Restoration of renal function by a novel prostaglandin EP$_4$ receptor-derived peptide in models of acute renal failure

Martin Leduc,$^{1,2}$ Xin Hou,$^1$ David Hamel,$^{1,3}$ Melanie Sanchez,$^4$ Christiane Quiniou,$^{1,2}$ Jean-Claude Honoré,$^4$ Olivier Roy,$^2$ Ankush Madaan,$^{1,4}$ William Lubell,$^5$ Daya R. Varma,$^4$ Joseph Mancini,$^1$ François Duhamel,$^1$ Krishna G. Peri,$^6$ Vincent Pichette,$^3$ Nikolaus Heveker,$^{1,2}$ and Sylvain Chemtob$^{1,3,4}$

$^1$Research Center/University Hospital Centre Sainte-Justine, Montréal, Québec, Canada; $^2$Department of Biochemistry, Université de Montréal, Montréal, Québec, Canada; $^3$Department of Pharmacology, Université de Montréal, Montréal, Québec, Canada; $^4$Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada; and $^5$Department of Chemistry, Université de Montréal, Montréal, Québec, Canada; and $^6$Theratechnologies, Incorporated, Montréal, Québec, Canada

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Acute renal failure (ARF) is characterized by an abrupt and sustained decline in the glomerular filtration rate (46). Despite significant advances in supportive therapy over the last few decades, ARF remains a serious medical condition associated with high levels of morbidity and mortality (7, 18). Depending on the definition used, ARF has been reported to affect from 1% to 25% of patients admitted to intensive care units and has led to mortality rates from 15% to 60% (17). On the basis of the progress made in understanding the pathophysiology of ARF, many therapeutic agents have been developed and tested in animal models of ARF (24). However, only limited success has been achieved in human clinical trials with pharmacological agents, such as loop diuretics, mannitol, dopamine receptor agonists, atrial natriuretic peptide, adenosine antagonists, N-acetylcysteine, IGF-1, and calcium entry blockers (24, 46). The lack of effective treatments for ARF warrants the identification of new molecular targets and approaches to develop efficient therapeutic agents for the treatment of renal insufficiency.

Prostaglandins, which are derivatives of the cyclooxygenase-catalyzed conversion of arachidonic acid, are potent mediators of renal function and hemodynamics (3, 8). PGE$_2$ is one of the major prostanoids found in the kidney, and it exerts its biological actions through one of the four EP receptors, EP$_1$–EP$_4$. The EP$_4$ receptors are predominantly expressed in the glomerulus and in pregglomerular vessels, but they are also expressed in the distal convoluted tubule, cortical collecting duct, and outer medullary vasa recta (4, 16). Activation of EP$_4$ by PGE$_2$ has a vasodilatory effect on renal vascular tone (41), stimulates renin release in juxtaglomerular cells (10), and promotes cell survival of podocytes (1). An EP$_4$-specific orthosteric (natural ligand binding site) agonist was found to reduce nephrotoxic injury and increased the survival rate of rats with mercury chloride-induced ARF (49), and an EP$_4$ agonist prevented the development of glomerulonephritis (34). These studies suggest that modulating EP$_4$ receptor activity could improve renal function in ARF.

In contrast to orthosteric ligands, functionally selective allosteric modulators of G protein-coupled receptors (GPCRs) offer advantages by exerting effects on some (desirable) receptor-mediated pathways without interfering with other pathways that are best left intact (12, 13, 19, 25, 45); hence, orthosteric ligands exhibit undesirable properties by lacking selectivity. There is increasing evidence that peptides derived from various extracellular and juxtamembranous regions of GPCRs can interfere with their respective activities, as demonstrated, for example, by the prostaglandin F$_2$$_\alpha$ (13, 38), vasopressin type 2...
(40), and β2 adrenergic (14) receptors. Peptides that target regions of the receptor remote from the (natural ligand) orthosteric binding site often exhibit noncompetitive allosteric properties and modulate only a subset of the receptor-mediated activities, allowing more specific pharmacological intervention targeting some, but not all, receptor signaling pathways. This concept, referred to as functional selectivity, has been described for numerous compounds (12, 45), including noncompetitive antagonists (19, 25).

The aim of the present work is to study the efficacy of a new (D-) peptidomimetic EP4 receptor modulator in tempering ARF; described for numerous compounds (12, 45), including noncompetitive antagonists (19, 25).

**MATERIALS AND METHODS**

**Animals and reagents.** Animals were used according to a protocol of the Ste-Justine Hospital Animal Care Committee and along the principles of the Guide for the Care and Use of Experimental Animals of the Canadian Council on Animal Care. THG213.29 was a kind gift from Theratechnologies (Montreal, QC, Canada); peptides were synthesized using F-moc chemistry and the solid phase Merrifield method, and peptide purity was assessed by HPLC and mass spectrometry. PGE2, U-46619, and GW627368X were purchased from Cayman Chemical (Ann Arbor, MI). L-902688 (51) was a kind gift from Merck Frosst (Kirkland, QC, Canada). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

**Vascular ring preparation.** Newborn piglets (1–4 days old) were anesthetized with halothane (1.5%) and the lower external saphenous vein (D-)-peptidomimetic EP4 receptor modulator in tempering ARF; described for numerous compounds (12, 45), including noncompetitive antagonists (19, 25).

**Renal function.** All experimental procedures were performed under anesthesia. Male Sprague-Dawley adult rats (250–300 g) were anesthetized using 100 mg/kg of ketamine/xylazine (9:1, w:w) injected intraperitoneally; female Beagle dogs were anesthetized with an intravenous injection of thiopental (5 mg/kg), and the anesthesia continued under isoflurane, while juvenile pigs (6–10 kg) were anesthetized using 100 mg/kg of ketamine/xylazine (9:1, w:w) injected intravenously of the weight of the animal. GFR and RPF were calculated as the ratio of urinary [3H]inulin to plasma [3H]inulin or the ratio of urinary [14C]aminohippuric acid to plasma [14C]aminohippuric acid, respectively, corrected for the volume of the urine and the weight of the animal. Intrafemoral arterial blood pressure was also measured. Moreover, blood urea nitrogen and serum creatinine levels were measured using QuantiChrom assay kits (BioAssay Systems, Hayward, CA).

**Cisplatin-induced model of acute renal failure.** ARF and tubular necrosis were induced by intraperitoneal injection of 17.5 mg/kg of cisplatin to Sprague-Dawley male rats on day 1. Rats were treated (intravenously) with saline or THG213.29, either 1 mg/kg on day 5 or 5 mg/kg three times per day from days 2 to 5. Renal function was then assessed on day 5, as described above.

**Ischemic-renal artery occlusion model of acute renal failure.** Male Sprague-Dawley rats were cannulated, and basal GFR was determined as described above. The left and right renal arteries were then clamped for a period of 60 min to induce acute renal ischemia. Following the ischemic period, clamps were removed, and the animals were immediately treated with THG213.29 (1 mg/kg iv bolus), fenoldopam (0.6 μg/kg bolus dose, followed by 0.6 μg·kg⁻¹·h⁻¹) or saline via the jugular vein. Sham-operated rats underwent the same procedure without out renal artery clamping. At the end of their assigned period, the animals were either killed to obtain tissue samples or anesthetized to measure renal parameters as described above.

**Histology.** Kidneys were fixed in 10% formalin and paraffin-embedded; then, the specimens were stained with periodic acid-Schiff stain. Histological changes were evaluated by quantitative measurements of glomerular structural changes, as well as obstruction of collecting ducts and tubules—performed in a blinded manner relative to treatment assignment (by X. Hou and D. R. Varma).

**Cell culture.** Human embryonic kidney (HEK) 293E (Invitrogen) and EP4 stably transfected HEK293 cells (HEK293/EP4) were cultured in DMEM supplemented with 10% FBS (Wisent, Saint-Jean-Baptiste, QC, Canada), 100 units/ml penicillin/streptomycin, and 2 mM L-glutamine (Invitrogen, Carlsbad, CA) and 200 μg/ml G418. Transient transfections were performed in 6-well or 10-cm dishes using the polyethyleneimine (Polysciences, Warrington, PA) method, as described previously (26).

**Plasmids.** The expression vectors containing human EP4 receptor and GY2 were obtained from the Missouri S&T DNA Resource Center (www.cdna.org). Plasmids encoding GFP10-Epac-Rluc3, Gα12/13-Rluc, YFP-Gb1, EP4-YFP, and Rluc-β-arrestin2 have been described previously (26).

**Radioligand binding.** HEK293/EP4 cells were incubated for 2 h at 4°C in PBS/0.5% BSA (wt/vol) buffer with 10 nM of [3H]PGE2, in the presence or absence of 10 μM unlabeled PGE2 to determine specific binding. Cells were washed three times with PBS/0.5% BSA and lysed with 0.2 N NaOH/0.1% Triton X-100. Bound radioactivity was measured on cell lysates with a liquid scintillation counter. For [3H]PGE2 dissociation rate experiments, a time course of displacement was performed by removing [3H]PGE2 (10 nM) after 2 h and replacing it with 1 μM unlabeled PGE2 in the presence or absence of THG213.29 (50 μM); reaction was stopped by removing the buffer and lysing the cells.

**Bioluminescence resonance energy transfer measurement.** Bioluminescence resonance energy transfer (BRET) measurements were performed as described previously (26). Briefly, transiently transfected HEK293E cells were seeded in 96-well white clear bottom microplates (ViewPlate, PerkinElmer) coated with poly-d-lysine and left in culture for 24 h. Cells were washed once with PBS and the Rluc

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substrates coelenterazine h (for BRET\textsuperscript{1} experiments; NanoLight Technology, Pinetop, AZ) or coelenterazine 400A (for BRET\textsuperscript{2} experiments; Biotium, Hayward, CA) added at a final concentration of 5 \(\mu\)M to BRET buffer (PBS, 0.5 mM \(\text{MgCl}_2\), 0.1% glucose). BRET readings were collected using a Mithras LB940 plate reader (Berthold) and MicroWin2000 software. BRET\textsuperscript{1} measurement between Rluc and YFP was obtained by sequential integration of the signals detected in the 460–500 nm (luciferase) and 510–550 nm (YFP) windows, whereas BRET\textsuperscript{2} readings between Rluc3 and GFP10 were collected by sequential integration of the signals detected in the 365–435 nm (Rluc3) and 505–525 nm (GFP10) windows. The BRET signal was calculated as the ratio of light emitted by acceptor (YFP or GFP10) over the light emitted by donor (Rluc or Rluc3). The values were corrected to net BRET by subtracting the background BRET signal obtained in cells transfected with Rluc (BRET\textsuperscript{1}) or Rluc3 (BRET\textsuperscript{2}) constructs alone. Cells were pretreated or not for 10 min with THG213.29 or GW627368X and treated with agonists at room temperature for 3 (G\textsubscript{\alpha\textsubscript{11}}), 10 (Epac) or 15 (\(\beta\)-arrestin) minutes prior to BRET measurements.

**CRE prediction.** Prediction of cAMP response element was carried out with CREB target DB of the Salk Institute using the human database (http://natural.salk.edu/CREB/) (52).

**Gene expression.** HEK293E and HEK293/EP\textsubscript{4} cells were stimulated with L-902688 (1 \(\mu\)M) in the presence or absence of THG213.29 (100 \(\mu\)M) for 4 h. Similarly, explants from rat kidney were prepared from young adult rats (~300 g) (Charles River). Tissues were minced in pieces.

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**Fig. 1.**

**A:** schematic two-dimensional representation of human EP\textsubscript{4} receptor indicating the juxtamembranous region from which peptide THG213 was derived. Structure of THG213 ([ftsyecl; \(\beta\)] and THG213.29 [L-(4,4)-biphenyl alanine-tsyeal; \(\gamma\)] peptides.

**B**

**C**
of ~25 mg, plated in 96-well plates in DMEM and treated with THG213.29 (100 μM) or PGE₂ (1 μM) for 4 h. Cells and explants were collected in TRZol (Invitrogen) and total RNA was isolated. Prior to extraction, explants were homogenized. Five hundred nanograms of RNA was combined with qScript cDNA SuperMix (Quanta Bioscience, Gaithersburg, MD), and cDNA synthesis was performed following the manufacturer’s protocol. Quantitative real-time PCR was performed on MxPro3000 (Strategene, La Jolla, CA) using iTaq SYBR Green SuperMix with ROX (Bio-Rad, Hercules, CA). Primers were synthesized by AlphaDNA (Montreal, QC, Canada) and sequences were: hIL6-F: TTCCTCACACGCTTGCCT; hIL6-R: GTGCTTGTTGGGGGCGCC TACA; hFadd-F: GCCCGTGGAGGAAAGACCTGT; hFadd-R: CAGGTGGGCCACTGTGGTGT; hDaxx-F: GCCAGGGGTGTTGA CCCCAGAC; hDaxx-R: GCCATCCACTGGGGCCCTCAC; hBAD-F: ATCTTTGCTCTACAGGGCAGCA; hBAD-R: ACGGCCCAGGCCCTCAATGAT; rFGF2-R: CACACTTAGAAGCCAGGC; rBcl2-F: GATAACGGAGGCTGGGGCG; rBcl2-R: ATCCAGGGCCTTCGGT; hIL6-R: GTCTGTGTGGGGCGGCGTC; hBAD-F: ATCTTGTCCTCACAGCCCAGAGCA; hBAD-R: ACAGCCAGGGCCCTCAATGAT; rFGF2-F: GACGCACAAGGCGGCTGA TGC; rFGF2-R: GATAACGGAGGCTGGGGCG; hBAD-F: ATCTTGTCCTCACAGCCCAGAGCA; hBAD-R: ACAGCCAGGGCCCTCAATGAT; rFGF2-F: GACGCACAAGGCGGCTGA TGC; rFGF2-R: GATAACGGAGGCTGGGGCG; hBAD-F: ATCTTGTCCTCACAGCCCAGAGCA; hBAD-R: ACAGCCAGGGCCCTCAATGAT; rFGF2-F: GACGCACAAGGCGGCTGA TGC; rFGF2-R: GATAACGGAGGCTGGGGCG.

**RESULTS**

THG213.29 is derived from an EP₄ extracellular juxtamembranous region and acts via EP₄. Initially, the all D-peptide THG213 (itseyecl; Fig. 1B) was derived from the first extra-cellular loop and transmembrane-3 junction of the human EP₄ receptor (Fig. 1A), as reported for other GPCRs (13, 38, 40). In the process of optimizing THG213 for efficacy (and solubility), derivatives were generated and tested for their ability to modulate PGE₂- induced vasodilation of piglet saphenous veins; this complex multisignal-dependent physiological functional assay was deliberately used to avoid a screening bias toward classical but potentially nonrelevant signaling pathways and because PGE₂-induced vasorelaxation of porcine saphenous vein is foremost EP₄-dependent (50), whereas stimulation of EP₂ and to an even lesser extent of EP₃ hardly relaxes saphenous veins (27, 33). Relevantly in the present context, we also tested THG213 derivatives on GFR. The peptidomimetic THG213.29 [L-(4,4)-biphenyl alanine-(all D) tseyecl-(all L) LKK; Fig. 1C] induced the most robust increase in rat GFR; THG213.29 (1 μM) also significantly suppressed PGE₂- and EP₂-selective agonist L-902688 (51) (10 nM)-mediated relaxation of saphenous vein (by nearly 50%; Fig. 2), suggesting that THG213.29 acts via EP₄.

Effect of THG213.29 on PGE₂ binding and dissociation kinetics. To ascertain the interaction of THG213.29 on the EP₄ receptor and determine its receptor-coupled mode of action, we performed competitive binding experiments and investigated major EP₄-mediated signaling pathways, including ones relevant to cytotoxicity and cytoprotection as in ARF. Unlabeled PGE₂ dose-dependently displaced bound [³H]PGE₂ in HEK293 cells specifically expressing only EP₃ but not the other PGE₂ receptors (Fig. 2B). As expected, on the basis of the EP₃ receptor region (juxtamembranous) from where the original peptide was derived, THG213.29 did not displace bound [³H]PGE₂, indicating that it did not compete for the EP₄ orthosteric natural ligand binding site, in line with an allosteric modulator (see below). THG213.29 was able to modulate the [³H]PGE₂ dissociation rate in radioligand dissociation kinetics experiments (Fig. 2C). Indeed, the addition of excess unlabeled PGE₂ resulted in bound [³H]PGE₂ dissociation with a longer half-life (t½) and a corresponding smaller kₘ½ in the presence of THG213.29. These results are in line with the properties of an allosteric ligand, as a key manifestation of an allosteric interaction is an alteration of the affinity of an orthosteric ligand for its binding site, and thus of its association and/or dissociation rates (31).

Functional selectivity of THG213.29. As EP₄ is classically described to couple to Go/ adenylate cyclase activation, we measured cAMP production in EP₄-transfected HEK293E cells coexpressing the previously characterized BRET-based Epac biosensor (26) that allows measurement of changes in intracellular cAMP levels in living cells. Stimulation with PGE₂ resulted in a dose-dependent increase in cAMP production (pEC₅₀ of 9.63 ± 0.16), while pretreatment of cells with THG213.29 (100 μM) reduced the E₅₀ of PGE₂ by 23% without affecting PGE₂ potency (pEC₅₀ of 9.74 ± 0.19) (Fig. 2D). The potency of THG213.29 to inhibit EP₄-mediated cAMP production was low (pIC₅₀ of 5.52 ± 0.68; Fig. 2E) but specific, as neither inhibition of PGE₂-mediated cAMP production was observed in HEK293E cells transfected with the closely related prostaglandin EP₂ receptor, nor of (unrelated) AVP-mediated cAMP production by the vasopressin 2 receptor (Fig. 2F). Moreover, THG213.29 did not exhibit any intrinsic effects on cAMP production in EP₃-transfected cells when administered alone (Fig. 2F). As EP₄ signaling has also been shown to involve coupling to pertussis toxin-sensitive Go/ proteins (11, 26) and β-arrestin-mediated effects (5, 26), we verified the effect of THG213.29 on these two other EP₄ signaling outcomes in living cells, using previously characterized BRET assays (26). THG213.29 had no intrinsic effect or no modulatory effect of PGE₂-induced structural rearrangements within the Go₁β₁γ₂ heterotrimeric G protein (reflecting activation of Go/; Fig. 2G) or on β-arrestin recruitment to the receptor (Fig. 2H) in EP₄-expressing HEK293E cells; conversely, the orthosteric EP₄-specific antagonist GW627368X completely abrogated the PGE₂-induced response in these assays. The results reveal the pharmacological selectivity of THG213.29, as it only partially inhibited specifically EP₄-mediated cAMP production without affecting two other signaling outcomes of the receptor.

Effect of THG213.29 on normal dog, rat, and pig renal function. Because EP₄ exhibits a role on kidney function (41), we tested whether THG213.29 affects renal function of different species, notably Beagle dogs, juvenile pigs, and Sprague-Dawley rats. GFR essentially doubled with a maximal effect found at a dose of 2 mg/kg in rats and pigs and at 5 mg/kg in dogs (Fig. 3A). Renal plasma flow and urine output were also augmented by THG213.29 administration in normal dogs and pigs, with a borderline increase for renal plasma flow in rats (Fig. 3, B and C); mean arterial blood pressure remained stable.
after injection of THG213.29, as shown on representative averaged tracing of blood pressure in dogs (Fig. 3D) and on compiled data in rats, dogs, and pigs (Fig. 3, E–G). Thus, effects of THG213.29 on renal functional parameters tested were species-independent.

**Efficacy of THG213.29 in a cisplatin-induced nephrotoxic acute renal failure rat model.** Nephrotoxic effects resulting from the use of antineoplastic, radiocontrast, and antimicrobial agents can result in ARF (35). Cisplatin is a common and effective chemotherapeutic agent used for the treatment of various malignancies with unfortunate dose-limiting nephrotoxicity observed in 25–35% of treated patients (39). We tested the efficacy of THG213.29 in a cisplatin-induced nephrotoxicity rat model of acute tubular necrosis and renal failure. Five days after administration of cisplatin to Sprague-Dawley rats, there was a dramatic decline in GFR, renal plasma flow, and urine output (Fig. 4A), and, consequently, a progressive increase of serum blood urea nitrogen (BUN) and creatinine levels (Fig. 4B); levels of plasma proteins, bicarbonate, sodium, and chloride did not change (results not shown). Late administration of a single dose of THG213.29 (1 mg/kg) on day 5 (after cisplatin) improved renal function, while three times per day (5 mg/kg) treatment with THG213.29 from days 2 to 5 (Fig. 4A) further restored measured renal function parameters; BUN and serum creatinine levels measured on day 5 also decreased in animals treated from day 2 to 5 after cisplatin (Fig. 4C). Histological assessment of kidneys of cisplatin-injected rats 72 h after treatment with saline revealed hyper trophy and/or erythrocyte extravasation in the majority of glomeruli, as well as occlusion of a small portion of collecting ducts; treatment with THG213.29 (5 mg/kg, three times a day) dramatically reduced glomerular hypertrophy and glomerular erythrocyte extravasation; collecting duct obstruction (percent) was unaffacted by THG213.29 (Fig. 4, D and E). Together, these results reveal that THG213.29 improves renal function and glomerular structure in a model of nephrotoxin-induced ARF.

**Efficacy of THG213.29 in a renal artery occlusion rat model of acute renal failure.** A variety of clinical conditions, including hemorrhage, heart failure, volume depletion, and shock can lead to compromised renal blood flow and ischemia, which accounts for the largest number of cases of ARF (44). We assessed the efficacy of THG213.29 in the widely used ischemic renal artery occlusion (RAO) rat model of ARF. In this model, the effect of THG213.29 was compared with fenoldopam, a selective dopamine D1 receptor agonist that has been shown to improve renal perfusion and decrease serum creatinine in limited clinical studies (46). Bilateral renal artery clamping was carried out for 60 min to induce acute renal ischemia, leading subsequently to a marked decrease in renal function. As illustrated in Fig. 5A, administration of either THG213.29 (1 mg/kg) or fenoldopam (0.6 µg/h/kg) increased urine output, but only THG213.29 was effective in significantly raising GFR and renal plasma flow 2 h after renal artery clamping; THG213.29 and fenoldopam were comparably effective in decreasing BUN and serum creatinine 72 h after RAO (Fig. 5B). Histological examination of kidneys collected 24 and 72 h after renal ischemia revealed structural damage compared with sham-operated rats, showing glomerular erythrocyte extravasation and tubular obstruction with cell debris (Fig. 5, C–G). These histological anomalies were considerably attenuated in rats treated with THG213.29. Results indicate that THG213.29 improves renal function and protects against tissue damage in a model of ischemia-induced ARF.

**Effects of THG213.29 on EP4-dependent gene expression.** In an attempt to understand the effects of THG213.29 in renal function and structure in ARF models, we tested in renal cortex whether THG213.29 can specifically induce genes consonant with its protective actions. Interestingly, THG213.29 stimulation of renal cortex induced mRNA expression (including on renal tissue) of the anti-inflammatory heme-oxygenase-1 (9), the anti-apoptotic Bcl2, and the cytoprotective FGF-2, whereas another gene of potential relevance, namely, endothelial nitric oxide synthase (eNOS), was unaffected (Fig. 6A). To further illustrate that the effects of THG213.29 in altering expression of cytoprotective, inflammatory, and proapoptotic genes are specifically mediated via EP4, we treated HEK293 cells stably transfected with EP4, with the EP4 orthosteric agonist L-902688 in the absence or presence of THG213.29. L-902688 slightly increased mRNA expression of FGF-2, and this was further augmented by cotreatment with THG213.29 (Fig. 6), consistent with findings on the renal cortex. Also, treatment with THG213.29 reduced L-902688-induced expression of proinflammatory cytokine IL-6, and of apoptotic factors BCL2-associated agonist of cell death (BAD) and death do-
main-associated protein (DAXX) (Fig. 6B); importantly, no response to L-902688 and/or THG213.29 was observed in HEK293 cells devoid of EP4, confirming that the effects of THG213.29 are EP4 dependent. The data also reveal that THG213.29 exhibits biased functional selectivity, depending as illustrated on the type of gene affected, resulting in inhibition of some and stimulation of others, in line with allosteric modulators (13, 20, 25, 45).

Fig. 4. Efficacy of THG213.29 in a cisplatin-induced nephrototoxic model of acute renal failure. Sprague-Dawley rats were injected with cisplatin (17.5 mg/kg ip) on day 1 to induce acute tubular necrosis and renal failure. A: renal function (GFR, renal plasma flow, and urine output) were measured in normal untreated rats or 5 days after administration of cisplatin. A single administration of THG213.29 on day 5 (1 mg/kg iv) improved kidney function compared with saline-treated rats; these parameters were normalized in rats treated with THG213.29 three times a day (5 mg/kg iv) from day 1 to day 5. Data are expressed as means ± SE from two rats per treatment group. **P < 0.01 compared with saline-treated rats. B: BUN and serum creatinine were measured in saline-treated rats daily for 5 days after administration of cisplatin. C: animals were injected with cisplatin on day 1, treated with saline or THG213.29 three times a day (5 mg/kg iv) from days 2 to 5 after cisplatin, and BUN, and serum creatinine were measured on day 6 (n = 4). Data are expressed as means ± SE. *P < 0.05 compared with saline-treated rats (ANOVA followed by trend analysis). D: hypertrophic glomeruli and/or erythrocyte extravasation and collecting duct obstruction were quantified from histology kidney sections of cisplatin-injected rats collected 72 h after treatment with saline or THG213.29 (5 mg/kg ip, three times a day from days 2 to 5 after cisplatin). Data are expressed as means ± SE from two rats per treatment group. **P < 0.01 compared with saline-treated rats. E: kidney sections from normal (control) or cisplatin-injected rats collected 72 h after treatment with saline or THG213.29 (5 mg/kg ip, three times a day from days 2 to 5 after cisplatin).
DISCUSSION

Despite improved supportive care, ARF remains a serious clinical condition associated with high mortality and morbidity, which have not significantly decreased over the past few decades; accordingly, new therapeutic modalities are needed for this medical condition. Approximately 85% of ARF is of ischemic or nephrotoxic origin (44). PGE2 and its receptors, including EP4, are widely expressed in the kidney and regulate renal function (3, 16, 24). Orthosteric EP4 agonists have been
shown to improve renal function in models of nephrotoxicity and glomerulonephritis (34, 49). However, functionally selective allosteric modulators of GPCRs offer numerous advantages over orthosteric ligands by exerting effects on some (desirable) receptor-mediated functions but not others best left intact. We, hereby, describe the effects of a first functionally selective EP4 modulator, THG213.29, which is effective in preserving renal function and structural integrity in distinct models of ARF.

Extracellular loops and the extracellular portions of transmembrane helices play an important role in the overall pharmacology of GPCRs (6), and there is a conformational coupling between the extracellular surface and the orthosteric binding site (2). THG213.29 was derived from a juxtaglomerular region of the EP4 receptor that is distinct from the presumed orthosteric binding site, which is located in the case of the closely related prostacyclin receptor, mostly within the transmembrane core (42). Consequently, THG213.29 exhibited noncompetitive properties toward EP4 as it did not displace bound PGE2 but modulated PGE2 binding dissociation kinetics (Fig. 2, B and C). The noncompetitive nature of THG213.29 is corroborated by the inability of high concentrations of PGE2 to overcome the partial inhibitory effects of THG213.29 on PGE2-induced cAMP production (insurmountable antagonism) (Fig. 2, D and E); in contrast, competitive orthosteric antagonism by definition can be surmounted by increasing concentrations of the natural ligand (30). THG213.29 exerted specificity for the EP4 receptor, as it inhibited the EP4-specific agonist L-902688-mediated piglet saphenous vein vasodilation (Fig. 2A, ii); 2 attenuated cAMP response in EP4, but not EP2 or V2R-expressing HEK293 cells (Fig. 2F); 3 modulated PGE2 binding dissociation kinetics to the EP4 receptor (Fig. 2C); and 4 modulated the L-902688-induced increased expression of various mRNAs in EP4-expressing cells only (Fig. 6B).

In the past several years, it has become increasingly clear that G protein-coupled receptors can activate independently a variety of signaling effectors and that the ability of a compound to modulate a given signaling pathway cannot be extrapolated to another signaling pathway triggered by the same receptor. The conceptual basis for this is that GPCRs do not have merely “inactive” and “active” conformations but that ligands can stabilize distinct receptor conformations, which are more or less potent and efficient in activating a given readout (21). This infers that a drug can affect some, but not all, functions evoked by a receptor, a concept referred to as functional selectivity (45). The difficulty of accommodating functional selectivity in screening campaigns has been pointed out (22); hence, a complex isolated-tissue physiological functional assay (piglet saphenous vein vasorelaxation) was initially used to determine the efficacy of peptides to avoid overseeing signaling responses based on reductionist in vitro recombinant systems. THG213.29 inhibited both PGE2 and L-902688-mediated vasorelaxation in this system. In an effort to decipher the effect of THG213.29 on EP4 activities, we investigated more specific signaling pathways using BRET-based assays in living HEK293 cells. EP4-mediated cAMP production was partially, but specifically, inhibited by THG213.29, while the Goi activation and β-arrestin recruitment pathways activated by PGE2 were unaffected by THG213.29 (Fig. 2, F–H). The observed functional selectivity is likely made possible by THG213.29-induced stabilization of a unique receptor conformation that, in essence, creates a “modified” receptor conformation with distinct functional properties, characteristic of allosteric modulators. The modulation of specific receptor activities may confer greater selectivity and possibly reduce undesirable side effects, compared with orthosteric antagonists that block all (beneficial and pathological) responses of the receptor to stimuli (19).

A significant feature of this study relates to the in vivo effects of THG213.29 on renal integrity following insults that lead to ARF. THG213.29 attenuated renal dysfunction associated with two separate models of ARF. Using the EP4-selective agonist CP-044,519–02, Vukicevic and colleagues (49) have previously shown that EP4 agonism reduces serum creatinine levels, proximal tubular necrosis, and abundance of apoptotic cells in a nephrotoxic mercury chloride rat model of ARF; fenoldopam has also been shown to be tubulo-protective in nephrotoxic and ischemic ARF (29, 32), which may explain its significant improvement in urine output, BUN, and creatinine with marginal effects on GFR (Fig. 5). We have shown that THG213.29 is a partial negative modulator of EP4-mediated cAMP production and saphenous vein vasorelaxation but has no effect on EP4-mediated Goi activation or β-arrestin recruitment; whether these cell signaling pathways operate in renal function is unclear. On the other hand, data at the transcriptional level are consistent with beneficial effects of THG213.29. In the renal cortex, THG213.29 induced expression of cytoprotective and anti-inflammatory genes (Fig. 6). In cultured cells specifically expressing EP4, THG213.29 correspondingly abrogated the expression of cytotoxic (proapoptotic) and proinflammatory genes, potentially regulated by cAMP and known to be involved in tissue degeneration following kidney injury (28, 43) (47, 48), and again increased expression of cytoprotective FGF-2 (Fig. 6). It has been shown that members of the extrinsic death receptor-dependent (DAXX) and the intrinsic mitochondrial (BAD) apoptosis pathways are rapidly induced following ischemic ARF in animal models and may be involved in tubule cell loss following ischemia-reperfusion injury (28, 43). Recovery from tubular damage requires regenerative mechanisms implicating the paracrine or autocrine action of growth factors, including FGF-2, which has been shown to induce a reexpression of
morphogenic proteins and accelerates the recovery process after renal damage in a model of ischemic ARF (47, 48). It has also been reported that increased IL-6 production by infiltrating macrophages exacerbates ischemic murine acute renal failure (23). Hence, the EP4-dependent modulation of inflammatory and cytotoxic factors by THG213.29 can explain the relative preservation of renal structural integrity observed after acute renal insults.

In addition, THG213.29 was found to augment renal hemodynamics (renal plasma flow) and GFR resulting in increased urine output. A dilation of the afferent arterioles with or without relaxation of the efferent arterioles would be required to induce these functional changes. Although, EP4 stimulation generally induces relaxation of vascular tissue [including sapheous vein (Fig. 2A)], in kidneys, EP4-induced renin release may counter these effects (41), but effects of renin are foremost only on efferent arterioles. In addition to the complex actions of the biased-signaling modulator THG213.29 on EP4 likely provide it with its intricate properties.

In summary, we have documented the characterization of a small peptidomimetic, THG213.29, which is derived from a juxtamembranous region of the prostaglandin EP4 receptor and is effective in models of ischemia- and nephrotoxic-induced ARF. THG213.29 exhibited properties consistent with those of a noncompetitive, functionally selective allosteric modulator. The use of therapeutic peptides [particularly D-peptides (as is the case herein) and/or peptidomimetics] has become increasingly attractive in recent years, mainly due to improvements in half-life and bioavailability (37), which may be of lesser concern in seriously ill (hospital-bound) patients with ARF; in addition, peptides can be used as scaffolds for subsequent conversion into small molecules. Moreover, peptides that tar-

dilatory functions acting through the EP4 receptor. PKA activation can also result in stimulation of hyaluronic acid formation, which forms a basis for extracellular matrix and DA closure. Signaling pathways downstream of the EP4 receptor can clearly be divergent dependent on timing and the environment of the challenge, adding to the complexity of THG213.29 actions. Altogether, the complex actions of the biased-signaling modulator THG213.29 on EP4 likely provide it with its intricate properties.

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get regions of the receptor remote from the orthosteric natural ligand-binding site might exhibit noncompetitive allosteric properties and modulate only a subset of the receptor-mediated activities, allowing more specific pharmacological intervention targeting some, but not all, receptor signaling pathways (37). Hence, THG213.29, a novel class allosteric modulator of the EP4 receptor effective in increasing renal function and diuresis in different species, may in the future yield promise in the clinical setting.

Perspectives and Significance

Drug treatments for ARF have so far not been successful. Historically, drug discovery efforts have been optimized for the discovery of standard orthosteric agonists and antagonists. However, one drawback often encountered with orthosteric ligands is undesirable side effects. Allosteric ligands, which bind to a receptor region remote from the natural ligand orthosteric site, have the potential to provide more versatility in receptor signaling modulation through functional selectivity and separate control of efficacy and affinity. We have exploited these properties with the characterization of a small peptidomimetic, THG213.29, which is derived from a juxtamembrane region of the prostaglandin EP4 receptor. The peptide exhibited pharmacological properties consistent with those of a noncompetitive, functionally selective allosteric modulator, and most relevantly improved renal function in two different models of ARF. THG213.29 is a novel class of allosteric modulator of the EP4 receptor with diuretic properties, which may have potential as therapy for acute kidney injury.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


