JPET Fast Forward. Published on May 14, 2004 as DOI:10.1124/jpet.104.068320

Pharmacology of the Urotensin-II Receptor Antagonist ACT-058362: First Demonstration of a Pathophysiological Role of the Urotensin System

Martine Clozel, Christoph Binkert, Magdalena Birker-Robaczewska, Céline Boukhadra, Shuang-Shuang Ding, Walter Fischli, Patrick Hess, Boris Mathys, Keith Morrison, Celia Müller, Claus Müller, Oliver Nayler, Changbin Qiu, Markus Rey, Michael W. Scherz, Jörg Velker, Thomas Weller, Jian-Fei Xi, and Patrick Ziltener.

Actelion Pharmaceuticals Ltd, Innovation Centre, Allschwil, Switzerland

(a) Running title: Urotensin receptor antagonist ACT-058362 in renal ischemia

(b) Corresponding author: Martine Clozel, MD, Actelion Pharmaceuticals Ltd,
Innovation Centre, Gewerbestrasse 16, Allschwil, CH-4123, Switzerland.
(Tel) +41 61 487 45 13, (Fax) +41 61 487 45 00.
e-Mail: Martine.Clozel@Actelion.com

(c) Number of:

- Text pages: 35
- Figures: 8
- Tables: 3
- References: 40

Number of words:

- Abstract: 489
- Introduction: 261
- Discussion: 1,676

(d) List of non-standard abbreviations: U-II, urotensin-II; UT, urotensin receptor; CHO, Chinese hamster ovary; GFR, glomerular filtration rate; $U_{Na}V$, urinary sodium excretion; FE_{Na}, fractional excretion of sodium; MRBF, mean renal blood flow.

(e) Recommended section assignment: best appears under the section heading "Cardiovascular"

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. 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 ^{125}I-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 (MAPK) 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 pD'_2 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 non-competitive 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). Since it is a tissular system, studies which rely on injecting exogenous U-II in the blood stream 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, effects of inconsistent amplitude.

We describe here the discovery and characterization of 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}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 previously described (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 / 95% air) using Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin or Ham's F12 medium supplemented with 10% FBS, 300 μg/ml G418, 100 U/ml penicillin, and 100 μg/ml streptomycin, respectively.

Radio-iodinated human U-II (^{125}I-U-II) and unlabeled human U-II were used in binding experiments. Radioligand binding assays with ^{125}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, USA) and then resuspended in binding buffer consisting of DMEM, pH 7.4 25 mM HEPES, 0.5% (w/v) BSA Fraction V. CHO cells at a density of 1×10^5 cells/well in 96-well plates were incubated for 4 h at 4 °C with shaking, in the presence of 20 pM (14,000 cpm) ^{125}I-U-II in 250 μl binding buffer in the presence or absence of test substances. Binding assays in TE-671 cells were performed identically but using 3×10^5 cells/well incubated at room temperature and in the presence of 28,000 cpm of ^{125}I-U-II. Minimum and maximum binding was derived from samples with and without 1 μM of unlabeled U-II, respectively. Dilutions of compounds were performed in DMSO, peptides were diluted in binding buffer. After a 4-h incubation period, the cells were filtered through GF/C filterplates pre-equilibrated with binding buffer (Packard Bioscience, CT, USA) using cold PBS pH 7.4, 0.1% (w/v) BSA Fraction V. After drying of the filterplates, 50 µl of scintillation cocktail (MicroScint 20, Packard Bioscience, CT, USA) was added to each well, and the filterplates were counted in a microplate counter.

Microsomal cell membranes were prepared according to Breu et al. (Breu et al., 1993). Membrane binding assays were performed in 200 µl PBS, pH 7.4, 1 mM EDTA, 0.5% (w/v) BSA Fraction V, 2.5% DMSO, in polypropylene microtiter plates. Membranes containing 2 to 5 µg of protein, were incubated for 4 h at room temperature with 20 pM ^{125}I-U-II and then filtered as described above.

Dissociation rate of human ^{125}I-U-II was determined in membranes from recombinant CHO cells expressing the human UT receptor. ^{125}I-U-II (50 pM) was bound to membranes for 2 h at room temperature. Dissociation was induced by the addition of 1 µM cold U-II. Reactions were stopped by filtration at various time points after induction of dissociation. Data were evaluated using KELL software, version 6 (Biosoft, Cambridge, UK).

For Scatchard analysis, the membrane binding conditions were modified to 38 h of incubation, at room temperature, in the presence of 0.01 nM to 1 nM ^{125}I-U-II. Non-specific binding was determined in the presence of 1 µM unlabeled U-II. Scatchard transformation of the binding data was performed with KELL software, version 6 (Biosoft, Cambridge, UK).

In Vitro Functional Inhibitory Potency

Calcium Measurements with Fluorometric Image Plate Reader (FLIPR). Cytoplasmic calcium mobilization was detected using the fluorescent indicator Fluo-3 and FLIPR as previously described (Ziltener et al, 2002). Briefly, cells were seeded into 96-well plates at a density of 4×10^5 cells/well, incubated overnight, and then loaded with Fluo-3 (Molecular Probes, OR, USA). Serial dilutions of ACT-058362 in HBSS, including 0.35 g/l $NaHCO_3$, 20 mM Hepes, 0.1% BSA and 0.5% DMSO final concentration, were added to the cells, and the cells incubated for 20 min. Human U-II was then added at a final concentration of 30 nM and the fluorescence output was measured at 500–560 nm. IC_{50} values were calculated using IC_{50} WITCH^{TM} (Actelion Pharmaceuticals Ltd, Allschwil, Switzerland).

MAPK Phosphorylation. TE-671 cells or recombinant CHO cells carrying the human UT receptor were seeded in 12-well plates and grown overnight (1×10^5 cells/well). Cells were starved for 4 h by incubating in standard medium without FBS but with 1 mg/ml fatty acid free BSA. Cells were subsequently incubated for 5 min with different concentrations of human U-II (for EC_{50} determination) or pre-incubated with different concentrations of ACT-058362 for 20 min and then incubated for 5 min with 50 nM (TE-671 cells) or 3 nM (CHO cells) U-II (for IC_{50} determination). ELISA experiments with pERK1/2 (pTpY185/187) and ERK1/2 (total) were then performed according to the manufacturers' protocol (BIOSOURCE International, Nivelles, Belgium). pERK1/2 levels were normalized against total ERK1/2 and results were expressed as the mean ± S.E.M. of 3 separate experiments with each data point in duplicate.

Isolated Rat Aortic Rings. Functional antagonism by ACT-058362 of the vasoconstriction induced by human U-II was assayed on isolated rat aortic rings. Male Wistar rats (14 to 16 weeks old) were sacrificed by exposure to CO_2. An aortic segment immediately distal to the left sub-clavian arterial branch was isolated, and rings (3 mm 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)

JPET Fast Forward. Published on May 14, 2004 as DOI: 10.1124/jpet.104.068320

containing Krebs-Henseleit solution of the following composition (mM): NaCl 115; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.5; $CaCl_2$ 2.5; $NaHCO_3$ 25; glucose 10. 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 (KCl, 60 mM). Removal of endothelium was confirmed by the inability of acetylcholine (10 μM) to relax vessels constricted with phenylephrine (1 μM). Following washout and a further equilibration of 30 min, tissues were exposed to either vehicle (control) or ACT-058362 (1–100 μM) for 20 min. A cumulative concentration-response curve to U-II (0.03 nM–0.3 μM) was then obtained.

In separate experiments, aortic rings were exposed to ACT-058362 (10 μM) for 20 min, before cumulative concentrations of potassium chloride (KCl, 5–50 mM), norepinephrine (0.1 nM–3 μM), 5-hydroxytryptamine (10 nM–100 μM) or endothelin-1 (10 pM–0.1 μM) 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 (g). Since ACT-058362 was shown to be an insurmountable antagonist of U-II induced contraction, its functional inhibitory potency (pD'_2 value) was calculated according to van Rossum (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: $pD'_2 = pD'_x + \log(X\text{-}1)$, where pD'_x is the negative logarithm of the concentration of ACT-058362 and X is the ratio of maximal contraction to U-II in the absence and presence of ACT-058362.

Acute effects of ACT-058362 on post-ischemic renal vasoconstriction 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 one week. Rats were anesthetized with 150 mg/kg, i.p. thiobutabarbital-Na (Inactin, Altana Pharma, Konstanz, Germany) and placed on a thermostatically controlled heating table to maintain body temperature at 36–38 °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, USA). Tracings were recorded on a PowerLab (IOX Data acquisition, Emka Technologies, Paris, France) connected to a Dell Dimension 733R computer with the Datanalyst software (version 1.5, Emka Technologies, Paris, France). 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 ACT-058362 (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

ACT-058362 was infused for 30 min before renal occlusion, during the 45 min of 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 ACT-058362 on ischemia-induced acute renal failure was evaluated.

Effects of ACT-058362 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 one week. After baseline serum and 24-h urine samples were collected, the rats were anesthetized with a mixture of 50 mg/kg Ketamin-HCl (Ketavet, Parke-Davis, Berlin, FRG) and 5 mg/kg xylazin (Rompun, Bayer, Leverkusen, FRG) i.p. Supplemental injection of anesthesic mixture was administered when required during surgery. Under sterile conditions, after a midline laparotomy, a right nephrectomy was performed. A non-traumatic 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 ACT-058362 (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 ACT-058362 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 ACT-058362 (n = 10); renal ischemic rats treated with vehicle (n = 12); renal ischemic rats treated with ACT-058362 (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, St. Louis, MO, USA). Sodium concentrations were measured using an ion-selective electrode (Automatic Biochemical Analyzer, Hitachi 7150, Japan). Glomerular filtration rate (GFR) was determined by the clearance of creatinine. Urinary sodium excretion ($U_{Na}V$) was calculated by: $U_{Na}V = U_{Na} \times V$. The fractional excretion of sodium (FE_{Na}) by: FE_{Na} (%) = $(U_{Na} \times V)/(S_{Na} \times GFR)$. The net tubular reabsorption of sodium (T_{Na}) by: $T_{Na} = (S_{Na} \times GFR) - (U_{Na} \times V)$ (Roux et al., 1999), where U_{Na} and S_{Na} are sodium concentrations in urinary and serum samples respectively, and V is urine flow rate.

Histological evaluation

In sham-operated rats and in post-ischemic renal failure rats treated with ACT-058362 or vehicle, the left kidney was isolated, embedded in paraffin, cut and stained with hematoxylin eosin (H + E). The severity of histological changes was determined under light microscopy and was graded semiquantitatively as previously described (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 to 3): grade 0, normal; 1, lesions involving < 25%; 2, lesions involving 25–50%; 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. Since 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 (ANOVA) using Statistica (StatSoft). 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

ACT-058362 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. ACT-058362 was dissolved in water immediately before use. ^{125}I-U-II (2,130 Ci/mmol) was obtained from Anawa Trading SA

(Wangen, Switzerland). U-II (Bachem AG, Bubendorf, 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, L-phenylephrine hydrochloride and acetylcholine chloride were obtained from Sigma (St Louis, MO, USA). KCl, HEPES, BSA Fraction V, DMSO and EDTA were obtained from Fluka (Buchs, Switzerland). DMEM and PBS were from Gibco Laboratories (Basel, Switzerland) and Invitrogen (Carlsbad, CA, USA), and Ham’s F12 medium was from Invitrogen (Carlsbad, CA, USA).

Results

Chemical structure of ACT-058362

The chemical structure of ACT-058362 (1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulfate salt) is shown in Figure 1.

Inhibition of urotensin-II binding by ACT-058362

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 nM 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).

ACT-058362 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 ACT-058362 against human UT receptor was lower than on membranes (IC_{50} = 46.2 ± 13 nM on TE 671 cells, 86 ± 30 nM on recombinant CHO cells). Compared to the human UT receptor, the binding inhibitory potency of ACT-058362 against the rat UT receptor was lower in membrane preparation (400 fold), as well as in cells (>120-fold) (Table 1).

Mode of ACT-058362 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 ACT-058362 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_{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_{off} in the order of 0.02 min^{-1}, and a slow dissociation site with a k_{off} 0.0015 min^{-1} (data not shown). The very low k_{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 ($T_{eq} = 3.5/k_{off}$) 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 ACT-058362 as compared to 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 (Figure 2 and Table 2). Eighty % of the ^{125}I-U-II binding sites were of low affinity with K_D value of 155 pM. The remaining 20% of the binding sites were of high affinity with K_D value of 1.9 pM. Addition of ACT-058362 resulted in an increase in the apparent K_D and B_{max} values (Table 2). Since the increase of K_D was large and that of the B_{max} was within the range of experimental error, our data appear to be consistent with a competitive mode of antagonism by ACT-058362 on both receptor sites. However, inhibition after 8 h of incubation, which is more reminiscent of the situation in biological assays, did not appear to be competitive, presumably because equilibrium was not reached (data not shown).

Functional antagonism by ACT-058362 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 nM and 6.5 ± 0.45 nM, respectively. ACT-058362 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 nM and > 10,000 nM, respectively (Table 3). This effect was specific, since ACT-058362 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 ACT-058362 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, ACT-058362 inhibited human U-II induced MAPK phosphorylation in a dose-dependent manner with an IC_{50} of 150 nM (Fig. 3). Similarly, ACT-058362 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 ACT-058362 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. ACT-058362 did not change baseline force, but reduced contraction to U-II in a concentration-dependent manner (Fig. 4). A linear relationship between the concentration of ACT-058362 and the decrease in maximal contraction to U-II was observed ($r^2 = 0.93$). The pD'$_2$ value for ACT-058362 was calculated to be 5.23 ± 0.11.

Functional selectivity of ACT-058362 for the UT receptor in rat aortic rings

Contraction of rat aortic rings was used to assess the functional selectivity of ACT-058362 for the UT receptor versus other receptors involved in vascular tone regulation. While 1 µM ACT-058362 significantly decreased U-II induced contraction (see Fig. 4), there was no inhibition of norepinephrine, 5-hydroxytryptamine and endothelin-1-induced contraction detectable at a concentration of 10 µM ACT-058362 (data not shown). These results indicated that in rat the selectivity ratio of ACT-058362 for the UT receptor vs. alpha-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 ACT-058362 on post-ischemic 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. ACT-058362 had no significant effect on renal blood flow (Fig. 5A), and no effect on MAP and HR. Fourty-five min renal ischemia in vehicle-treated rats resulted in a 15 ± 6% decrease at 60 min in renal blood flow as compared to baseline values. In contrast, ACT-058362 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 thus fully prevented the decrease in renal blood flow after ischemia. ACT-058362 did not significantly alter MAP and HR (data not shown).

Effects of ACT-058362 on post-ischemic 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 vs 0.84 ± 0.06 mg/dl, $p < 0.01$) and a significant 55 ± 6% decrease in GFR at 48 h post reperfusion in vehicle-treated rats (Fig. 6). Treatment with ACT-058362 prevented the increase in serum creatinine concentration (1.08 ± 0.16 vs 1.80 ± 0.27 mg/dl, $p < 0.01$), and reduced the decrease in GFR (– 33 ± 5% vs – 55 ± 6%, $p < 0.01$) (Fig. 6). GFR was improved but not normalized and remained significantly lower than the pre-ischemia levels (0.29 ± 0.02 vs 0.44 ± 0.03 ml/min/100g, $p < 0.001$). ACT-058362 had no effect on serum creatinine concentration and GFR in sham-operated rats.

Neither renal artery clamping nor ACT-058362 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 vs 198 ± 24 μmol/min, $p < 0.001$) (Fig. 7B). This led to a significant increase in the fractional excretion of sodium (Fig. 7C). ACT-058362 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, ACT-058362 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. Semi-quantitative histological lesion grading revealed that there were markedly more tubulointerstitial lesions in the untreated renal failure rats as compared to sham-operated rats. Treatment with ACT-058362 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 U-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, 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; Watanabe et al., 2001b; Zou et al., 2001; Maguire and Davenport, 2002). In order to understand the pathophysiological role of endogenous U-II, it was important to develop specific UT receptor antagonists. ACT-058362 is a non-peptide, specific UT receptor antagonist suitable for delineating the physiological and pathological roles of the urotensin system.

ACT-058362 is a potent inhibitor of the human UT receptor as demonstrated in radio-ligand binding experiments using ^{125}I-U-II in cell and membrane preparations expressing the human UT receptor. Binding studies showed that the binding inhibitory potency of ACT-058362 is > 100-fold higher on human UT receptor as compared with the rat UT receptor. In contrast, U-II showed only 10-fold higher binding potency on the human UT receptor compared with the rat receptor.

ACT-058362 inhibited U-II induced contraction of rat aortic rings in a concentration dependent manner. At 10^{-4} M, ACT-058362 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, ACT-058362 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 authors 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 peptidic UT receptor antagonist, inhibited Ca^{2+} mobilization in HEK293 cells expressing UT receptors in a competitive manner, while 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 ACT-058362 in the isolated rat aortic ring system. First, the interaction between ACT-058362 and the UT receptor on the surface of rat native cells is indeed of noncompetitive manner. Second, the binding of ACT-058362 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 ACT-058362 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 ACT-058362 is a specific antagonist of UT receptors, and does not antagonize the action of other vasoconstrictor agents such as KCl, endothelin-1, 5-hydroxytryptamine and norepinephrine. Therefore, ACT-058362 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, since 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 endocrine 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 spill-over from tissular U-II, more than reflecting the circulation of an endocrine hormone.

ACT-058362 was very effective in a rat model of renal ischemia, both for preventing the post-ischemic renal vasoconstriction and for reducing the post-ischemic acute renal failure. The profile of ACT-058362 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) while 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 ACT-058362 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 ACT-058362 for preventing post-ischemic renal failure. In a rat model of post-ischemic ARF, ACT-058362 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), ACT-058362 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, bladder) (Loretz and Bern, 1980; Loretz and Bern, 1981; Conlon et al., 1997), and exposing fish (flounder) to an osmotic stress (freshwater vs saltwater environment) elevated circulating U-II-like immunoreactive peptides, concomitant with alterations in plasma osmolarity 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. ACT-058362 significantly attenuated renal glomerular and tubular dysfunction. Histological examination of the kidney of ischemic ARF rats revealed massive tubular damage at 48 h post reperfusion. ACT-058362 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 ACT-058362 fully restored post-ischemic renal blood flow. This suggests that there may be other factors involved in the pathogenesis of post-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 ACT-058362 is entirely secondary to the prevention of post-ischemic vasoconstriction, or whether a direct tubular effect is involved. The fact that ACT-058362 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 ACT-058362 is a preferential afferent or efferent vasodilator in rats and if 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, since 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 reno-protection, such as an antiproliferative or anti-inflammatory effect of ACT-058362 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 ACT-058362 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 (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 endothelium 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 ACT-058362 support the possibility of U-II as a causal factor of ischemic ARF.

In conclusion, ACT-058362 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 ACT-058362 reduced the glomerular and tubular dysfunction and renal tissue injury induced by renal ischemia/reperfusion. In sham-operated rats, ACT-058362 increased urinary sodium excretion and fractional excretion of sodium. ACT-058362 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 ACT-058362 may be useful in the treatment of renal diseases.

References

Ajis A, Bagnall NM, Collis MG and Johns EJ (2003) Effect of endothelin antagonists on the renal haemodynamic and tubular responses to ischaemia-reperfusion injury in anaesthetised rats. *Exp Physiol* **88**:483-490.

Ames RS, Sarau HM, Chambers JK, Willette RN, Aiyar NV, Romanic AM, Louden CS, Foley JJ, Sauermelch CF, Coatney RW, Ao Z, Disa J, Holmes SD, Stadel JM, Martin JD, Liu WS, Glover GI, Wilson S, McNulty DE, Ellis CE, Elshourbagy NA, Shabon U, Trill JJ, Hay DW, Douglas SA and et al. (1999) Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* **401**:282-286.

Arendshorst WJ, Finn WF and Gottschalk CW (1975) Pathogenesis of acute renal failure following temporary renal ischemia in the rat. *Circ Res* **37**:558-568.

Birker-Robaczewska M, Boukhadra C, Studer R, Mueller C, Binkert C and Nayler O (2003) The expression of urotensin II receptor (U2R) is up-regulated by interferon-gamma. *J Recept Signal Transduct Res* **23**:289-305.

Breu V, Loffler BM and Clozel M (1993) In vitro characterization of Ro 46-2005, a novel synthetic non-peptide endothelin antagonist of ETA and ETB receptors. *FEBS Lett* **334**:210-214.

Brodsky SV, Yamamoto T, Tada T, Kim B, Chen J, Kajiya F and Goligorsky MS (2002) Endothelial dysfunction in ischemic acute renal failure: rescue by transplanted endothelial cells. *Am J Physiol Renal Physiol* **282**:F1140-1149.

Chatterjee PK, Cuzzocrea S, Brown PA, Zacharowski K, Stewart KN, Mota-Filipe H and Thiemermann C (2000) Tempol, a membrane-permeable radical scavenger, reduces oxidant stress-mediated renal dysfunction and injury in the rat. *Kidney Int* **58**:658-673.

Conlon JM, Tostivint H and Vaudry H (1997) Somatostatin- and urotensin II-related peptides: molecular diversity and evolutionary perspectives. *Regul Pept* **69**:95-103.

Coulouarn Y, Lihrmann I, Jegou S, Anouar Y, Tostivint H, Beauvillain JC, Conlon JM, Bern HA and Vaudry H (1998) Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc Natl Acad Sci U S A* **95**:15803-15808.

Criscione L, de Gasparo M, Buhlmayer P, Whitebread S, Ramjoue HP and Wood J (1993) Pharmacological profile of valsartan: a potent, orally active, nonpeptide antagonist of the angiotensin II AT1-receptor subtype. *Br J Pharmacol* **110**:761-771.

Douglas SA, Ashton DJ, Sauermelch CF, Coatney RW, Ohlstein DH, Ruffolo MR, Ohlstein EH, Aiyar NV and Willette RN (2000a) Human urotensin-II is a potent vasoactive peptide: pharmacological characterization in the rat, mouse, dog and primate. *J Cardiovasc Pharmacol* **36**:S163-166.

Douglas SA and Ohlstein EH (2000) Human urotensin-II, the most potent mammalian vasoconstrictor identified to date, as a therapeutic target for the management of cardiovascular disease. *Trends Cardiovasc Med* **10**:229-237.

Douglas SA, Sulpizio AC, Piercy V, Sarau HM, Ames RS, Aiyar NV, Ohlstein EH and Willette RN (2000b) Differential vasoconstrictor activity of human urotensin-II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey. *Br J Pharmacol* **131**:1262-1274.

Herold CL, Behm DJ, Buckley PT, Foley JJ, Wixted WE, Sarau HM and Douglas SA (2003) The neuromedin B receptor antagonist, BIM-23127, is a potent antagonist at human and rat urotensin-II receptors. *Br J Pharmacol* **139**:203-207.

Koelz AM, Bertschin S, Hermle M, Mihatsch M, Brunner FP and Thiel G (1988) The angiotensin converting enzyme inhibitor enalapril in acute ischemic renal failure in rats. *Experientia* **44**:172-175.

Liu Q, Pong SS, Zeng Z, Zhang Q, Howard AD, Williams DL, Jr., Davidoff M, Wang R, Austin CP, McDonald TP, Bai C, George SR, Evans JF and Caskey CT (1999)

Identification of urotensin II as the endogenous ligand for the orphan G-protein-coupled receptor GPR14. *Biochem Biophys Res Commun* **266**:174-178.

Loretz CA and Bern HA (1980) Ion transport by the urinary bladder of the gobiid teleost, Gillichthys mirabilis. *Am J Physiol* **239**:R415-423.

Loretz CA and Bern HA (1981) Stimulation of sodium transport across the teleost urinary bladder by urotensin II. *Gen Comp Endocrinol* **43**:325-330.

MacLean MR, Alexander D, Stirrat A, Gallagher M, Douglas SA, Ohlstein EH, Morecroft I and Polland K (2000) Contractile responses to human urotensin-II in rat and human pulmonary arteries: effect of endothelial factors and chronic hypoxia in the rat. *Br J Pharmacol* **130**:201-204.

Maguire JJ and Davenport AP (2002) Is urotensin-II the new endothelin? *Br J Pharmacol* **137**:579-588.

Maguire JJ, Kuc RE and Davenport AP (2000) Orphan-receptor ligand human urotensin II: receptor localization in human tissues and comparison of vasoconstrictor responses with endothelin-1. *Br J Pharmacol* **131**:441-446.

Matsushita M, Shichiri M, Imai T, Iwashina M, Tanaka H, Takasu N and Hirata Y (2001) Co-expression of urotensin II and its receptor (GPR14) in human cardiovascular and renal tissues. *J Hypertens* **19**:2185-2190.

Molitoris BA (1991) New insights into the cell biology of ischemic acute renal failure. *J Am Soc Nephrol* **1**:1263-1270.

Mori M, Sugo T, Abe M, Shimomura Y, Kurihara M, Kitada C, Kikuchi K, Shintani Y, Kurokawa T, Onda H, Nishimura O and Fujino M (1999) Urotensin II is the endogenous ligand of a G-protein-coupled orphan receptor, SENR (GPR14). *Biochem Biophys Res Commun* **265**:123-129.

Munda R and Alexander JW (1980) Failure of saralasin in preventing renal failure in ischemic transplanted kidneys. *Am Surg* **46**:637-639.

Nothacker HP, Wang Z, McNeill AM, Saito Y, Merten S, O'Dowd B, Duckles SP and Civelli O (1999) Identification of the natural ligand of an orphan G-protein-coupled receptor involved in the regulation of vasoconstriction. *Nat Cell Biol* **1**:383-385.

Pearson D, Shively JE, Clark BR, Geschwind, II, Barkley M, Nishioka RS and Bern HA (1980) Urotensin II: a somatostatin-like peptide in the caudal neurosecretory system of fishes. *Proc Natl Acad Sci U S A* **77**:5021-5024.

Roux S, Qiu C, Sprecher U, Osterwalder R and Clozel JP (1999) Protective effects of endothelin receptor antagonists in dogs with aortic cross-clamping. *J Cardiovasc Pharmacol* **34**:199-205.

Sauzeau V, Le Mellionnec E, Bertoglio J, Scalbert E, Pacaud P and Loirand G (2001) Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. *Circ Res* **88**:1102-1104.

Shenouda A, Douglas SA, Ohlstein EH and Giaid A (2002) Localization of urotensin-II immunoreactivity in normal human kidneys and renal carcinoma. *J Histochem Cytochem* **50**:885-889.

Shibouta Y, Inada Y, Ojima M, Wada T, Noda M, Sanada T, Kubo K, Kohara Y, Naka T and Nishikawa K (1993) Pharmacological profile of a highly potent and long-acting angiotensin II receptor antagonist, 2-ethoxy-1-[[2'-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl]-1H-benzimidazole-7-carboxylic acid (CV-11974), and its prodrug, (+/-)-1-(cyclohexyloxycarbonyloxy)-ethyl 2-ethoxy-1-[[2'-(1H-tetrazol-5- yl)biphenyl-4-yl]methyl]-1H-benzimidazole-7-carboxylate (TCV-116). *J Pharmacol Exp Ther* **266**:114-120.

Stein JH, Lifschitz MD and Barnes LD (1978) Current concepts on the pathophysiology of acute renal failure. *Am J Physiol* **234**:F171-181.

Totsune K, Takahashi K, Arihara Z, Sone M, Satoh F, Ito S, Kimura Y, Sasano H and Murakami O (2001) Role of urotensin II in patients on dialysis. *Lancet* **358**:810-811.

van Rossum JM (1963) Cumulative dose-response curves. Techniques for the making of dose-response curve in isolated organs and evaluation of drug parameters. *Arch Int Pharmacodyn Ther* **143**:229-330.

Veniant M, Heudes D, Clozel JP, Bruneval P and Menard J (1994) Calcium blockade versus ACE inhibition in clipped and unclipped kidneys of 2K-1C rats. *Kidney Int* **46**:421-429.

Watanabe T, Pakala R, Katagiri T and Benedict CR (2001a) Synergistic effect of urotensin II with mildly oxidized LDL on DNA synthesis in vascular smooth muscle cells. *Circulation* **104**:16-18.

Watanabe T, Pakala R, Katagiri T and Benedict CR (2001b) Synergistic effect of urotensin II with serotonin on vascular smooth muscle cell proliferation. *J Hypertens* **19**:2191-2196.

Winter MJ, Hubbard PC, McCrohan CR and Balment RJ (1999) A homologous radioimmunoassay for the measurement of urotensin II in the euryhaline flounder, Platichthys flesus. *Gen Comp Endocrinol* **114**:249-256.

Ziltener P, Mueller C, Haenig B, Scherz MW and Nayler O (2002) Urotensin II mediates ERK1/2 phosphorylation and proliferation in GPR14-transfected cell lines. *J Recept Signal Transduct Res* **22**:155-168.

Zou Y, Nagai R and Yamazaki T (2001) Urotensin II induces hypertrophic responses in cultured cardiomyocytes from neonatal rats. *FEBS Lett* **508**:57-60.

Legends for figures

Fig. 1. Chemical structure of ACT-058362 sulfate salt, $C_{25}H_{32}N_4O_6S$ (MW = 516.6).

Fig. 2. Saturation binding curves and Scatchard transformation (inserts) of ^{125}I-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 ACT-058362. Curves were fitted directly using the KELL software (see Material and Methods).

Fig. 3. Inhibition of human U-II induced MAPK phosphorylation by 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 ACT-058362.

Fig. 5. (A) effect of ACT-058362 (10 mg/kg/h, i.v.) or vehicle on mean renal blood flow (MRBF) in sham-operated rats; (B) effect of ACT-058362 or vehicle on MRBF in rats subjected to renal ischemia by renal artery clamping for 45 min. **$p < 0.01$ compared to vehicle treated rats.

Fig. 6. Effect of ACT-058362 (10 mg/kg/h, i.v.) or vehicle on glomerular filtration rate estimated by creatinine clearance in sham-operated rats and in rats with post-ischemic renal failure. Pre: before sham operation or renal clamping; Post: after sham operation or renal clamping. ** $p < 0.01$, ***$p < 0.001$.

Fig. 7. Effect of ACT-058362 (10 mg/kg/h, i.v.) or vehicle on (A) serum sodium concentration (Serum Na), (B) tubular sodium re-absorption (T_{Na}), (C) fractional excretion of sodium (FE_{Na}) and (D) 24-h urinary sodium excretion ($U_{Na}V$), in sham-operated rats (Sham, n = 10) and in rats with post-ischemic renal failure (ARF, n = 12) at 48 h after renal clamping. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

JPET Fast Forward. Published on May 14, 2004 as DOI: 10.1124/jpet.104.068320
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 8. Effect of ACT-058362 or vehicle on the histological scores of tubulointerstitial lesions in rats with post-ischemic renal failure (ARF) as compared to sham-operated rats (Sham). *** $p < 0.001$.

TABLE 1

Binding inhibitory potency of human U-II and ACT-058362 in cell- and membrane-binding assays. Binding experiments were performed by incubating 20 pM of human ^{125}I-U-II for 8 h with whole cells or membrane preparations in the presence of increasing concentration of cold ligands (human U-II or ACT-058362).

	Cell binding IC$_{50}$ (nM)			Membrane binding IC$_{50}$ (nM)	
Ligand	TE 671	CHO human UT	CHO rat UT	CHO human UT	CHO rat UT
Human U-II	0.27 ± 0.08 (n = 10)	0.73 ± 0.09 (n = 22)	7.2 ± 3.6 (n = 2)	1.2 ± 0.1 (n = 22)	6.7 ± 0.4 (n = 24)
ACT-058362	46.2 ± 13 (n = 9)	86 ± 30 (n = 4)	> 10,000 (n = 2)	3.6 ± 0.2 (n = 40)	1475 ± 70 (n = 40)

U-II, urotensin-II; UT, urotensin receptor; CHO, Chinese hamster ovary cells

TABLE 2

The effect of ACT-058362 on U-II binding kinetics. Scatchard analysis of saturation binding experiments 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 IC_{50} value estimated for the same membrane preparation at 38 h.

Binding sites		ACT-058362 (0 nM)	ACT-058362 (5 nM)
High affinity site	K_D (pM)	1.9	28
	B_{max} (pM)	1.7	3.7
Low affinity site	K_D (pM)	155	776
	B_{max} (pM)	6.8	7.7

TABLE 3

Potency and species selectivity of ACT-058362 on U-II induced intracellular Ca^{2+} mobilization in CHO cells expressing human or rat UT receptor. Urotensin-II induced Ca^{2+} mobilization in the recombinant CHO cells was measured by the Fluorometric Image Plate Reader (FLIPR) assay system. The potency of U-II (EC_{50}) was determined in the absence of ACT-058362. The inhibitory potency of ACT-058362 (IC_{50}) was determined on Ca^{2+} mobilization induced by 30 nM U-II.

Ligand	Human UT receptor	Rat UT receptor
Human U-II (EC_{50}, nM)	23 ± 2 (n = 10)	6.5 ± 0.45 nM (n = 7)
ACT-058362 (IC_{50}, nM)	17 ± 0.63 (n = 17)	> 10,000 (n = 4)

U-II, urotensin-II; UT, urotensin receptor

Acknowledgment:

We would like to warmly thank Beat Steiner for his helpful comments.

JPET Fast Forward. Published on May 14, 2004 as DOI: 10.1124/jpet.104.068320

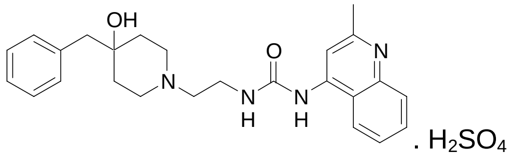

Fig. 1

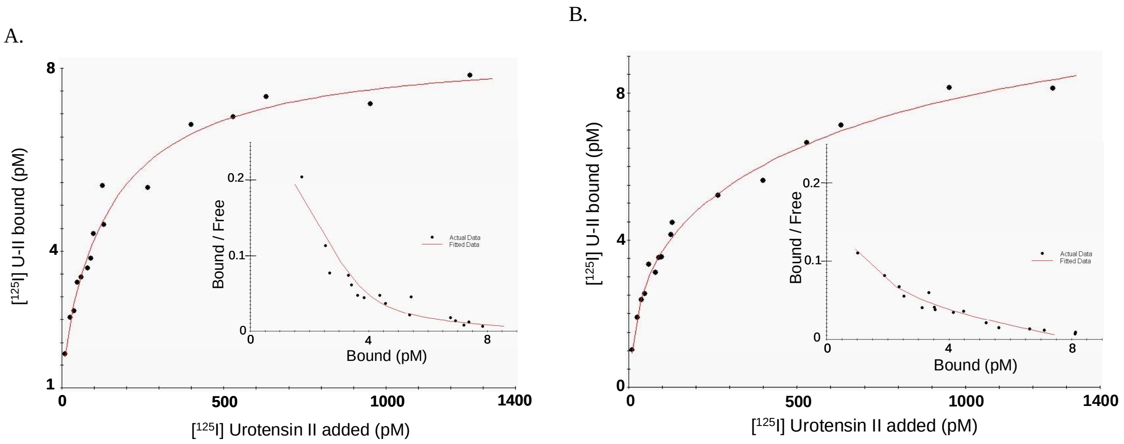
A.
B.
[125I] U-II bound (pM)
8
4
1
0
0
500
1000
1400
[125I] Urotensin II added (pM)
Bound / Free
0.2
0.1
0
4
8
Bound (pM)
Actual Data
Fitted Data

Fig. 2

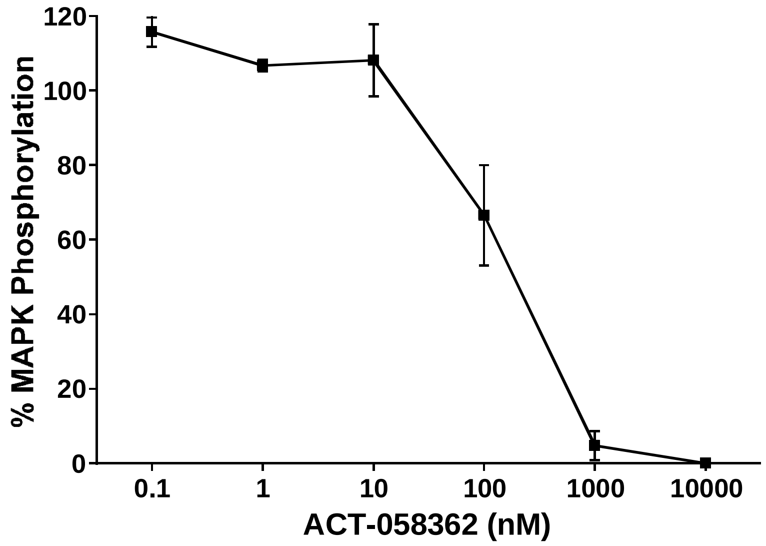
% MAPK Phosphorylation
120
100
80
60
40
20
0
0.1
1
10
100
1000
10000
ACT-058362 (nM)

Fig. 3

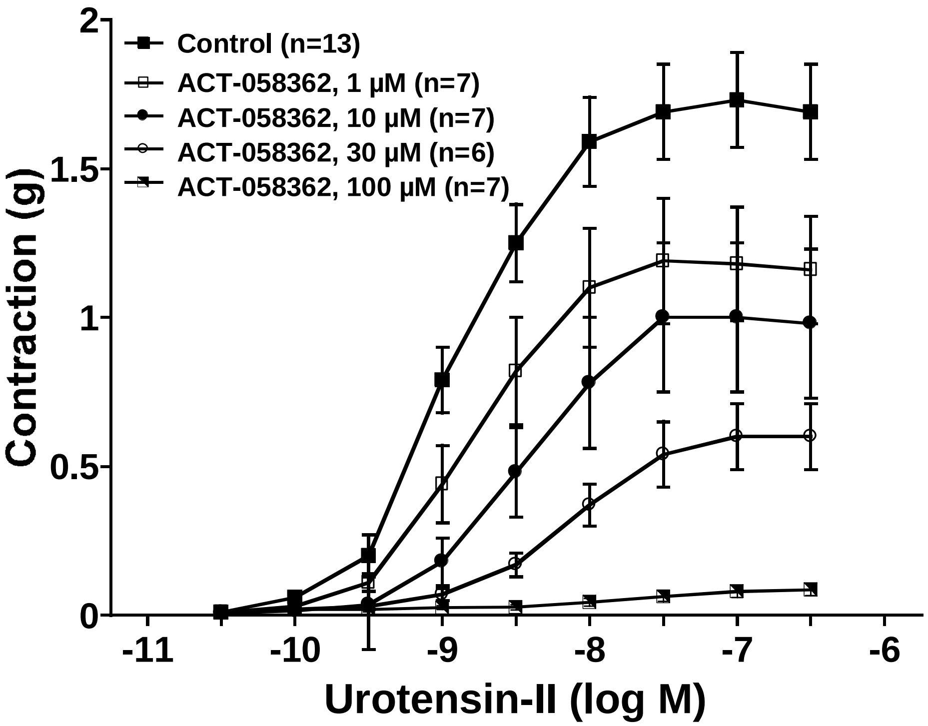
Control (n=13)
ACT-058362, 1 μM (n=7)
ACT-058362, 10 μM (n=7)
ACT-058362, 30 μM (n=6)
ACT-058362, 100 μM (n=7)
Contraction (g)
2
1.5
1
0.5
0
-11
-10
-9
-8
-7
-6
Urotensin-II (log M)

Fig. 4

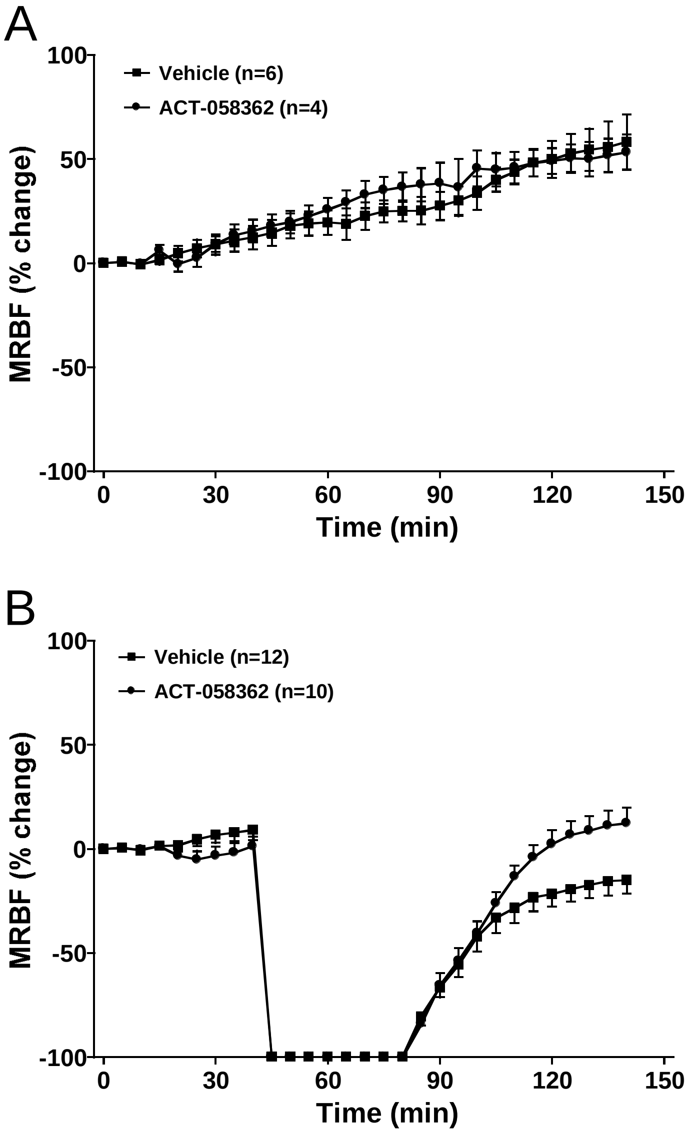
A
Vehicle (n=6)
ACT-058362 (n=4)
MRBF (% change)
100
50
0
-50
-100
0
30
60
90
120
150
Time (min)
B
Vehicle (n=12)
ACT-058362 (n=10)
MRBF (% change)
100
50
0
-50
-100
0
30
60
90
120
150
Time (min)

Fig. 5

JPET Fast Forward. Published on May 14, 2004 as DOI: 10.1124/jpet.104.068320

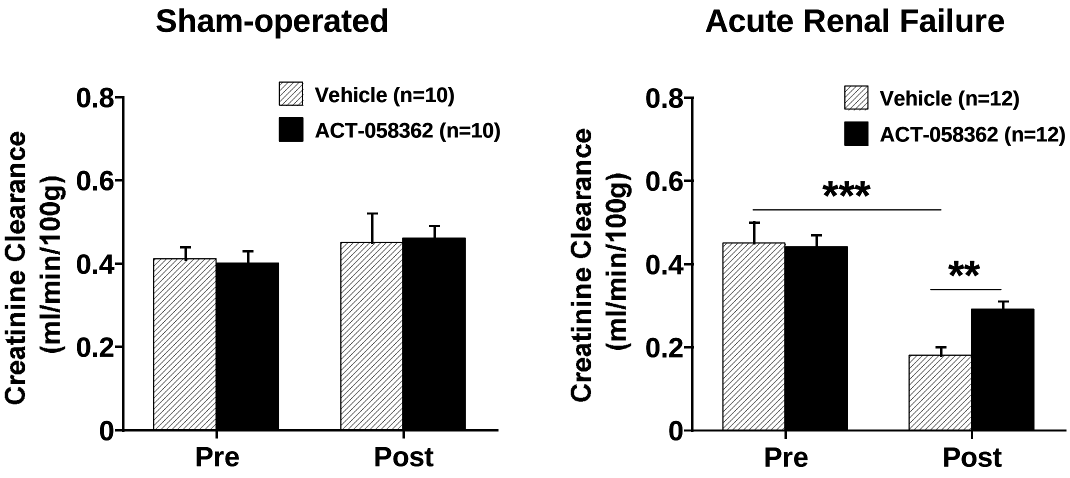

Fig. 6

JPET Fast Forward. Published on May 14, 2004 as DOI: 10.1124/jpet.104.068320
This article has not been copyedited and formatted. The final version may differ from this version.

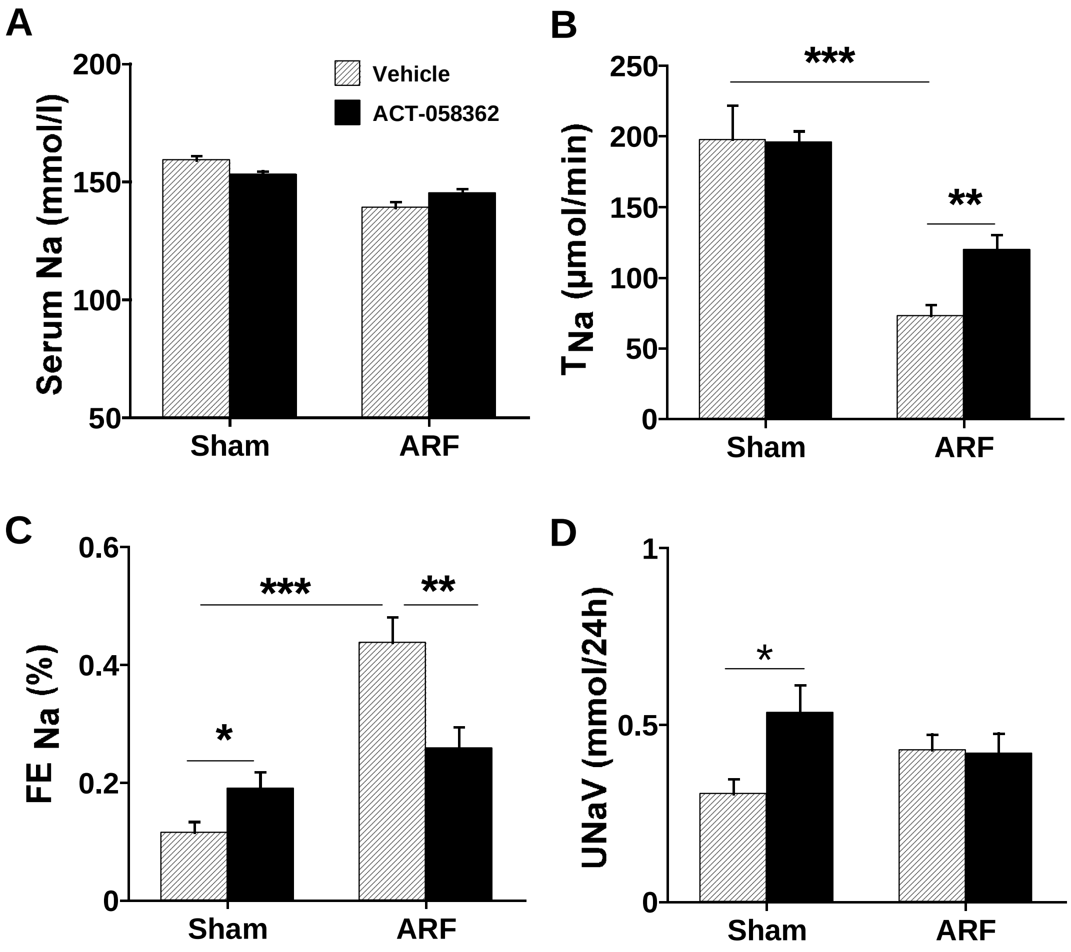

Fig. 7

JPET Fast Forward. Published on May 14, 2004 as DOI: 10.1124/jpet.104.068320
This article has not been copyedited and formatted. The final version may differ from this version.

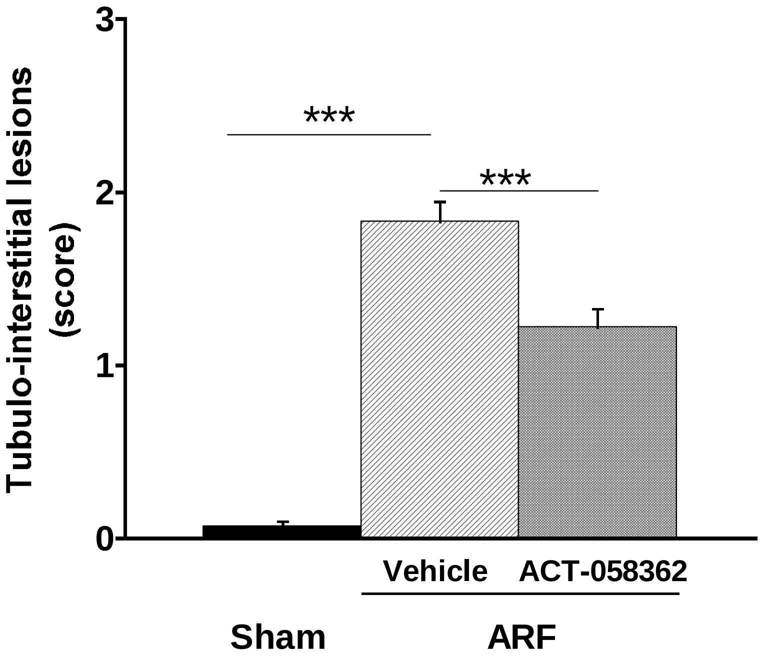

Fig. 8