Identification of a New Class of Prostaglandin Transporter Inhibitors and Characterization of Their Biological Effects on Prostaglandin E\textsubscript{2} Transport

Yuling Chi, Sonya M. Khersonsky, Young-Tae Chang, and Victor L. Schuster

Departments of Medicine and Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York (Y.C., V.L.S.); and New York University, Department of Chemistry, New York, New York (S.M.K., Y.-T.C.)

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ABSTRACT

Prostaglandins (PGs) are involved in several major signaling pathways. Their effects are terminated when they are transported across cell membranes and oxidized intracellularly. The transport step of PG metabolism is carried out by the prostaglandin transporter (PGT). Inhibition of PGT would therefore be expected to change local or circulating concentrations of prostaglandins, and thus their biological effects. To develop PGT-specific inhibitors with high affinity, we designed a library of triazine compounds and screened 1842 small molecules by using Madin-Darby canine kidney cells stably expressing rat PGT. We found several effective PGT inhibitors. Among them, the most potent inhibitor had a $K_i$ of $3.7 \pm 0.2$ nM. These inhibitors allowed us to isolate the efflux process of PGE\textsubscript{2} and to demonstrate that PGT does not transport PGE\textsubscript{2} outwardly under physiological conditions.

Prostaglandins (PGs) are synthesized from arachidonic acid by cyclooxygenases (COX1 and COX2) and corresponding synthases (Helliwell et al., 2004). PGs play an important role in physiology and clinical settings. Their biological effects include triggering inflammation, fever, and pain (Blatteis and Sehic, 1997; Bley et al., 1998; Vanegas and Schaible, 2001; Samad et al., 2002); induction of labor (Ullmann et al., 1992); modulation of renal hemodynamics and of water and solute reabsorption (Epstein, 1986; Wang et al., 1998; Yokoyama et al., 2002); and arterial vasodilatation (Clyman et al., 1978; Ceeiani and Olley, 1988; Smith et al., 1994). PG analogs, such as latanoprost and unoprostone, have been used to treat glaucoma (Stjernschantz, 1995, 2004; Alm, 1998; Susanna et al., 2002). At the cellular level, PGs are involved in several major signaling pathways, including the mitogen-activated protein kinase and protein kinase A pathways by up-regulation of cAMP (Narumiya et al., 1999; Bos et al., 2004).

The magnitude of PG effects depends not only on their production but also on their metabolism. We identified the prostaglandin transporter (PGT) (Kanai et al., 1995) and have reported that PGT removes PGs from the extracellular compartment and thereby terminates their interactions with receptors on cell membranes. PGT delivers PGs to cytoplasmic 15-OH PG dehydrogenase (Schuster, 2002; Nomura et al., 2004), resulting in oxidation and inactivation.

Because PGT is highly expressed in the tissues and organs where PGs are synthesized (Bao et al., 2002), and because PGT regulates a broad and complex PG signaling system, an inhibitor of PGT would be important for manipulating signaling. Known PGT blockers include inhibitors of the organic anion transporters, such as bromcresol green and bromosulfophthalein, and some COX2 inhibitors, such as indomethacin and ibuprofen (Bito and Salvador, 1976; Kanai et al., 1995). One of the main problems with these inhibitors is that they are not specific for PGT (Jacquemin et al., 1994; Sweet et al., 1997). Thus, a search for additional PGT inhibitors is indicated.

To develop high affinity, PGT-specific inhibitors, we screened compounds from a triazine library. Using MDCK cells stably expressing PGT (Endo et al., 2002), screening of 1842 small molecules yielded several effective inhibitors. The most potent inhibitor in this group of compounds, TGBz T34, has a $K_i$ of $3.7 \pm 0.2$ nM. This compound also permitted us to isolate the efflux process of PGE\textsubscript{2} and to demonstrate that PGT does not transport PGE\textsubscript{2} outwardly under physiological conditions.

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ABBREVIATIONS: PG, prostaglandin; COX, cyclooxygenase; PGT, prostaglandin transporter; MDCK, Madin-Darby canine kidney.

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Materials and Methods

Materials. The cell lines used in this study were 3T3 cells that express endogenous PGT and MDCK cells stably transfected with the green fluorescent protein-tagged PGT in our laboratory (Endo et al., 2002). Tritium-labeled PGE₂ (\(^{[3}H\)PGE₂) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Unlabeled PGE₂ was obtained from Cayman Chemical (Ann Arbor, MI).

Synthesis of 1842 Small Molecule Compounds. The methods and procedures for synthesis of 1842 compounds were reported previously (Moon et al., 2002; Bork et al., 2003a,b; Khersonsky et al., 2003; Uttamchandani et al., 2004).

PGE₂ Transport Measurement. MDCK or 3T3 cells were seeded at 15 to 20% confluence on 24-well plates. The day on which the cells were seeded was considered day 1. PGE₂ uptake experiments were conducted on day 4. All of the PGE₂ uptake experiments were conducted at room temperature. On day 4, cells were washed twice with Waymouth buffer (135 mM NaCl, 13 mM H-HEPES, 13 mM Na-HEPES, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 0.8 mM MgSO₄, 5 mM KCl, and 28 mM D-glucose). Then, 200 µl of Waymouth buffer containing \(^{[3}H\)PGE₂ was added to each well. At the designed time, the uptake of \(^{[3}H\)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 \(^{[3}H\)PGE₂ was counted by MicroBeta counter (Perkin Elmer Life and Analytical Sciences).

For preliminary screening of the compounds, 25 µl of Waymouth buffer containing small organic compounds was added to each well; this was immediately followed by the addition of 175 µl of Waymouth buffer containing \(^{[3}H\)PGE₂. In each well, the total volume of uptake medium was 200 µl. Organic compounds were first dissolved in dimethyl sulfoxide and then diluted in Waymouth buffer. The percentage of inhibition of \(^{[3}H\)PGE₂ uptake by compounds was calculated as [(uptake\(_{\text{vehicle}} \) - uptake\(_{\text{inhibitor}}\) ) / (uptake\(_{\text{vehicle}}\) )] × 100.

Measurements of \(K_i\) Values. The initial velocities at various initial extracellular concentrations of PGE₂ were determined from the PGE₂ uptake in the first 2 min; these were linear over the time course of PGE₂ uptake. \(K_i\) 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 \(^{[3}H\)labeled PGE₂, which has a specific activity of 500 Ci/mol. At high concentrations of PGE₂, we made a mixture of \(^{[3}H\)labeled and unlabeled PGE₂ to a final specific activity of 35 Ci/mol.

Results

Screening of Small Molecules for Inhibition of PGE₂ Uptake. The small molecule triazine library compounds (1842 members) were synthesized following reported procedures (Moon et al., 2002; Bork et al., 2003a,b; Khersonsky et al., 2003; Uttamchandani et al., 2004). The main scaffolds of the compounds are depicted in Fig. 1 with our codes of AA, BN, EA, RT, TF, and TGBz. The full structural information on R1 and R2 groups is provided in Supplemental Figs. 1S and 2S. Among the 1842 compounds tested, the six compounds with the highest inhibitory activities were all from the TGBz scaffold. The T substituent at the R1 position and an acidic group (COOH or phenol) at the R2 position constitute important motifs for activity.

Determination of \(K_i\) Values of Inhibitors and Their Modes of Inhibition. Of the six initial compounds, we chose TGBz T34, T07, and T41 (Fig. 2) to further determine their inhibition kinetic parameters. Structures of the other three compounds are given in Supplemental Fig. 3S. The inhibition constant of TGBz T34 and its mode of inhibition, as determined by varying the PGE₂ concentrations at fixed levels of TGBz T34, are shown in Fig. 3. The pattern was characteristic for competitive inhibition. The same experiments were conducted for TGBz T41 and T07; the \(K_i\) values are listed in Table 1. All of these compounds are competitive inhibitors of PGT. TGBz T34 is the most potent inhibitor with a \(K_i\) of 3.7 ± 0.2 µM. In separate experiments, when cells were preincubated in TGBz T34 for 10 or 20 min, the \(K_i\) was not significantly different from that obtained by adding TGBz T34 simultaneously with PGE₂ (0-min preincubation \(K_i\) = 1.22 µM; 10-min preincubation \(K_i\) = 1.63 µM; and 20-min preincubation \(K_i\) = 1.41 µM; N.S. to each other). These data suggest that there is no significant time dependence of binding of the inhibitor to PGT.

TGBz T34 Specifically Inhibits PGE₂ Uptake by PGT. A typical time course of PGE₂ uptake in the absence of inhibitor is shown in Fig. 4A (squares). In the absence of TGBz T34, intracellular PGE₂ rapidly accumulated, reaching a peak within 9 or 10 min. After this overshoot, a plateau was obtained, indicating that the rate of uptake equalled the rate of efflux. These data are similar to those previously published from our laboratory (Chan et al., 1998, 2002).

To further test the inhibition effect of TGBz T34, we measured the time course of PGE₂ uptake in the presence of various concentrations of TGBz T34 added at the beginning of uptake. As shown in Fig. 4A, as the concentration of TGBz T34 increased, the peak level of intracellular PGE₂ accumulation decreased, and the time point for reaching the peak PGE₂ level shifted, such that it took a shorter time for intracellular PGE₂ to reach its peak level at higher concentrations of TGBz T34. In addition, as the concentration of TGBz T34 increased, the overshoot phenomenon diminished. When the concentration of TGBz T34 was 25 µM (i.e., 8-fold higher than its \(K_i\)), the overshoot phenomenon completely disappeared. The residual uptake reflects PGE₂ entry by diffusion; it is similar to the curve of PGE₂ uptake by wild-type MDCK cells before they were transfected with PGT (Endo et al.,...
2002). Similar overshoot data and inhibition by TGBz T34 were obtained in Swiss 3T3 cells expressing endogenous PGT (data not shown).

**PGT Does Not Export PGE$_2$.** Since the discovery of PGT, the issue of whether it transports PGs in both directions has been unsettled (Chan et al., 1998; Schuster, 2002; Banu et al., 2003). To resolve this issue, we applied TGBz T34 after loading intracellular PGE$_2$ to a peak level (9 min) so as to block all PGE$_2$ transport by PGT, and then monitored the efflux of PGE$_2$. As shown in Fig. 4B, addition of TGBz T34 at 25 mM induced a rapid depletion of intracellular PGE$_2$. Intracellular PGE$_2$ fell to baseline within 5 min and remained at that level for the rest of the time course. When there was no addition of T34, intracellular PGE$_2$ stayed at a much higher level. This result strongly suggests that PGT does not participate in PGE$_2$ efflux. Instead, efflux occurs by either simple diffusion or by a combination of diffusion and another very low-affinity carrier.

**PGE$_2$ Efflux Occurs by Simple Diffusion.** We used TGBz T34 to further isolate the efflux process of PGE$_2$. As shown in Fig. 4C, we allowed PGE$_2$ uptake to proceed to different time points (3, 6, 9, 20, and 35 min) and then added 25 mM T34 to stop the accumulation. The intracellular concentrations of PGE$_2$ at different time points were calculated by dividing the total amount of intracellular PGE$_2$ on the dish by the total volume of cells, based on cell number counts and published individual cell volume (Schneider et al., 2000; Hill et al., 2004). Since the addition of inhibitor involved removing extracellular tracer PGE$_2$, the intracellular PGE$_2$ concentration at the point of inhibitor addition approximates the outwardly directed PGE$_2$ gradient. Initial PGE$_2$ efflux rates at various PGE$_2$ outward gradients are shown in Fig. 4D.

Initial PGE$_2$ efflux rates from Fig. 4, C and D, were linear as a function of the outwardly directed PGE$_2$ gradients over the range of 0 to 30 nM, with a "y"-intercept not significantly different from zero (efflux rate = (0.0106(gradient) + 0.048; $r^2$ = 0.98; $p < 0.05$). This linearity held true even when the intracellular PGE$_2$ concentration was extended to almost 800 nM; i.e., 10-fold the $K_m$ of PGT for PGE$_2$ (data not shown). From the slope of the relationship, we generated a range of permeability coefficients for PGE$_2$ efflux of 1.2 to 5.3 cm/s, based on the range of MDCK cell volumes reported in the literature (Schneider et al., 2000; Hill et al., 2004). Using wild-type MDCK cells that do not express PGT, we obtained a permeability coefficient for PGE$_2$ influx (by simple diffusion) of 0.45 cm/s (data not shown). The ratio of the influx-to-efflux permeability coefficients was thus in the range of 2.7 to 11.7.

**Discussion**

Organic dyes and nonsteroidal anti-inflammatory drugs have been known to inhibit PGT for some time (Kanai et al., 1995). This is the first report of a new class of PGT inhibitors developed by screening small molecules. The compound library was built by solid-phase combinatorial chemistry and screened by MicroBeta scintillation counting on multiwell plates. This strategy allowed us to find a PGT inhibitor, TGBz T34, with a $K_i$ value of 3.7 ± 0.2 μM.

![Fig. 3. Inhibitory effect of TGBz T34 on PGT-mediated PGE$_2$ uptake.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (μM)</th>
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<tr>
<td>T34</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>T41</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>T07</td>
<td>12.5 ± 1.5</td>
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The $K_i$ of TGBz T34 is similar to that of bromcresol green.
TGBz T34 has the potential to be improved because there are three moieties around the scaffold that can be modified. Native substrates of PGT all possess a COOH group and are negatively charged at physiological pH (Schuster, 1998). The carboxylic group at carbon 1 is critical for PG binding to PGT (Eling et al., 1977; Schuster et al., 2000), which is probably why group B (Supplemental Fig. 3S) was associated with inhibition.

Some investigators have hypothesized that PGT represents the mechanism by which PGs efflux from cells (Funk, 2001; Banu et al., 2003), whereas others have invoked active pumps such as multidrug resistance protein 4 (Reid et al., 2003). Identification of these inhibitors enabled us to investigate the mechanism of PGE\textsubscript{2} transport in a more refined way. As reported previously by our laboratory (Chan et al., 1998; Schuster, 2002) and in the present study, a normal time course of PGE\textsubscript{2} transport is divided into three phases (Fig. 4). Phase 1 is rapid uptake, phase 2 is overshoot, and phase 3 is equilibrium phase. The addition of T34 at the point of peak intracellular PGE\textsubscript{2} accumulation demonstrated that PGE\textsubscript{2} efflux is ongoing during PGT-mediated uptake (Fig. 4) (i.e., the accumulation of intracellular PGE\textsubscript{2} in phase 1) and that the maintenance of the equilibrium in phase 3 is due to the active pumping of PGE\textsubscript{2} into the cells by PGT against a background efflux.

Using TGBz T34, we were able to isolate the components of PGE\textsubscript{2} efflux. After loading cells with PGE\textsubscript{2} and blocking PGT-mediated uptake with T34, the PGE\textsubscript{2} efflux rate was linear as a function of the estimated outwardly directed PG gradient, even at high concentrations. These data indicate that PGE\textsubscript{2} efflux, at least from the compartment loaded by PGT, most likely occurred by simple diffusion.

The hypothesis that PGE\textsubscript{2} efflux occurs by simple diffusion is further supported by our calculated permeability coefficients. At physiological pH, PGs are negatively charged. Because the cell interior is electrically negative, the electrical driving force for simple diffusion is in favor of PGE\textsubscript{2} efflux. The theoretical ratio of the permeability coefficients for diffusional efflux compared with diffusional influx, based on the membrane potential, is in the range of 2 to 11 (Schuster, 2002). The ratios we generated agree with this range. Together, our data support a model of PGE\textsubscript{2} transport as a pump (PGT-mediated influx)-leak (diffusional efflux) system.

In summary, we have reported developing a new class of PGT inhibitors by screening a library of small molecules. The most potent of these allowed us to clarify the mechanisms for influx and efflux of PGE\textsubscript{2}. This compound and others should form the basis for further pharmacological investigation of PG transport and should serve as lead compounds in developing therapeutic agents.

References