Restoration of renal function by a novel prostaglandin EP₄ receptor-derived peptide in models of acute renal failure

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Abstract

Acute renal failure (ARF) is a serious medical complication characterized by an abrupt and sustained decline in renal function. Despite significant advances in supportive care there is currently no effective treatment to restore renal function. Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) is a lipid hormone mediator abundantly produced in the kidney where it acts locally to regulate renal function; several studies suggest that modulating EP\textsubscript{4} receptor activity could improve renal function following kidney injury. An optimized peptidomimetic ligand of EP\textsubscript{4} receptor, THG213.29, was tested for its efficacy to improve renal function (glomerular filtration rate, renal plasma flow and urine output) and histological changes in a model of ARF induced by either cisplatin or renal artery occlusion in Sprague-Dawley rats. THG213.29 modulated PGE\textsubscript{2} binding dissociation kinetics, indicative of an allosteric binding mode. Consistently, THG213.29 antagonized EP\textsubscript{4}-mediated relaxation of piglet saphenous vein rings, partially inhibited EP\textsubscript{4}-mediated cAMP production, but did not affect G\textsubscript{ai} activation or \beta-arrestin recruitment. In vivo, THG213.29 significantly improved renal function and histological changes in cisplatin- and renal artery occlusion-induced ARF models. THG213.29 increased mRNA expression of heme-oxygenase 1, Bcl2 and fibroblast growth factor 2 (FGF-2) in renal cortex; correspondingly in EP\textsubscript{4}-transfected HEK293 cells, THG213.29 augmented FGF-2, and abrogated EP\textsubscript{4}-dependent overexpression of inflammatory IL-6 and of apoptotic DAXX and BAD. Our results demonstrate that THG213.29 represents a novel class of diuretic agent with noncompetitive allosteric modulator effects on EP\textsubscript{4} receptor resulting in improved renal function and integrity following acute renal failure.

Keywords: allosteric modulator, cisplatin, renal artery occlusion.
**Introduction**

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). Based on 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, insulin-like growth factor-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 preglomerular 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 EP4 agonist prevented the development of glomerulonephritis (34). These studies suggest that modulating EP4 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 which 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 for the prostaglandin F2α (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 non-competitive 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 non-competitive 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; in this process we investigated its mode of action. We hereby describe that the optimized peptidomimetic THG213.29, which possesses functional selectivity towards the EP4 receptor, proved to be efficacious in improving renal function and structural outcome in ischemia- and nephrotoxin-induced rat models of ARF.
**Material 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. PGE$_2$, U-46619 and GW627368X were purchased from Cayman Chemical (Ann Arbor, MI). L-902688 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 veins removed and placed in cold Krebs buffer (120 mM NaCl, 4.5 mM KCl, 2.5 mM CaCl$_2$, 1 mM MgSO$_4$, 27 mM NaHCO$_3$, 1 mM KH$_2$PO$_4$, 10 mM glucose, pH 7.4) to which 1.5 U/ml heparin was added. The veins were cleaned of extraneous tissue and cut in 3-4 mm rings which were placed in individual jacketed 15 ml organ baths containing Krebs buffer saturated with 95% O$_2$/5% CO$_2$ and maintained at 37°C. The rings were equilibrated for 60 minutes under 2 g tension and were pre-treated or not with THG213.29 for 30 min prior to addition of U-46619 (0.2 µM). When the response to U-46619 reached a steady state, PGE$_2$ or L-902688 was added. Tension was measured by force-displacement transducers and was recorded using Work Bench software (Kent Scientific, Litchfield, CT).
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 intra-peritoneally (i.p.), female Beagle dogs were anesthetized with an i.v. injection of thiopental (5 mg/kg) and the anesthesia continued under isoflurane, while juvenile pigs (6-10 kg) were anesthetized with 1.5% halothane. The animals were kept warm and their body temperature monitored every 15 minutes. Renal function were determined as previously described (36). Briefly, the carotid artery was cannulated to measure the arterial blood pressure with a pressure transducer (Gould Easy Graph) and to collect blood samples. A catheter was inserted at the tip of the bladder to collect urine. The jugular (rats), cephalic (dogs) or femoral (pigs) vein was also catheterized for infusion of saline containing the anesthetics and $[^3]$H inulin (8 µCi/h for rats, 10 µCi/h for dogs and pigs, $[^1]$C] aminohippuric acid (0.8 µCi/h for rats, 1 µCi/h for dogs and pigs). The radiolabeled compounds were allowed to equilibrate for 40 minutes. Two urine samples were collected over 10 minute periods (from 40 to 50 minutes, and from 50 to 60 minutes) while blood samples were collected at 45 and 55 minutes to assess the stability of the basal GFR. Saline or THG213.29 was administered i.v. via the jugular (rats), cephalic (dogs) or femoral (pigs) vein. Urine and blood samples were then collected every 20 minutes for an additional period of 2 h. The radioactivity in the blood and urine samples was measured with a liquid scintillation counter. Urine flow rates, GFR and RPF were determined at different times and averaged for a 60 min period starting 20 min after drug administration. Urinary volume (UV) was calculated and expressed as µl of urine/min corrected for the weight of the animal. GFR and RPF were calculated as the ratio of urinary $[^3]$Hinulin to plasma $[^3]$Hinulin or the ratio of urinary $[^1]$C] aminohippuric acid to plasma $[^1]$C] 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 was induced by i.p. injection of 17.5 mg/kg of cisplatin to Sprague-Dawley male rats on day 1. Rats were treated (i.v.) with saline or THG213.29, either 1 mg/kg on day 5 or 5 mg/kg three times a 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 determined as described above. The left and right renal arteries were then clamped for a period of 60 minutes to induce acute renal ischemia. Following the ischemic period, clamps were removed and the animals were immediately treated with THG213.29 (1 mg/kg i.v. bolus), fenoldopam (0.6 µg/kg bolus dose, followed by 0.6 µg/kg/h) or saline via the jugular vein. Sham-operated rats underwent the same procedure without renal artery clamping. At the end of their assigned period, the animals were either sacrificed to obtain tissue samples or anesthetized to measure renal parameters as described above.

*Histology* – Kidneys were fixed in 10% formalin, paraffin-embedded and the specimens 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 XH and DRV).
Cell culture – Human embryonic kidney (HEK) 293E (Invitrogen) and EP4 stably transfected HEK293 cells (HEK293/EP4) were cultured in Dulbecco’s modified Eagle's medium supplemented with 10% fetal bovine serum (Wisent), 100 units/ml penicillin/streptomycin and 2 mM L-glutamine (Invitrogen) and 200 μg/ml G418. Transient transfections were performed in 6-well or 10 cm dishes using the polyethylenimine (Polysciences, PA) method, as described previously (26).

Plasmids – The expression vectors containing human EP4 receptor and Gγ2 were obtained from the Missouri S&T cDNA Resource Center (www.cdna.org). Plasmids encoding GFP10-Epac-Rluc3, Gαi1-91Rluc, YFP-GBP1, EP4-YFP, and Rluc-β-arrestin 2 have been described previously (26).

Radioligand binding – HEK293/EP4 cells were incubated for 2h at 4°C in PBS/0.5% BSA (w/v) buffer with 10nM of [3H]PGE2, in the presence or absence of 10μM 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 hours 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.
**BRET measurement** – 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 24h. Cells were washed once with PBS and the Rluc substrates coelenterazine h (for BRET\(^1\) experiments; NanoLight Technology) or coelenterazine 400A (for BRET\(^2\) experiments; Biotium) added at a final concentration of 5 µM to BRET buffer (PBS, 0.5 mM MgCl\(_2\), 0.1% glucose). BRET readings were collected using a Mithras LB940 plate reader (Berthold) and MicroWin2000 software. BRET\(^1\) 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\(^2\) 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\(^1\)) or Rluc3 (BRET\(^2\)) constructs alone. Cells were pre-treated or not for 10 minutes with THG213.29 or GW627368X and treated with agonists at room temperature for 3 (G\(_\alpha_{i1}\)), 10 (Epac) or 15 (β-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/](http://natural.salk.edu/CREB/)) (52).

**Gene expression** – HEK293E and HEK293/EP\(_4\) cells were stimulated with L-902688 (1 µM) in the presence/absence of THG213.29 (100 µM) for 4 h. Similarly, explants from rat kidney
were prepared from young adult rats (approximately 300g) (Charles River). Tissues were minced in pieces of approximately 25mg, placed 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 TRIzol (Invitrogen) and total RNA isolated. Prior to extraction, explants were homogenized. 500 ng of RNA was combined to qScript cDNA SuperMix (Quanta Bioscience) and cDNA synthesis performed following the manufacturer’s protocol. Quantitative real-time PCR was performed on MxPro3000 (Stratagene) using iTaq SYBR Green SuperMix with ROX (Bio-Rad).

Primers were synthesized by AlphaDNA (Montreal, QC) and sequences were: hIL6-F: TCTCCACAACGGCCTTCGGTC; hIL6-R: GTCTGTGTGGGGCGGCCTACA; hFadd-F: GCGCCTGGGGAAGAAGACCTGT; hFadd-R: CAGGTGGGCCACTGTTGCGT; hDaxx-F: GCCAGGCGTTGACCCCTGCAC; hDaxx-R: GCCATTCCACTAGG GCCCCTCCACC; hBAD-F: ATCTTGTCCTCACAGCCAGCA; hBAD-R: ACAGCCCCACGCCTCCATGT; rBcl2-F: GATAACGGAGGCTGGGATGC; rBcl2-R: ATGCACCCAGAGCTGACG; hFGF2-F: GACCCCAAGCGGCTGTACTGC; hFGF2-R: TTGTAGCTTGATGAGGGTTCG; rHMOX1-F: GGGAAGGCTTTAAGCTGGTGA; rHMOX1-R: TGGCTGGTGTGTAAGGGATG; rFGF2-F: GATCCCAAGCGGCTCTACTG; rFGF2-R: CACACTTAGAAGCCAGCAGC; reNOS-F: GGCTGAGTACCCAAGCTGAG; reNOS-R: ATTGTGGCTCGGGTGGATTT. mRNA expression levels were normalized against 18S rRNA endogenous control levels in each sample and calculated relative to control vehicle-treated cells.

**Statistical analysis** – Values are presented as mean ± S.E.M. Curve-fitting and statistical analysis was conducted by using GraphPad Prism 4 software. Statistical difference between
two groups was measured using Student’s t-test, and for differences between more than two groups by one-way ANOVA followed by Dunnett’s post-test. For comparison between treatments and cell line for gene expression levels, two-way ANOVA followed by Bonferroni’s post-test was used. Statistical significance was set at $P < 0.05$. 
Results

THG213.29 is derived from an EP4 extracellular juxtamembranous region and acts via EP4

Initially, the all d-peptide THG213 (iftsyecl; Fig. 1b) was derived from the first extracellular 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 multi-signal-dependent physiological functional assay was deliberately used to avoid a screening bias towards classical but potentially non-relevant 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) tseya-(all L) LKK; Fig. 1C] induced the most robust increase in rat GFR; THG213.29 (1 μM) also significantly suppressed PGE2- and EP4-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 respectively competitive binding experiments and investigated major EP4-mediated signaling pathways including ones relevant to cytotoxicity/cytoprotection as in ARF. Unlabeled PGE2 dose-dependently displaced bound [3H]PGE2 in HEK293 cells specifically expressing only EP4 but not the other PGE2 receptors (Fig. 2B). As expected
based on the EP4 receptor region (juxtamembranous) from where the original peptide was derived, THG213.29 did not displace bound [$^3$H]PGE$_2$, 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 [$^3$H]PGE$_2$ dissociation rate in radioligand dissociation kinetics experiments (Fig. 2C). Indeed, addition of excess unlabeled PGE$_2$ resulted in bound [$^3$H]PGE$_2$ dissociation with a longer half life ($t_{1/2}$) and a corresponding smaller $k_{off}$ 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 G$\alpha_s$/adenylate cyclase activation, we measured cAMP production in EP4-transfected HEK293E cells co-expressing the previously characterized bioluminescence resonance energy transfer (BRET)-based Epac biosensor (26) that allows measurement of changes in intracellular cAMP levels in living cells. Stimulation with PGE$_2$ resulted in a dose-dependent increase in cAMP production ($pEC_{50}$ of 9.63 ± 0.16), while pre-treatment of cells with THG213.29 (100 μM) reduced the $E_{max}$ of PGE$_2$ by 23% without affecting PGE$_2$ potency ($pEC_{50}$ of 9.74 ± 0.19) (Fig. 2D). The potency of THG213.29 to inhibit EP4-mediated cAMP production was low ($pIC_{50}$ of 5.52 ± 0.68; Fig. 2E) but specific, as neither inhibition of PGE$_2$-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 EP4-transfected cells when administered alone.
(Fig. 2F). As EP₄ signaling has also been shown to involve coupling to pertussis toxin (PTX)-sensitive Gαᵢ 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 Gαᵢ₁β₁γ₂ heterotrimeric G protein (reflecting activation of Gαᵢ₁; 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 if 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. 3B, 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. 3E-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 (post-cisplatin) improved renal function, while three times a 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 2-5 after cisplatin (Fig. 4C). Histological assessment of kidneys of cisplatin-injected rats 72 h after treatment with saline revealed hypertrophy 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 unaffected by THG213.29 (Fig. 4 D,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 haemorrhage, 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 to 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 to sham-operated rats, showing glomerular erythrocyte extravasation and tubular obstruction with cell debris (Fig. 5C-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 if 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 fibroblast growth factor (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 pro-apoptotic 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 co-treatment with THG213.29 (Fig. 6), consistent with findings on renal cortex. Also, treatment with THG213.29 reduced L-902688-induced expression of proinflammatory cytokine interleukin (IL)-6, and of apoptotic factors BCL2-associated agonist of cell death (BAD) and death-domain 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).
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). PGE$_2$ and its receptors, including EP$_4$, are widely expressed in the kidney and regulate renal function (3, 16, 24). Orthosteric EP$_4$ agonists have been shown to improve renal function in models of nephrotoxicity and glomerulonephritis (34,49). However, functionally selective allosteric modulators of GPCRs offers 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 EP$_4$ 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 juxtamembranous region of the EP$_4$ 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 non-competitive properties towards EP$_4$ as it did not displace bound PGE$_2$ but modulated PGE$_2$ binding dissociation kinetics (Fig. 2 B, C). The non-competitive nature of THG213.29 is corroborated by the inability of high concentrations of PGE$_2$ to overcome the partial inhibitory effects of THG213.29 on PGE$_2$-induced cAMP production (insurmountable
antagonism) (Fig. 2 D,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: i) inhibited the EP4-specific agonist L-902688-mediated piglet saphenous vein vasodilation (Fig. 2A (ii)); ii) attenuated cAMP response in EP4 but not EP2 or V2R-expressing HEK293 cells (Fig 2F); iii) modulated PGE2 binding dissociation kinetics to the EP4 receptor (Fig. 2C); and iv) 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 to accommodate 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 Gαi1 activation and β-arrestin recruitment
pathways activated by PGE₂ were unaffected by THG213.29 (Fig. 2F-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, as compared to 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 Gaᵢ 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 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 (pro-apoptotic) and pro-inflammatory 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 para- or autocrine action of growth factors, including FGF-2, which has been shown to induce a re-expression 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 saphenous 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 EP₄ stimulation on vasomotricity, THG213.29 caused a modest decrease in cAMP, which cannot explain enhanced in vivo renal hemodynamics. However, THG213.29 triggered expression of the inducible heme-oxygenase-1 (9) - a primary generator of the potent vasorelaxant carbon monoxide, in renal cortex; this could explain augmented renal hemodynamics in vivo (Fig. 6A).
Other than the mechanisms described in the two previous paragraphs, additional EP₄ receptor activities not yet characterized may also be modulated (positively or negatively) by THG213.29 and may be implicated in the efficacy of the peptide. The EP₄ receptor data in the kidney has a corollary in the control of patent ductus arteriosus (DA) (15). The use of an EP₄ agonist maintains the DA patent even when dosed with indomethacin. The quandary in this case is that the EP₄ receptor null mice also results in a patent DA. PGE₂ has been shown to have both constrictor and dilatory functions acting through the EP₄ receptor. PKA activation after EP₄ stimulation can directly promote vasodilatation by inhibiting myosin light chain kinase and maintains the DA patent during fetal development. 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.

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 non-competitive, 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 target
regions of the receptor remote from the orthosteric natural ligand-binding site might exhibit non-competitive 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 treatment 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 are 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 juxtamembranous region of the prostaglandin EP4 receptor. The peptide exhibited pharmacological properties consistent with those of a non-competitive, 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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References


Figure legends

**Figure 1.** (A) Schematic two dimensional representation of human EP4 receptor indicating the juxtamembranous region from which peptide THG213 was derived. (B, C) Structure of THG213 (iftsyec, B) and THG213.29 (L-(4,4)-biphenyl alanine-tsyeaLKK, C) peptides.

**Figure 2.** A) Inhibition of EP4-mediated vasomotor response in isolated piglet saphenous vein rings by THG213.29. Saphenous vein rings pre-exposed or not to THG213.29 (1 µM) were contracted with the TP receptor agonist U-46619 (0.2 µM) and were then exposed to 1 µM PGE2 (i) or to 10 nM of the EP4-specific agonist L-902688 (ii). Results are the mean ± S.E.M. of 22 (PGE2-treated) or 9 (L-902688-treated) rings. **P <0.01, ***P <0.0001 compared to saline. B,C) Effects of THG213.29 on the specific binding and dissociation kinetics of [3H]PGE2 to EP4 receptors. (B) Displacement of bound [3H]PGE2 (10 nM) by increasing concentrations of unlabeled PGE2 or THG213.29 in HEK293/EP4 cells. Data represent the mean ± S.E.M. of three experiments, each performed in duplicate. (C) Dissociation kinetics of [3H]PGE2 (10 nM) binding by excess unlabeled PGE2 (1 µM) in the presence or absence of THG213.29 (213.29; 50 µM) in HEK293/EP4 cells. Data from one representative experiment are shown. Inset shows mean ± S.E.M. of dissociation half life and koff from 4 experiments, each performed in duplicate; **P <0.1 compared to PGE2. (D) Functional selectivity of THG213.29. PGE2 concentration-response curves of EP4 receptor-mediated cAMP production in the presence or absence of THG213.29 (100 µM) assessed by BRET in living HEK293E cells co-transfected with Epac biosensor and EP4. Data from one representative experiment of four (each performed in triplicate) are shown. (E) THG213.29 inhibition curve of PGE2–induced cAMP production assessed by BRET in living HEK293E...
cells co-transfected with Epac biosensor and EP₄. Data from one representative experiment of four (each performed in triplicate) are shown (to avoid visual cluttering). (F) Selectivity of THG213.29 for EP₄. Effect of THG213.29 on cAMP production assessed by Epac-based BRET assay. HEK293E cells co-transfected with Epac sensor and either EP₄, EP₂ or vasopressin type 2 (V2R) receptors were pre-treated or not with THG213.29 (100 μM) and stimulated with PGE₂ or AVP (0.1 μM). Data represent the mean ± S.E.M. of 3-6 experiments, each performed in triplicate; ***P <0.001). (G) BRET assay to monitor EP₄-mediated Gαᵢ₁ activation was performed in living HEK293E cells co-transfected with Gαᵢ₁-Rluc, YFP-GBP₁, Gγ₂ and EP₄. Variation of the BRET signal compared to basal conditions was measured in cells pre-treated or not with THG213.29 (100 μM) or EP₄ antagonist GW627368X (1 μM) and stimulated or not with PGE₂ (0.1 μM). Data represent the mean ± S.E.M. of 5 experiments, each performed in triplicate. (H) β-arrestin recruitment to EP₄ receptor was monitored by BRET in living HEK293E cells co-expressing EP₄-YFP and Rluc-β-arrestin 2. Ligand-promoted BRET variation was measured in cells pre-treated or not with THG213.29 (100 μM) or EP₄ antagonist GW627368X (1 μM) and stimulated or not with PGE₂ (0.1 μM). Data represent the mean ± S.E.M. of 4 experiments, each performed in triplicate.

**Figure 3.** Effects of THG213.29 on kidney function in normal rats, dogs and pigs. THG213.29 administration (i.v. bolus dose; rats and pigs: 2 mg/kg, dogs: 5 mg/kg) significantly increased GFR (A), renal plasma flow (B) and urine output (C) in normal Sprague-Dawley rats, Beagle dogs and pigs. Data represent the mean ± S.E.M. from 4 (rats) or 6 (dogs, pigs) animals; *P <0.05, ***P <0.001 compared to saline. (D) Averaged
representative tracing of mean arterial blood pressure in dogs, before and after i.v. injection of THG213.29 (at dose indicated above). Data are mean ± S.E.M.; n=4. (E-G) Mean arterial blood pressure at baseline, and at 30 and 60 min after i.v. THG213.29 in rats, dogs and pigs (at doses indicated above). Data are mean ± S.E.M.; n=4 per species.

**Figure 4.** Efficacy of THG213.29 in a cisplatin-induced nephrotoxic model of acute renal failure. Sprague-Dawley rats were injected with cisplatin (17.5 mg/kg i.p.) 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 five days after administration of cisplatin. A single administration of THG213.29 on day 5 (1 mg/kg i.v.) improved kidney function compared to saline-treated rats; these parameters were normalized in rats treated with THG213.29 three times a day (5 mg/kg i.v.) from days 2-5. Data are the mean ± S.E.M. from 106 normal untreated rats or 4-6 rats per treatment group, *P <0.05, **P <0.01 compared to cisplatin protocol rats treated with saline; (B) BUN and serum creatinine were measured in saline-treated rats daily for five 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 i.v.) from days 2 to 5 after cisplatin, and BUN and serum creatinine were measured on day 6 (n = 4); data represent mean ± S.E.M. *P <0.05 compared to 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 i.p., three times a day from days 2 to 5 after cisplatin). Data represent mean ± S.E.M. from 2 rats per treatment group, **P <0.01 compared to 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 i.p., three times a day from days 2 to 5 after cisplatin).

**Figure 5.** Efficacy of THG213.29 in the ischemic renal artery occlusion (RAO) model of acute kidney injury. RAO model rats (clamped renal arteries) were treated with THG213.29 (1 mg/kg i.v. bolus dose), fenoldopam (0.6 µg/kg bolus dose, followed by 0.6 µg/h/kg for 2 hours) or saline immediately after renal artery unclamping; GFR, renal plasma flow and urine output were then measured over the next two hours (A) and BUN and serum creatinine levels were measured after 72 h (B). Data are the mean ± S.E.M. from 4-6 animals per treatment group, *P <0.05, **P <0.01 compared to renal artery clamping with saline treatment; (C-F) kidney sections from RAO model rats were examined 24 (C, D) or 72 h (E, F) after treatment with saline (C, E) or THG213.29 (1 mg/kg, i.v. bolus dose) (D, F); long arrows point to glomerular erythrocyte extravasation; short arrowheads point to tubular occlusion with cell debris; glomerular erythrocyte extravasation (24 h) and tubular occlusion (72 h) were quantified from histology sections (G). Data represent mean ± S.E.M. from 4 rats per treatment group.

**Figure 6.** (A) Gene expression of Heme oxygenase type 1, Bcl2, FGF-2 and eNOS. Rat renal tissue was exposed to THG213.29 (1 µM) 4 h before RNA extraction was performed and followed with qPCR. Data represent the mean ± S.E.M. of 3 experiments, each performed in triplicate. (B) Modulation of L-902688-induced gene expression by THG213.29 (referred to as THG). HEK293/EP₄ and parental HEK293 cells were stimulated or not with L-
902688 (1 µM) in the presence or absence of THG213.29 (100 µM) for 4 h, and mRNA expression level of the indicated genes was determined by qPCR. Data represent the mean ± S.E.M. of 3 experiments, each performed in triplicate; *P <0.05, **P <0.01, ***P <0.001.
Figure 1
Figure 2
Figure 3
Figure 5

A

GFR (ml/min/kg)

Saline  213.29  Fenold

Renal Plasma Flow (ml/min/kg)

Saline  213.29  Fenold

Urine Output (µl/min)

Saline  213.29  Fenold

B

Blood Urea Nitrogen (mmol/l)

Saline  213.29  Fenold

Creatinine (µmol/l)

Saline  213.29  Fenold

C

Glomeruli with erythrocyte extravasation (%)

Saline  213.29

Tubules with obstruction (%)

Saline  213.29

C

24h

E

Saline

THG213.29

72h

D

F
Figure 6