Developement 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 an 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 (T26A), a competitive inhibitor of PGT, with a Ki 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; EC 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$_2$-M), 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$_2$. The work was supported by the National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases (Grant 5R01-DK049688) and the American Heart Association (Grant 0830336N).

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The best hit of that screening was T34 (Fig. 1) with a binding affinity of 3.7 μM. Although the IC50 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)-6,6-(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 has a specific activity 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 μl 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 μl of Waymouth buffer containing [3H]PGE2. In each well, the total volume of uptake buffer was 200 μl. 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 μM 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 μl of chilled Waymouth buffer. Cells were then lysed with 100 μl 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 ([uptakevehicle - uptakeinhibitor] / (uptakevehicle)) × 100.

Kᵢ 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. Kᵢ 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 Ci/mol. At high concentrations of PGE2, we made a mixture of 3H-labeled and unlabeled PGE2 to a final specific activity of 25 μCi/mol.

Kᵢ 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 Kᵢ 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 μl) and 5 μl of m-PGES (1 unit/μl) were added to 99.5 μl 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 μM (at final concentration of 40 μM) prostaglandin H₂ was added to the reaction mixture to initiate the reaction. At 30 s, 5 μl of reaction mixture was taken to 45 μl 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 PGE were seeded onto 24-well plates at 30% confluence. Three days later, they were treated with 1 μM arachidonic acid (AA) in the presence of either vehicle (DMSO) or 5 μM 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 μM bradykinin (to increase endogenous PGE₂ synthesis) in the presence of either vehicle (DMSO) or 5 μM 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 μM 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 μl 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 inserted into the right carotid artery, and then 100 ng of PGE₂ in 200 μl 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 μM) 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 compounds. 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>
<thead>
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<th>Code</th>
<th>Structure of R₂</th>
<th>Inhibition ± S.D. (n = 3) %</th>
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<td>T34</td>
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<td>80 ± 6</td>
</tr>
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percentage inhibition of tracer PGE\textsubscript{2} uptake at 5 \textmu 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 R\textsubscript{2} moiety. The charge of the group on the aromatic ring had significant impact on inhibitory activity (-OH or -H or -F > -NH\textsubscript{2}), 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 R\textsubscript{2} and from 0 to 4 for the aromatic R\textsubscript{2}. When R\textsubscript{2} is an aliphatic straight chain ending with a -CH\textsubscript{3}, the inhibition increases as \(n\) increases and reaches the highest level at \(n = 7\); thereafter, the inhibition decreases as \(n\) increases further (Fig. 2A). When R\textsubscript{2} 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 R\textsubscript{2} 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 R\textsubscript{2} group is in favor of inhibition. These results show that the inhibitory activity is sensitive to the structure of the R\textsubscript{2} moiety, suggesting that R\textsubscript{2} 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-(2-azidoethoxy)ethoxy)ethyl)ethylamino)-6-(phenylamino)-1,3,5-triazin-2-yl)amino)benzamide (T28A), and \(N-(2-(2-(2-azidoethoxy)ethoxy)ethyl)ethylamino)-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 PGE\textsubscript{2} uptake by PGT at various concentrations of PGE\textsubscript{2} 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 PGE\textsubscript{2} 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 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 -OCH\textsubscript{3} (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
-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 PGE$_2$ signaling pathway involved in PGE$_2$ 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 PGE$_2$ Synthesis in MDCK Cells in the Absence of PGT.** As an integrative test of whether T26A affects PGE$_2$ synthesis, and to confirm the results shown in Figs. 4 and 5, we measured PGE$_2$ 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 PGE$_2$ synthesis and release at any time point, arguing against any additional T26A effects on m-PGES-2 and/or cytosolic PGE$_2$.

Together, these results indicate that T26A is a highly selective inhibitor of PGT, which is the influx step for PGE$_2$ that is required for PGE$_2$ intracellular metabolism.

**Effects of PGT Inhibition on PGE$_2$ Metabolism in Cell Culture.** We next determined whether PGT inhibition with T26A affects the metabolism of PGE$_2$ 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 PGE$_2$ and intracellular PGE$_2$-M.

As shown in Fig. 7, A and B), a result consistent with the lack of a PGE$_2$ 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 PGE$_2$ from the extracellular compartment back into the intracellular compartment and deliver it to 15PGDH for subsequent oxidation to PGE$_2$-M. Thus, extracellular PGE$_2$ was 2- to 3-fold lower in WT-MDCK cells than in WT-MDCK cells, whereas intracellular PGE$_2$-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 PGE$_2$ influx and subsequent oxidation, such that PGE$_2$ 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 PGE$_2$, we applied 10 nM exogenous PGE$_2$ to cells, together with vehicle or T26A. As was the case with T26A on endogenous PGE$_2$ metabolism, in WT-MDCK...
cells almost all of the exogenously added PGE$_2$ remained in the extracellular compartment as intact PGE$_2$ over 3 h (Fig. 7, C and D). In contrast, PGT-MDCK cells eliminated 95% of the added PGE$_2$ from the medium; this resulted in a rise of intracellular PGE$_2$-M (Fig. 7, C and D). With time, extracellular PGE$_2$ gradually increased, whereas intracellular PGE$_2$-M gradually decreased, a result of the pump leak-mediated overshoot of PGE$_2$ that we have described previously (Chan et al., 1998; Chi et al., 2006). It is noteworthy that T26A was able to abrogate the PGE$_2$ metabolism caused by PGT (Fig. 7, C and D). These results demonstrate that extracellular PGE$_2$, even at concentrations much higher than endogenous levels, is rapidly internalized by PGT and is subsequently metabolized to PGE$_2$-M, and that this PGE$_2$ metabolism can be effectively prevented by inhibiting PGT with T26A.

T26A on PGE$_2$ Metabolism In Vivo. Endogenous and exogenous PGE$_2$ 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 PGE$_2$ 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 PGE$_2$ in anesthetized rats injected intravenously with T26A peaked at more than 200% that of rats injected with vehicle. Conversely, the concentration of endogenous arterial PGE$_2$ was reduced 50% by intravenous T26A (Fig. 8B). T26A also slowed the elimination of exogenously added PGE$_2$. 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 PGE$_2$ concentration after an intravenous PGE$_2$ injection. In accord with these results, the concentration of arterial PGE$_2$-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 PGE$_2$ uptake and its subsequent metabolism to PGE$_2$ metabolites including 15-keto-PGE$_2$. When injected intravenously into rats, T26A raises the plasma level of endogenous PGE$_2$ and reduces the plasma level of the PGE$_2$ metabolites. Rats preinjected with T26A demonstrate impaired metabolism of exogenously administered PGE$_2$. T26A does not affect the activities of purified COX1, COX2, and m-PGES, nor does it affect overall PGE$_2$ synthesis in cultured cells. Thus, T26A increases PGE$_2$ via inhibition of metabolism rather than activation of synthesis.

PGE$_2$ 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 prostaglandin 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 PGE$_2$ added to the perfusate of the isolated, perfused lung or kidney disappears within 5 min and reappears as the 15-keto-PGE$_2$ metabolite in the perfusion collections (Bito and Baroody, 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 PGE$_2$ appeared intact in the perfusion collections, suggesting that PGE$_2$ 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 PGE$_2$ uptake carrier (PGT) convert PGE$_2$ 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 PGE$_2$ uptake before enzymatic oxidation (Schuster, 1998, 2002). We have pre-
Previously 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.

**Fig. 7.** 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 µl of vehicle (Veh; 4% DMSO + 4% cremophor) or 25 mM T26A via the jugular vein. C and D, rats were preinjected with 100 µl of vehicle or 25 mM T26A via jugular vein and 13 min later were injected with 100 ng of exogenous PGE₂ (in 100 µl 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 ± 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 µl of vehicle (Veh; 4% DMSO + 4% cremophor) or 25 mM T26A via the jugular vein. C and D, rats were preinjected with 100 µl of vehicle or 25 mM T26A via jugular vein and 13 min later were injected with 100 ng of exogenous PGE₂ (in 100 µl 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 ± S.E.M. (*) p < 0.05 by unpaired t test.
more strongly than T26A-3 does (Table 3). \(R_1\) and \(R_3\) possibly resemble the two acyl chains of PGE\(_2\). In this regard, replacing the -OH group of \(R_2\) with a -COOH did not improve affinity (Table 3), suggesting that a -COOH in the end of either \(R_1\) or \(R_3\) 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, \(R_3\), 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 lipids of plasma protein binding.

In addition, we determined whether T26A directly interacts with PGE\(_2\) 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 (Parrish et al., 2003) and pulmonary hypertension (Gomberg-Maitland and Olszewski, 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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