Development of a High-Affinity Inhibitor of the Prostaglandin Transporter

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ABSTRACT

Prostaglandin E_2 (PGE_2) triggers a vast array of biological signals and physiological events. The prostaglandin transporter (PGT) controls PGE_2 influx and is rate-limiting for PGE_2 metabolism and signaling termination. PGT global knockout mice die on postnatal day 1 from patent ductus arteriosus. A high-affinity PGT inhibitor would thus be a powerful tool for studying PGT function in adult animals. Moreover, such an inhibitor could be potentially developed into a therapeutic drug targeting PGT. Based on structure-activity relationship studies that built on recently identified inhibitors of PGT, we obtained *N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-4-((4-((2-(2-(2-benzamidoethoxy)ethoxy)ethyl)amino)-6-((4-hydroxyphenyl)amino)-1,3,5-triazin-2-yl)amino)benzamide (T26A), a competitive inhibitor of PGT, with a K_i of 378 nM. T26A seems to be highly selective for PGT, because it

neither interacts with a PGT homolog in the organic anion transporter family nor affects PGE_2 synthesis. In Madin-Darby canine kidney cells stably transfected with PGT, T26A blocked PGE_2 metabolism, resulting in retention of PGE_2 in the extracellular compartment and the negligible appearance of PGE_2 metabolites in the intracellular compartment. Compared with vehicle, T26A injected intravenously into rats effectively doubled the amount of endogenous PGE_2 in the circulation and reduced the level of circulating endogenous PGE_2 metabolites to 50%. Intravenous T26A was also able to slow the metabolism of exogenously injected PGE_2 . These results confirm that PGT directly regulates PGE_2 metabolism and demonstrate that a high-affinity inhibitor of PGT can effectively prevent PGE_2 metabolism and prolong the half-life of circulating PGE_2 .

Introduction

Prostaglandins such as PGE_2 are autocrine and paracrine lipid mediators that trigger a wide variety of signals (Miller, 2006). These signals are terminated by the metabolism of PGE_2 (Ferreira and Vane, 1967; Hamberg and Samuelsson, 1971; Schuster, 1998). Oxidative inactivation of PGE_2 is catalyzed by 15-hydroxyprostaglandin dehydrogenase (15PGDH; E.C.

1.1.1.141), which is localized in the cytoplasm (Casey et al., 1980, 1982; Kobayashi et al., 1992). Thus, PGE_2 has to be translocated from the outside to the inside of cells for it to be metabolized to PGE_2 metabolites (PGE_2 -Ms), including 15-keto PGE_2 . The prostaglandin transporter (PGT), a member of the organic anion transporter (OATP) family (Kanai et al., 1995), mediates the energetically active influx of PGE_2 (Chi et al., 2006), accounting for almost 100% of PGE_2 internalization (Kanai et al., 1995; Chi and Schuster, 2010). In cell culture, we have demonstrated that PGT-mediated PGE_2 influx is required and is rate-limiting for PGE_2 metabolism (Nomura et al., 2004). In accord with these findings, we have reported that global deletion of PGT in mice results in elevated systemic PGE_2 and reduced plasma PGE_2 -M levels (Chang et al., 2010). Taken

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ABBREVIATIONS: PGE_2 , prostaglandin E_2 ; PGE_2 -M, PGE_2 metabolite; m-PGES, microsomal PGE_2 synthase; 15PGDH, 15-hydroxyprostaglandin dehydrogenase; PGT, prostaglandin transporter; OATP, organic anion transporter; SAR, structure-activity relationship; MDCK, Madin-Darby canine kidney; WT, wild type; AA, arachidonic acid; COX, cyclooxygenase; T26A, *N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-4-((4-((2-(2-(2-benzamidoethoxy)ethoxy)ethyl)amino)-6-((4-hydroxyphenyl)amino)-1,3,5-triazin-2-yl)amino)benzamide; DMSO, dimethyl sulfoxide; EIA, enzyme immunoassay; T34, *N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-4-((4-((2-(2-(2-benzamidoethoxy)ethoxy)ethyl)amino)-6-(phenethylamino)-1,3,5-triazin-2-yl)amino)benzamide; T25A, *N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-4-((4-((2-(2-(2-benzamidoethoxy)ethoxy)ethyl)amino)-6-((4-fluorophenyl)amino)-1,3,5-triazin-2-yl)amino)benzamide; T28A, *N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-4-((4-((2-(2-(2-benzamidoethoxy)ethoxy)ethyl)amino)-6-(phenylamino)-1,3,5-triazin-2-yl)amino)benzamide.

Fig. 1. Structures of PGE₂ (C) and PGT inhibitors T34 (A top) and T26A (A bottom and B).

together, these data indicate that PGT plays a central role in PGE₂ metabolism and signal termination.

As an experimental tool, however, such a genetic approach has limitations, because PGT global knockout mice cannot survive past postnatal day 1 because of patent ductus arteriosus unless they are rescued by administration of indomethacin to the mother before parturition (Chang et al., 2010). A chemical agent that specifically inhibits PGT would be advantageous, both to rapidly assess PGT functions in a variety of different physiological and pathophysiological models and further investigate the role of PGT in controlling PGE₂ metabolism and thus signaling.

Previously, we reported a new class of PGT inhibitors obtained by screening a small molecular library (Chi et al., 2006). The best hit of that screening was T34 (Fig. 1) with a binding constant (K_i) of 3.7 μ M. Although the K_i of T34 on PG uptake is almost identical to that of bromocresol green (Bito and Salvador, 1976, Kanai et al., 1995), *N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-4-((4-((2-(2-(2-benzamidoethoxy)ethoxy)ethyl)amino)-6-(phenethylamino)-1,3,5-triazin-2-yl)amino)benzamide (T34) has considerable potential to be optimized into a high-affinity inhibitor by virtue of its three side chains, R₁, R₂, and R₃ (Fig. 1). Accordingly, we conducted extensive structure-activity relationship (SAR) studies starting with the T34 platform, which allowed us to obtain *N*-(2-(2-(2-azidoethoxy)ethoxy)ethyl)-4-((4-((2-(2-(2-benzamidoethoxy)ethoxy)ethyl)amino)-6-(4-hydroxyphenyl)amino)-1,3,5-triazin-2-yl)amino)benzamide (T26A) (Fig. 1), a competitive inhibitor of PGT, with a K_i of 378 nM (10-fold more potent than T34). It is noteworthy that we show that T26A is specific for PGT and in whole-animal studies document the ability of T26A to systemically inhibit PGE₂ metabolism.

Materials and Methods

Materials. The cell lines used in this study were wild-type MDCK (WT-MDCK) cells that do not express endogenous PGT and MDCK cells stably transfected with the green fluorescent protein-tagged rat PGT (PGT-MDCK) as generated in the laboratory of author V.L.S. (Endo et al., 2002). Tritium-labeled PGE₂ (³H]PGE₂) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Unlabeled PGE₂, ovine cyclooxygenase (COX) 1, ovine COX2, human recombinant COX2, and human recombinant microsomal PGE₂ synthase (m-PGES) were obtained from Cayman Chemical (Ann Arbor, MI).

Synthesis of Small-Molecule Compounds. The small-molecule triazine compounds were synthesized following reported procedures (Min et al., 2007).

Inhibitory Effects of Small Molecules on PGT Activity.

These procedures were as described previously (Chi et al., 2006). In brief, PGT-MDCK cells were seeded on 24-well plates. Seventy two hours later, cells were washed twice with chilled Waymouth buffer. Then 25 μ l of Waymouth buffer containing either vehicle (DMSO) or small organic compounds was added to each well; this was immediately followed by the addition of 175 μ l of Waymouth buffer containing [³H]PGE₂. In each well, the total volume of uptake buffer was 200 μ l. Organic compounds were first dissolved in DMSO and then diluted in Waymouth buffer. The final concentration of small molecules and [³H]PGE₂ in the uptake buffer were 5 μ M and 1 nM, respectively. At 10 min, the uptake of [³H]PGE₂ was stopped by aspiration of uptake buffer; this was followed by immediate washing twice with 500 μ l of chilled Waymouth buffer. Cells were then lysed with 100 μ l of lysis buffer containing 0.25% SDS and 0.05 N NaOH. Scintillation solution (1.5 ml) was added to each well, and intracellular [³H]PGE₂ was counted by MicroBeta Counter (PerkinElmer Life and Analytical Sciences). The percentage inhibition of [³H]PGE₂ uptake by compounds was calculated as $[(\text{uptake}_{\text{vehicle}} - \text{uptake}_{\text{inhibitor}}) \div (\text{uptake}_{\text{vehicle}})] \times 100$.

K_i Values of Inhibitors. The procedures were as described previously (Chi et al., 2006). We first measured IC₅₀ of an inhibitor. We then obtained the initial velocities of PGE₂ uptake at various initial extracellular concentrations of PGE₂ in the presence of various concentrations of the inhibitor. The concentrations of the inhibitor were equal to 1- to 2-fold and 2- to 3-fold the IC₅₀. K_i values were obtained by curve-fitting the reciprocal of initial velocities of PGE₂ uptake versus the reciprocal of extracellular PGE₂ concentrations at various concentrations of the inhibitors. At low PGE₂ concentrations, the extracellular concentrations were taken as ³H-labeled PGE₂, which has a specific activity of 500 μ Ci/mol. At high concentrations of PGE₂, we made a mixture of ³H-labeled and unlabeled PGE₂ to a final specific activity of 25 μ Ci/mol.

K_i of T26A to OATPc. HeLa cells stably transfected with pMEP4-OATPc were obtained from Dr. Allan Wolkoff's laboratory at the Albert Einstein College of Medicine (Bronx, NY). Culture of these cells and induction of OATPc were conducted as described previously (Wang et al., 2003). The initial rates of [³⁵S]sulfobromophthalein (a gift from Dr. Wolkoff's laboratory) uptake by OATPc at various concentrations of [³⁵S]sulfobromophthalein in the presence of various concentrations of T26A were determined by methods similar to those described previously (Chi et al., 2006), and the K_i of T26A to OATPc was calculated accordingly (Chi et al., 2006).

T26A Effects on Cyclooxygenase Activity. The effects of T26A on the activities of ovine COX1, ovine COX2, and human recombinant COX2 enzymes were measured by using a kit from Cayman Chemical.

T26A Effects on m-PGES Activity. DMSO or various concentrations of T26A dissolved in DMSO (2.5 μ l) and 5 μ l of m-PGES (1 unit/ μ l) were added to 99.5 μ l of reaction buffer containing 100 mM

NaH₂PO₄ at pH 7.2, 0.1% Triton X-100, 1 mM EDTA, and 2.5 mM glutathione. The reaction mixture was incubated at room temperature for 15 min. Eighteen microliters of 283.7 μ M (at final concentration of 40 μ M) prostaglandin H₂ was added to the reaction mixture to initiate the reaction. At 30 s, 5 μ l of reaction mixture was taken to 45 μ l of quench buffer containing 50 mg/ml stannous chloride in 0.1 N HCl. All of these procedures were conducted at room temperature. Synthesized PGE₂ in the reaction buffer was analyzed by using the PGE₂ EIA kit from Cayman Chemical.

T26A Effects on PGE₂ Synthesis. Wild-type MDCK cells that do not express endogenous PGT were seeded onto 24-well plates at 30% confluence. Three days later, they were treated with 1 μ M arachidonic acid (AA) in the presence of either vehicle (DMSO) or 5 μ M T26A at 37°C for various durations. Cell media were collected at various time points. PGE₂ concentrations in the media were assayed by an EIA kit from Cayman Chemical.

Extracellular PGE₂ and Intracellular PGE₂-M. MDCK cells were seeded onto six-well plates at 30% confluence. Three days later, they were treated with 10 μ M bradykinin (to increase endogenous PGE₂ synthesis) in the presence of either vehicle (DMSO) or 5 μ M T26A at 37°C for various durations. In separate experiments, exogenous PGE₂ was added to the medium at the same time as either vehicle or 5 μ M T26A was added, and cells were treated for various durations. At various time points, media were collected for measurements of extracellular concentrations of PGE₂. Cells were washed with phosphate-buffered saline twice, lysed with 250 μ l of phosphate-buffered saline containing 0.1 M HCl and 0.1% Triton X-100 at room temperature for 15 min, and scraped off the plates. Cell suspensions were pipetted up and down for several times to ensure thorough lysing. Cell lysates were collected and centrifuged at 10,000g, 4°C for 10 min. Supernatants were collected. PGE₂-M in the supernatants was measured using an EIA kit from Cayman Chemical, which measures the sum of PGE₂ metabolites, including 15-keto PGE₂ and 13,14-dihydro-15-keto PGE₂.

Acute Effects of T26A on PGE₂ Metabolism In Vivo. Sprague-Dawley male rats (Charles River Laboratories, Inc., Wilmington, MA) weighing 300 to 350 g were anesthetized with xylazine (10 mg/kg)-ketamine (50 mg/kg) and were then administered 2000 U heparin (Sigma-Aldrich, St. Louis, MO). After stable anesthesia was obtained, a polyethylene catheter (PE 50; 0.97 mm OK, 0.58 mm i.d.) was advanced into the right ventricle via the right jugular vein for the administration of compounds. A Millar catheter (SPR-249; Millar Instruments, Inc., Houston, TX) was inserted into the right carotid artery and connected to a syringe for blood withdrawal. Thereafter, 200 μ l of vehicle (4% DMSO + 4% cremophor) or 25 mM T26A were injected into the jugular vein, 13 min later 1 ml of blood was withdrawn from the carotid artery, and then 100 ng of PGE₂ in 200 μ l of saline was immediately injected intravenously. The time at which PGE₂ was injected was considered 0 min in Fig. 8, C and D. Thereafter, 1 ml of arterial blood was withdrawn at 1, 3, 5, and 10 min. Indomethacin (10 μ M) was added to blood immediately after withdrawal to block further PG synthesis, and the blood was immediately centrifuged at 15,000 rpm and 4°C for 15 min. Plasma was collected and kept at -80°C for PGE₂ and PGE₂-M measurements. PGE₂ and PGE₂-M were measured using PGE₂ and PGE₂-M EIA kits from Cayman Chemical.

Results

Structure-Activity Relationship Studies of T34. As shown in Fig. 1, T34 has three side-chain moieties, R₁, R₂, and R₃, that could be modified. We designed and synthesized the second generation of this class of compounds by varying the R₁, R₂, and R₃ chain length, charge, and aromaticity/aliphaticity. Modification of R₁ and R₃ did not significantly improve affinity (data not shown). However, changes in R₂ profoundly affected the inhibitory activities of the com-

pounds. We therefore focused on further modification of R₂. The structure-activity relationships of selected second-generation PGT inhibitors are summarized in Table 1. The per-

TABLE 1

Structures and inhibition activities of some of TGBz T34 derivatives

The inhibitory activities of small molecules were determined by measurements of PGT activity of [³H]PGE₂ uptake in PGT-MDCK cells in the presence of either vehicle (DMSO) or 5 μ M small molecules. The percentage inhibition of [³H]PGE₂ uptake was calculated as [(uptake_{vehicle} - uptake_{inhibitor})/uptake_{vehicle}] $\times$ 100.

Code	Structure of R ₂	Inhibition $\pm$ S.D. (n = 3) %
T34		80 $\pm$ 6
T26A		94 $\pm$ 9
T28A		83 $\pm$ 7
T25A		82 $\pm$ 5
T22A		73 $\pm$ 6
T07A		70 $\pm$ 4
T18A		69 $\pm$ 6
T21A		69 $\pm$ 7
T08A		57 $\pm$ 6
T27A		54 $\pm$ 4
T03A		52 $\pm$ 6
T15A		49 $\pm$ 6
T14A		42 $\pm$ 3
T20A		41 $\pm$ 5
T11A		39 $\pm$ 5
T13A		24 $\pm$ 3
T09A		24 $\pm$ 2
T04A		23 $\pm$ 3
T02A		19 $\pm$ 1

Fig. 2. Inhibitory activities of selected T34 derivatives at 5 μ M with an aliphatic R₂ and 0 to 10 carbons in R₂ (A), or an aromatic benzene R₂ end and 0 to 4 carbons between the main scaffold and R₂ (B), or a phenol R₂ ends with 0 or 2 carbons between the main scaffold and R₂ (C). Inhibition = $[(\text{uptake}_{\text{vehicle}} - \text{uptake}_{\text{inhibitor}}) / \text{uptake}_{\text{vehicle}}] \times 100$. Values are mean $\pm$ S.D. ($n = 3$).

centage inhibition of tracer PGE₂ uptake at 5 μ M concentration was calculated as $[(\text{uptake}_{\text{vehicle}} - \text{uptake}_{\text{inhibitor}}) / \text{uptake}_{\text{vehicle}}] \times 100$. Overall, the compounds containing an aromatic benzene ring showed better inhibitory activity than the compounds containing an aliphatic chain in the R₂ moiety. The charge of the group on the aromatic ring had significant impact on inhibitory activity (-OH or -H or -F > -NH₂), indicating that positive charge decreases inhibition.

Figure 2 shows the effect of chain length on inhibition. We varied the number of carbons from 0 to 10 for the aliphatic R₂ and from 0 to 4 for the aromatic R₂. When R₂ is an aliphatic straight chain ending with a -CH₃, the inhibition increases as n increases and reaches the highest level at n of 7; thereafter, the inhibition decreases as n increases further (Fig. 2A). When R₂ ends with an aromatic benzene, only a benzyl type of substituent, which has one carbon between the benzene ring and the main scaffold, reduces the inhibitory potency dramatically (Fig. 2B). The inhibitions with $n = 0$ and 2 carbons between the benzene ring and the main scaffold are almost equal and are higher than all of the others (Fig. 2B). When R₂ ends with a phenol group, the inhibition is higher when $n = 0$ than $n = 2$ carbons between the benzene ring and the main scaffold (Fig. 2C), implying that -OH enhances inhibition and that rigidity in the R₂ group is in favor of inhibition. These results show that the inhibitory activity is sensitive to the structure of the R₂ moiety, suggesting that R₂ is critical for binding to PGT.

Kinetics of PGT Inhibitors. We next determined the binding constants of the top three inhibitors listed in Table 1, namely, T26A, *N*-(2-(2-azidoethoxy)ethoxy)ethyl-4-((4-((2-(2-benzamidoethoxy)ethoxy)ethyl)amino)-6-(phenylamino)-1,3,5-triazin-2-yl)amino)benzamide (T28A), and *N*-(2-(2-azidoethoxy)ethoxy)ethyl-4-((4-((2-(2-benzamidoethoxy)ethoxy)ethyl)amino)-6-((4-fluorophenyl)amino)-1,3,5-triazin-2-yl)amino)benzamide (T25A), by measuring the initial velocities of PGE₂ uptake by PGT at various concentrations of PGE₂ in the presence of various concentrations of one of these inhibitors. We generated K_i values and determined the type of inhibition of these inhibitors by fitting Lineweaver-Burk curves, as shown in Fig. 3. The resulting K_i values of these three inhibitors are listed in Table 2. They all are competitive inhibitors. T26A has the highest affinity with a K_i of 378 nM, a value reasonably close to the binding constant of the substrate PGE₂ to PGT (90 nM) (Kanai et al., 1995).

T26A Derivatives. Modification of T34 as above had revealed that aromaticity was in favor of inhibition. We therefore increased aromaticity of T26A by replacing the phenol with a naphthol group. As shown in Table 3, the K_i of T26A-1 is slightly higher than that of T26A, suggesting that either the interaction between PGT and the inhibitor does not require aromaticity higher than that possessed by the phenyl

Fig. 3. Lineweaver-Burk plot of TGBz T26A. Initial velocities of [³H]PGE₂ uptake by PGT in PGT-MDCK cells at various concentrations of [³H]PGE₂ in the presence of various concentrations of T26A were measured during the first 2 min of [³H]PGE₂ uptake (the linear range). K_i value and type of inhibition were determined by fitting Lineweaver-Burk curves.

TABLE 2

K_i values of T26A, T28A, and T25A

Initial velocities of [³H]PGE₂ uptake by PGT in PGT-MDCK cells at various concentrations of [³H]PGE₂ in the presence of various concentrations of one of these inhibitors were measured during the first 2 min of [³H]PGE₂ uptake (the linear range). K_i values were determined by fitting Lineweaver-Burk curves. For each compound, K_i was measured three times. Values are mean $\pm$ S.D.

Code	Structure of R ₂	$K_i \pm$ S.D. ($n = 3$)
		nM
T26A		378 $\pm$ 25
T28A		3631 $\pm$ 291
T25A		3929 $\pm$ 302

ring or that the potential higher affinity caused by higher aromaticity of the naphthalene ring was cancelled by the bulkiness of the naphthalene ring.

Comparison of the best three compounds in Table 2 indicates that -OH on the benzene ring enhances binding affinity. To determine whether the position of -OH mattered, we moved -OH from the *para* to the *meta* position (T26A-2). Table 3 shows that this reduced inhibition.

The -OH of T26A can be either a donor or a receiver of protons. To address this issue, we replaced it with -OCH₃ (T26A-3). The methoxy group of T26A-3 slightly reduced the inhibitory activity compared with T26A, suggesting that this -OH is possibly a proton donor, and therefore the amino acid of PGT that interacts with T26A is possibly a proton receiver.

The native substrates of PGT contain a -COOH at the end of the carbon chain, and PGT functions as an anion transporter (Chan et al., 1998, 2002). Thus, we hypothesized that adding a -COOH could increase binding to PGT substrate sites as well as increasing water solubility. However, a

TABLE 3

T26A derivatives and their K_i values

Initial velocities of [³H]PGE₂ uptake by PGT in PGT-MDCK cells at various concentrations of [³H]PGE₂ in the presence of various concentrations of one of these inhibitors were measured during the first 2 min of [³H]PGE₂ uptake (the linear range). K_i values were determined by fitting Lineweaver-Burk curves. For each compound, K_i was measured three times. Values are mean ± S.D.

Code	Structure of R ₂	$K_i \pm \text{S.D. (n = 3)}$
		<i>nM</i>
T26A		378 ± 25
T26A-1		477 ± 37
T26A-2		766 ± 59
T26A-3		663 ± 62
T26A-4		4118 ± 325

-COOH did not increase affinity (Table 3), nor did it affect solubility (data not shown).

Specificity of T26A. For any chemical modulator, specificity is always an issue. PGT is an organic anion transporter and belongs to the larger OATP family (Hagenbuch and Meier, 2004). Because it is not feasible to test T26A specificity against all of members of this transporter superfamily, we chose OATPc, which has high homology to PGT (Kanai et al., 1995). We measured the K_i of T26A against this transporter using its substrate sulfobromophthalein (Wang et al., 2003). The K_i of T26A for OATPc is approximately 10 μM, i.e., almost 30-fold higher than that for PGT, suggesting that T26A has high specificity in favor of PGT compared with other organic anion transporters.

T26A on COX Activity. Another potential concern is whether T26A interacts with components of the PGE₂ signaling pathway involved in PGE₂ production, such as COX1/2. To directly determine whether T26A had any effect on COX activity, we obtained commercially available ovine COX1, ovine COX2, and human recombinant COX2 enzymes and measured PG synthesis upon addition of AA in the absence and presence of various concentrations of T26A. Figure 4 shows that T26A did not have any significant effects on the activities of those three enzymes, either at a concentration close to the K_i of T26A to PGT or at a concentration 100-fold higher than the K_i of T26A to PGT.

T26A on PGES Activity. PGE₂ synthesis also relies on the activity of PGE₂ specific synthases, such as m-PGES. We further determined that T26A did not have significant effect on m-PGES activity (Fig. 5).

T26A on Overall PGE₂ Synthesis in MDCK Cells in the Absence of PGT. As an integrative test of whether T26A affects PGE₂ synthesis, and to confirm the results shown in Figs. 4 and 5, we measured PGE₂ synthesis/release in response to AA addition in WT-MDCK cells (which lack PGT expression), in both the presence and absence of T26A. As shown in Fig. 6, T26A did not affect PGE₂ synthesis and release at any time point, arguing against any additional T26A effects on m-PGES-2 and/or cytosolic PGES.

Together, these results indicate that T26A is a highly selective inhibitor of PGT, which is the influx step for PGE₂ that is required for PGE₂ intracellular metabolism.

Effects of PGT Inhibition on PGE₂ Metabolism in Cell Culture. We next determined whether PGT inhibition with T26A affects the metabolism of PGE₂ generated endogenously in response to bradykinin. We treated either WT-MDCK cells or PGT-MDCK cells with bradykinin and either vehicle or T26A and measured extracellular PGE₂ and intracellular PGE₂-M. In WT-MDCK cells, the absence of PGT resulted in almost all of the released PGE₂ remaining in the extracellular compartment (Fig. 7A). Extracellular PGE₂ accumulated as time went on, whereas the amount of PGE₂-M in the intracellular compartment remained low (Fig. 7B). In these WT-MDCK cells, T26A did not affect PGE₂ metabolism (Fig. 7, A and B), a result consistent with the lack of a PGE₂ uptake mechanism in these wild-type cells. In contrast, the expression of PGT at the plasma membrane enabled PGT-MDCK cells to transport newly released medium PGE₂ from the extracellular compartment back into the intracellular compartment and deliver it to 15PGDH for subsequent oxidation to PGE₂-M (Fig. 7, A and B). Thus, extracellular PGE₂ was 2- to 3-fold lower in PGT-MDCK cells than in WT-MDCK cells, whereas intracellular PGE₂-M was 2- to 3-fold higher in PGT-MDCK cells than in WT-MDCK cells (Fig. 7, A and B). These results are similar to those previously reported from the laboratory of author V.L.S. (Nomura et al., 2004). It is noteworthy that T26A almost completely blocked PGE₂ influx and subsequent oxidation, such that PGE₂ was retained in the extracellular compartment at the levels similar to those in WT-MDCK cells (Fig. 7, A and B).

To determine whether PGT is capable of regulating the metabolism of exogenous PGE₂, we applied 10 nM exogenous PGE₂ to cells, together with vehicle or T26A. As was the case with T26A on endogenous PGE₂ metabolism, in WT-MDCK

Fig. 4. T26A on purified COX activities. COX activity was measured as the COX-catalyzed PGE₂ synthesis, which was initiated by adding AA to the reaction mixtures containing ovine COX1 (A), ovine COX2 (B), or human recombinant COX2 (C) in the presence of vehicle (Ctl; DMSO) or various concentrations of T26A. Experiments were conducted in triplicate. Values are mean ± S.E.M. No significant differences were found between experiments conducted with vehicle or T26A.

Fig. 5. T26A on purified m-PGES activity. m-PGES activity was measured as m-PGES catalyzed PGE₂ synthesis, which was initiated by adding prostaglandin H₂ to the reaction mixture containing human recombinant m-PGES in the presence of vehicle (DMSO) or various concentrations of T26A. These experiments were conducted in triplicate. Values are mean ± S.E.M. No significant differences were found between experiments conducted with vehicle or T26A.

Fig. 6. T26A on PGE₂ synthesis in intact cells. Wild-type MDCK cells that do not express endogenous PGT were treated with 1 μM AA in the presence of either vehicle (Veh; DMSO) or 5 μM T26A for various durations at 37°C. Cell media were collected at the indicated time points. PGE₂ in media was assayed by an EIA kit from Cayman Chemical. Experiments were conducted in triplicate. Values are mean ± S.E.M. No significant difference was found between experiments conducted with or without T26A.

cells almost all of the exogenously added PGE₂ remained in the extracellular compartment as intact PGE₂ over 3 h (Fig. 7, C and D). In contrast, PGT-MDCK cells eliminated 95% of the added PGE₂ from the medium; this resulted in a rise of intracellular PGE₂-M (Fig. 7, C and D). With time, extracellular PGE₂ gradually increased, whereas intracellular PGE₂-M gradually decreased, a result of the pump leak-mediated overshoot of PGE₂ influx that we have described previously (Chan et al., 1998; Chi et al., 2006). It is noteworthy that T26A was able to abrogate the PGE₂ metabolism caused by PGT (Fig. 7, C and D). These results demonstrate that extracellular PGE₂, even at concentrations much higher than endogenous levels, is rapidly internalized by PGT and is subsequently metabolized to PGE₂-M, and that this PGE₂ metabolism can be effectively prevented by inhibiting PGT with T26A.

T26A on PGE₂ Metabolism In Vivo. Endogenous and exogenous PGE₂ are nearly completely metabolized in a single passage through the lung (Ferreira and Vane, 1967; Piper et al., 1970; Dawson et al., 1975; Schuster, 1998), and PGT is very strongly expressed in the lung (Kanai et al., 1995; Lu et al., 1996; Pucci et al., 1999). To assess the ability of T26A to block PGE₂ metabolism in vivo, we injected vehicle/T26A into

the jugular vein of rats and withdrew blood via the carotid artery. Figure 8A shows that the arterial concentration of endogenous PGE₂ in anesthetized rats injected intravenously with T26A peaked at more than 200% that of rats injected with vehicle. Conversely, the concentration of endogenous arterial PGE₂-M was reduced 50% by intravenous T26A (Fig. 8B). T26A also slowed the elimination of exogenously added PGE₂. As shown in Fig. 8C, compared with rats previously injected with vehicle alone, those injected with T26A achieved a 3- to 4-fold higher arterial PGE₂ concentration after an intravenous PGE₂ injection. In accord with these results, the concentration of arterial PGE₂-M was 3- to 4-fold lower in T26A-treated rats compared with that of vehicle-treated controls.

Discussion

The present studies describe the development, using structure-function analysis, of a high-affinity inhibitor, T26A, of the PGT. In cultured MDCK cells, T26A blocks PGE₂ uptake and its subsequent metabolism to PGE₂ metabolites including 15-keto-PGE₂. When injected intravenously into rats, T26A raises the plasma level of endogenous PGE₂ and reduces the plasma level of the PGE₂ metabolites. Rats preinjected with T26A demonstrate impaired metabolism of exogenously administered PGE₂. T26A does not affect the activities of purified COX1, COX2, and m-PGES, nor does it affect overall PGE₂ synthesis in cultured cells. Thus, T26A increases PGE₂ via inhibition of metabolism rather than activation of synthesis.

PGE₂ triggers a vast variety of signals including inflammation, vasodilation, and angiogenesis (Weeks, 1972; Clyman et al., 1978; Tsujii et al., 1998). The extent of PG signaling depends, to a large degree, on its concentration at cell-surface receptors, which is determined, in turn, by the relative rates of synthesis and metabolism. Although prostanoid investigators historically focused on synthetic pathways, rapid metabolic clearance of PGs was reported as early as the 1960s (Ferreira and Vane, 1967). Subsequent work demonstrated that PGE₂ added to the perfusate of the isolated, perfused lung or kidney disappears within 5 min and reappears as the 15-keto-PGE₂ metabolite in the perfusion collections (Bito and Barody, 1975; Dawson et al., 1975; Anderson and Eling, 1976; Bito, 1976; Bito et al., 1976, 1977; Eling and Anderson, 1976; Eling et al., 1977; Hawkins et al., 1977, 1978). These groundbreaking early studies further revealed that when bromocresol green, a nonspecific organic anion transport inhibitor, was applied to the perfusion system PGE₂ appeared intact in the perfusion collections, suggesting that PGE₂ metabolism depended on its transport. These earlier studies are consistent with the present data as presented in Fig. 7, C and D, namely, MDCK cells [which express 15PGDH (Nomura et al., 2005)] that have been engineered to also express a PGE₂ uptake carrier (PGT) convert PGE₂ to the 15-keto metabolite.

Despite this extensive prior literature, the molecular mechanism of PG transport and its role in regulating PG signal termination have been appreciated only recently. We molecularly identified the PGT (Kanai et al., 1995) and postulated that it is responsible for PGE₂ uptake before enzymatic oxidation (Schuster, 1998, 2002). We have pre-

Fig. 7. Effects of PGT inhibition on PGE₂ metabolism in MDCK cells. WT-MDCK and PGT-MDCK cells were seeded at 37°C for 72 h and treated with 10 μ M bradykinin (A and B) or 10 nM exogenous PGE₂ (C and D) in the presence of either DMSO [as vehicle (Veh)] or 5 μ M T26A for various durations at 37°C. Cell media were collected for extracellular PGE₂ measurements, and cells were lysed for intracellular PGE₂-M measurements. Experiments were conducted in triplicate. Values are mean $\pm$ S.E.M. *, $p < 0.05$ by unpaired t test.

Fig. 8. Effects of PGT inhibition on PGE₂ metabolism in vivo. A and B, endogenous PGE₂ and PGE₂-M levels in blood withdrawn from carotid artery of rats 13 min after injection of 100 μ l of vehicle (Veh; 4% DMSO + 4% cremophor) or 25 mM T26A via the jugular vein. C and D, rats were preinjected with 100 μ l of vehicle or 25 mM T26A via jugular vein and 13 min later were injected with 100 ng of exogenous PGE₂ (in 100 μ l of saline) intravenously. Data show time course of the resulting PGE₂ and PGE₂-M arterial blood levels. The time at which PGE₂ was injected was set at 0 min. Values are mean $\pm$ S.E.M. ($n = 5$). *, $p < 0.05$ by unpaired t test.

viously shown in culture cells that PGT is critical for PGE₂ metabolism (Nomura et al., 2004), because PGT-mediated PGE₂ reuptake is the main pathway for PGE₂ influx (Chi and Schuster, 2010) and is a prerequisite for PGE₂ intracellular metabolism (Nomura et al., 2004). We have also reported that adult mice rendered null at the PGT locus have elevated systemic (urinary) PGE₂ levels and lowered plasma PGE₂ metabolite levels compared with wild-type mice (Chang et al., 2010), indicating that PGT plays an important role in regulating PG levels in the circulation. That said, because in those genetic studies PGT was knocked out from the single-cell stage, those studies did not allow us to determine whether the increased PGE₂ in plasma was a direct result of PGT deletion or rather was caused by one or more biological modulations that resulted indirectly from PGT deletion. The fact that pharmacological inhibition of PGT with T26A in the present experiments increased plasma PGE₂ within several minutes (Fig. 8) indicates that PGT does indeed directly modulate extracellular PGE₂ levels. The present results also suggest that pharmacological PGT inhibition is a powerful tool for studying the biological roles of PGT.

The SAR studies described in this article allowed us to obtain an inhibitor, T26A, that is 10-fold more potent than its precursor (Chi et al., 2006). There is some structural resemblance between T26A and PGE₂ (Fig. 1, B and C). R₂ of T26A resembles the five-member ring in PGE₂. The -OH on the ring of PGE₂ is important for binding, and it is probably a proton donor, rather than a receiver (Chi et al., 2010). Likewise, the -OH of R₂ is important for binding and is probably a proton donor because T26A binds to PGT

more strongly than T26A-3 does (Table 3). R₁ and R₃ possibly resemble the two acyl chains of PGE₂. In this regard, replacing the -OH group of R₂ with a -COOH did not improve affinity (Table 3), suggesting that a -COOH in the end of either R₁ or R₃ could possibly improve solubility without adversely affecting affinity.

The results of SAR studies of T34 and T26A provide a basis for speculating about the binding site of PGT. T34 has three moieties, only one of which, R₂, is sensitive to inhibition, suggesting that PGT possibly has only one binding site for its substrates. The present studies suggest that the amino acids of PGT that binds to the substrates is probably positively charged, because a negative charge on the inhibitor site increases affinity (Table 1). These results are in accord with our previous studies demonstrating that PGT is inhibited by disulfonic stilbenes, niflumic acid, and the thiol reactive anion MTSES [Na(2-sulfonatoethyl) methanethiosulfonate] (Chan et al., 1998); by our cysteine-scanning mutagenesis and molecular modeling of putative transmembrane 10, which indicated that the substrate binding of PGT is formed among its membrane-spanning segments, with four residues along the cytoplasmic end of helix 10 contributing to one surface of the binding site (Chan et al., 1999); and by our site-directed mutagenesis studies indicating a critical role for arginine 560 in substrate translocation (Chan et al., 2002).

In addition, we hypothesize that the docking pocket for T26A probably has limited space to accommodate a substrate, because replacing phenol with naphthol did not increase the affinity (Table 3), even though naphthol has higher aromaticity than phenol, which is presumably in favor of binding (Table 1).

All small-molecule inhibitors suffer from possible lack of specificity. We have addressed the issue of specificity by examining the affinity of T26A for a closely related gene family member, OATPc (SLCO1B1) that has high homology with PGT (Kanai et al., 1995). SLCO1B1 is a particularly relevant homolog for this study because it mediates the hepatic uptake of various drugs, including most statins and statin acids (König et al., 2006), and because genetic variations in the SLCO1B1 gene have been associated with both statin-associated myopathy and reductions in the lipid-lowering effect of statins (SEARCH Collaborative Group, 2008). The 10-fold weaker binding of T26A to OATPc compared with PGT suggests that T26A is a selective inhibitor of PGT within the OATP gene family.

In addition, we determined whether T26A directly interacted with synthases of PGE₂. Insignificant effects of T26A on the activities of COX1, COX2, and m-PGES (Figs. 4 and 5) eliminated the concern that increased PGE₂ by T26A could be caused by activation of those synthases. This was further confirmed by the fact that T26A did not affect overall PGE₂ production in cells that did not express PGT (Fig. 6). That T26A does not affect PGE₂ synthesis is also supported by the in vivo data showing that endogenous serum PGE₂ levels were more than doubled within short period of time (15 min) after T26A injection (Fig. 8A).

In addition to being a powerful basic research tool for investigating the fundamental role of PGT in PG metabolism and signal termination, a potent PGT inhibitor such as T26A provides a starting point for the development of therapeutic agents targeting PGT. Because PGE₂ and

other PGs trigger a vast array of beneficial physiological events, and PGT regulates the metabolism of PGs, a specific inhibitor of PGT could potentially be developed for clinical applications, such as open-angle glaucoma (Parish et al., 2003) and pulmonary hypertension (Gomberg-Maitland and Olschewski, 2008), among others.

Authorship Contributions

Participated in research design: Chi and Schuster.

Conducted experiments: Chi and Jasmin.

Contributed new reagents or analytic tools: Chi, Min, Lisanti, and Chang.

Performed data analysis: Chi, Jasmin, and Schuster.

Wrote or contributed to the writing of the manuscript: Chi, Min, and Schuster.

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