Identification of a New Class of Prostaglandin Transporter Inhibitors and Characterization of Their Biological Effects on Prostaglandin E₂ Transport

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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 $3.7 \pm 0.2 \, \mu M$. These inhibitors allowed us to isolate the efflux process of PGE₂ and to demonstrate that PGT does not transport PGE₂ 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; Coccani 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 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 $3.7 \pm 0.2 \, \mu M$. This compound also permitted us to isolate the efflux process of PGE₂ and to demonstrate that PGT does not transport PGE₂ outwardly under physiological conditions.

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ABBREVIATIONS: PG, prostaglandin; COX, cyclooxygenase; PGT, prostaglandin transporter; MDCK, Madin-Darby canine kidney.
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 PGE₂ uptake in the first 2 min; these were linear over the time.

Inhibitors. At low PGE₂ concentrations, the extracellular concentrations were conducted on day 4. All of the PGE₂ uptake experiments were conducted without PGE₂. 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 l of chilled Waymouth buffer. Cells were then lysed with 100 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 \(^{3}H\)PGE₂ was counted by MicroBeta counter (Perkin Elmer Life and Analytical Sciences).

Fig. 1. Structures of the main scaffolds.

For preliminary screening of the compounds, 25 l of Waymouth buffer containing small organic compounds was added to each well; this was immediately followed by the addition of 175 l of Waymouth buffer containing \(^{3}H\)PGE₂. In each well, the total volume of uptake medium was 200 l. 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 \((\text{uptake}_{\text{vehicle}} - \text{uptake}_{\text{inhibitor}}) / \text{uptake}_{\text{vehicle}}) \times 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. The initial extracellular concentrations of PGE₂ were varied from our laboratory (Chan et al., 1998, 2002).

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 \(\mu\)M. In separate experiments, when cells were preincubated in 10 or 20 min, the \(K_i\) was not significantly different from that obtained by adding TGBz T34 simultaneously with PGE₂ (0-min preincubation \(K_i = 1.22\) \(\mu\)M; 10-min preincubation \(K_i = 1.63\) \(\mu\)M; and 20-min preincubation \(K_i = 1.41\) \(\mu\)M; 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 equaled 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 \(\mu\)M (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 PGE2 to a peak level (9 min) so as to block all PGE2 transport by PGT, and then monitored the efflux of PGE2. As shown in Fig. 4B, addition of TGBz T34 at 25 mM induced a rapid depletion of intracellular PGE2. Intracellular PGE2 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 PGE2 stayed at a much higher level. This result strongly suggests that PGT does not participate in PGE2 efflux. Instead, efflux occurs by either simple diffusion or by a combination of diffusion and another very low-affinity carrier.

**PGE2 Efflux Occurs by Simple Diffusion**. We used TGBz T34 to further isolate the efflux process of PGE2. 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 μM 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 PGE2 concentration at the point of inhibitor addition approximates the outwardly directed PGE2 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 PGE2 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 PGE2 concentration was extended to almost 800 nM; i.e., 10-fold the K_{m} for PGE2 (data not shown). From the slope of the relationship, we generated a range of permeability coefficients for PGE2 efflux of 1.2 to 5.3×10^{-6} 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 PGE2 influx (by simple diffusion) of 0.45×10^{-6} 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, after screening fewer than 2000 compounds. At 25 μM, TGBz T34 exerted full inhibition of PGE2 transport by PGT. Double reciprocal analysis revealed that T34 is a competitive inhibitor of PGT. Because T34 eliminated PGT transport activity rapidly, it probably inhibits PGT directly rather than indirectly via metabolic effects.

The K_{i} of TGBz T34 is similar to that of bromcresol green
(Kanai et al., 1995). 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 PGE2 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 PGE2 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 PGE2 accumulation demonstrated that PGE2 efflux is ongoing during PGT-mediated uptake (Fig. 4) (i.e., the accumulation of intracellular PGE2 in phase 1) and that the maintenance of the equilibrium in phase 3 is due to the active pumping of PGE2 into the cells by PGT against a background efflux.

Using TGBz T34, we were able to isolate the components of PGE2 efflux. After loading cells with PGE2 and blocking PGT-mediated uptake with T34, the PGE2 efflux rate was linear as a function of the estimated outwardly directed PG gradient, even at high concentrations. These data indicate that PGE2 efflux, at least from the compartment loaded by PGT, most likely occurred by simple diffusion.

The hypothesis that PGE2 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 PGE2 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 PGE2 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 PGE2. 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


