Pharmacology of the Urotensin-II Receptor Antagonist Palosuran (ACT-058362; 1-[2-(4-Benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea Sulfate Salt): First Demonstration of a Pathophysiological Role of the Urotensin System

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ABSTRACT

Urotensin-II (U-II) is a cyclic peptide now described as the most potent vasoconstrictor known. U-II binds to a specific G protein-coupled receptor, formerly the orphan receptor GPR14, now renamed urotensin receptor (UT receptor), and present in mammalian species. Palosuran (ACT-058362; 1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulfate salt) is a new potent and specific antagonist of the human UT receptor. ACT-058362 antagonizes the specific binding of 125I-labeled U-II on natural and recombinant cells carrying the human UT receptor with a high affinity in the low nanomolar range and a competitive mode of antagonism, revealed only with prolonged incubation times. ACT-058362 also inhibits U-II-induced calcium mobilization and mitogen-activated protein kinase phosphorylation. The binding inhibitory potency of ACT-058362 is more than 100-fold less on the rat than on the human UT receptor, which is reflected in a pEC₅₀ value of 5.2 for inhibiting contraction of isolated rat aortic rings induced by U-II. In functional assays of short incubation times, ACT-058362 behaves as an apparent noncompetitive inhibitor. In vivo, intravenous ACT-058362 prevents the no-reflow phenomenon, which follows renal artery clamping in rats, without decreasing blood pressure and prevents the subsequent development of acute renal failure and the histological consequences of ischemia. In conclusion, the in vivo efficacy of the specific UT receptor antagonist ACT-058362 reveals a role of endogenous U-II in renal ischemia. As a selective renal vasodilator, ACT-058362 may be effective in other renal diseases.

Urotensin-II (U-II) is a cyclic peptide, isolated in 1980 from fish urophysis (Pearson et al., 1980). The successive discoveries that U-II exists in humans (in 1998) (Coulouarn et al., 1998), acts via binding to a human G protein-coupled receptor (in 1999) (Ames et al., 1999), and is the most potent vasoconstrictor ever described (in 2000) (Douglas and Ohlstein, 2000), suggest that U-II may be a fundamental peptide in human physiology and pathology.

The evaluation of the pathophysiological role of U-II is difficult. First, because it is a tissular system, U-II is produced by endothelial cells, renal epithelial cells, spinal cord neurons, and atherosclerotic plaques (Ames et al., 1999; Maguire et al., 2000; Matsushita et al., 2001), and its receptor (the former orphan receptor GPR14, now renamed UT receptor) is expressed in skeletal muscle, cerebral cortex, kidney cortex, vascular smooth muscle, and heart (Ames et al., 1999; Maguire et al., 2000). Because it is a tissular system, studies that rely on injecting exogenous U-II in the bloodstream may be misleading. Second, the U-II system seems to have a low level of expression in physiological situations, with low receptor capacity and low U-II production rate. For these reasons, variable results, difficult to interpret, have been described: vasodilator and vasoconstrictor
effects of U-II, natriuresis and antinatriuresis, and effects of inconsistent amplitude.

We describe here the discovery and characterization of palosuran (ACT-058362), a potent and specific antagonist of the human UT receptor, and its use as a pharmacological tool to study the role of endogenous U-II in physiological and pathological situations in models of kidney diseases.

Materials and Methods

\(^{125}\text{I}\)-Urotensin II Binding Assays

We have previously shown that functional UT receptor is constitutively expressed in the human rhabdomyosarcoma-derived cell line TE-671 (Birker-Robaczewska et al., 2003). Recombinant Chinese hamster ovary (CHO)-K1 cells expressing human or rat UT receptors were generated, grown, and characterized as described previously (Ziltener et al., 2002). TE-671 cells and recombinant CHO-K1 cells expressing rat or human UT receptor were cultured in a humidified atmosphere (5% CO\(_2\) and 95% air) using Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% FBS, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin or Ham's F-12 medium supplemented with 10% FBS, 300 \(\mu\)g/ml G418, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin, respectively.

Radio-iodinated human U-II \(^{125}\text{I}\)-U-II and unlabeled human U-II were used in binding experiments. Radioligand binding assays with \(^{125}\text{I}\)-U-II were established in whole cells and membrane preparations. For whole cell binding assays, cells were detached from the cell culture plate with enzyme-free PBS-based cell dissociation buffer (Invitrogen, Carlsbad, CA), and then resuspended in binding buffer consisting of DMEM, pH 7.4, 25 mM HEPES, and 0.5% (w/v) BSA (Invitrogen, Carlsbad, CA), and aerated with 95% O\(_2\), 5% CO\(_2\), pH 7.4. A resting force of 0.3 g (9.8 mN) was applied to the aortic ring, and changes in force were recorded using an automated system (Emka Technologies, Paris, France). The viability of each aortic ring was determined as the negative logarithm of the concentration causing a 50% reduction in the maximum force generated by U-II (EC\(_{50}\) value) was calculated according to van Rossum (1963)\(\times\). Isolated Rat Aortic Rings. Functional antagonism by palosuran of the vasoconstriction induced by human U-II was assayed on isolated rat aortic rings. Male Wistar rats (14–16 weeks old) were sacrificed by exposure to CO\(_2\). An aortic segment immediately distal to the left subclavian arterial branch was isolated, and rings (3 mm in length) were prepared (Douglas et al., 2000a). The endothelium was removed by gentle rubbing of the intimal surface, and aortic rings were suspended in tissue baths (10 ml) containing Krebs-Henseleit solution of the following composition: 115 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO\(_4\), 1.5 mM KH\(_2\)PO\(_4\), 2.5 mM CaCl\(_2\), 25 mM NaHCO\(_3\), and 10 mM glucose. The bathing solution was maintained at 37°C and aerated with 95% O\(_2\), 5% CO\(_2\), pH 7.4. A resting force of 1 g (9.8 mN) was applied to the aortic ring, and changes in force generation were recorded using an automated system (Emka Technologies, Paris, France). The viability of each aortic ring was determined by measuring contraction to potassium chloride (60 mM KCl). Removal of endothelium was confirmed by the inability of acetylcholine (10 \(\mu\)M) to relax vessels constricted with phenylephrine (1 \(\mu\)M). After washout and a further equilibration of 30 min, tissues were exposed to either vehicle (control) or palosuran (1–100 \(\mu\)M) for 20 min. A cumulative concentration-response curve to U-II (0.03–0.3 \(\mu\)M) was then obtained.

In separate experiments, aortic rings were exposed to palosuran (10 \(\mu\)M) for 20 min before cumulative concentrations of potassium chloride (5–50 mM KCl), norepinephrine (0.1–3 \(\mu\)M), 5-hydroxytryptamine (10 nM–100 \(\mu\)M), or endothelin-1 (10 \(\mu\)M–0.1 \(\mu\)M) were added.

The maximum force was defined as the force generated by the highest concentration of U-II causing a maximal effect, and from this the agonist concentration yielding a half-maximal effect (EC\(_{50}\)) was calculated. Contractile responses are expressed as absolute units of tension (grams). Because palosuran was shown to be an insurmountable antagonist of U-II induced contraction, its functional inhibitory potency (pD\(_{2}\)\textsubscript{A} value) was calculated according to van Rossum (1963) and was defined as the negative logarithm of the concentration causing a 50% reduction in the maximum force generated by U-II: pD\(_{2}\textsubscript{A} = pD_{A}^{*} + \log(X - 1)\), where pD\(_{A}^{*}\) is the negative logarithm of the
concentration of palosuran, and X is the ratio of maximal contraction to U-II in the absence and presence of palosuran.

**Acute Effects of Palosuran on Postischemic Renal Vasocostriction in Rats**

The study was performed in male Wistar rats weighing 200 to 300 g. The rats were handled according to the Position of the American Heart Association on Research Animal Use adopted November 11, 1984, by the American Heart Association. All rats were housed in climate-controlled conditions with a 12-h light/dark cycle and free access to normal pelleted rat chow and drinking water. The experiments were performed after an adaptation period of at least 1 week. Rats were anesthetized with 150 mg/kg i.p. thiobutabarbitral-Na (Inactin; Altana Pharma, Konstanz, Germany) and placed on a thermally controlled heating table to maintain body temperature at 36 to 38°C. A tracheotomy tube was put in place, and a catheter was inserted into the left jugular vein for drug infusion. A polyethylene cannula was placed in the right carotid artery and connected to a pressure transducer (MLT1050 precision BP transducer; AD Instruments, Hastings, UK) for recording of arterial blood pressure. Through a midline abdominal incision, the right kidney was removed and a Doppler flow probe was placed around the left renal artery for measurement of renal blood velocity. The probe was connected to a pulsed Doppler flowmeter (Triton Technology, San Diego, CA). Tracings were recorded on a PowerLab (IOX Data acquisition; Emka Technologies, Paris, France) connected to a Dell Dimension 733R computer with the Datalnalysis software (version 1.5; Emka Technologies). After a stabilization period, mean arterial blood pressure (MAP), heart rate (HR), and renal blood flow were continuously recorded. After baseline measurements, rats randomly received a continuous infusion of palosuran (10 mg/kg/h i.v.) or vehicle (saline; 2 ml/kg/h). This rate of infusion resulted in plasma concentrations of around 5 μM. Thirty minutes after starting infusion, renal ischemia was induced by a 45-min clamping of the left renal artery with a snare placed around the artery at its origin and was followed by a reperfusion period of 1 h. Vehicle or palosuran was infused for 30 min around the artery at its origin and was followed by a 45-min renal artery clamping, and for the 60 min of reperfusion. Sham-operated rats were subjected to the same procedure but the left renal artery was not clamped.

The right nephrectomy was performed in order to match the experimental situation of the next study, where the effect of palosuran on ischemia-induced acute renal failure was evaluated.

**Effects of Palosuran on Ischemic Renal Failure in Rats**

The study was also performed in male Wistar rats weighing 200 to 300 g, with similar housing conditions as in the first study. The experiments were performed after an adaptation period of at least 1 week. After baseline serum and 24-h urine samples were collected, the rats were anesthetized with a mixture of 50 mg/kg ketamine-HCl (Ketavet; Parke-Davis, Berlin, Germany) and 5 mg/kg xylazine (Rompun; Bayer, Leverkusen, Germany) i.p. Supplemental injection of anesthetic mixture was administered when required during surgery. Under sterile conditions, after a midline laparotomy, a right nephrectomy was performed. A nontraumatic microvascular clip was placed across the left renal artery to induce renal ischemia. At the end of a 45-min ischemia, the vascular clip was removed and the left kidney was visually inspected to insure reperfusion. The incision was immediately closed. In sham-operated rats, the right kidney was removed, but no clamping of the left renal artery was applied.

The rats subjected to renal artery clamping and the sham-operated rats were randomly assigned to receive either an intravenous infusion of palosuran (10 mg/kg/h) or vehicle (saline; 2 ml/kg/h), starting 30 min before renal ischemia. The infusion was continued during the 45-min renal artery occlusion and for 60 min after clamping. The choice of the dose of palosuran was based on the previous study demonstrating that a dose of 10 mg/kg/h fully prevented the decrease in renal blood flow after renal ischemia. Four groups of animals were used in this study: sham-operated rats treated with vehicle (n = 10), sham-operated rats treated with palosuran (n = 10), renal ischemic rats treated with vehicle (n = 12), and renal ischemic rats treated with palosuran (n = 12).

At 24 h after reperfusion, rats were placed in metabolic cages for 24-h urine sample collection. At the end of this period (48 h after ischemia), rats were anesthetized and serum samples were obtained. The left kidney was removed and preserved in phosphate-buffered 10% formalin for morphological studies.

**Analytical Procedures**

The volume of urine samples was determined gravimetrically. Serum and 24-h urine samples were assayed for creatinine and Na⁺ concentrations. Creatinine concentrations were measured colorimetrically using a commercially available kit (Sigma-Aldrich, St. Louis, MO). Sodium concentrations were measured using an ion-selective electrode (automatic biochemical analyzer, Hitachi 7150; Hitachi, Tokyo, Japan). Glomerular filtration rate (GFR) was determined by the clearance of creatinine. Urinary sodium excretion (UNaV) was calculated by UNaV = UNa × V and the fractional excretion of sodium (FENa) by FENa (%) = (UNa/V)/(SNa × GFR). The net tubular reabsorption of sodium (TRNa) was calculated by TRNa = (SNa × GFR) – (UNa × V) (Roux et al., 1999), where UNa and SNa are sodium concentrations in urine and serum samples, respectively, and V is urine flow rate.

**Histological Evaluation**

In sham-operated rats and in postischemic renal failure rats treated with palosuran or vehicle, the left kidney was isolated, embeded in paraffin, cut, and stained with hematoxylin eosin. The severity of histological changes was determined under light microscopy and was graded semiquantitatively as described previously (Veniant et al., 1994). Briefly, tubulointerstitial lesions (interstitial inflammation and fibrosis, tubular atrophy, and dilation with casts) were assessed at 100 × magnification in every third field of each kidney (total of 10 fields/kidney) and assigned an injury grade (0–3; grade 0, normal; 1, lesions involving <25%; 2, lesions involving 25 to 50%; and 3, lesions involving >50% of the field). A score for tubulointerstitial lesions for each kidney was obtained by averaging the grades given to all fields. The evaluations were performed in a blinded manner. Because there were no visible glomerular lesions, glomerular scores were not evaluated.

**Statistical Analysis**

Data are expressed as mean ± S.E.M. Statistical analyses were performed by analysis of variance using Statistica (StatSoft, Tulsa, OK). Where a significant F was observed, the data were further analyzed with a Student-Newman-Keuls procedure. Statistical significance is defined where p < 0.05.

**Drugs**

Palosuran was synthesized in the course of a chemical optimization effort of UT receptor antagonists identified by random screening of the Actelion compound collection with radioligand binding technologies. Palosuran was dissolved in water immediately before use. 1.5% U-II (2130 C/mmol) was obtained from Anawa Trading SA (Wangen, Switzerland). U-II (Batch AO: BBendorf, Switzerland) and endothelin-1 (Alexis Biochemicals, Lausen, Switzerland) were prepared as stock solutions in methanol/water (1:1) and stored at −25°C. Dilutions were made using 0.1% BSA on the day of each experiment. Norepinephrine bitartrate, 5-hydroxytryptamine hydrochloride, 1-phenylethylamine hydrochloride, and acetylcholine chloride were obtained from Sigma-Aldrich. KCl, HEPES, BSA fraction V, DMSO, and EDTA were obtained from Fluka (Buchs, Switzerland). DMEM, PBS were from Invitrogen (Basel, Switzerland) and Invitrogen (Carlsbad, CA), and Ham’s F-12 medium were from Invitrogen (Carlsbad, CA).
Results

Chemical Structure of Palosuran. The chemical structure of palosuran is shown in Fig. 1.

Inhibition of Urotensin-II Binding by Palosuran. Binding of $^{125}$I-U-II to TE-671 cells and to recombinant CHO cells expressing the human UT receptor was potently inhibited by unlabeled U-II, with IC$_{50}$ values of 0.27 ± 0.08 and 0.73 ± 0.09 nM, respectively. The inhibitory potency of unlabeled human U-II was 10-fold lower in recombinant CHO cells expressing the rat UT receptor. Similar results were obtained in the respective membrane preparations (Table 1).

Palosuran inhibited 125I-U-II binding to human UT receptors in membrane preparations from CHO cells carrying the human UT receptors almost as potently as cold U-II, with an IC$_{50}$ of 3.6 ± 0.2 nM. On cells, the inhibitory binding potency of palosuran against human UT receptor was lower than on membranes (IC$_{50}$ = 46.2 ± 13 nM on TE 671 cells and 86 ± 30 nM on recombinant CHO cells). Compared with the human UT receptor, the binding inhibitory potency of palosuran against the rat UT receptor was lower in membrane preparation (400-fold), as well as in cells (>120-fold) (Table 1).

Mode of Palosuran and UT Receptor Interaction. Scatchard analysis was performed on human UT receptors expressed on membrane preparations from recombinant CHO cells to determine human U-II and palosuran binding kinetics. Initial experiments were performed at room temperature with incubation time of 8 h where the binding of $^{125}$I-U-II reached a plateau. The experimental curve in the Scatchard analysis was linear and compatible with one binding site with an apparent $K_D$ of 50 pM and $B_{\text{max}}$ of 2.4 pM corresponding to 3600 receptors per cell. However, ligand-dissociation experiments indicated the presence of two binding sites for $^{125}$I-U-II: a fast dissociation site with $k_{\text{off}}$ in the order of 0.02 min$^{-1}$ and a slow dissociation site with a $k_{\text{off}}$ of 0.0015 min$^{-1}$ (data not shown). The very low $k_{\text{off}}$ rate of the slow dissociation site indicated that the time required to reach binding equilibrium may not have been sufficient in the initial binding studies where an incubation time of 8 h was used. Indeed, the calculated time to reach equilibrium ($T_{\text{eq}} = 3.5/k_{\text{off}}$) for the slow site was 39 h. Therefore, binding experiments were performed at room temperature for 38 h and showed unchanged maximum binding and unaltered IC$_{50}$ values of U-II and palosuran compared with 8 h of incubation. Saturation binding experiments performed for 38 h at room temperature, and Scatchard analyses of these data confirmed the presence of two binding sites in membranes from recombinant CHO cells expressing the human UT receptor (Fig. 2 and Table 2). Eighty percent of the $^{125}$I-U-II binding sites were of low affinity with a $K_D$ value of 155 pM. The remaining 20% of the binding sites were of high-affinity with a $K_D$ value of 1.9 pM. Addition of palosuran resulted in an increase in the apparent $K_D$ and $B_{\text{max}}$ values (Table 2). Because the increase of $K_D$ was large and that of the $B_{\text{max}}$ was within the range of experimental error, our data seem to be consistent with a competitive mode of antagonism by palosuran on both receptor sites. However, inhibition after 8 h of incubation, which is more reminiscent of the situation in biological assays, did not seem to be competitive, presumably because equilibrium was not reached (data not shown).

Functional Antagonism by Palosuran in Cell Preparations. Human U-II induced intracellular Ca$^{2+}$ mobilization in CHO cells expressing human or rat UT receptors with EC$_{50}$ values of 23 ± 2 and 6.5 ± 0.45 nM, respectively. Palosuran inhibited Ca$^{2+}$ mobilization in response to human U-II in CHO cells expressing human and rat UT receptor with IC$_{50}$ values of 17 ± 0.63 and >10,000 nM, respectively (Table 3). This effect was specific, because palosuran did not alter endothelin-1 induced intracellular Ca$^{2+}$ mobilization in CHO cells overexpressing the endothelin ET$_A$ receptor (data not shown). The species differences of palosuran in the Ca$^{2+}$ mobilization experiments confirmed the results obtained in binding experiments.

In recombinant CHO cells expressing the human UT receptor, human U-II increased MAPK phosphorylation concentration dependently with an EC$_{50}$ value of around 3 nM (Ziltener et al., 2002). In the same cells, palosuran inhibited human U-II induced MAPK phosphorylation in a dose-dependent manner with an IC$_{50}$ value of 150 nM (Fig. 3). Similarly, palosuran reduced the increase in MAPK phosphorylation induced by human U-II in human TE-671 cells, which express endogenous UT receptors (data not shown).

Functional Antagonism by Palosuran in Rat Aortic Rings. U-II induced potent and concentration-dependent contraction of rat aortic rings, yielding an EC$_{50}$ value of 1.34 ± 0.17 nM. The maximal response to U-II was 1.73 ± 0.16 g. Palosuran did not change baseline force, but it reduced contraction to U-II in a concentration-dependent manner (Fig. 4). A linear relationship between the concentration of palosuran and the decrease in maximal contraction to U-II was observed ($r^2 = 0.93$). The pD$_{2}$ value for palosuran was calculated to be 5.23 ± 0.11.

### Table 1

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Cell Binding IC$_{50}$</th>
<th>Membrane Binding IC$_{50}$</th>
</tr>
</thead>
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<tr>
<td></td>
<td>TE 671 (n = 10)</td>
<td>CHO Human UT (n = 22)</td>
</tr>
<tr>
<td>Human U-II</td>
<td>0.27 ± 0.08</td>
<td>0.73 ± 0.09</td>
</tr>
<tr>
<td>ACT-058362</td>
<td>46.2 ± 13</td>
<td>86 ± 30 (n = 4)</td>
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Functional Selectivity of Palosuran for the UT Receptor in Rat Aortic Rings. Contraction of rat aortic rings was used to assess the functional selectivity of palosuran for the UT receptor versus other receptors involved in vascular tone regulation. Although 1 μM palosuran significantly decreased U-II induced contraction (Fig. 4), there was no inhibition of norepinephrine, 5-hydroxytryptamine, and endothelin-1-induced contraction detectable at a concentration of 10 μM palosuran (data not shown). These results indicated that in rat the selectivity ratio of palosuran for the UT receptor versus α-1 adrenoceptor, 5-hydroxytryptamine 2A receptor, and endothelin receptor A is >10, despite the rather low affinity of the compound to the rat UT receptor.

Acute Effects of Palosuran on Postischemic Renal Vasoconstriction in Rats. Baseline values for MAP, HR, and mean renal blood flow were similar in all groups. In sham-operated rats, there was a small progressive increase in renal blood flow, due to the right nephrectomy. Palosuran had no significant effect on renal blood flow (Fig. 5A) and no effect on MAP and HR. Forty-five-minute renal ischemia in vehicle-treated rats resulted in a 15 ± 6% decrease at 60 min in renal blood flow compared with baseline values. In contrast, palosuran restored renal blood flow to baseline values at 30 min after reperfusion and by 60 min increased renal blood flow by 12 ± 7% above baseline values (Fig. 5B) and

Fig. 2. Saturation binding curves and Scatchard transformation (insets) of [125I]-U-II binding to membrane preparations from CHO cells expressing recombinant human UT receptor. The CHO membranes were incubated for 38 h at room temperature in the absence (A) and presence (B) of 5 nM palosuran. Curves were fitted directly using the KELL software (see Materials and Methods).

TABLE 2
Effect of palosuran (ACT-058362) on U-II binding kinetics
Scatchard analysis of saturation binding experiments (values are in picomolar concentration) performed for 38 h at room temperature in membrane preparations of CHO cells expressing the human UT receptor in the absence or presence of ACT-058362. The concentration of ACT-058362 added corresponds to its IC50 value estimated for the same membrane preparation at 38 h.

<table>
<thead>
<tr>
<th>Binding Sites</th>
<th>ACT-058362 (0 nM)</th>
<th>ACT-058362 (5 nM)</th>
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<tbody>
<tr>
<td>High-affinity</td>
<td>$K_D$</td>
<td>$K_D$</td>
</tr>
<tr>
<td></td>
<td>1.9</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>$B_{max}$</td>
<td>$B_{max}$</td>
</tr>
<tr>
<td></td>
<td>1.7</td>
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<td>Low-affinity</td>
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<td>155</td>
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<td>$B_{max}$</td>
<td>$B_{max}$</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>7.7</td>
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TABLE 3
Potency and species selectivity of palosuran (ACT-058362) on U-II induced intracellular Ca²⁺ mobilization in CHO cells expressing human or rat UT receptor
Urotensin-II-induced Ca²⁺ mobilization in the recombinant CHO cells was measured by the FLIPR assay system. The potency of U-II (EC₅₀) was determined in the absence of ACT-058362. The inhibitory potency of ACT-058362 (IC₅₀) was determined on Ca²⁺ mobilization induced by 30 nM U-II.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Human UT Receptor</th>
<th>Rat UT Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human U-II (EC₅₀, nM)</td>
<td>23 ± 2 (n = 10)</td>
<td>6.5 ± 0.45 nM (n = 7)</td>
</tr>
<tr>
<td>ACT-058362 (IC₅₀, nM)</td>
<td>17 ± 0.63 (n = 17)</td>
<td>&gt;10,000 (n = 4)</td>
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</table>

Fig. 3. Inhibition of human U-II induced MAPK phosphorylation by palosuran (ACT-058362) in CHO cells expressing recombinant human UT receptors.

Fig. 4. Concentration-response curve of contraction induced by U-II on isolated rat aortic rings in the absence and presence of palosuran (ACT-058362).
thus fully prevented the decrease in renal blood flow after ischemia. Palosuran did not significantly alter MAP and HR (data not shown).

Effects of Palosuran on Postischemic Renal Failure in Rats. Baseline values were similar in all experimental groups. Clamping of the left renal artery for 45 min in association with right nephrectomy caused acute renal failure, as shown by a statistically significant increase in serum creatinine concentration (1.80 ± 0.27 versus 0.84 ± 0.06 mg/dl; p < 0.01) and a significant 55 ± 6% decrease in GFR at 48 h postreperfusion in vehicle-treated rats (Fig. 6). Treatment with palosuran prevented the increase in serum creatinine concentration (1.08 ± 0.16 versus 1.80 ± 0.27 mg/dl; p < 0.01) and reduced the decrease in GFR (−33 ± 5 versus −55 ± 6%; p < 0.01) (Fig. 6). GFR was improved but not normalized and remained significantly lower than the preischemia levels (0.29 ± 0.02 versus 0.44 ± 0.03 ml/min/100 g; p < 0.001). Palosuran had no effect on serum creatinine concentration and GFR in sham-operated rats.

Neither renal artery clamping nor palosuran had any significant effect on serum sodium concentration (Fig. 7A). In vehicle-treated rats, renal artery clamping resulted in a significant decrease in tubular sodium reabsorption (73 ± 8 versus 198 ± 24 μmol/min; p < 0.001) (Fig. 7B). This led to a significant increase in the fractional excretion of sodium (Fig. 7C). Palosuran significantly reduced the consequences of renal ischemia and attenuated both the decrease in tubular sodium reabsorption (Fig. 7B) and the increase in fractional excretion of sodium (Fig. 7C). In sham-operated rats, but not in acute renal failure rats, palosuran significantly increased 24-h urinary sodium excretion (p < 0.05) and sodium fractional excretion (p < 0.05) (Fig. 7D).

Clamping the renal artery for 45 min induced massive tubular damage characterized by acute tubular necrosis and tubular obstruction by urinary casts at 48 h post-reperfusion. Semiquantitative histological lesion grading revealed that there were markedly more tubulointerstitial lesions in the untreated renal failure rats compared with sham-operated rats. Treatment with palosuran decreased the severity of tubular changes, as shown by a statistically significant decrease in the score of tubulointerstitial lesions (Fig. 8).

Discussion

Since the discovery of UT-II as an endogenous ligand of the orphan G protein-coupled receptor GPR14, now called UT receptor (Ames et al., 1999; Liu et al., 1999; Mori et al., 1999; Nothacker et al., 1999), extensive research has focused on the UT-II system as a potential therapeutic target. It has been shown that exogenous U-II exhibits a cardiovascular profile similar to that of endothelin-1. Both peptides mediate vasoconstriction and vasodilation, cell proliferation, and cardiac hypertrophy and may modulate cardiac function (Ames et al., 1999; Douglas et al., 2000b; Maguire et al., 2000; Sauzeau et al., 2001; Watanabe et al., 2001a,b; Zou et al., 2001; Maguire and Davenport, 2002). To understand the pathophysiological role of endogenous U-II, it was important to develop specific...
Differences in the pharmacological nature of antagonists, palosuran interacted competitively with its receptor. Using membrane preparations expressing human UT receptor, antagonism of the contraction induced by U-II. In binding studies, palosuran almost completely inhibited the maximal contractile response of the rings, demonstrating insurmountable antagonism of the contraction induced by U-II. In binding studies using membrane preparations expressing human UT receptors, palosuran interacted competitively with its receptor. Differences in the pharmacological nature of antagonism observed between functional assays and receptor binding studies are not uncommon. In fact, a similar discrepancy in the mode of inhibition between the vasoconstriction assay and the binding assay was described e.g., for CV-11974, an angiotensin II receptor antagonist (Shibouta et al., 1993). These studies reported that CV-11974 displayed noncompetitive inhibition of contraction to angiotensin II in rabbit aorta, whereas the inhibition of CV-11974 binding to rabbit aortic membranes was of competitive nature. Furthermore, BIM-23127, a peptide UT receptor antagonist, inhibited Ca\(^{2+}\) mobilization in human embryonic kidney 293 cells expressing UT receptors in a competitive manner, whereas this compound displayed noncompetitive antagonism of contraction to U-II in isolated rat aorta (Herold et al., 2003). There are at least two hypotheses to explain the data generated with palosuran in the isolated rat aortic ring system. First, the interaction between palosuran and the UT receptor on the surface of rat native cells is indeed of noncompetitive manner. Second, the binding of palosuran to the aortic rings induced internalization of the UT receptors reducing the number of cell surface receptors available for binding of U-II. Subsequent challenges with U-II did not result in maximal contraction anymore, because the number of UT receptors in the palosuran-pretreated tissue was too low. Further studies will be required to better understand the complex nature of the inhibition in intact vessels.

The functional assays using rat aortic rings demonstrate that palosuran is a specific antagonist of UT receptors and does not antagonize the action of other vasoconstrictor agents such as KCl, endothelin-1, 5-hydroxtryptamine, and norepinephrine. Therefore, palosuran is a valid tool to evaluate the role of endogenous U-II in disease models, in particular in kidney pathologies. Recent studies have demonstrated that both U-II and the UT receptor are expressed in the kidney (Matsushita et al., 2001). The kidney indeed may synthesize U-II, because urinary fractional excretion of U-II exceeds the glomerular filtration rate (Matsushita et al., 2001). Urotensin-II is expressed in epithelial cells of tubules and collecting ducts, capillary and glomerular endothelial cells, and in endothelial and smooth muscle cells of renal arteries (Shenouda et al., 2002). The UT receptor, in contrast, is found in kidney cortex (Maguire et al., 2000; Matsushita et al., 2001; Shenouda et al., 2002). Therefore, U-II acting on UT receptors may play an important role in renal physiology/pathophysiology through an autocrine or paracrine action. An endothrine function seems less likely, in view of the very low concentration of circulating U-II-like immunoreactive peptide, suggesting that U-II concentrations in plasma may represent a spillover from tissular U-II, more than reflecting the circulation of an endocrine hormone.

Palosuran was very effective in a rat model of renal ischemia, both for preventing the postischemic renal vasoconstriction and for reducing the postischemic acute renal failure. The profile of palosuran was very peculiar, because the increase in renal blood flow seen after ischemia was not accompanied by any systemic vasodilation, suggesting a selective renal vasodilating effect. The “no-reflow” phenomenon has been shown to be associated with endothelial dysfunction and an increase in the production of endothelin and free radicals (Brodsky et al., 2002). Endothelin receptor antagonists and antioxidants have demonstrated efficacy in preventing no-reflow (Chatterjee et al., 2000; Ajis et al., 2003),
whereas vasodilators such as dopamine, atrial natriuretic peptide, and calcium channel blockers were unsuccessful (Munda and Alexander, 1980; Koelz et al., 1988). Here, we show that endogenous U-II plays a role in mediating the abnormal renal vasoconstriction after ischemia. In contrast, the absence of effect of palosuran on renal blood flow in sham-operated rats suggests that U-II does not participate in the control of normal renal blood flow under physiological conditions.

The no-reflow phenomenon is known to play a fundamental role in the secondary development of ARF after ischemia (Arendshorst et al., 1975). It was therefore tempting to evaluate the efficacy of palosuran for preventing posts ischemic renal failure. In a rat model of posts ischemic ARF, palosuran attenuated the increase in serum creatinine and reduced the decrease in GFR. It also attenuated the loss of sodium reabsorption, a well known phenomenon after renal ischemia (Stein et al., 1978; Molitoris, 1991). This effect is opposite to the observations made in normal sham-operated rats. In uninephrectomized rats (sham controls), palosuran significantly increased 24-h urinary sodium excretion. These results suggest that U-II may play a physiological role in sodium balance. Alternatively, after ischemia, U-II may contribute to abnormal vasoconstriction and secondary deficient sodium reabsorption. Previous studies have shown that U-II modulates transepithelial sodium ion transport in fish (skin epithelia, operculum, intestine, and bladder) (Loretz and Bern, 1980; Loretz and Bern, 1981; Conlon et al., 1997), and exposing fish (flounder) to an osmotic stress (freshwater versus saltwater environment) elevated circulating U-II-like immunoreactive peptides, concomitant with alterations in plasma osmolality and sodium (Winter et al., 1999). Thus, U-II may possess osmoregulatory functions in aquatic animals, although little is known to date about its role in mammals.

Treatment of ischemic ARF rats with i.v. palosuran significantly attenuated renal glomerular and tubular dysfunction. Histological examination of the kidney of ischemic ARF rats revealed massive tubular damage at 48 h postreperfusion. Palosuran administration significantly decreased renal tubular lesions in this rat model of ARF, although this effect on the renal lesions is of a lesser magnitude than might have been expected, given that palosuran fully restored posts ischemic renal blood flow. This suggests that there may be other factors involved in the pathogenesis of posts ischemic renal tubular injury. To our knowledge, it is the first evidence that a UT receptor antagonist improves renal dysfunction and tissue injury induced by ischemia/reperfusion, revealing a role of U-II in the pathogenesis of ischemic ARF in rats. Further studies are required to evaluate whether a UT receptor antagonist can reverse the ischemia/reperfusion-induced renal dysfunction and tissue injury when given after reperfusion.

It is not clear from the present study whether the beneficial effect of palosuran is entirely secondary to the prevention of posts ischemic vasoconstriction, or whether a direct tubular effect is involved. The fact that palosuran was able to fully prevent the decrease in renal blood flow after renal ischemia suggests that the beneficial effect on renal function may be mainly due to inhibition of U-II-mediated renal vasoconstriction. Our study, however, does not elucidate whether palosuran is a preferential afferent or efferent vasodilator in rats and whether it modifies filtration fraction. The observation of an improvement in renal tubular handling of sodium indicates that the improvement in renal function may also be due to a direct tubulo-protective effect of the substance, although the tubular effects may be secondary to an increased renal perfusion. A combination of both mechanisms is also a possibility, because both U-II and UT receptor have been found in both vascular and renal tissue (Maguire et al., 2000; Matsushita et al., 2001; Shenouda et al., 2002). Furthermore, other mechanisms of renoprotection, such as an antiproliferative or anti-inflammatory effect of palosuran, cannot be excluded. The U-II-induced MAPK phosphorylation and the induction of U-II high-affinity binding sites by interferon-γ support a role of the U-II system in inflammation (Birker-Robaczewska et al., 2003), and the ability of palosuran to inhibit U-II-induced MAPK phosphorylation suggests a potential anti-inflammatory effect.

Our findings in the present study strongly suggest that endogenous U-II contributes to the pathogenesis of ischemic ARF. Plasma concentrations of U-II are significantly elevated in experimental and human renal failure (our unpublished observations; Matsushita et al., 2001; Totsune et al., 2001; Shenouda et al., 2002). Vasoconstrictor responses to U-II in pulmonary arteries are enhanced by endothelin removal or by chronic hypoxia (MacLean et al., 2000). Thus, it is likely that increased local production of U-II in the kidney during ischemia and/or after reperfusion has detrimental consequences. The present pharmacological studies using the UT receptor antagonist palosuran support the possibility of U-II as a causal factor of ischemic ARF.

In conclusion, palosuran is a novel UT receptor antagonist that represents an important research tool for evaluating the pathophysiological role of endogenous U-II. Short-term intravenous administration of palosuran reduced the glomerular and tubular dysfunction and renal tissue injury induced by renal ischemia/reperfusion. In sham-operated rats, palosuran increased urinary sodium excretion and fractional excretion of sodium. Palosuran is more potent on the human UT receptor than on the rat receptor. If the findings resulting from the experimental models translate into clinical efficacy, UT receptor antagonists such as palosuran may be useful in the treatment of renal diseases.

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References


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