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Synthetic Modification of Prostaglandin $F_{2\alpha}$ Indicates Different Structural Determinants for Binding to the Prostaglandin F Receptor Versus the Prostaglandin Transporter

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ABSTRACT

Several principles governing the binding of E series prostaglandins to EP receptors have emerged in recent years. The C-1 carboxyl group binds electrostatically to a conserved arginine residue in the seventh transmembrane segment of the receptor. Prostaglandin E analogs involving bioisosteric replacements of the carboxyl group, such as acylsulfonamide, are also active. In addition, structurally similar esters may also exhibit similar affinity, presumably by virtue of hydrogen bonding. Other regions of the substrate molecule appear to bind to other domains of EP receptors, either via hydrophobic interactions or by hydrogen bonding. Less information is available about the structural requirements for substrate binding to FP receptors. Prostanoids also bind to the prostaglandin transporter PGT. In this case, a conserved C-1 carboxyl group is critically important, since C-1 esters exhibit little affinity. Here we examined the binding of chemically diverse $PGF_{2\alpha}$ structural analogs to the FP receptor and compared these with binding by the PG transporter. PGT recognized a wide range of anionic substituents. In contrast, the carboxylic acid group was essential for optimal binding to the FP receptor, since replacement by larger moieties with a similar pK_a, such as acylsulfonamide and tetrazole, substantially decreased binding affinity. Interestingly, insertion of cyclic substituents in the omega chain increased binding to the FP receptor but reduced affinity for PGT, and substitution for the 15-hydroxyl group produced only a modest reduction in FP receptor binding, but eliminated binding by PGT. Because extracellular $PGF_{2\alpha}$ may compete for binding between FP receptors and PGT, these findings have implications for designing $PGF_{2\alpha}$ analogs for treating disease states.

Extensive structural information is available for substrate binding to G-protein-coupled receptors. In general, residues in the extracellular half of the transmembrane domain bind small ligands such as biogenic amines, neurotransmitters, opsin, and nucleotides (Baldwin, 1994). Similar models have been put forth for the binding of thromboxane A_2 to its receptor (Yamamoto et al., 1993).

Binding analyses using primarily recombinant prostaglandin (PG) E (EP) receptors and various ligands have revealed the importance of electrostatic interactions between the substrate C-1 carboxylate and a highly conserved arginine in putative transmembrane segment 7. Mutagenesis of this conserved arginine, or elimination of the C-1 negative charge on the substrate, results in a marked reduction in binding affinity for many, but not all, ligands (Tsai et al., 1991; Huang and Tai, 1995; Negishi et al., 1995; Breyer et al., 1996b; Audoly and Breyer, 1997b). More recent data indicate that hydrogen bonds and/or hydrophobic interactions may play equally important roles in substrate binding (Negishi et al., 1995; Chang et al., 1997a,b). Domains outside the transmembrane segments are also claimed as important for substrate-receptor interaction (Audoly and Breyer, 1997a).

The first carrier (PGT) shown to catalyze the transport of PGE_2 and $PGF_{2\alpha}$ was recently identified (Kanai et al., 1995; Lu et al., 1996). The substrate specificity of PGT (Kanai et al., 1995), and the observation that it functions as a reversible anion exchanger (Chan et al., 1998), suggests that PGT may function to release newly synthesized PGs and/or to take up PGs before their intracellular metabolism (Schuster, 1998). Consistent with such roles, the tissue distribution of PGT expression is quite broad (Lu et al., 1996; Schuster et al., 1997).

The interaction of PG substrates with PGT appears to be similar to that of substrates with EP receptors in certain respects. First, the substrate binding site of PGT lies, at a minimum, within transmembrane span 11 (Chan et al., 1996). Second, PGT has two highly conserved cationic resi-

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ABBREVIATIONS: PG, prostaglandin; FP, prostaglandin F; PGT, prostaglandin transporter; PCR, polymerase chain reaction.

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TABLE 1
Comparison of the affinities of various $PGF_{2\alpha}$ analogs for PGT and the FP receptor

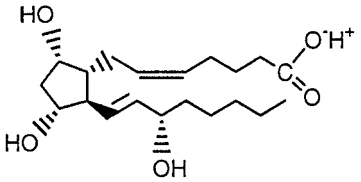
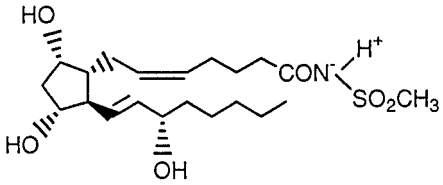
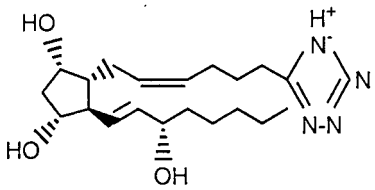
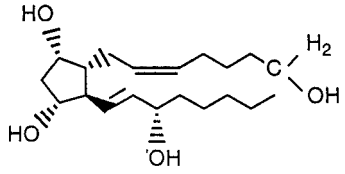
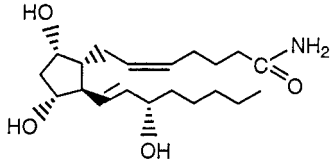
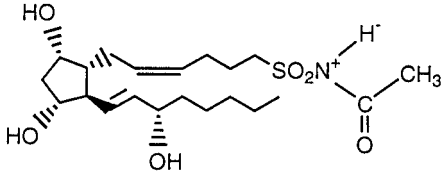
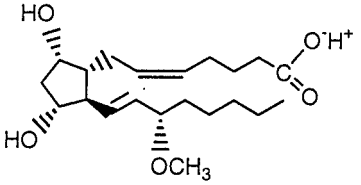
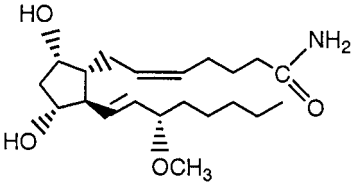
Compound Structure	AGN Number	pK_a	IC_{50} (nM) PG Transporter	IC_{50} (nM) FP Receptor
	Prostaglandin $F_{2\alpha}$	4.5	45	48
	AGN 191365	4.5	307	>710,000
	AGN 191366	4.5	71	8,128
	AGN 190910	Neutral	Not determined	>10,000
	AGN 190911	Neutral	7,300	>10,000
	AGN 194394	2.0	34	>10,000
	AGN 191995	4.5	330,000	228
	AGN 192151	Neutral	630,000	>10,000

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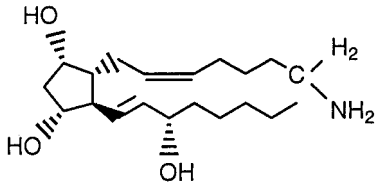
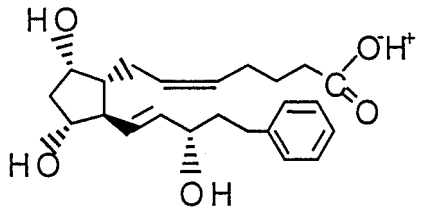
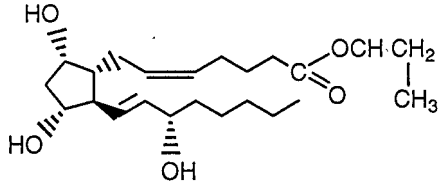
TABLE 1
(*Continued*)

Compound Structure	AGN Number	pK_a	IC_{50} (nM) PG Transporter	IC_{50} (nM) FP Receptor
	AGN 191088	10.0	710,000	>10,000
	17-Phenyl $PGF_{2\alpha}$	4.5	168	18
	Prostaglandin $F_{2\alpha}$ 1-isopropyl ester	Neutral	>5,000	3,200

dues (arginine and lysine) in the transmembrane spanning segments (Kanai et al., 1995; Lu et al., 1996; Pucci et al., 1999) and these residues are important in binding and transport (Chan et al., 1996). However, characterization of substrate interactions with PGT revealed weak binding of $PGF_{2\alpha}$ isopropyl ester ($K_i > 5000$ nM) relative to that of $PGF_{2\alpha}$ ($K_i \sim 50$ nM), suggesting that electrostatic interactions with the C-1 carboxyl group may play a major role in substrate binding (Itoh et al., 1996). Complementary inhibitor data from our laboratory are also consistent with substrate recognition by the transporter as the anion (Chan et al., 1998).

In the studies described herein, we performed a direct comparison of the structural requirements for substrate binding to PGT versus the FP receptor, especially with regard to the C-1 moiety, to gain insights into the respective binding sites of the two proteins. Our findings indicate that the substrate requirements for the FP receptor differ from those of PGT in many important respects.

Experimental Procedures

Transient Expression of Human FP Receptor in COS-7 Cells. A cDNA sequence containing the entire coding region of the human FP receptor was generated by PCR, using primers designed on the basis of the known sequence (Abramovitz et al., 1994). The nucleotide sequence of the cDNA was determined using a Silver Sequencing Kit (Promega, Madison, WI). Two nucleotide changes were observed: at codon 71, AGT, and at codon 199, AGG. Neither change results in an alteration of the deduced amino acid sequence. The FP receptor cDNA was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) and utilized for heterologous expression.

COS-7 cells were transiently transfected using Lipofectin (Life Technologies, Inc., Gaithersburg, MD) according to manufacturer's protocols. For binding studies, 2×10^6 cells were plated onto 150-mm dishes 24 h before transfection. Each plate was transfected with 50 μg of plasmid DNA and 50 μl of Lipofectin. Cells were collected and membranes prepared (Regan et al., 1988) at 72 h post-transfection, and frozen at −80°C until use.

Radioligand Binding Studies on FP Receptor. Radioligand binding studies were performed in a 200-μl volume using 5 nM ^{3}H-17-phenyl $PGF_{2\alpha}$. The reaction was started by adding protein that achieved a final concentration of 1 mg/ml. Nonspecific binding was determined using 10^{-5} M 17-phenyl $PGF_{2\alpha}$. Incubation was terminated after 60 min by the addition of 4 ml of ice-cold Tris-HCl buffer and vacuum filtration through Whatman GF/B glass fiber filters using a Brandel cell harvester. The filters were washed three times with ice-cold buffer. The filters were then oven dried and 5 ml of scintillation fluid was added to each dried, singular segment before counting.

Transient Expression of PGT in HeLa Cells. For HeLa cell expression, cells were grown to 80% confluence on 35-mm dishes and were infected with recombinant vaccinia virus vTF7–3 (10 plaque-forming units/cell) (Fuerst et al., 1986). Thirty minutes after infection, cells were transfected with the full-length PGT cDNA (10 μg/ml) plus Lipofectin (20 μg/ml) as described elsewhere (Blakely et al., 1991; Kanai et al., 1995). After 3 h of incubation, vaccinia virus and the DNA-Lipofectin complex were removed, and the cells were maintained in Dulbecco's modified Eagle's medium with 5% fetal bovine serum overnight until the uptake assay.

Tracer PGE_2 Uptake. Tracer PGE_2 uptakes in HeLa cells were performed 19 h after transfection. Monolayers were washed twice with Waymouth solution, and timed uptakes over 5 min at 27°C were performed by adding tracer prostanoid to the Waymouth solution [tracer prostanoid uptake is linear over the initial 5 min (Kanai et al., 1995)]. [^{3}H]PGE_2 (171 Ci/mmol, PerkinElmer Life Sciences, Wilmington, DE) was added at 0.1 μCi/ml per dish, total concentration ~0.6 nM. Tracer uptake was stopped by washing with ice-cold Waymouth solution containing 5% bovine serum albumin once and then four times with Waymouth solution lacking bovine serum albumin. Cells were scraped and counted by liquid scintillation.

Inhibition Constants. For substrate K_i determinations, [^{3}H]PGE_2 uptakes were determined in duplicate on a given transfec-

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tion in the presence of various concentrations (10 nM to 10 μM) of unlabeled prostanoids. Inhibition dose responses were curve-fitted, and IC_{50} values were calculated from three to five separate transfections.

Materials. The $PGF_{2\alpha}$ analogs were all synthesized at Allergan, and the structures are given in Table 1 (see *Results*). [^{3}H]AGN 190910, [^{3}H]AGN 192151, and [^{3}H]17-phenyl $PGF_{2\alpha}$ were all prepared by Amersham Pharmacia Biotech (Buckinghamshire, UK). All cell culture media, serum, and reagents, including Lipofectin, were obtained from Life Technologies, Inc. with the exception of charcoal-treated serum, which was obtained from Gemini Products, Inc. (Calabasas, CA). COS-7 cells were obtained from Dr. John Regan, University of Arizona. Expression vector pcDNA3 was obtained from Invitrogen. All PCR reagents and enzymes were obtained from PE Applied Biosystems (Foster City, CA). PCR primers were purchased from Genosys Biotechnologies (The Woodlands, TX). All reagents, acrylamide gel mixes, and buffers for DNA sequencing were purchased from Promega.

Results

Substrate Affinities for the FP Receptor. The structures of the compounds tested and the results of radioligand binding competition studies versus [^{3}H]17-phenyl $PGF_{2\alpha}$ are summarized in Table 1. The data indicate that a carboxylic acid group is essential for recognizing the FP receptor. Only $PGF_{2\alpha}$ and 17-phenyl $PGF_{2\alpha}$ had high affinity. Replacement of the carboxylic acid moiety by other substituents with a similar pK_a (AGN 191365, AGN 191366), with a lower pK_a (AGN 194394), with an alkaline pK_a (AGN 191088), or with nonionizable moieties (AGN 190910, AGN 190911, and AGN 192151) resulted in compounds with reduced or minimal affinity for the FP receptor. With regard to the 15-position hydroxyl group, the 15-R-$PGF_{2\alpha}$ isomer had an IC_{50} for the FP receptor of 5400 nM.

Substrate Affinities for PGT. Figure 1 shows a representative dose-dependent inhibition of tracer PGE_2 uptake by rat PGT transiently expressed in HeLa cells using the following analogs of $PGF_{2\alpha}$: AGN 191995, 191365, and 192151. The data are curve-fitted with a single exponential (lines). In this example, AGN 191995 and AGN 191365 have poor affinity, whereas AGN 192151 has moderate affinity for PGT.

Table 1 presents affinity constants for PGT of the various C-1 substituted substrates [the affinity constants for 17-phenyl $PGF_{2\alpha}$ and $PGF_{2\alpha}$-1-isopropyl ester in Table 1 are taken from our previous paper (Itoh et al., 1996)]. It is clear that neutralizing the C-1 position anion, even with a sterically small moiety (AGN 190910 or AGN 190911), abolishes affinity for PGT. Substitution of a somewhat bulkier anionic group at the C-1 position (AGN 191365) restores a substantial proportion of PGT affinity, although not back to that of the native $PGF_{2\alpha}$. In contrast, substitution of an alternative large anionic group (AGN 194394) leaves the affinity for PGT intact. Interestingly, it appears that replacement of the carbon atom at position 1 with a sulfur atom has little effect on transporter affinity (AGN 194394), whereas substitution at more distal positions with an *S*-containing group (AGN 191365) tends to reduce PGT affinity. Predictably, replacement of the –COOH moiety with an amine resulted in greatly decreased affinity for both PGT and the FP receptor.

Insertion of cyclic substituents in the omega chain (17-phenyl $PGF_{2\alpha}$) increased binding to the FP receptor from 48 nM to 18 nM but reduced affinity for PGT. Substitution of the 15-hydroxyl group (AGN 191995) produced only a modest reduction in FP receptor binding (228 nM), but eliminated binding by PGT.

To evaluate the relationship between the affinity of PGT for various substrates and their actual translocation, we measured the transport rates of three radioactive compounds ([^{3}H]AGN 190910, [^{3}H]AGN 192151, and [^{3}H]17-phenyl $PGF_{2\alpha}$) and compared these to the transport rate of [^{3}H]PGE_2. As shown in Fig. 2, 17-phenyl $PGF_{2\alpha}$ was transported by PGT at about 40% the rate of PGE_2, in keeping with the 2- to 3-fold higher K_i of 17-phenyl $PGF_{2\alpha}$ compared with PGE_2 (Itoh et al., 1996). In contrast, neither AGN 190910 nor AGN 192151 were transported. The low transport rate of AGN 192151 is in agreement with the poor affinity (Table 1). Although the affinity of nonradioactive AGN 190910 was not determined, the absence of transport strongly suggests that its affinity for PGT is also very poor.

Discussion

In the present study, we found that the C-1 carboxylic acid group was essential for optimal substrate binding to the FP

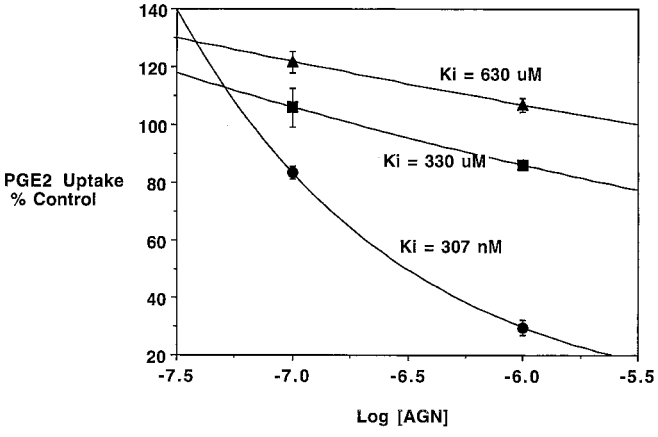

Fig. 1. Representative dose-dependent inhibition of tracer PGE_2 uptake by rat PGT transiently expressed in HeLa cells using analogs of $PGF_{2\alpha}$. The data are curve-fitted with a single exponential (lines). ■, AGN191995; ●, AGN191365; ▲, AGN192151.

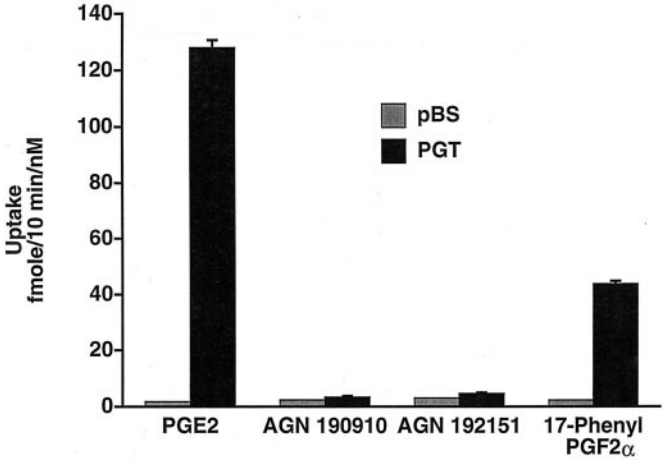

Fig. 2. Ten minute uptakes of radioactive PGE_2, AGN 190910, AGN 192151, and 17-phenyl $PGF_{2\alpha}$ by dishes of HeLa cells (1 mg of protein per dish) transiently transfected with either control plasmid (pBluescript, pBS) or the rat prostaglandin transporter PGT cDNA. Uptakes are normalized for 1 nM substrate concentration.

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receptor. Replacement of the carboxylic acid group by larger moieties with a similar pK_a, such as acylsulfonamide and tetrazole, substantially decreased binding affinity. The prostaglandin transporter PGT, on the other hand, recognized a fairly wide range of anionic substrates at C-1. In contrast, insertion of cyclic substituents in the omega chain increased binding to the FP receptor but reduced affinity for PGT, and substitution for the 15-hydroxyl group produced only a modest reduction in FP receptor binding but eliminated binding by PGT.

Prostaglandins and thromboxanes bind to a number of structurally diverse molecules, such as their cell-surface receptors (EP, FP, IP, DP, and TP) (Breyer et al., 1996a), the prostaglandin transporter PGT (Itoh et al., 1996), peroxisome proliferator-activated receptors α and γ (Forman et al., 1995; Kliewer et al., 1995), and prostaglandin 15 dehydrogenase (Nakano et al., 1969; Wright and Corder, 1979; Uchida et al., 1988). Because these proteins may compete for a given prostanoid molecule, it is important to understand the molecular nature of the substrate binding.

The binding of PGE_2 to its receptor is perhaps the best studied of these molecular interactions. PGE_2 has several structural features participating in its agonist activity, including the α-carboxylic acid, the 15-hydroxyl group, the 9-carbonyl group, and the cyclopentane ring (Negishi et al., 1995). Of particular note is that EP receptors contain a highly conserved arginine in transmembrane span 7, mutations of which cause loss of receptor activation (Huang and Tai, 1995; Negishi et al., 1995; Audoly and Breyer, 1997b). The arginine might electrostatically bind the C-1 carboxylic acid directly, or might act as an electrostatic "bait" to attract substrate into the receptor.

Considerably less information is available about the molecular nature of substrate binding to the FP receptor. The highly conserved arginine of the EP and TP receptors is present in the FP receptor (Abramovitz et al., 1994). Our data suggest that the C-1 group interacts with the FP receptor in an ionic- and stereo-specific fashion, since substitution with bulkier anionic or neutral groups abrogated binding (Table 1). It is possible that the FP receptor has a sterically restrictive "pocket" for the C-1 carboxyl group that does not allow access by bulkier groups.

In the case of the EP receptor, the conserved arginine residue can form not only an ion pair with the negatively charged carboxylic acid group of the ligand but also can donate a hydrogen bond to the carbonyl group of the ligand (Ippolito et al., 1990; Chang et al., 1997a). Experimentally, C-1 esters activate both the wild type EP_3 receptor and receptors in which the conserved arginine is mutagenized to other polar residues (Chang et al., 1997a). On the other hand, 1-OH PGE_2, which can accept as well as donate hydrogen but prefers to donate, is a very weak agonist at the EP_3 receptor (Chang et al., 1997b). Our results, showing lack of binding of neutral C-1 substitutions, and particularly of the 1-OH substitution (AGN 190910), to the FP receptor are comparable with these latter findings. Furthermore, studies with the FP receptor indicate that C-1 esters are low affinity ligands.

As with prostanoid receptors, there appears to be a role of electrostatic forces in the binding of substrates by PGT, since neutralizing the C-1 anionic charge abrogates binding (Table 1) (Itoh et al., 1996). However, sterically the requirements at C-1 by the FP receptor and by PGT differ substantially, in that the FP receptor has a strict requirement for a carboxylate group, whereas PGT allows bulkier C-1 anionic groups (Table 1). Recent data from our laboratory indicate that, as with prostanoid receptors, a conserved lysine in transmembrane span 12 of PGT may function to electrostatically attract anionic substrates into PGT, if not to directly bind the C-1 anionic group (Chan et al., 1996).

The FP receptor has considerable tolerance for substitution for the 15-hydroxyl group and insertion of cyclic substituents in the omega chain. Although PGT tolerates the cyclic substituents fairly well, PGT does not tolerate any substitution for the 15-hydroxyl group. This is in keeping with our previous results, which indicated that the specificity of PGT for the 15-position is extremely exacting, since switching between the 15-hydroxy (*R*) and (*S*) isoforms causes a dramatic change in binding affinity (Itoh et al., 1996). Of interest, the FP receptor also will not tolerate this geometric change (the affinity goes from 48 nM for 15-*S*-$PGF_{2\alpha}$ to 5400 nM for 15-*R*-$PGF_{2\alpha}$). Although we do not know exactly which amino acids of PGT contact the C-15 hydroxyl group of PGT, we recently used cysteine-scanning mutagenesis to show that four amino acids along one face of transmembrane span 10 likely contact the substrate (Chan et al., 1999). Given the generally moderate polarity of these amino acids (Ala-526, Ala-529, His-533, and Cys-530), these would appear to be reasonable candidates for interaction with C-15.

The exact physiological role of PGT remains to be determined. Immunocytochemical localization of PGT in rat tissues has shown its expression most strongly in cells that synthesize prostaglandins (Bao et al., 1998), suggesting a role in the release of newly synthesized prostanoids across the plasma membrane. On the other hand, the substrate specificity of rat PGT closely matches that described for the metabolic clearance of prostaglandins by the isolated, perfused rat lung (Itoh et al., 1996). Since PGT mRNA expression is high in the rat lung (Kanai et al., 1995), PGT may play a role in cellular uptake and signal termination (these roles are not mutually exclusive). If the latter is the case, then PGT and the FP receptor would compete for binding newly released $PGF_{2\alpha}$. Although the transporter and the receptor have similar affinities for native $PGF_{2\alpha}$ (both about 50 nM), given that metabolism of $PGF_{2\alpha}$ occurs predominantly by dehydrogenation at the C-15 hydroxyl group (Devereux et al., 1987), it is possible that C-15 metabolites might still stimulate the receptor but would not be transported by PGT.

In summary, the FP receptor has a strict requirement for a carboxyl group at the C-1 position but a broader acceptance of substitutions at C-15 and the omega chain, whereas PGT accepts bulkier C-1 anionic groups but is intolerant of changes to the C-15 hydroxyl.

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