glucogen synthase kinase-3; HMLC, human lung mast cells; K_i, binding affinity; IL, interleukin; IP_3, inositol triphosphate; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MKP-1, MAPK phosphatase 1; PGE_2, prostaglandin E_2; PKA, protein kinase A; PLC, phospholipase C; PI3K, phosphatidylinositiol 3-kinase; PRP, platelet-rich plasma; TCF, T-cell factor; TRP, Transient Receptor Potential; VEGF, vascular endothelial growth factor.

Introduction

Prostaglandin E_2 (PGE_2) is an important lipid mediator that activates four different prostanoid E (EP) receptors, termed EP_1, EP_2, EP_3, and EP_4. Along with other important prostanoids, including PGD_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and thromboxane A_2 (TxA_2), PGE_2 is synthesized from arachidonic acid by cyclooxygenase (COX) enzymes via the unstable intermediate forms PGG_2 and PGH_2 [1, 2]. $EP_{1\text{-}4}$ are G protein-coupled receptors (GPCR) that form part of prostanoid receptor family that include receptors for other endogenous prostanoid ligands, including $DP_{1\text{-}2}$ (for PGD_2), FP (for $PGF_{2\alpha}$), IP (for PGI_2) and TP (for TxA_2).

PGE_2 exhibits various functions in the human body which can be either beneficial or disadvantageous. With the relatively recent development of selective EP receptor antagonists and agonists it will be possible to examine ligand-mediated cell signalling pathways triggered via the EP receptors, well as the effects of the individual EP receptors more closely. This is especially important in the respiratory setting, where targeting the EP receptor system offers great potential as novel therapeutics to combat chronic respiratory diseases and lung conditions [3]. To date however, the EP receptor antagonists and agonists available have shown varying degrees of potency and selectively. Some of these have been used in the respiratory setting and offer a convenient pharmacological means to target EP receptors and demonstrate functional outcomes. However, these compounds do not always target the receptors they say they target. Often receptor identification has been incorrectly assigned based on the promiscuous nature of the agonist/antagonist.

Given that prostanoids are currently enjoying a renaissance, and that ligand-mediated cell signalling pathways triggered via EP receptors have broad implications in health and disease, it is important to have an understanding of the EP receptor family and to utilize selective

pharmacological agents. We address this herein and review the EP receptor family and the signalling pathways that are activated upon ligand binding (PGE_2 in particular). We will then provide a summary of some of the $EP_{1\text{-}4}$ receptor agonists and antagonists and offer an assessment of their selectivity. We will then focus on the respiratory setting and provide an overview of the agonists/antagonists used to implicate EP receptor function in the context of respiratory health and disease. Finally, we will highlight some of the more selective pharmacological reagents that recently been developed and underscore the promise of utilizing transgenic mice where a particular EP receptor has been knocked-out. These murine models are able to distinctly implicate a particular EP receptor in a precise manner. When coupled with validated experimental models of respiratory disease, these tools will allow researchers to more clearly define molecular mechanisms responsible for disease pathogenesis and enable improved treatments in the future.

I. Ligand-mediated cellular signalling via EP receptors

A. EP_1 receptor

1. Receptor

EP_1 was the first characterized EP receptor. Compared to other EP receptor subtypes, EP_1 is not widely distributed and its expression in animal tissue is relatively low, mostly occurring in kidney and lung tissue [1]. The human EP_1-receptor consists of 402 amino acids. In terms of amino acid sequence identity, the human EP_1 receptor is more closely related to the TXA_2 receptor, followed by EP_3, EP_2 and EP_4 [4]. The human EP_1 receptor is 86% homologous with the rat EP_1-receptor (rEP_1). In rats, Okuda-Ashitaka *et al.* [5] identified a variant: when the mRNAs of rEP_1 and of the rEP_1-variant were compared it became clear that the mRNA of the

rEP_1-variant is the unspliced form of the rEP_1-mRNA [5]. The mouse and rat EP_1 receptors share a sequence homology of 96% [5].

2. Signalling

One of the major signalling outcomes from engagement of PGE_2 with the EP_1 receptor is increased levels of intracellular Ca^{2+} [6]. When both receptors, rEP_1 and its variant, were expressed in Chinese hamster ovary (CHO) cells and the rEP_1-receptor activated, Ca^{2+} mobilization was significantly subdued. Co-expression of EP_4 and the rEP_1-variant in CHO cells led to a blockade of cAMP formation after activation of EP_4 [5]. These findings suggest that the rat EP_1 receptor variant is able to suppress the signalling of other receptors for PGE_2.

Although it is generally well accepted that EP_1 stimulation generates intracellular Ca^{2+} increase resulting in smooth muscle contraction, there are differing opinions about the coupling of the receptor, the activation of phospholipase C (PLC) and the hydrolysis of phosphatidylinositol [7]. Coleman *et al.* [1] stated that Ca^{2+} mobilization from intracellular stores is mediated via an inositol triphosphate (IP_3)-independent mechanism via EP_1. This was due to the fact that PGE_2-induced Ca^{2+} mobilization is not blocked by pertussis toxin and does not require extracellular Ca^{2+} [1]. Moreover, changes in cAMP were negligible in CHO-cells expressing recombinant murine EP_1 receptors. In contrast, Watabe *et al.* [8] showed that the increase of intracellular Ca^{2+} by engagement of the EP_1 receptor was completely abolished by removal of extracellular Ca^{2+}; therefore suggesting that the PGE_2- induced increase in Ca^{2+} is due to the entry of extracellular Ca^{2+} [8]. Additionally, Katoh *et al.* observed two different ways of increasing intracellular Ca^{2+}. Stimulation of mouse EP_1- receptor in cDNA-transfected CHO cells leads to an entry of extracellular Ca^{2+} inside the cell which again results in hydrolysis of phosphatidylinositol. This process is independent of PLC. There is also Ca^{2+}- mobilization from internal stores, but in

contrast to extracellular Ca^{2+}- influx, it is relatively weakly induced [9]. This process is sensitive to PLC inhibitors [10]. Protein kinase C affects the regulation of EP_1 signal transduction as it induces short- and long-term desensitization of this receptor. Further research is required to reconcile these cellular signalling differences.

There is also evidence that the EP_1 receptor also couples to $G_{q/11}$ and $G_{i/o}$. Tests with antisense oligodeoxynucleotides in the Xenopus oocyte expression system performed by Tabata *et al.* demonstrated that EP_1 receptors can induce Ca^{2+} mobilization via $G_{q/11}$ [10]. Ji *et al.* [7] showed that PGE_2 clearly stimulated intracellular IP_3 accumulation in HEK293 cells expressing the human EP_1 receptor via coupling to $G_{q/11}$. They also reported that the EP_1 receptor could couple to $G_{i/o}$ to activate multiple signalling pathways (ribosomal S6 kinases, phosphatidylinositol-3-kinase (PI3K), Akt and mammalian target of rapamycin) in a pertussin toxin-sensitive manner [7].

Moreover, it has been demonstrated that the interaction time between PGE_2 and the EP_1 receptor, compared to some of the other EP receptors, is relatively rapid. Association and dissociation of the ligand PGE_2 takes 30 seconds on EP_1, whereas association with and dissociation from EP_2 takes 10 and 5 minutes [9]. In EP_3 receptors these processes requires 10 minutes and 80 minutes, respectively [9].

B. EP_2 and EP_4 receptors

1. Receptors

EP_2 and EP_4 receptors will be discussed together in this section due to their similarities. Until 1995 the EP_4 receptor was unknown and only three forms of EP receptors had been described in the literature. The discovery of the EP_4 receptor was driven by a mistake, as the receptor first thought to be the EP_2 receptor was in fact the EP_4 receptor [11]. The true identity was soon to be

published and further investigations of this new receptor were performed [12-14]. EP_2 and EP_4 receptors differ in the length of the C-terminal sequence, as the EP_2 receptor has a rather short C-terminal sequence while the EP_4 receptor has a long C-terminal sequence [2]. EP_2 and EP_4 receptors show similar binding profiles and both mediate cellular signalling via adenylate cyclase activity resulting in increased levels of intracellular cAMP [15]. Although they demonstrate similarities in pharmacology and functional coupling, EP_2 and EP_4 receptors do not share high levels of amino acid identity: only ~38% homology in the transmembrane domain [16]. Phylogenetic analysis shows that the EP_2 receptor is more closely related to other relaxant prostanoid receptors, such as the IP and DP receptors [17], than the other EP receptors. Overall, there exists about ~30% identity with the other receptors, i.e. ~37% with EP_1 and ~ 34% with EP_3 [12, 16].

2. Signalling

Engagement of EP_2 and EP_4 receptors activate adenylate cyclase activity [1] resulting in cAMP increase [15]. When cAMP activates protein kinase A (PKA), the release of the PKA catalytic subunits allows them to phosphorylate a wide range of cellular targets, resulting in myriad cellular effects [1, 18]. Adenylate cyclase/cAMP/PKA signalling after PGE_2 stimulation of EP_2 and EP_4 receptors occurs in a G_s-coupled manner and can induce phosphorylation of the transcription factor, cAMP response element binding protein (CREB) at serine-133 resulting. This results in transcriptional regulation of genes containing promoters containing CRE consensus sequences. As EP_2 and EP_4 receptors are widely distributed throughout the body [19], receptor response to mediators is widespread: for example, relaxation in smooth muscle cells, non-acid secretion on epithelial cells and inhibition of mediator release in inflammatory cells, to name a few [1, 18].

But EP_2 and EP_4 receptors do show differences in their regulation following treatment with PGE_2. While the EP_4 receptor undergoes short term agonist-induced desensitization and internalization, the EP_2 receptor does not; although receptors show downregulation upon long-term exposure (>2 hour) to an agonist [20, 21]. Additionally, there are differences in the amount of cAMP produced upon stimulation. When receptors are stably expressed in HEK293 cells (at similar levels of receptor expression), the maximal amount of cAMP formation by stimulated EP_4 receptors is only 20 to 50% of that achieved amount by EP_2 stimulation [22, 23].

In addition to the well-recognized role played by adenylate cyclase in mediating the EP_2 and EP_4-mediated cellular signalling responses to ligands, other pathways have been reported. A notable example is the activation of T-cell factor (TCF) signalling via glucogen synthase kinase-3 (GSK-3), the enzyme responsible for phosphorylating and thus destabilizing β-catenin. GSK-3 can be inactivated by phosphorylation, this process can be induced by EP_2 and EP_4-mediated cellular signalling [22, 24, 25]. The common canonical signalling pathway starts with the Wnt ligand binding to the Frizzled receptor. Dishevelled is then recruited and results in the inhibition of the kinase activity of GSK-3. Active GSK-3 promotes the instability of β-catenin via phosphorylation and thus addition of ubiquitin, leading to degradation by the proteasome. When stable, β-catenin can interact with TCF DNA-binding factors to form a transcriptionally active complex that can alter gene expression. The involvement of EP_2 and EP_4 in this pathway was implicated by Fujino *et al.* [22] through utilization of TCF-responsive luciferase reporter genes in HEK293 cells transfected with human EP_2 and EP_4 receptors. They showed that activation of these receptors with the ligand PGE_2 can activate TCF signalling. Furthermore, stimulation of reporter genes with PGE_2 was aligned with phosphorylation of GSK-3, PI3K/Akt and activation of TCF signalling. Moreover, EP_2 receptor signalling occurred primarily through a PKA-

dependent pathway, while EP_4 receptors activate TCF signalling primarily by a PI3K-dependent pathway [22].

Additional studies provide further evidence linking stimulation of EP_4 receptors with the PI3K pathway. PGE_2 treatment leads to PI3K-dependant phosphorylation of extracellular signal-regulated kinase (ERK). This PI3K-dependent pathway can only be induced by EP_4 receptors, not EP_2 receptors [26]. As EP_2 and EP_4 receptors differ in their agonist-induced internalization and desensitization and since internalization of GPCRs has been described to be associated with a transactivation of the mitogen-activated protein kinase (MAPK) pathway there may be a link between the activation of ERK and the selective internalization [26-28].

As outlined earlier, phosphorylation of CREB was originally be shown to occur via the classical cAMP-dependent PKA pathway, but further experiments with pharmacological inhibitors proposed that PKA is not the only kinase with the ability of phosphorylating CREB after EP_2/EP_4 ligation. Phosphorylation of CREB was reported to linked to increased PI3K activity after activation of the ERKs and Akt signalling pathways [29-31]. Fujino *et al.* tested the phosphorylation of CREB after activation of EP_2 and EP_4 receptors and studied the activation of cAMP/PKA- and the PI3K-dependent signalling pathways. They showed that the mechanism of phosphorylating CREB is in EP_2-expressing cells mostly cAMP/PKA-driven, while the mechanism in EP_4-expressing cells involves a PI3K-dependent pathway. They further stated that stimulation of EP_4 receptors via PGE_2 negatively regulates the activity of PKA. Using H-89, a pharmacological inhibitor of PKA, it was shown that an inhibition of PKA decreases the phosphorylation of CREB in EP_2-expressing cells, but not in EP_4 [31]. However, Clarke *et al.* stated in 2005 [32] that H-89 is rather unsuitable as a tool for pharmacological research as it is quite non-selective. Instead, they transfected human airway smooth muscle (ASM) cells with an

adenovirus vector containing the PKA inhibitor, PKIα [32] and showed that EP_2 and EP_4 signalling occurred via the PKA-pathway, not via the PI3K pathway [32]. These data question the involvement of the PI3K pathways in ligand-mediated cellular signalling via EP2/EP_4 receptors and further investigations are warranted.

C. EP_3 receptor

1. Receptor

Amongst all the EP receptor family members, the EP_3 receptor is the only one known to express various isoforms [1]. It is understood that cows have four EP_3- receptor isoforms called EP_{3A}, EP_{3B}, EP_{3C} and EP_{3D} [33]. The bovine EP_{3D} is homologous to the human EP_{3D} whereas the human EP_{3A} receptor is a homolog of the mouse $EP_{3\alpha}$ isoform [4]. Additionally, there are two more isoforms known in mice which are $EP_{3\beta}$ and $EP_{3\gamma}$ [33]. Rabbits express five isoforms named $EP_{3\text{-}72A}$, $EP_{3\text{-}74A}$, $EP_{3\text{-}77A}$, $EP_{3\text{-}80A}$ and $EP_{3\text{-}NT}$ [34]. According to Israel and Regan [35], there have been ten different mRNA splicing variants identified in humans, resulting in eight different EP_3 isoforms, named $EP_{3\text{-}I}$, $EP_{3\text{-}II}$, $EP_{3\text{-}III}$, $EP_{3\text{-}IV}$, $EP_{3\text{-}V}$, $EP_{3\text{-}VI}$, $EP_{3\text{-}e}$ and $EP_{3\text{-}f}$ [36]. Among the isoform $EP_{3\text{-}I}$ there are three different splice variants which vary in the 3′-untranslated region, termed $EP_{3\text{-}Ia}$, $EP_{3\text{-}Ib}$ and $EP_{3\text{-}Ic}$. The nomenclature of the EP_3 isoforms is not uniform and different publications have sometimes used different terms for the same isoform. Table 1 attempts to harmonize the nomenclature for the various EP_3 receptor isoforms.

Table 1. Human EP_3 receptor isoform nomenclature

Carboxyl-terminal domains [2], [37]	Nomenclature according to Kotani *et al.* [38] [37]	Nomenclature according to Schmid *et al.* [39]	Nomenclature according to Regan *et al.* [4]

IRYHTNNYASSSTSLPC QCSSTLMWSDHLER	$EP_{3\text{-}I}$	EP_{3a}	EP_{3A}
VANAVSSCSNDGQKGQ PISLSNEIIQTEA	$EP_{3\text{-}II}$	EP_{3c}	EP_{3D}
EEFWGN	$EP_{3\text{-}III}$	EP_{3b}	EP_{3E}
MRKRRLREQEEFWGN	$EP_{3\text{-}IV}$	EP_{3d}	EP_{3C} [35]
MRKRRLREQEMGPDGR CFCHAWRQVPRTWCSS HDREPCSVQLS	$EP_{3\text{-}V}$		
EMGPDGRCFCHAWRQ VPRTWCSSHDREPCSV QLS	$EP_{3\text{-}VI}$		
MRKRRLREQLICSLRTL RYRGQLHIVGKYKPIVC	EP_{3e}	EP_{3e}	
MRKRRLREQAPLLPTPT VIDPSRFCAQPFRWFLD LSFPAMSSSHPQLPLTL ASFKLLREPCSVQLS	EP_{3f}	EP_{3f}	

As detailed in Table 1, the EP_3 isoforms differ in the amino acid sequence of the C-terminal region. Across the seven transmembrane helices of all EP_3 isoforms, the first 359 amino acids are identical. However the carboxyl terminal of each isoform differs, being 6 to 65 amino acids long [40]. Thus, as a consequence of different mRNA splicing variants, the resulting protein isoforms have distinct signal transduction pathways [39].

2. Signalling

In humans, the EP_3 receptor isoforms can be G_i-, G_s-, G_q- or $G_{12/13}$-coupled [35], with the main signalling pathway for EP_3 considered to occur in a G_i-mediated manner [41]. Because of this, ligand engagement of the EP_3 receptor (via either the endogenous ligand PGE_2 or other EP_3-agonists) can result in a myriad of intracellular signalling pathways: some of these will be summarized below.

One of the major outcomes of activation of the EP_3 receptor is inhibition of adenylate cyclase and reduction of cAMP levels via G_i and enhancement of intracellular Ca^{2+} [1, 41]. Schmid *et al.* [39] also reports that EP_{3a}, EP_{3b} and EP_{3c} enhance Ca^{2+} concentration and EP_{3d}, EP_{3e} and EP_{3f} reduce cAMP via G_i.

The MAPK pathway can also be activated via EP_3 receptor isoforms. In cells transfected with human EP_{3A}, PGE_2 was shown to stimulate MAPK activity dose-dependently in a manner linked to G_i-protein activation [42]. The $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$ receptors (expressed in HEK293 cells) can also trigger a MAPK-dependent transcriptional activation, although different signalling intermediaries are involved [35]. However, in contrast to the central role played by MAPK after activation of $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$, PGE_2 activation of $EP_{3\text{-}Ia}$ caused a negligible phosphorylation of ERK [35].

Other second messengers have also been shown to be involved in EP_3 activation: i.e. engagement of EP_{3A1}, EP_{3C} and EP_{3D} in primary human keratinocytes leads to an increase in diacylglycerol and ceramide production [43]. EP_3 receptors have also been shown to be constitutively active although isoform specific differences exist: while $EP_{3\text{-}I}$ and $EP_{3\text{-}II}$ are not significantly constitutively active, $EP_{3\text{-}III}$ and $EP_{3\text{-}IV}$ do exhibit constitutive activity [40].

Small changes in amino acid sequence have also been shown to result in changes to signal transduction. For example, activation of the $EP_{3\text{-}V}$ and $EP_{3\text{-}VI}$ receptors (mainly detected in human uterus) results in production of cAMP via $EP_{3\text{-}VI}$, but not via $EP_{3\text{-}V}$ receptor [36]. Interestingly, both receptors feature nearly the same amino acid sequence with the exception of nine amino acid residues [36] – see Table 1. The different carboxy termini of the EP_3 isoforms have also an impact on the respective receptor's internalization in response to PGE_2. Furthermore, the isoforms are internalized by different mechanisms. Under influence of PGE_2, the $EP_{3\text{-}I}$ receptor is internalized most rapidly and completely whereas $EP_{3\text{-}II}$, $EP_{3\text{-}V}$, $EP_{3\text{-}VI}$ and EP_{3f} are only internalized to a certain extent. Moreover, $EP_{3\text{-}II}$ is internalized independently of β-arrestin. Usually, β-arrestin binds to phosphorylated serine or threonine in the C-termimal tails. An explanation for why the $EP_{3\text{-}III}$ and $EP_{3\text{-}IV}$ isoform are not internalized could be due to their comparatively short carboxy termini and the absence of the amino acids serine and threonine [36]. The consequence of different rates of receptor internalization impacts on the physiological functions of the various members of the EP_3 receptor isoform family [37].

II. Selective and non-selective EP agonists and antagonists

The design of potent and selective agonists and antagonists are essential research tools in prostanoid biology. For many years now, classification of the prostanoid receptors has been based mainly on the pharmacological actions of agonists and antagonists. However, in some cases, the selectivity of the pharmacological agents may have been limited. The following sections will summarize some of the commonly used agonists and antagonists for each member of the EP receptor family and provide an assessment of their selectivity.

A. EP_1 receptor agonists and antagonists

1. EP_1 agonist: ONO-DI-004

ONO-DI-004 [(17S)-2,5-ethano-6-oxo-17,20-dimethyl PGE_1] from Ono Pharmaceuticals is a derivate of PGE_1 and a selective EP_1 agonist [44]. In CHO cells expressing EP receptor subtypes [45], the K_i values have been shown to be 0.15 μM for the EP_1 receptor and >10 μM for other EP receptor subtypes. However, ONO-DI-004 appears to have only moderate affinity with the receptor, and its agonist effects have been demonstrated to be dependent on species and tissues.

2. EP_1 antagonist: ONO-8711

ONO-8711 is a competitive antagonist at EP_1 receptors that has been shown to inhibit PGE_2-induced increases in cytosolic Ca^{2+} concentration with inhibitory concentrations of 0.21 μM for mouse EP_1 and 0.05 μM for human EP_1 receptors [46]. In terms of pharmacological profile, ONO-8711 has K_i values of 1.7 nM at mouse EP_1 and 0.6 nM at human EP_1 receptors expressed in CHO cells, while K_i values of 67 nM for mouse EP_3 and 76 nM for human TP receptors were reported. K_i values for other prostanoid receptors were >1,000 nM [46].

3. EP_1 antagonist: ONO-8713

ONO-8713 is a selective EP_1 antagonist that is similar to ONO-8711 but with greater selectivity for the EP_1 receptor. Tested in CHO cells stably expressing different EP receptor subtypes, ONO-8713 has K_i values of 0.3 nM for both the human and mouse EP_1 receptor and >1,000 nM for all other prostanoid receptors. This is in comparison to ONO-8711, where the K_i value for mouse and human EP_1 receptor is 1.7 and 0.6 nM, respectively, and 67 nM and 76 nM for mouse EP_3 and human TP receptor, respectively [46, 47]. Thus, ONO-8713 is more selective and potent than ONO-8711.

4. EP_1 antagonist: GW848687X

GW848687X is a potent and selective EP_1 receptor antagonist developed by GSK. Due to its favourable profile in models of inflammatory pain it was selected as development candidate [48]. It is a competitive antagonist at the EP_1 receptor with a pA_2 of 9.1 and a selectivity >400-fold for EP_1 receptor to other EP receptor subtypes, DP and IP receptors. It has 30-fold selectivity over the TP receptor, while not characterized at the FP receptor [48].

5. EP_1 antagonist: SC-51089

SC-51089 is an acylhydrazide developed by the Searle group that was considered to be a potential clinical candidate as an analgesic but was subsequently dropped from the development pipeline when issues around its metabolism appeared, i.e. release of hydrazine [49]. In 293E/CRE-SEAP reporter cells expressing human EP_1 receptor, SC-51089 had a pK_B value of 6.9 (K_B of 115 nM) but no activity at the DP receptor [50]. Abramovitz *et al.* [51] utilized radioligand binding assays in HEK293 cells expressing EP receptors and found K_i values of 1,332±187 nM for EP_1, 17,500 ±6,122 nM for $EP_{3\text{-III}}$, 1,175 nM for TP and >100,000 nM for other prostanoid receptors [51]. These results are approximately one order of magnitude lower than that determined by Durocher *et al.* [50].

6. EP_1 antagonist: SC-51322

SC-51322 is the most potent of the Searle series of EP_1 receptor antagonists [52] with pK_B values of 8.8 (K_B of 1.60 nM) in 293E/CRE-SEAP cell reporter assays [50]) and a K_i value of 13.8 nM in the radioligand binding assay [51]. These are compared to K_i values of 698±122 nM for $EP_{3\text{-III}}$ and 507±47 nM for TP receptors, with K_i for other prostanoid receptors >10,000 nM. These data show that SC-51322 has high affinity for EP_1 with a selectivity of 50-fold over EP_3 and TP, and essentially no activity at the other prostanoid receptors.

7. EP_1 antagonist: SC-19220

SC-19220 is a dibenzoxazepine hydrazide derivative and one of the oldest prostanoid receptor antagonists. In 1971 Sanner *et al.* described the inhibitory effect of SC-19220 on PGE_2-induced contraction of guinea pig isolated ileum [53]. At doses between 0.3 and 300 μM, SC-19220 acted as a competitive antagonist in isolated guinea pig gastric muscle [54]. SC-19220 was also shown to antagonize PGE_2-induced contractions mediated by the EP_1 receptor in guinea pig trachea [55]. In a radioligand binding assay in cells expressing the human EP_1 receptor [6], SC-19220 was shown to compete for [^{3}H]-PGE_2-specific binding with an IC_{50} value of 6.7 μM. SC-19220 has no binding affinity to human EP_2 receptors expressed under the same conditions [11]. However, SC-19220 has no affinity for the mouse EP_1 receptor, suggesting that there are some species differences [15].

B. EP_2 receptor agonists and antagonists

There are two main classes of EP_2 receptor agonists available: those molecules that are structurally related to the endogenous ligand PGE_2 (see 1-4) and the pyridyl-sulfonamines (see 5 & 6). While EP_2 receptor agonists have been available for some time, more selective and potent antagonists for the EP_2 receptor have emerged recently.

1. EP_2 agonist: Butaprost

Butaprost is one of the older synthetic EP_2 receptor agonists, being less potent than the native PGE_2 with a K_i of 2,400 nM [56]. In terms of selectivity, the K_i for $EP_{1,3}$ and EP_4 are higher than 10,000 nM [56]. The ester group of butaprost is rather unstable and the free acid form metabolite is more potent (with an EP_2 receptor K_i of 73); but butaprost does activate IP receptors as well [57]. The free acid and the methyl ester forms of butaprost have been shown to be 2- and 30-fold less potent than the natural ligand PGE_2 at the EP_2 receptor [56].

2. EP_2 agonist: 9b-Chloro-16-hydroxy-17,17-trimethylene-ω-nor-PGF_2

The 16-hydroxy-17,17-trimethylene 9beta-chloro PGF_2 derivative, a product of Ono pharmaceuticals, has been reported to be a potent EP_2 receptor agonist with K_i for the EP_2 receptor of 3.3 nM [58]. It is specific for EP_2 receptors and shows nearly the same potency as PGE_2 in functional assays [58, 59].

3. EP_2 agonist: ONO-AE1-259

ONO-AE-259 shows a high binding affinity for EP_2 with K_i of 1.7 nM, a 22-fold higher affinity than PGE_2 for the EP_2 receptor. It also shows 700-1,500-fold selectivity for EP_2 over other prostanoid receptors, namely $EP_{1, 3, 4}$ and IP [58, 59]. This specific agonist is often used to define EP_2 receptor-mediated effects experimentally in *in vitro*, *ex vivo* and *in vivo* models. For example *in vitro*, ONO-AE1-259 can increase cytosolic cAMP levels in human ASM cells and significantly inhibit ASM cell migration [60].

4. EP_2 agonist: AH13205

AH13205 is a relatively non-selective EP_2 receptor agonist with a comparatively low potency. Tani *et al.* published the following K_i values: EP_1 receptor 2,800 nM, EP_2 receptor 320 nM, EP_3 receptor 49 nM, EP_4 receptor 2,200 nM [58]. Early studies by Nials *et al.* in 1993 [61] showed that AH13205 relaxes airway smooth muscle from cats and guinea pigs *in vitro* and works as a potent bronchodilator in guinea pigs on inhalation. Despite its promising preclinical profile it did not appear to have any bronchodilator activity in humans [61] and is largely superseded by other agonists.

5. EP_2 agonist: CP-533536

CP-533536 is a highly potent and selective EP_2 agonist with a high binding affinity for the EP_2 receptor (K_i of 50 nM) and good selectivity, i.e. CP533536 is 16-fold higher selective for EP_2 than DP and 50 to 60-fold higher selective for EP_2 to $EP_{1, 3, 4}$ and IP receptors..

6. EP_2 agonist: CP-544326

CP-544326 is at least 270 times more selective for human EP_2 receptors, compared to other human EP receptors: i.e. receptor affinity to EP_2 is 10 nM, while the affinity to $EP_{1,3}$ and EP_4 is higher than 3,200 nM [62]. In rat and human cells expressing EP_2, CP-544326-mediated cAMP production with average EC_{50} of 1.9 nM and 2.8 nM, respectively [62]. This was similar to that observed for PGE_2 (EC_{50}=2.6 nM) [62].

7. EP_2 antagonist: AH-6809

AH-6809 is one of the longest used EP receptor antagonists. AH-6809, previously considered as an EP_1 antagonist, was proven to have affinity to EP_2 as well [63]. It was characterized as being non-selective, having similar affinity for EP_2, EP_3 and DP [51]. Abramovitz *et al.* [51] demonstrated the following binding affinities for AH-6809 in assays using HEK293 cells expressing EP receptors: K_i (EP_1) 1,217±98 nM, K_i (EP_2) 1,150±36 nM, K_i (EP_3) 1,597±140 nM and K_i (DP) 1,415±104 nM. Despite the relatively weak antagonistic activity and a rather non-selective profile, AH-6809 is commercially available and has been commonly used for probing EP_2 receptor function, due to unavailability of other selective EP_2 antagonists for some time.

8. EP_2 antagonist: PF-04418948

PF-04418948 is azeditine-carboxylic acid derivative and selective EP_2 antagonist developed by Pfizer. It has performed well in EP_2 antagonist affinity and potency experiments in cell systems and *in vivo* analysis in rodent models [64]. Investigators reported that PF-04418948 inhibited

PGE_2-induced increase in cAMP in cells expressing EP_2 receptors with a functional K_b value of 1.8 nM and also reversed PGE_2-induced relaxation in the mouse trachea with an IC_{50} of 2.7 nM [64]. Given orally, PF-04418948 attenuated the butaprost-induced cutaneous blood flow response in rats [64]. PF-04418948 is a EP_2 selective antagonist (IC_{50} (EP_2) is 16 nM while IC_{50} ($EP_{1, 3, 4}$, IP, DP) >10,000) [64] and the compound has little activity against a diverse panel of GPCRs and ion channels at concentrations much higher than needed to antagonize EP_2 receptors [65]. Birrell *et al.* [66] have also conducted further investigations that demonstrate the selectivity profile of PF-04418948 using functional bioassay systems. They tested PF-04418948 in a range of tissue-based assays to confirm selectivity against the native EP_2 receptor: testing the EP_2 receptor in mouse and guinea pig isolated tracheal tissue, EP_1-mediated responses of guinea pig isolated tracheal tissue, EP_3 receptor-mediated responses of guinea pig isolated vagal tissue and EP_4 receptor-mediated contraction of rat and human isolated tracheal tissue [66]. They showed that PF-04418948 only acted as an antagonist in EP_2-mediated events, competitively inhibited relaxations of murine and guinea pig trachea induced by the EP_2 agonist ONO-AE1-259 and the endogenous ligand PGE_2. Notably, the affinity of the compound was not equal in the two preparations, the guinea pig trachea showed atypically low affinity compared to the mouse. The authors interpret the data as a question whether the guinea pig trachea is a usable EP_2 receptor assay system [66]. In summary, PF-04418948 may be the long needed tool for further discoveries about the EP_2 receptor, as research about this subtype of EP receptor was hampered due to the lack of a specific antagonist.

9. EP_2 antagonist: TG6-129, TG4-155, TG6-10-1

Emory University has published a novel class of EP_2 antagonists, structurally distinct from PF-04418948. TG4-155 has an EP_2 Schild K_B of 2.4 nM and displays selectivity for EP_2 over EP_1,

EP_3, EP_4 and IP (550-4750-fold) but only 14-fold selectivity against the DP_1 receptor. TG6-10-1 has an EP_2 Schild K_B of 17.8 nM and shows a 300-fold selectivity against EP_3, EP_4 and IP receptors, but only 100-fold selectivity against EP_1, FP and TP receptors and only 10-fold selectivity against DP_1 receptor [59, 67]. TG6-129 was developed by Emory University as another lead component for another class of EP_2 antagonists. The compound shows nanomolecular potency and competitive antagonism for the EP_2 receptor. Unlike TG4-155 and TG6-10-1, TG6-129 has shown high selectivity against DP_1 (1660-fold) and EP_4 receptors (440-fold), but only 22-fold selectivity against the IP receptor [59, 68]. Although the selectivity of these compounds is not as high as PF-04418948, these compounds provide another option to further research about EP_2 receptor and its role in health and disease.

C. EP_3 receptor agonists and antagonists

Given that the EP3 receptor has a number of isoforms, many of the pharmacological studies designed to determine the selectivity and potency of agonists and antagonists against the EP3 receptors have also explored the relative efficacy against some of the important isoforms.

1. EP_3 agonist: Sulprostone

Sulprostone (16-phenoxy PGE_2) is a synthetic selective EP_1 and EP_3 agonist [69]. In radioligand binding assays, performed in HEK293 cells expressing EP receptors, Sulprostone has K_i values of 0.35±0.11 nM for $EP_{3\text{-III}}$, 198±10 nM for FP, 107±15 nM for EP_1 and >7,740 nM for EP_4, EP_2, DP, IP and TP [51]. Okada *et al.* [70] examined the contractile actions of prostanoid analogues on longitudinal smooth muscles of the murine gastric fundus and ileum, where tensions induced by sulprostone (10 μM) were 126±5 and 95±5% respectively. These values were significantly greater than those induced by another EP_3 agonist (ONO-AE-248: see 3 below), leading the authors to suggest that sulprostone may act on other receptors in addition to EP_3 [70].

2. EP_3 agonist: Misoprostol

Misoprostol is a methyl ester analog of PGE_1. Misoprostol (methyl ester) showed K_i values of 10,249 ±1343 nM at EP_2, 319±15 nM at EP_3, 5,499±1102 nM at EP_4 and 35,675±9,577 nM at EP_1 receptors, while misoprostol (free acid) has K_i values of 34±5 nM at EP_2, 7.9±1.0 nM at EP_3, 23±2 nM at EP_4 and >10,000 nM at EP_1 receptors: measured in radioligand binding assays, performed in HEK293 cells expressing EP receptors [51]. The de-esterification of misoprostol (methyl ester) produces drastic changes in the pharmacological profile with changes in affinity and selectivity for EP receptors: affinity changes of at least 350-fold at EP_2, 40-fold at EP_3 and 250-fold at EP_4 receptors; modified selectivity from EP_3 preferable to equipotent at EP_2, EP_3 and EP_4 [51].

3. EP_3 agonist: ONO-AE-248

ONO-AE-248 is a selective EP_3 agonist with close structural similarities to PGE_2, being O-dimethylated at position 11 and 15 [11,15-*O*-dimethyl-PGE_2]. Binding affinities of ONO-AE-248 were tested on CHO cells transfected with mouse EP-receptors 1-4 and have shown a K_i value of 15 nM for the mouse EP_3 receptor. The K_i values for other mouse EP-receptors are >3,700 nM, confirming that ONO-AE-248 is specific for EP_3. Furthermore, the agonistic activity of ONO-AE-248 at the EP_3-receptor is 5.2 nM whereas it is >10,000 nM for the other three EP-receptors [44]. ONO-AE-248 induced dose-dependent contractions in human pulmonary artery rings with pEC_{50} value of 6.34±0.35 [44]. In contrast, in human pulmonary veins, ONE-AE-248 did not cause any contraction; this was considered due to the absence or presence of EP_3 receptors in the selected tissue [44]. *In vitro*, ASM cell migration, assessed by a chemotaxis assay, showed that ONO-AE-248 acts as a modest, but significant chemoattractant and enhancer of baseline cell migration [60].

4. EP_3 agonist: M&B28767

M&B28767 is a relatively non-selective agonist for EP_3. It shows best binding affinities to the human EP_3 and EP_4 receptors, but also binds to the other two EP receptors as well. K_i (EP_1)=508 nM, K_i (EP_2)=1,370 nM, K_i (EP_3)=0.3, K_i (EP_4)=24 nM [71]. *In vitro*, Kiriyama *et al*. performed radioligand binding assays in CHO cells expressing mouse EP receptors, showing K_i values of 0.68 nM for mEP_3 receptor, while 120 nM and 124 nM for mEP_1 and mFP receptors, respectively [15]. Perhaps M&B28767 demonstrates greater utility as a mEP_3 receptor agonist? Accordingly, M&B28767 and sulprostone were compared in an experimental model examining morphine withdrawal 'jumps' caused by naloxone in morphine-dependant mice. M&B2767 produced an inhibitory effect on morphine withdrawal jumps at lower doses than sulprostone [72]. M&B28767 can also exert differential effects on EP_3 isoforms: M&B28767 was shown to increase basal cAMP level and inhibited the forskolin-induced increase in the cAMP level induced via $EP_{3\gamma}$ stimulation, while and decreasing both the basal and forskolin-elevated cAMP level induced through $EP_{3\alpha}$ and $EP_{3\beta}$ [73].

5. EP_3 agonist: TEI-3356

TEI-3356 is an isocarbacyclin analogue and a selective antagonist for EP_3 receptor. Pharmacological characterizations of TEI-3356 was performed with [^{3}H]-PGE_2 binding assays to test receptor affinity in CHO cells stably expressing EP receptors: TEI-3356 shows IC_{50} values of 33 nM for EP_1, 450 nM for EP_2 and 0.1 nM for EP_3 on displacement of [^{3}H]-PGE_2 binding and 90 nM for IP on displacement of [^{3}H]-iloprost binding [74]. TEI-3356 (IC_{50}= 0.01 µM) is stated as being a less potent EP_3 agonist than M&B28767 (IC_{50}= 0.001 µM), but showing higher selectivity for EP_3 than M&B28767 [74].

6. EP_3 agonist: GR63799X

GR63799X was demonstrated in a radioligand binding assays performed in CHO cells expressing mouse EP receptors to have high selectivity for the mouse EP_3 receptor with K_i values of 1.9 nM for mEP_3 and 480 nM for the mEP_4 receptors [15].

7. EP_3 antagonist: ONO-AE3-240

ONO-AE3-240 was developed by Ono Pharmaceuticals as a selective EP_3 antagonist. Binding affinities were tested with radioligand competition-binding assays and Amano *et al.* have published Ki values for EP_1 (590 nM), EP_3 (0.23 nM), EP_4 (58 nM), FP (1,500 nM) and >10,000 nM for EP_2, DP, TP and IP [75]. This selective antagonist has been used to implicate a role for EP_3 receptor-mediated signalling in tumor development and angiogenesis. This was demonstrated in mice, where subcutaneously injected dilutions of ONO-AE3-240 inhibited tumour growth and angiogenesis in a sarcoma model typified by new vessel growth [75].

8. EP_3 antagonist: L-826266

L-826266 is a selective EP_3 antagonist from Merck Frosst, Canada. Maher *et al.* have shown that PGE_2 mediates cough via the EP_3 receptor using a range of *in vitro* and *in vivo* techniques utilizing the EP_3 receptor antagonist L-826266 [76]. *In vivo* studies showed that L826266 attenuated the depolarization to PGE_2 in the isolated guinea pig vagus nerve and significantly inhibited the PGE_2-induced depolarization of mouse vagus nerve (64.8±2.8%), while also showing similar effects in human vagus nerve [76]. Guinea pigs were exposed to an aerosol of PGE_2 in *in vivo* studies, where the use of EP_3 antagonist L826266 significantly decreased the number of coughs in response to aerosolized PGE_2 [76]. However, L-826266 is unable to distinguish between the EP_3 receptor splice variants, according to Oliva *et al.* [77].

9. EP_3 antagonist: CM9

Binding studies performed with CM9 in a wide range of cells, including human osteosarcoma cells expressing human EP_{3C} and EP_{3E} and DP receptors, rat EP_{3A} and EP_{3C} receptors as well as CHO cells expressing hEP_{3A}, hEP_1, hEP_2 and hTP receptors and HEK293 cells expressing hEP_4, hFP and hIP receptors. Collectively, these data demonstrate that CM9 shows K_i values of 0.21±0.17 nM in hEP_{3A}, 0.24 nM in hEP_{3E}, 0.25±0.03 nM in rEP_{3A}, 0.78±0.17 in rEP_{3C} and 570 nM in hTP. K_i values of CM9 on the other prostanoid receptors are >2,390 nM [78]. These data shows that CM9 is a suitable tool for the evaluation of a broad range of EP_3 receptor isoforms.

10. EP_3 antagonist: DG-041

DG-041 is selective EP_3 antagonists developed by deCODE Chemistry to serve as a novel antiplatelet agent, as it shows significant antiplatelet activity with only minimal impact on bleeding time. *In vitro* pharmacology was published by Singh *et al.* [79]: DG-041 shows good selectivity in radioligand [^{3}H]-PGE_2 displacement binding assay with IC_{50} values of 4.6 nM for EP_3, 4,169 nM for EP_2, 8,039 nM for EP_4, 14,414 nM for IP and >20,000 nM for EP_1. Thus, DG-041 shows more than 1,000-fold selectivity for the EP_4 receptor over other prostanoid receptors [79].

D. EP_4 receptor agonists and antagonists

The discovery and utility of selective EP_4 agonists and antagonists has advanced since 2000, when Abravotiz *et al.* stated “there are no selective agonists or antagonists for EP_4…” [51]. Today, there are many selective EP_4 agonists and antagonists commercially available and these will be summarized below.

1. EP_4 agonist: TCS2510

TCS2510 was developed in the early 2000's by the Merck Frosst as a chemically modified analogue of PGE_2 containing a lactam and a tetrazole structure [80]. Billot *et al.* [80] measured potency and selectivity over other prostanoid receptors in HEK293 transfectants and measured binding properties in cell cAMP assays. The K_i of TCS2510 for the EP_4 receptor was 1.2±0.2 nM, while the K_i to the other prostanoid receptors was >13,000 nM. TCS2510 is a full agonist in the cell efficacy assay with an EC_{50} of 2.5±1.0 nM, and this was comparable to the EC_{50} of PGE_2 itself (EC_{50}=3.0± 0.4 nM) [80].

2. EP_4 agonist: Compound 9b

Serono Research Institute published and tested analogs of PGE_2 with introduction of diene groups at the ω-side chain [81]. Using receptor binding assays on membranes prepared from HEK293 cells expressing $EP_{1\text{-}4}$ receptors, they identified compound 9b as having potency for the EP_4 receptor (K_i= 4 nM, EC_{50}= 0.1 nM), but also affinity to the EP_2 receptor (K_i=60 nM, EC_{50}=35 nM). Compound 9b had low affinity for EP_1 and EP_3 receptors (both K_i> 10,000 nM)[81].

3. EP_4 agonist: ONO-AE1-734

Competitive radioligand binding assays performed by Kabashima *et al.* [82] measured the *K*i values for ONO-AE1-734 as 0.7 nM for EP_4, 56 nM for EP_3, and 620 nM for EP_2. The rest of the prostanoid receptors have *K*i more than 10,000 nM [82].

4. EP_4 agonist: ONO-AE1-329

ONO-AE1-329 is selective agonist of the EP_4 receptor that is widely used in research. In 2000, Suzawa *et al.* tested binding affinities of different EP receptors expressed in CHO cells and reported: K_i (EP_1)>10 μM, K_i (EP_2)=2.1 μM, K_i (EP_3)=1.2 μM, K_i (EP_4)=0.0097 μM [45]. A

number of the experimental models have examined the efficacy of ONO-AE1-329 as a relaxant, but species differences exist. ONO-AE1-329 caused relaxation of human airway muscle (76.2±8.6% of maximum relaxation to 100 mM papaverine) with a pK_i for the EP_4 receptor of 8.0 [83]. Benyahia *et al.* showed similar effects of ONO-AE1-329; it induced potent relaxation of human bronchial preparations (pEC_{50}=7.0±0.3) and vasodilatation in human pulmonary veins [84]. The compound was also able to cause substantial relaxation of rat tracheal strips (36.7±4.0% of maximum relaxation to 100 μM papaverine) [83]. Notably, EP receptor-mediated relaxation of human airway muscle differs compared to the animal models. While bronchial relaxation in guinea pigs, mice and monkeys relies more on EP_2-receptor signals, while human and rat bronchial relaxation is mediated via EP_4 receptor [83]. ONO-AE1-329 has also been used on cells: Luschnig-Schratl *et al.* have shown that ligation of EP_4 receptors can inhibit eosinophil function [85], while Aso *et al.* have demonstrated that it enhanced release of IL-8 by human pulmonary microvascular endothelial cells via EP_4 [86].

5. EP_4 agonist: L-902688

L-902688 is a selective EP_4 agonist that was shown to relax human bronchial muscle (pEC_{50}= 7.8±0.2) and induce dilatation of human pulmonary vein preparations [84]. Used in experiments by Foudi *et al.* [87] L-902688 induced potent vasodilatation of the human pulmonary vein in manner similar to that of the native ligand PGE_2 (pEC_{50}=8.1± 0.1 compared to pEC_{50}=7.2±0.2, respectively). The introduction of a 6-tetrazole ring to the molecule prevents β-oxidation [88].

6. EP_4 antagonist: ONO-AE3-208

ONO-AE3-208 has *K*i values obtained by a competitive radioligand binding assay of: 1.3 nM for EP_4, 30 nM for EP_3, 790 nM for FP, 2.400 nM for TP and more than 10,000 nM for the other prostanoid receptors [82]. It has been shown to be an effective EP_4 antagonist in functional

assays as ONO-AE4-208 was able to inhibit relaxation of human airway muscle caused by PGE_2 and the EP_4 agonist ONO-AE1-329 (pA_2=8.1±0.2 and 8.8±0.3, respectively) [83].

7. EP_4 antagonist: GW627368X

GW627368X is a benzene sulphonamide developed by GSK and characterized as a potent and selective antagonist of EP_4 receptors with additional human TP receptor affinity [89]. The compound is reported to be without any agonist or antagonist activity for prostanoid EP_2, EP_3, IP and FP receptors, and while binding to human prostanoid TP receptors, but not to the TP receptors of other species.

8. EP_4 antagonist: AH23848

AH23848 has been widely used as an EP_4 antagonist and played an essential role in the early pharmacological definition of the EP_4 receptor. In competitive binding assays performed in 2000, it was shown to have a K_i of 2,690±232 nM for EP_4 [90]. More recently it was confirmed to have a comparatively low affinity antagonist at both the EP_4 and TP receptors (pK_i 4.9-5.6 and 6.2, respectively) [83]. Although it was the prototype of EP_4 antagonist its use has been largely overtaken by more selective and potent antagonists [88].

9. EP_4 antagonist: CJ-023423

CJ-023,423 is a potent and selective prostaglandin EP_4 receptor antagonist with antihyperalgesic properties from Pfizer. In radioligand binding studies using membranes prepared from HEK293 cells stably expressing human EP_4 or rat EP_4 receptors, CJ-023,423 displaced [^{3}H]-PGE_2 binding with an IC_{50} value of 14±4 nM and 27±1 nM , respectively [91]. CJ-023,423 was 200-fold more selective for the human EP_4 receptor than other prostanoid receptors, showing IC_{50} >20 μM for $EP_{1,2,3}$, FP, IP and TP and IC_{50} of 4.3 μM for DP [91]. These results suggest that CJ-023,423 is a

competitive antagonist for human and rat EP_4 receptors and in *in vivo*, orally given doses of CJ-023,423 reduced thermal hyperalgesia induced by intraplanar injection of PGE_2 [91].

10. EP_4 antagonist: CJ-042794

Pfizer's CJ-042794 could be seen as an improvement on CJ-023423 based on potency. In *in vitro* pharmacological characterization utilizing binding assays in membrane samples of HEK293 cells overexpressing human EP receptors CJ-042794 displaced [^{3}H]-PGE_2 binding to each receptor EP_1, EP_2, EP_3, and EP_4 at 5.0 nM, 5.5 nM, 2.8 nM, and 0.4 nM, respectively, and the binding affinity of CJ-042794 was at least 200-fold more selective for human EP_4 receptor than for other human receptors [92]. Moreover, when the selectivity of the antagonist was tested toward 63 proteins, CJ-042794 did not show any affinity to these GPCRs, transporters and ion channels [92].

11. EP_4 antagonist: MF-766

MF-766 was designed by Merck Frosst and Colucci *et al*. [93] utilised the rat adjuvant-induced arthritis model to demonstrate *in vitro* potency and selectivity: high affinity at the EP_4 receptor (K_i 0.23 nM) and good selectivity against other prostanoid receptors with 7,000-fold higher affinity for EP_4 receptors (closest was DP_1 with a K_i of 1,800 nM). It has shown to be a full antagonist with an IC_{50} of 9.5 nM in the functional assay and the affinity to the EP_4 receptor (i.e. K_i 0.23 nM) was not significantly shifted in the presence of 10% human serum (K_i now 0.34 nM). The authors also evaluated the pharmacokinetic profile in rats and beagle dogs and MF-766 showed good pharmacokinetic properties with high oral bioavailability (74-86%) and low to moderate clearance rate (half-life was between 2.6 and 4.6 hours) [93].

12. EP_4 antagonist: L-161982 / EP_{4A}

L-161982 (also known as EP_{4A}) contains a diaryl-acylsulfonamide structure and in radioligand binding assays in HEK293 cells expressing human and rat EP_4 receptors have binding affinities (K_i) of 0.024 µM and 0.032 µM, respectively [94]. Affinities (K_i) to the other EP receptors are 1.9 µM for human EP_3 and 0.7 µM for human TP receptors: this shows a selectivity of roughly 30-fold for the human EP_4 receptor. L-161982 is at least 200-fold more selective for rat EP_4 than the other rat EP receptor subtypes [94]. L-161982 has been utilized to implicate a role for EP_4 in bone anabolic effects of PGE_2 in RP-1 periosteal cells [95]. Clarke *et al.* have used L-161982 as a pharmacologically active antagonist in human airway smooth muscle cells to inhibit the augmentation of cytokine release by EP_4 agonism [32].

13. EP_4 antagonist: BGC20-1531

The pharmacological characterisation of BGC20-1531 was published in 2009 [96] and showed that BGC20-1531 was able to displace [^{3}H]-PGE_2 from human recombinant EP_4 receptor with a pK_i of 7.9±0.1. This antagonist showed negligible affinity to other prostanoid receptors: measured pK_i values were EP_1 < 5, EP_2 < 5, EP_3 < 5, EP_4 7.9, TP < 5, DP 5.6 and IP < 5, showing that BGC20-1531 has good affinity and selectivity for the human recombinant EP_4 receptor [96]. The affinity and potency demonstrated in cell lines expressing human EP_4 receptors (pK_i=7.9 and pK_b=7.6) were comparable with the results of the *ex vivo* assays (p*K*b= 7.6 - 7.8).

III. Transgenic mice

A real step-change in the field commenced when transgenic mice in which the genes for each of the four EP receptors has been knocked-out began to be utilized in the respiratory context. The Narumiya lab from Kyoto University [19] were first to generate the mice by homologous

recombination [97] and the mice known as *Ptger1*$^{-/-}$, *Ptger2*$^{-/-}$, *Ptger3*$^{-/-}$, *Ptger4*$^{-/-}$ lack the EP_1, EP_2, EP_3 and EP_4 receptors. The first reports that used in the respiratory space came in Maher *et al. [76]*, who combined a pharmacological approach (using selective EP_3 receptor antagonists) and prostanoid receptor–deficient mice to clearly implicate the EP_3 receptor as mediating PGE_2-induced cough. Since that time, these mice have been used in a number of key studies that provide notable *in vivo* proof-of-concept data. For example, prostanoid receptor-deficient transgenic mice have been used to show that EP_4 receptor activation is responsible for the anti-inflammatory activity of PGE_2 [98] and to implicate EP_2 and EP_4 receptors in PGE_2-induced microvascular leak [99]. Further studies that utilize these mice are warranted.

IV. Role and function of EP receptors in lung health and disease

EP receptors have been identified in playing a critical role in chronic respiratory diseases, including asthma and COPD. In the following sections we will highlight some notable examples where EP receptor agonists and antagonists, or prostanoid receptor-deficient mice, have been utilized to implicate EP receptors in pathophysiological processes in respiratory conditions. Moreover, these tools have highlighted novel pharmacotherapeutic strategies and targets that could yield beneficial outcomes in the future.

A. Migration of airway smooth muscle cells

In addition to regulating airway calibre and mediating bronchospasm, ASM cells fulfil different tasks such as proliferation resulting in hypertrophy and hyperplasia, as well as contribution to airway inflammation through the synthesis of a range of different mediators [100]. A subset of EP receptors has been demonstrated as being present in human ASM cells: i.e. the mRNA of EP_2, EP_3 and EP_4 are detected in human ASM cells, while EP_1 mRNA was not represented in this type of cells [101-103]. Migration is also an important function of ASM cells that may have

significant impact on asthma pathogenesis. PGE_2, and the selective EP_2 agonist ONO-AE1-259 and EP_4 agonist ONO-AE1-329 were shown to decrease migration of human ASM cells induced by platelet-derived growth factor BB *in vitro* [60]. This process occurs via activation of cAMP/PKA. Thus, it follows that since activation of EP_2 and EP_4 receptors attenuate ASM cells migration [60], selective EP agonists may represent novel pharmacotherapeutic strategies to reverse or prevent development of airway remodelling in asthma.

B. Airway microvascular leak

Airway microvascular leakage has been clinically shown to be an early sign of asthma impairment, even when no signs of inflammation, such as increased cellular influx, had yet developed [104]. Moreover microvascular leak has also been shown to exist in COPD [105]. In 2016, Jones *et al.* [99] utilized mouse and guinea pig allergic asthma models to demonstrate the influence of EP_2 and EP_4 receptors on microvascular leakage. Activating the EP_2 and EP_4 receptors with PGE_2, or selective agonists ONO-AE1-259 (EP_2) and ONO-AE1-329 (EP_4) induces microvascular leakage. In contrast, the response to PGE_2 was greatly decreased when the EP_2 and EP_4 receptor was knocked out in *Ptger2*$^{-/-}$ and *Ptger4*$^{-/-}$ mice, respectively [99].

C. Pulmonary blood vessels

By engaging with different prostanoid receptors, prostaglandins can exert differential effects in the pulmonary vasculature, including both vasoconstriction [106] and vasorelaxation [87]. Among the four EP receptors, only EP_1 and EP_4 were found in smooth muscle cells of human pulmonary veins [87]. Vasoconstrictive activity of EP_1 receptors was proven by use of 17-phenyl-PGE_2, sulprostone (a non-selective EP_1 and EP_3 agonists) and iloprost, a PGI_2 analogue and agonist at the IP-and EP_1 receptors. The antagonist AH6809 ($DP/EP_1/EP_2$ receptor antagonist) and SC19220 (EP_1 antagonist) reversed the agonists' effect on human pulmonary

veins [106]. Additionally, EP_1 antagonists (ONO-8711, ONO-8713) reversed sulprostone-induced contractions in human pulmonary vessels [44]. On the other hand, the selective EP_4 agonists ONO-AE1-329 and L-902688 induced relaxation of human pulmonary veins while the selective EP_4 antagonist GW627368X led to vasoconstriction. Thus, it follows that EP_2 receptors induce vasoconstriction [44, 106], and EP_4 receptors mediate vasorelaxation in human pulmonary veins [87], although other non-EP prostanoid receptors are involved. The situation is different in human pulmonary arteries where vasoconstriction is mediated via EP_3 and TP receptors. This was confirmed by the use of the EP_3 agonist ONO-AE-248 and the TP-antagonist BAY u3405 [44].

D. Mast cell degranulation

Mast cells are recruited to the lung in asthma and are critical for the initiation and perpetuation of inflammation and disease pathogenesis [107-109]. Mast cells degranulate in an asthma attack in an IgE-mediated manner and notably, PGE_2 has been shown to inhibit IgE-mediated release of histamine in cultured human lung mast cells (HMLC) *in vitro* [110]. More precisely this process is mediated via activation of the EP_2 receptor. This was proven in experiments using several EP agonists and antagonists where only the EP_2 agonist butaprost was able to inhibit histamine degranulation from HLMC whereas sulprostone (EP_1/EP_3 agonist), 17-phenyl-trinor-PGE_2 (EP_1 agonist) and agonists of DP, FP, IP or TP failed to inhibit the histamine release [111]. AH6809 (a DP, EP_1, EP_2 antagonist) was able to antagonize the inhibitory effect of PGE_2. The EP_4 antagonist AH23848 did not succeed in inhibiting the effect of PGE_2 on HLMCs [111]. In 2013, Kay *et al*. extended their earlier study to reappraise the effects of PGE_2 on HLMC degranulation because at this point in time more selective EP_2 and EP_4 agonists and antagonists were available. Non-selective (PGE_2, Misoprostol), selective EP_2 agonists (ONO-AE1-259, AH13205,

butaprost-free acid), selective EP_2 antagonists (PF-04418948, PF-04852946), selective EP_4 agonists (L-902,688, TCS251) and selective EP_4 antagonists (CJ-042794, L-161,982) were utilized in the study [112]. The outcome was that while IgE-dependent histamine release in HLMC can be mediated by both EP_2 and EP_4 receptor, EP_2 receptor-mediated release predominates. A number of other interesting observations were made in this study [112]: the EP_2 selective receptor agonist ONO-AE1-259 behaved as a partial agonist relative to PGE_2; HLMCs express mRNA for EP_2 and EP_4 receptors, while the human mast cell line LAD2 doesn't and expresses EP_3 instead; and the inhibitory effect of selective EP_4 agonist L-902,688 could not be reversed by specific EP_2 and EP_4 antagonists leading to the conclusion that L-902,688 influences the degranulation of HLMC via another unknown mechanism [112].

In 2015, Säfholm *et al.* [113] investigated the relationship between mast cell degranulation and bronchoconstriction in human small airways *ex vivo* in order to more directly investigate the correlation between mast cell activity and muscular bronchial activity. Säfholm *et al.* used intact sections of human small airways in order to mirror the processes in the human body more precisely and showed that PGE_2 mediates bronchorelaxation via EP_4 receptor and bronchoconstriction via the TP receptor. Furthermore, PGE_2 inhibits IgE-mediated release of histamine and other mediators. Using the EP_2 receptor antagonist PF-04418948 it was shown that the inhibiting effect of PGE_2 on mast cell degranulation occurs via activation of EP_2 receptors [113]. These *ex vivo* studies, in part, mimic the *in vitro* analyses where EP_2 receptors were shown to play an important role [111, 112].

A further study describes a protective role for PGE_2 in exercise-induced bronchoconstriction. Because of hyperventilation while exercising a loss of airway water occurs leading to a higher osmolality in the bronchi. This augmented osmolality in airway surface liquid is thought to cause

activation of mast cells and subsequent bronchoconstriction [114]. To mimic the effects of exercise on airway fluid osmolarity, mannitol was used as a hyperosmolar stimulus [114, 115]. Utilizing the EP_2 (AH6809) and EP_4 (AH23848) antagonists, PGE_2 was shown to prevent hyperosmolar mast cells activation via EP_2 and EP_4 receptors [115]. Taken together, we can conclude that EP_2 and EP_4 agonists can exert beneficial actions in exercise-induced bronchoconstriction by reduction of human mast cell activity caused by higher osmolarity in the bronchi [115].

E. Bronchodilatation

In human airways, inhalation of PGE_2 results in a bronchodilatation [116]. Excitingly, the selective EP_4 receptor agonist ONO-AE1-329 can also lead to relaxation of human airways [83]. Moreover, the selective EP_4 receptor antagonist ONO-AE3-208 reversed the bronchodilatation achieved with PGE_2 and ONO-AE1-329. The same was observed in rat trachea [83]. In other species, specifically guinea pig, mouse and monkey, relaxation of trachea was mediated by the EP_2 receptor. This emphasizes important species differences and underscores that processes in animal models are not always congruent with processes in the human body [83].

The role of EP_4 receptors in mediating bronchodilatation was confirmed in human bronchial preparations by Benyahira *et al.* [84], where histamine-induced contraction could be reversed by the selective EP_4 agonists ONO-AE1-329 and L-902688. In addition, the specific EP_4 antagonist GW62768X blocked PGE_2-induced relaxations of human bronchial preparations which were pre-contracted with histamine [84].

However, the conclusions made in Buckley *et al.* [83] and Benyahia *et al.* [84] differ from that of Norel *et al.* [117] who showed that PGE_2-induced relaxation of human airway tissue could not be reversed by the EP_4 antagonist AH23848B. Instead AH6809 (DP, EP_1, EP_2-receptor antagonist)

decreased bronchodilatation [117]. This is good example that clearly shows the importance of using selective pharmacological tools to implicate a particular EP receptor and how better reagents have become available over time. It has to be remarked that the later studies [83, 84] had more specific EP receptor agonists and antagonists available than Norel *et al.* [117]. The specific EP_2 receptor agonists AH13205 and ONO-AE1-259, as well as specific EP_4 agonist ONO-AE1-329, L-902688 and selective EP_4 antagonist ONO-AE3-208 and GW627368X were applied [83, 84]; whereas Norel *et al.* used Misoprostol, AH23848B (TP, EP_4 antagonist) and AH6809 (DP, EP_1, EP_2 receptor antagonist) which are not as specific [117].

F. Cough

Inhaled PGE_2 induces cough in humans [118, 119]. Maher *et al.* [76] utilized a sensory nerve activation model using isolated vagus nerves of human, guinea pig and mouse to show that depolarization after PGE_2 challenge indicated cough. When the isolated vagus nerves of guinea pigs were exposed to the selective EP_3 antagonist L826266, PGE_2-mediated depolarization due was attenuated. These data demonstrate that cough induced by PGE_2 mediated via the EP_3 receptor [76]. More recently, the Transient Receptor Potential (TRP) family of ion channels has been implicated as playing an important role as the common effectors of the cough response [120, 121] and TRP channels represent novel therapeutic targets.

G. Angiogenesis

Airway remodelling in asthma is typified by increased vascularization due to angiogenesis. This is promoted through production of vascular endothelial growth factor (VEGF) in airway resident cells. VEGF production is regulated by prostanoids. In human ASM cells, the expression of VEGF-A was increased by activation of EP_2 and EP_4 via PGE_2, ONO-AE1-259 and ONO-AE1-329 [102]. In human lung fibroblasts, Huang *et al.* [122] employed butaprost, ONO-AE3-248

and ONO-AE1-329 to come to the conclusion that PGE_2 induces apoptosis via activation of EP_2 and EP_4 receptors. Also in lung fibroblasts (human fetal), PGE_2 activation of the EP_2 receptor was shown to results in stimulation of VEGF production [123]. The researchers demonstrated that the interaction between the phosphodiesterase 4 inhibitor roflumilast with either PGE_2, ONO-AE1-259-01 (EP_2 agonist), or ONO-AE1-329 (EP_4 agonist), lead to an enhancement of VEGF production. The stimulatory effect of PGE_2 on VEGF production could be significantly inhibited by EP_2 antagonist AH6809. Nevertheless, a significant block of the stimulatory effect of roflumilast could only be achieved by combining EP_2, EP_3 (ONO-AE3-240) and EP_4 (ONO-AE3-208) antagonists. These data reinforce the notion that EP_2 and EP_4 receptors, and possibly the EP_3 receptor, are involved in the stimulatory effect of roflumilast in the presence of PGE_2. A further discovery was that the EP_1 antagonist ONO-8713 enhanced the VEGF release in presence of control media and PGE_2 suggesting that activation of EP_1 receptor could possibly stop VEGF release [123].

H. Senescence

Cellular senescence is considered a pathogenic feature of COPD [124]. An investigation of lung fibroblasts from COPD patients, smokers and non-smoker controls showed that lung fibroblasts of COPD patients express a higher amount of EP_2 and EP_4 receptors [125]. This was demonstrated via the use of several EP_2 and EP_4 receptor agonists (ONO-AE1-259) and antagonists (AH6809, PF-04418948, GW627368X, L-161982) which mimicked and accordingly reversed the effect of PGE_2 on lung fibroblasts. Furthermore, fibroblasts from people with COPD demonstrated enhanced senescence markers, as well as augmented COX-2, PGE_2, cytokine, chemokine, and matrix metalloproteinase expression. Notably, through enhanced PGE_2 secretion, senescent COPD lung fibroblasts exerted autocrine and paracrine effects on non-

senescent COPD lung fibroblasts via activation of the EP_2 and EP_4 receptors. This potentiating feedback loop leads to reinforcement and propagation of senescence and implicates a central role for prostanoids/EP receptors in COPD [125].

I. Airway inflammation

Inflammation drives disease progression and pathogenesis in several lung diseases and studies have shown that these pathways are driven via EP receptor ligand engagement. Of particular importance are the EP_2 and EP_4 receptors, predominately due to their links to adenylate cyclase and the ability to increase intracellular cAMP [126]. This has been demonstrated in *in vitro* and *in vivo* models of airway inflammation. In ASM cells, we [127] and others [128-131] have shown that mediators that increase cAMP (including PGE_2) can modulate cytokine expression *in vitro*. In 2000, we showed that PGE_2 increased cAMP in ASM cells and that this augmented cAMP has differential effects on cytokines: some were repressed, while some were augmented [127]. We [132] went onto to show that the outcome of cAMP elevation on the synthetic function of ASM cells could be explained by an understanding of the transcriptional regulation of the cytokine, *viz* whether the cytokine has a CRE in its 5′-promoter region (e.g. IL-6 does and hence cAMP alone will increase IL-6 secretion). Two studies by Clark *et al.* [101, 129] also showed that the impact of PGE_2 on airway inflammation *in vitro* could vary depending on cytokine examined. Clark *et al.* [129] demonstrated that the IL-1β-induced release of GM-CSF could be suppressed by PGE_2, ONO-AE1-259 (selective EP_2 agonist) and other non-selective EP_2 agonists, while the reverse was observed for EP_2 antagonist AH6890 but not for EP_4 antagonists (AH23848B, L-161,982). These findings lead to the conclusion that the release of GM-CSF can be inhibited by PGE_2 through activation of the EP_2 receptor [129]. A similar study investigating G-CSF discovered that PGE_2 increases elaboration of G-CSF which was promoted

by IL-1β. This transaction is performed by both EP_2 and EP_4 receptors. AH6809 (DP, EP_1, EP_2 antagonist) and L-161,982 (EP_4 antagonist) were not able to inhibit the PGE_2 induced G-CSF release completely. Both EP antagonists had to be added in combination in order to realize an effective antagonism [101].

However, in more recent studies we have shown that cAMP elevation can also increase anti-inflammatory proteins, namely, the MAPK deactivator and anti-inflammatory protein, MAPK phosphatase 1 (MKP-1) [133]. MKP-1upregulation is a common response to elevated cAMP induced by a diverse range of stimuli. In ASM cells we have shown that inflammatory stimuli [134], cAMP elevating agents [133], as well as respiratory medicines such as β_2-agonists [133, 135] and phosphodiesterase 4 inhibitors [136, 137], can all increase MKP-1 via CREB-mediated pathways and perhaps offset the impact of cAMP on cytokine production. In 2016, we used selective antagonists of EP_2 (PF-04418948) and EP_4 receptors (GW 627368X) to show that PGE_2 increases production of the anti-inflammatory protein MKP-1 via cAMP/CREB-mediated cellular signalling in ASM cells and that EP_2 may, in part, be involved, although further studies are warranted [138]

Other cellular models of airway inflammation have been utilized to demonstrate the impact of EP receptor engagement in the respiratory context. Taking macrophages as a key example with relevance to lung disease, Takayama *et al.* [94] showed that activation of the EP_4 receptor of human macrophages via PGE_2 leads to an inhibition of cytokine production, including IL-8. More recently, Gill *et al.* [139] evaluated the effects of EP_2 selective (PF-04852946, PF-04418948) and EP_4 -selective (L-161,982, CJ-042794) receptor antagonists on PGE_2 responses. They confirmed that the anti-inflammatory effects of PGE_2 on human lung macrophages are mediated by the EP_4 receptor [139].

In mice, intranasal lipopolysaccharide (LPS) challenge enhanced the neutrophil infiltration in bronchoalveolar (BAL) fluid which was reversed by additional application of PGE_2 or PGE_2 analogue misoprostol [140]. The reduction of neutrophils by PGE_2 was mimicked by EP_4 selective agonist ONO-AE1-329 and blocked by the EP_4 receptor antagonists GW627368X or ONO-AE3-208. LPS-induced pulmonary vascular leakage was reversed by PGE_2 or ONO-AE1-329 (specific EP_4 receptor antagonist). Hence the conclusion drawn was that the infiltration of neutrophils in *in vivo* models of airway inflammation can be inhibited by activation of EP_4 receptors [140].

Finally, the *in vivo* studies with the prostanoid receptor–deficient mice have been conducted. In 2015, Birrell *et al.* [98] performed murine respiratory models with $EP_{1\text{-}4}$ receptor knock-out mice (*Ptger1-4*$^{-/-}$), challenged either with LPS (as an innate stimulus), ovalbumin (as an allergic response, mimicking asthma) or cigarette smoke (as an inhaled pollutant, mimicking COPD). The outcome of this experiment was that increased levels of eosinophils (especially in ovalbumin-challenged mice) and elevated levels of neutrophils in LPS and smoke treated mice were found in the BAL of *Ptger4*$^{-/-}$ mice. There were no significant changes in the number of inflammatory cells found in the BAL in the $EP_{1\text{-}3}$ receptor knock-out mice. Coupled with cell-based assay systems, where murine and human monocytes were treated with LPS to stimulate cytokine production, PGE_2 exerted a concentration-dependent repression. The repressive effects of PGE_2 could be mimicked by the selective EP_4 agonist ONO-AE1-329 but not by the selective $EP_{1\text{-}3}$ agonists ONO-D1-004, ONO-AE1-259 and ONO-AE-248. These findings led the authors to assert that PGE_2 activated EP_4 receptors in the lung exert anti-inflammatory effects [98].

V. Summary and future directions

Targeting EP receptors represent a fast developing sector in research, especially in lung health, with exciting scope for development of efficacious pharmacotherapeutic agents in the future. The discovery of specific means to manipulate the EP receptor with agonists and antagonists, or through the use of genetically-modified prostanoid receptor–deficient mice, has resulted in more precise research results whereby the individual functions of EP receptors in specific tissues have now been clearly defined. A key example is the EP_4 receptor that has been now firmly entrenched as a target to induce bronchodilatation [83] and reduce inflammation [98]. Another key message that has emerged is the species differences in EP receptor-mediated cell signalling pathways. This is best highlighted in the example of the EP_2 receptor, where selectivity profiling and functional bioassays demonstrate distinct differences between commonly-used experimental models [66].

In summary, PGE_2 is an important bioactive prostanoid that has both 'bad' and 'good' effects because of myriad interactions with $EP_{1\text{-}4}$ receptors [3]. With the advent of more selective means to target individual EP receptors, comes the opportunity to precisely promote the beneficial effects of prostanoids while minimizing the unwanted effects. Although research challenges remain (such as understanding how the prostanoid receptors themselves are upregulated or desensitized, and the impact of cell signalling cross-talk on functional outcome), there is no doubt that novel and specific molecules targeting individual prostanoid receptors (or even isoforms) may represent efficacious drug entities in the future. Drug delivery through aerosolization technology may enable us to deliver these novel medicines (alone or in combination with current medicines) to the right place in the lung to achieve maximum benefit while minimize systemic effects. Thus, selectively targeting EP receptor-mediated cell signalling pathways will likely yield positive implications for lung health and disease in the future.

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