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RESEARCH PAPER

Palosuran inhibits binding to primate UT receptors in cell membranes but demonstrates differential activity in intact cells and vascular tissues

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Background and purpose: The recent development of the UT ligand palosuran (1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulphate salt) has led to the proposition that urotensin-II (U-II) plays a significant pathological role in acute and chronic renal injury in the rat.
Experimental approach: In the present study, the pharmacological properties of palosuran were investigated further using a series of radioligand binding and functional bioassays.
Key results: Palosuran functioned as a 'primate-selective' UT ligand in recombinant cell membranes (monkey and human UT K_i values of 4 ± 1 and 5 ± 1 nM), lacking appreciable affinity at other mammalian UT isoforms (rodent and feline K_i values $>1\,\mu$M). Paradoxically, however, palosuran lost significant (10- to 54-fold) affinity for native and recombinant human UT when radioligand binding was performed in intact cells (K_i values of 50 ± 3 and 276 ± 67 nM). In accordance, palosuran also exhibited diminished activity in hUT (human urotensin-II receptor)-CHO (Chinese hamster ovary) cells (IC_{50} 323 ± 67 nM) and isolated arteries ($K_b > 10\,\mu$M in rat aorta; $K_b > 8.5\,\mu$M in cat arteries; $K_b > 1.6\,\mu$M in monkey arteries; K_b $2.2 \pm 0.6\,\mu$M in hUT transgenic mouse aorta). Relative to recombinant binding K_i values, palosuran was subjected to a 392- to 690-fold reduction in functional activity in monkey isolated arteries. Such phenomena were peculiar to palosuran and were not apparent with an alternative chemotype, SB-657510 (2-bromo-*N*-[4-chloro-3-((*R*)-1-methyl-pyrrolidin-3-yloxy)-phenyl]-4,5-dimethoxybenzenesulphonamide HCl).
Conclusions and implications: Collectively, such findings suggest that caution should be taken when interpreting data generated using palosuran. The loss of UT affinity/activity observed in intact cells and tissues *cf.* membranes offers a potential explanation for the disappointing clinical efficacy reported with palosuran in diabetic nephropathy patients. As such, the (patho)physiological significance of U-II in diabetic renal dysfunction remains uncertain.
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Keywords: urotensin-II; UT; GPR14; SB-657510; palosuran; ACT-058362; G-protein-coupled receptor; diabetic nephropathy; antagonist

Abbreviations: CHO cells, Chinese hamster ovary cells; DPBS, Dulbecco's phosphate-buffered saline; palosuran, 1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulphate salt; SB-657510, 2-bromo-*N*-[4-chloro-3-((*R*)-1-methyl-pyrrolidin-3-yloxy)-phenyl]-4,5-dimethoxybenzenesulphonamide HCl; SJRH30, human rhabdomyosarcoma cell line; SPA, scintillation proximity assay; (h)U-II, (human) urotensin-II; (h)UT, (human) urotensin-II receptor

Introduction

Human urotensin-II (hU-II), cognate ligand for the 'orphan' G-protein-coupled receptor UT (Ames *et al.*, 1999; Douglas and Ohlstein, 2000; NC-IUPHAR database: http://www.iuphar-db.org/index.jsp), is purported to play a significant role in the aetiology of several cardiorenal and metabolic disease states, including heart failure (Douglas *et al.*, 2002; Richards *et al.*, 2002), atherosclerosis (Bousette *et al.*, 2004; Maguire *et al.*, 2004), hypertension (Cheung *et al.*, 2004) and diabetes (Totsune *et al.*, 2003, 2004; Wenyi *et al.*, 2003). Such a proposition has led to the rapid development in recent

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years of several distinct UT receptor antagonists (Dhanak *et al*., 2003; Douglas *et al*., 2004a).

Palosuran (ACT-058362; 1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulphate salt), one such high-affinity human UT ligand provides cardiorenal and metabolic protection in rat models of both ischaemic (Clozel *et al*., 2004) and diabetic (Clozel *et al*., 2006) nephropathy. Additional *in vitro* studies demonstrate that this moiety also potentiates glucose-induced insulin secretion in rat isolated, perfused pancreas (Egido *et al*., 2005; Marco *et al*., 2005) and, most significantly, reduces microalbuminuria in diabetic nephropathy patients (Sidharta *et al*., 2006). Recently, however, it was found that palosuran failed to provide substantial efficacy in diabetic nephropathy patients based on collective data compiled from three separate proof-of-concept clinical studies (http://www.actelion.com/uninet/www/www_main_p.nsf/Content/me+23+May+2005; Clozel *et al*., 2006). As such, the precise pathophysiological significance of U-II in the diabetic kidney remains unclear. The underlying reason for the 'insufficient' clinical efficacy reported is, at present, unknown but could reflect the (a) possibility that U-II is not a clinically significant pathological mediator in diabetic nephropathy, (b) use of inadequate dosage regimens (resulting in insufficient exposure to active drug) and/or (c) fact that palosuran is an ineffective antagonist of U-II function. Such potential explanations clearly have contrasting implications for the continued development of UT antagonists in this indication.

Despite the disappointing clinical data described above, palosuran has the potential to become a 'benchmark' UT antagonist, a standard pharmacological tool used extensively within the field. However, upon close inspection, it is apparent that conflicting efficacy data exist within the literature for this moiety. Whereas palosuran (1–10 μM) purportedly regulates angiogenesis and adrenocortical function in the rat (Albertin *et al*., 2006; Spinazzi *et al*., 2006), the antagonist concentrations employed in such studies (1–10 μM) are inactive in intact cells expressing rat recombinant UT isoform (IC_{50} values $>10\,\mu M$ in radioligand binding and $[Ca^{2+}]_i$-mobilization assays; Clozel *et al*., 2004). More alarmingly, such actions are not replicated by another UT antagonist, SB-710411 (Albertin *et al*., 2006; Spinazzi *et al*., 2006).

In view of these disparities, in the present study the pharmacological characteristics of palosuran were assessed in detail using a broad spectrum of radioligand binding (rodent, feline and primate recombinant UT whole cell and membrane-binding formats) and functional U-II assays. These properties were compared to those of a chemically distinct, sulphonamide UT antagonist, SB-657510 (2-bromo-*N*-[4-chloro-3-((*R*)-1-methyl-pyrrolidin-3-yloxy)-phenyl]-4,5-dimethoxybenzenesulphonamide HCl) (Douglas *et al*., 2005).

In accord with the results of Clozel *et al*. (2004), palosuran behaved as a high-affinity, 'primate-selective' UT ligand in recombinant membrane preparations. Subsequently, however, palosuran was found to be subject to a profound loss in affinity/activity when profiled in intact cells or isolated arteries. Such phenomena were specific for palosuran and were not evident with the alternative chemotype, SB-657510. It is, therefore, concluded that palosuran is not an optimal preclinical pharmacological tool for use in non-primate studies. Furthermore, such findings (loss of affinity/activity in intact cells and tissues) perhaps offer insight into the poor clinical efficacy attained in diabetic nephropathy subjects. On the basis of the present observations, a significant role for U-II in the aetiology of diabetic nephropathy cannot be dismissed.

Methods

The pharmacological properties of palosuran, a quinolinyl-urea, were assessed in both radioligand (membrane *cf*. whole cell)-binding studies and *in vitro* functional bioassays (isolated arterial contraction) and were compared directly to those of a 'reference' (chemically distinct sulphonamide) UT antagonist, SB-657510 (Figure 1; Douglas *et al*., 2005). Pharmacological properties were profiled across multiple mammalian species, including mouse, rat, cat, monkey and human.

All studies were performed in accredited facilities in accordance with institutional guidelines.

Radioligand binding at recombinant hUT in cell membrane preparations

Cell membranes were prepared from human embryonic kidney (HEK293) cells stably transfected with mouse or monkey UT or human osteosarcoma (U2OS) cells transiently expressing rat, cat or human UT (Ames *et al*., 1999; Behm *et al*., 2006). [^{125}I]hU-II competition binding assays (scintillation proximity assay, SPA) were performed using 400 pM [^{125}I(Tyr^{9})]hU-II in the presence or absence of 1 pM–1 μM cold hU-II, SB-657510 or palosuran in assay buffer (20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ and 0.05% bovine serum albumin) containing cell membranes pre-coupled to wheat germ agglutinin coated-SPA (WGA-SPA) beads. Nonspecific binding was defined using 1 μM unlabelled hU-II. Assay plates were sealed, shaken gently (45 min, 20 °C) and left overnight before scintillation counting (TopCount; Perkin Elmer, Shelton, CT, USA).

Radioligand binding at recombinant and native hUT in whole cell preparations

Whole cell [^{125}I]hU-II competition binding assays were performed using transient recombinant hUT (human

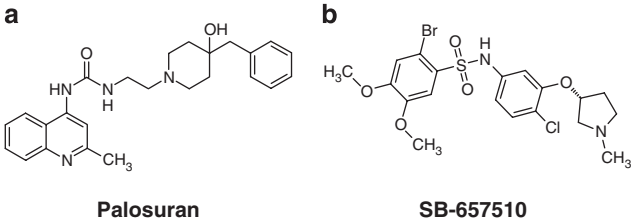

Figure 1 Structures of the UT receptor antagonists (**a**) palosuran (1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulphate salt; Aissaoui *et al*., 2003) and (**b**) SB-657510 (2-bromo-*N*-[4-chloro-3-((*R*)-1-methyl-pyrrolidin-3-yloxy)-phenyl]-4,5-dimethoxybenzenesulphonamide hydrochloride; Douglas *et al*., 2005).

urotensin-II receptor)-U2OS cells (Behm *et al*., 2006). Assays were performed with 125 pM [^{125}I(Tyr^{9})]hU-II in the presence or absence of 1 pM–1 μM unlabelled hU-II, SB-657510 or palosuran in Dulbecco's phosphate-buffered saline (DPBS; 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$) and 0.1% bovine serum albumin. Nonspecific binding was defined using 1 μM cold hU-II. Assay plates were incubated at 37 °C for 30 min. Following three washes with DPBS, the cells were lysed with 2 N NaOH and the resulting lysate was counted using a gamma counter (Wallac Wizard, Perkin Elmer).

Radioligand binding at native hUT in intact SJRH30 cells
Binding of [^{125}I]hU-II to native hUT receptors was studied according to Douglas *et al*. (2004b) using a whole cell-binding assay format in the human rhabdomyosarcoma cell line, SJRH30. Briefly, SJRH30 cells were washed once in DPBS buffer (with 10 mM $MgCl_2$, 0.7 mM $CaCl_2$, 1.4 mM glucose, 0.2% bovine serum albumin) immediately prior to exposure (37 °C for 1 or 4 h) to 180 pM [^{125}I]hU-II and competing ligands. Following three washes with DPBS, the cells were lysed with 2 N NaOH and the resulting lysate was counted using a gamma counter (Wallac Wizard).

Intracellular [Ca^{2+}]-mobilization assay
Intracellular [Ca^{2+}]-mobilization experiments were performed at 22 °C in hUT recombinant Chinese hamster ovary (CHO) cells using a fluorometric imaging plate reader (Cerep, Paris, France). Antagonists (palosuran, SB-657510; 0.1 nM–10 μM) were added 10 min prior to the addition of 10 nM hU-II and IC_{50} curves were generated by nonlinear regression analysis.

Assessment of contractility in mammalian isolated arteries
Endothelium-denuded arterial rings (approximately 3 mm in length) were cleaned of adherent tissues and suspended in Krebs solution of the following composition (mM): NaCl, 112.0; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; dextrose, 11.0. Indomethacin (0.01 mM) was included in all experiments unless stated otherwise. Isometric force responses (measured using MLT0201/D transducers) were recorded digitally (Chart 5.0 software; ADInstruments, Colorado Springs, CO, USA) under optimal resting tension (1.0, 2.0, 2.0 and 0.5 g in rat, cat, monkey and transgenic mouse vessels, respectively; Douglas *et al*., 2000; Behm *et al*., 2004). Following 1 h equilibration, vessels were treated with 60 mM KCl and 1 μM phenylephrine (subsequent responses were normalized to KCl). Functional endothelial loss was confirmed using 10 μM carbachol. Paired arteries were pretreated (30 min) with vehicle (0.1% dimethylsulphoxide), palosuran or SB-657510 following which cumulative concentration–response curves to hU-II were constructed (tissues were used to generate only one concentration–response curve).

Rat isolated aortae
Following induction of anaesthesia (5% isoflurane in O_2), male Sprague–Dawley or Wistar rats (400 g; Charles River, Raleigh, NC, USA) were killed by exsanguination and proximal thoracic aortae were isolated (Douglas *et al*., 2000). Care was taken to isolate consistently a ~15–20 mm length of proximal descending aorta and isolated rings were randomly assigned to organ baths (to minimize any 'reactivity bias' resulting from differential receptor distribution/spare receptor reserve or drug treatment; Itoh *et al*., 1988; Douglas *et al*., 2000; Maguire *et al*., 2000). The influence of cyclooxygenase inhibition was also investigated where Wistar rat studies were performed in the presence and absence of 10 μM indomethacin.

Cat isolated aortae and femoral arteries
Femoral arteries and thoracic aortae were isolated from adult male cats (4–5 kg; Liberty Research Inc., Waverly, NY, USA) following sodium pentobarbitone overdose (100 mg kg^{-1}, i.v. Fatal-Plus) as previously described (Behm *et al*., 2004).

Cynomolgus monkey isolated renal and mesenteric arteries
Renal and superior mesenteric arteries were isolated from male cynomolgus monkeys (4–7 kg; Primate Products, Miami, FL, USA; Covance, Alice, TX, USA: Charles River, Andover, MA, USA; Mannheimer, Homestead, FL, USA) following sodium pentobarbitone overdose (100 mg kg^{-1}, i.v. Fatal-Plus; Douglas *et al*., 2000).

hUT transgenic mouse isolated thoracic aorta
Male hUT transgenic mice (25–30 g) were anaesthetized (5% isoflurane: O_2) and thoracic aortae were isolated following exsanguination (Douglas *et al*., 2000). Transgenic mice were originally generated using an overexpression cassette consisting of the promoter of the SM22α gene (22 kDa smooth muscle-specific protein; *Msc*I restriction fragment containing 2736 bp of 5′-flanking region, the 65 bp first exon and 1188 bp of the first intron; Moessler *et al*., 1996) driving expression of the hUT cDNA linked to a *Lac*Z (gene encoding β-galactosidase) reporter gene through an internal ribosome entry site element. The SM22α promoter drives expression in vascular smooth muscle cells (Li *et al*., 1996; Moessler *et al*., 1996). The gene construct was microinjected into isogenic C57B1/6 single-celled mouse oocytes. Positive founder mice were identified by PCR (*Lac*Z reporter gene primers: 5 primer: 5-GACCAGCCCTTCCCGGCTGTGCCG-3; 3 primer: 5-GCC GACCACGGGTTGCCGTTTTCA-3; 30 cycles of 94 °C (45 s), 65 °C (45 s) and 72 °C (45 s) giving a product size of 300 bp) and numbers were expanded to produce experimental animals.

Reagents and materials
SB-657510 (Douglas *et al*., 2005; Figure 1) and palosuran (Figure 1) were synthesized at GlaxoSmithKline. Palosuran structure was confirmed through one-dimensional [^{1}H]NMR, two-dimensional NMR and liquid chromatography with mass spectroscopic detection. Fatal-Plus was supplied by Vortech Pharmaceuticals (Dearborn, MI, USA). Carbachol, indomethacin, phenylephrine and bovine serum albumin

were from Sigma (St Louis, MO, USA). All other reagents were of analytical grade. WGA-SPA beads were from Amersham (Arlington Heights, IL, USA); DPBS from Gibco (Grand Island, NY, USA). SJRH30, American Type Culture Collection number CRL-2061 was from ATCC (Manassas, VA, USA). The MLT0201/D transducers were from Letica (Barcelona, Spain) and the ADInstruments Chart 5.0 software from ADInstruments.

Data analysis

Binding curves were analysed by nonlinear regression (GraphPad Prism) and concentration–contraction curves were fitted to a logistic equation (Douglas *et al*., 2005). Antagonist affinity (K_b) was determined using the Schild equation (Jenkinson *et al*., 1998). All values are mean ± s.e.mean (*n* represents the number of independent triplicate experiments or the number of animals from which vessels were isolated). Statistical comparisons were made using a paired, two-tailed *t*-test or ANOVA (Dunnett's post-test analysis). Differences were considered significant when $P \leqslant 0.05$.

Results

The pharmacological properties of palosuran were assessed in both radioligand (membrane *cf*. whole cell) binding and *in vitro* functional bioassay ($[Ca^{2+}]_i$ mobilization, isolated arterial contraction) studies. Palosuran functioned as a high-affinity, 'primate-selective' UT ligand in membrane-binding assays but lost almost two orders of magnitude in affinity when profiled in a whole cell radioligand-binding format. Accordingly, palosuran lacked appreciable (that is, commensurate with primate membrane K_i values) activity in functional, intact tissue bioassays.

Radioligand-binding studies in cell membrane preparations demonstrate that palosuran exhibits primate UT selectivity

hU-II was a high-affinity UT ligand across species when assessed in a recombinant UT cell membrane-binding assay (K_i values of 2–8 nM at rat, cat and primate UT, K_i 28 nM at mouse UT; Table 1 and Figure 2a). Whereas the sulphonamide SB-657510 also functioned as a high-affinity, pan-species hUT ligand (K_i values of 17–65 nM, differing by <3-fold in mouse, rat, cat and monkey UT membranes *cf*. human UT K_i; Table 1 and Figure 2b), palosuran exhibited primate UT selectivity (>200-fold selectivity *cf*. non-primate receptors; Table 1 and Figure 2c) under identical conditions, that is, identical high-affinity binding was evident in primate and non-human primate UT cell membranes (K_i values of 4–5 nM) but no affinity was evident (K_i values >1 μM) at rat, mouse or cat UT.

Palosuran exhibits a selective loss in human recombinant UT affinity in an intact cell radioligand-binding assay

hU-II was a high-affinity human UT ligand irrespective of whether cell membrane- (K_i 2 nM) or whole cell (K_i 3 nM; Table 2 and Figure 3a)-binding formats were employed (1.5-fold difference). Similarly, SB-657510 K_i values were identical (0.8-fold difference) in cell membrane and whole cell-binding formats (K_i values of 61 and 46 nM, respectively; Table 2 and Figure 3b). In contrast, however, palosuran experienced a significant 54-fold loss in hUT affinity when profiled in a whole cell-binding format (0.3 μM K_i in intact cells *cf*. 5 nM K_i in hUT cell membranes; Table 2 and Figure 3c).

Palosuran lacks high affinity for native hUT receptor endogenously expressed in the rhabdomyosarcoma cell line SJRH30

hU-II and SB-657510 competed with [^{125}I]hU-II for binding at native hUT receptor in intact SJRH30 cells with affinities similar to those determined at human recombinant UT cell membranes (Table 3). In contrast, however, palosuran exhibited a ⩾10-fold reduction in affinity in intact SJRH30 cells as compared to human recombinant UT cell membranes. K_i values for all three ligands were not significantly altered by length of incubation (1–4 h; Table 3).

Palosuran inhibits hU-II-induced $[Ca^{2+}]_i$ mobilization in recombinant hUT-CHO cells with a diminished functional activity (IC_{50}) as compared to its binding affinity (K_i)

Pretreatment with SB-657510 inhibited $[Ca^{2+}]_i$ mobilization elicited by 10 nM hU-II with an IC_{50} of 180 ± 46 nM (Figure 4a), a value consistent with membrane-binding data (61 nM K_i at human UT membranes; Table 1). In contrast to SB-657510, however, palosuran was significantly (65-fold) less active at inhibiting $[Ca^{2+}]_i$ mobilization (IC_{50}

Table 1 Palosuran is a high-affinity, 'primate-specific' UT receptor ligand in recombinant cell membranes: radioligand-binding affinity constants (K_i values, [^{125}I]hU-II competition binding) for human urotensin-II (hU-II), palosuran (ACT-058362) and SB-657510 in mammalian recombinant UT receptor membranes

	hU-II		*SB-657510*		*Palosuran (ACT-058362)*	
	K_i *(nM)*	*Affinity relative to hUT*[a]	K_i *(nM)*	*Affinity relative to hUT*[a]	K_i *(nM)*	*Affinity relative to hUT*[a]
Human (*n*=7)	2 ± 1	—	61 ± 8	—	5 ± 1	—
Monkey (*n*=3)	5 ± 2	2.5	17 ± 5	0.3	4 ± 1	0.8
Cat (*n*=3)	8 ± 1	4.0	30 ± 7	0.5	>1000	>200.0
Rat (*n*=3)	5 ± 1	2.5	65 ± 7	1.1	>1000	>200.0
Mouse (*n*=3)	28 ± 5	14.0	56 ± 5	0.9	>1000	>200.0

Abbreviation: hUT, human urotensin-II receptor.
[a]Relative affinities for hU-II, SB-657510 and palosuran in non-human UT isoforms are expressed relative to their respective human UT K_i values. All values are expressed as mean ± s.e.mean.

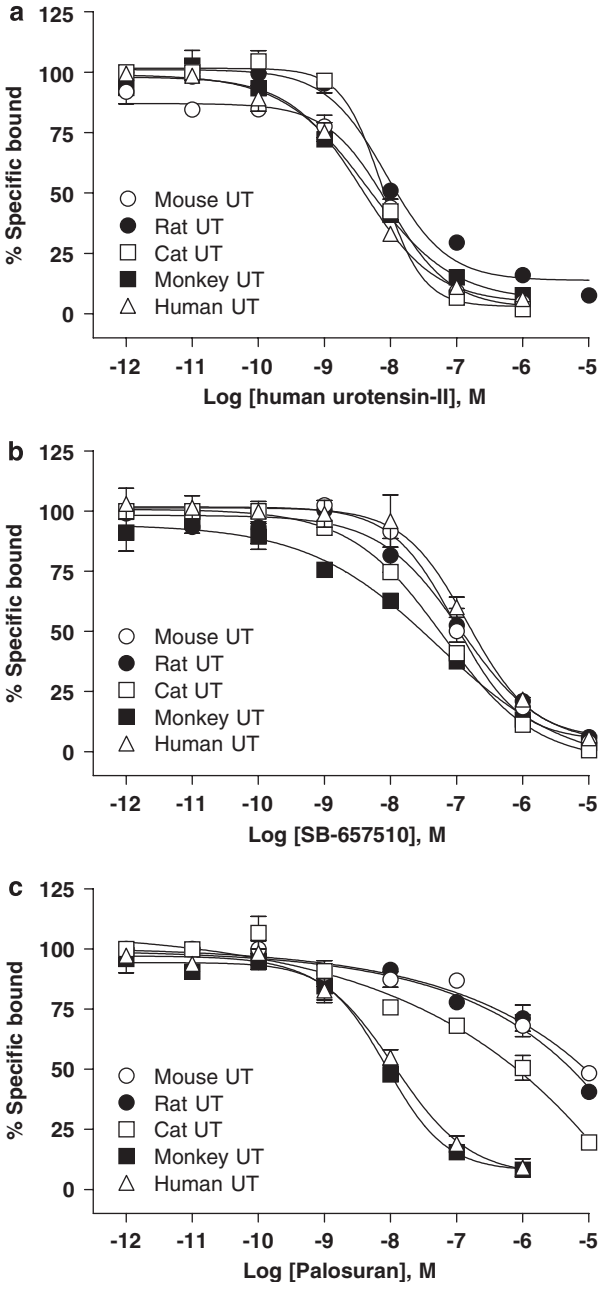

Figure 2 [^{125}I]hU-II (human urotensin-II) radioligand competition binding at recombinant mouse, rat, cat, monkey and human UT in membrane preparations. In contrast to cold hU-II and SB-657510, which exhibited similar affinities for rodent, feline and primate UT (**a**, **b**), palosuran (**c**) exhibited 'primate UT selectivity'.

323 ± 67 nM; Figure 4b) as compared to its membrane-binding affinity (5 nM K_i at human UT membranes; Table 1).

Palosuran is devoid of significant antagonist activity in rat intact, isolated aortae

SB-657510 (1 μM) functioned as an active UT antagonist in Sprague–Dawley and Wistar rat isolated aortae causing significant ($P<0.01$–0.001), ~20-fold rightward shifts in the hU-II concentration–response curve (Tables 4 and 6, and Figures 5 and 6). Antagonism was competitive with no alteration in E_{max}. In accord with the rat UT membrane K_i (65 nM; Table 1), SB-657510 K_b ranged from 49 to 79 nM in the rat aorta in both strains. In contrast, Tables 5 and 6 and Figures 5 and 6 demonstrate that palosuran was inactive in both Sprague–Dawley and Wistar rat isolated aortae (K_b values >10 μM), commensurate with rat UT binding $K_i>1$ μM (Table 1). As such, this quinolinyl-urea was >100-fold less active than SB-657510 in these two rat UT functional bioassays.

Palosuran was soluble under such conditions (5.0 ± 0.1 μM calculated organ bath concentration at 30 min following incubation).

The presence of indomethacin (10 μM) did not alter the contractile responses to hU-II or the inhibitory effects of the antagonists (Table 6 and Figure 6).

Table 2 Palosuran lacks high affinity for recombinant hUT receptor expressed in intact cells: radioligand-binding affinity constants (K_i values, [^{125}I]hU-II competition) for human urotensin-II (hU-II), palosuran and SB-657510 at the recombinant hUT receptor in membrane and whole cell preparations

	Membrane binding K_i *(nM; n = 7)*	*Whole cell binding* K_i *(nM; n = 4)*	*Relative affinity*[a]
Urotensin-II	1.9 ± 0.5	2.9 ± 0.5	1.5
SB-657510	61.4 ± 7.5	46.2 ± 3.0	0.8
Palosuran	5.1 ± 0.7	276.0 ± 66.9***	54.1

Abbreviation: hUT, human urotensin-II receptor.
[a]Relative affinity is expressed as the whole cell binding human UT K_i relative to the corresponding value obtained in hUT membrane preparations. All values are expressed as mean ± s.e.mean. Membrane data are from Table 1 and are included for ease of comparison. Statistical comparisons were made using an unpaired, two-tailed *t*-test where ***$P<0.001$ (versus K_i obtained for the corresponding ligand in membrane preparation).

Palosuran lacks appreciable activity as a hU-II antagonist in cat intact, isolated aortae and femoral arteries

SB-657510 (10 μM) antagonized the contractile actions of hU-II in both cat isolated femoral arteries and aortae, causing significant 154- ($P<0.05$) and 33-fold ($P<0.01$) rightward shifts in the agonist concentration–response curves, respectively (K_b values of 106 and 824 nM; Table 4 and Figure 7). Antagonism was competitive in nature (concentration–response curves were shifted in a parallel manner with no alteration in E_{max}).

In contrast, palosuran was inactive in cat isolated aortae ($K_b>10$ μM; Table 5 and Figure 7). This was in accord with the cat membrane binding ($K_i>1$ μM; Table 1). Although palosuran exhibited surmountable inhibitory activity in the cat isolated femoral artery, this was extremely weak (K_b ~9 μM; Table 5 and Figure 7).

The high-affinity primate UT ligand palosuran is a weak hU-II antagonist in monkey intact isolated renal and mesenteric arteries

SB-657510 (10 μM) functioned as a UT antagonist in both monkey isolated renal and mesenteric arteries causing significant ($P<0.05$) 209- and 90-fold rightward shifts in the hU-II concentration–response curves, respectively (Table 4 and Figure 8). Antagonism was competitive (concentration–response curves were shifted in a parallel,

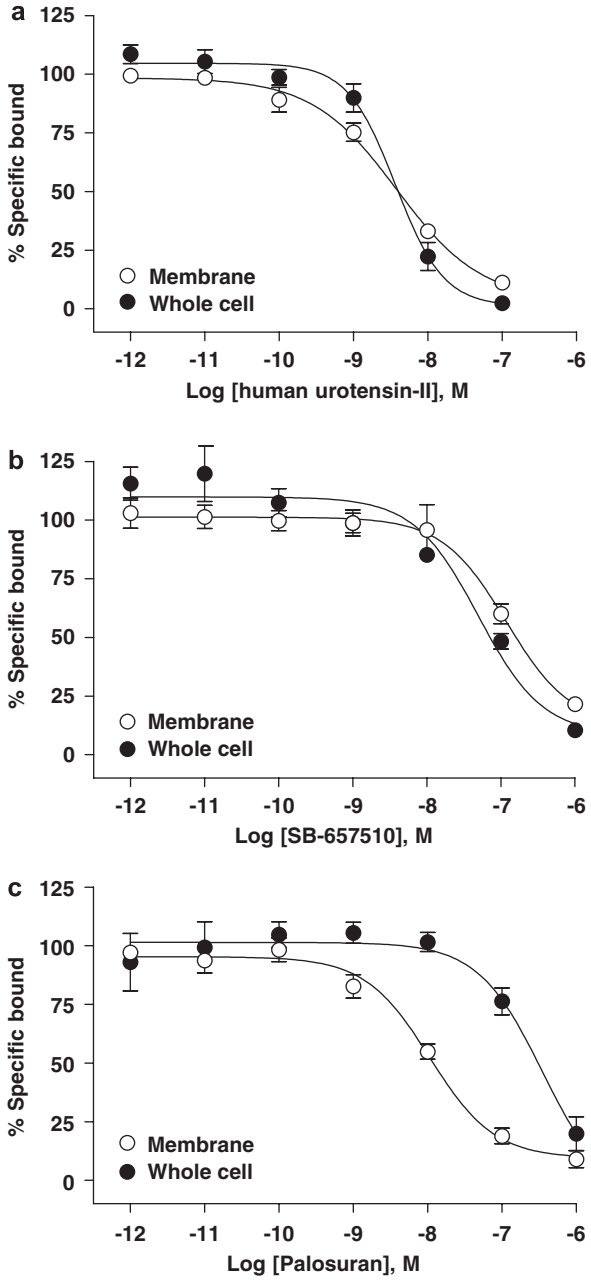

Figure 3 [^{125}I]hU-II (human urotensin-II) competed for binding at recombinant hUT (human urotensin-II receptor) in membrane and intact cell preparations with (**a**) hU-II, (**b**) SB-657510 and (**c**) palosuran. In contrast to both hU-II and SB-657510 where hUT-binding affinities were similar on membranes and intact cells, the inhibitory binding affinity of palosuran for hUT was 54-fold less potent in intact cells as compared to membrane preparations.

manner with no alteration in E_{max}). K_b values were 71 and 158 nM in renal and mesenteric vessels, respectively.

In contrast to the membrane-binding data (4 nM K_i at monkey UT membranes; Table 1), palosuran (10 μM) was significantly (392- to 690-fold) less active in monkey isolated renal and mesenteric arteries with K_b values of 1.6 and 2.8 μM, respectively (indeed, the modest 6- to 11-fold rightward shifts in the hU-II EC_{50}s were not statistically significant; Table 5, and Figures 8b and d).

Table 3 Palosuran lacks high affinity for native hUT receptor endogenously expressed in the rhabdomyosarcoma cell line SJRH30: radioligand-binding affinity constants (K_i values, [^{125}I]hU-II competition) for human urotensin-II (hU-II), palosuran and SB-657510 at the native hUT receptor in whole cell preparations following 1 and 4 h incubation periods

	K_i *(nM)*			*Relative affinity*[a]	
	1 h incubation	*4 h incubation*	*Ratio*	*1 h incubation*	*4 h incubation*
Urotensin-II	0.2 ± 0.0	0.3 ± 0.1	1.5	0.1	0.2
SB-657510	56.9 ± 9.7	213.0 ± 71.2	3.7	0.9	3.5
Palosuran	49.8 ± 3.3	192.5 ± 57.2	3.9	9.8	37.7

Abbreviation: hUT, human urotensin-II receptor.
[a]Relative affinity is expressed as the native human UT K_i relative to the corresponding value obtained in recombinant hUT membrane preparations (see Table 1). All values are expressed as mean ± s.e.m. Statistical comparisons were made using an unpaired, two-tailed *t*-test and no values were determined from 1 h ($n = 3$).

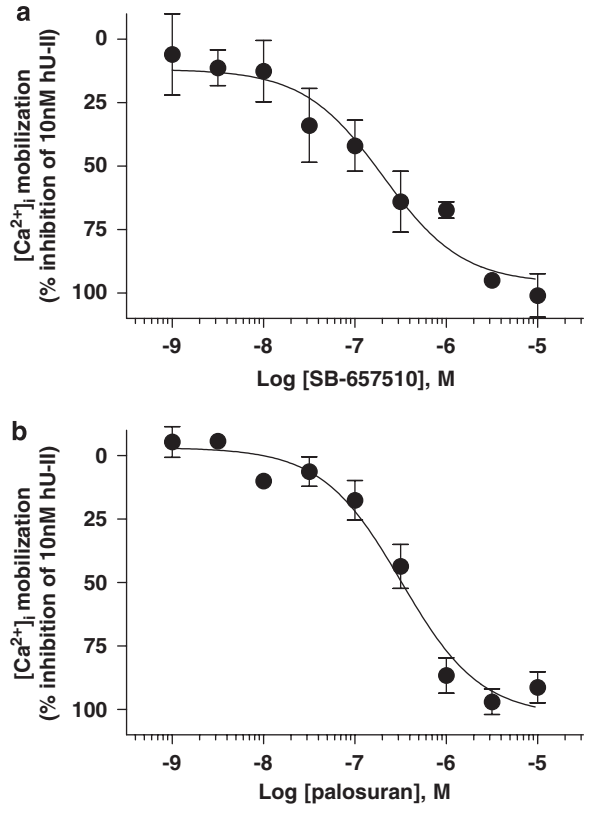

Figure 4 Inhibition of 10 nM hU-II (human urotensin-II)-induced $[Ca^{2+}]_i$ mobilization in recombinant hUT (human urotensin-II receptor)-CHO (Chinese hamster ovary) cells by (**a**) SB-657510 and (**b**) palosuran. SB-657510 inhibited $[Ca^{2+}]_i$ mobilization with an IC_{50} of 180 ± 46 nM, a value consistent with membrane-binding affinity (K_i 61 nM at human UT membranes; Table 1). In contrast, palosuran was significantly (65-fold) less active at inhibiting $[Ca^{2+}]_i$ mobilization (IC_{50} 323 ± 67 nM) as compared to its membrane-binding affinity (K_i 5 nM at human UT membranes; Table 1).

Antagonism in the human UT transgenic mouse isolated thoracic aorta

As predicted from the human recombinant UT membrane/intact cell-binding studies (K_i values of 61 and 46 nM, respectively; Tables 1 and 2), SB-657510 was an active (K_b 117 nM) UT antagonist in mouse isolated aortae expressing the human UT transgene (Table 4 and Figure 9). SB-657510

Table 4 SB-657510 is a competitive, 'pan-species' active UT antagonist in mammalian isolated arteries

	E_{max} (% KCl)		EC_{50} (nM)		SB-657510 K_b (nM)	K_b/K_i
	Vehicle-treated control	SB-657510-treated	Vehicle-treated control	SB-657510-treated		
Sprague–Dawley rat						
Thoracic aorta ($n=8$)	90 ± 11	89 ± 6	3.1 ± 0.6	59.5 ± 10.3***	62 ± 10	1.0
Cat						
Femoral artery ($n=4$)	206 ± 36	239 ± 43	0.9 ± 0.2	138.3 ± 53.8*	106 ± 47	3.5
Thoracic aorta ($n=5$)	126 ± 10	140 ± 10	1.5 ± 0.3	50.1 ± 9.9**	824 ± 446	27.5
Monkey						
Renal artery ($n=6$)	130 ± 10	143 ± 22	0.7 ± 0.1	146.5 ± 38.4*	71 ± 18	4.2
Mesenteric artery ($n=5$)	155 ± 24	135 ± 19	2.1 ± 0.8	189.3 ± 59.9*	158 ± 71	9.3
Human UT transgenic mouse						
Thoracic aorta ($n=5$)	68 ± 5	74 ± 10	1.7 ± 0.3	153.7 ± 22.9**	117 ± 23	1.9

All values are expressed as mean ± s.e.mean. Tissues were pretreated for 30 min with either 1 μM (rat aorta) or 10 μM SB-657510 prior to generating cumulative concentration–response curves to hU-II (human urotensin-II). K_b/K_i is a ratio comparing functional activity of SB-657510 in an isolated arterial preparation (K_b above) with the corresponding affinity (K_i, see Table 1) in the UT recombinant binding assay in the same species. Statistical comparisons were made using a two-tailed *t*-test where *$P<0.05$, **$P<0.01$ and ***$P<0.001$ versus vehicle.

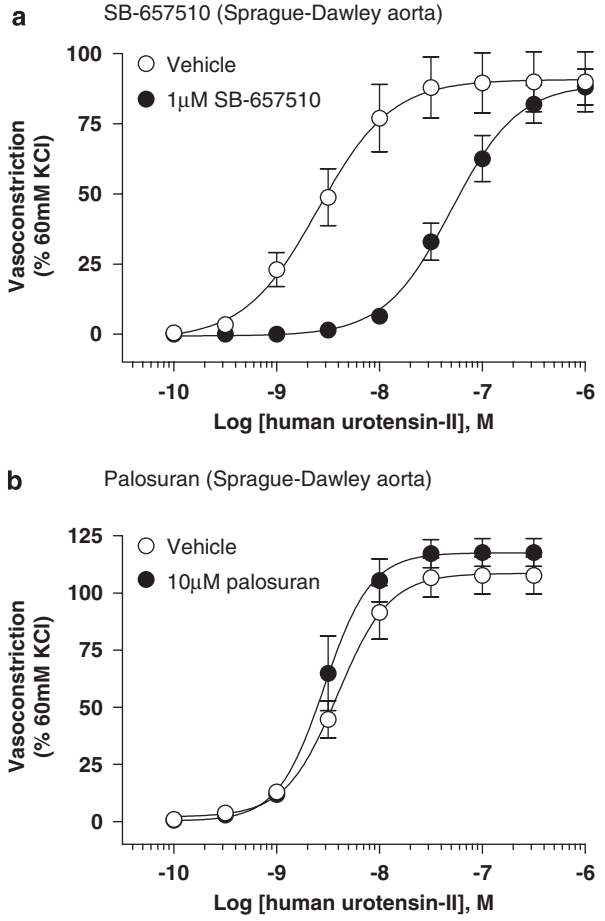

Figure 5 Inhibition of hU-II (human urotensin-II)-induced contraction of Sprague–Dawley rat isolated aorta by (**a**) 1 μM SB-657510 and (**b**) 10 μM palosuran. Whereas SB-657510 inhibited hU-II contraction with a K_b of 62 nM (Table 4), palosuran failed to elicit an inhibitory effect ($K_b>10\,000$ nM; Table 5).

(10 μM) caused a significant ($P<0.01$), parallel, 90-fold rightward shift in the hU-II concentration–response curve with no alteration in E_{max} consistent with a competitive mode of antagonism.

In contrast, however, palosuran was 19-fold weaker than SB-657510 in the same preparation (Table 5 and Figure 9b). Consistent with the differential membrane *cf.* intact cell hUT binding K_i, the resultant 2.2 μM K_b obtained in the mouse transgenic hUT isolated aorta was significantly lower than the hUT membrane K_i (5 nM). This corresponded to a 434-fold loss in activity in the transgenic hUT functional bioassay.

Discussion and conclusions

The present study demonstrates that palosuran is a sub-optimal pharmacological tool for use in preclinical (non-primate) studies. Palosuran lacks appreciable affinity ($K_b>1$ μM) in numerous functional bioassays, specifically isolated arterial contractility assays from a diverse range of species, including the rat, cat, monkey and transgenic hUT mouse. Such findings, perhaps, provide insight into the disappointing clinical efficacy data reported recently in diabetic nephropathy patients (see Clozel *et al.*, 2006). In this patient cohort, significant clinical exposure was most likely never attained, that is exposure was insufficient to provide adequate levels of hUT receptor occupancy. Specifically, the peak palosuran plasma concentration ($C_{max}\leqslant 260$ nM) recorded in humans (Sidharta *et al.*, 2006) was $\geqslant$10-fold lower than the UT affinity of palosuran determined in mammalian isolated arteries in the present study (K_b values of $\sim$2 to >10 μM across species). The peak palosuran concentrations attained in man would, therefore, equate to $\leqslant$10% total human UT receptor occupancy (estimated using the modified Langmuir–Clark equation where the fraction of total receptor occupancy is described by $R_T = 1/1+(K_D/[A])$; see Bowman and Rand, 1980).

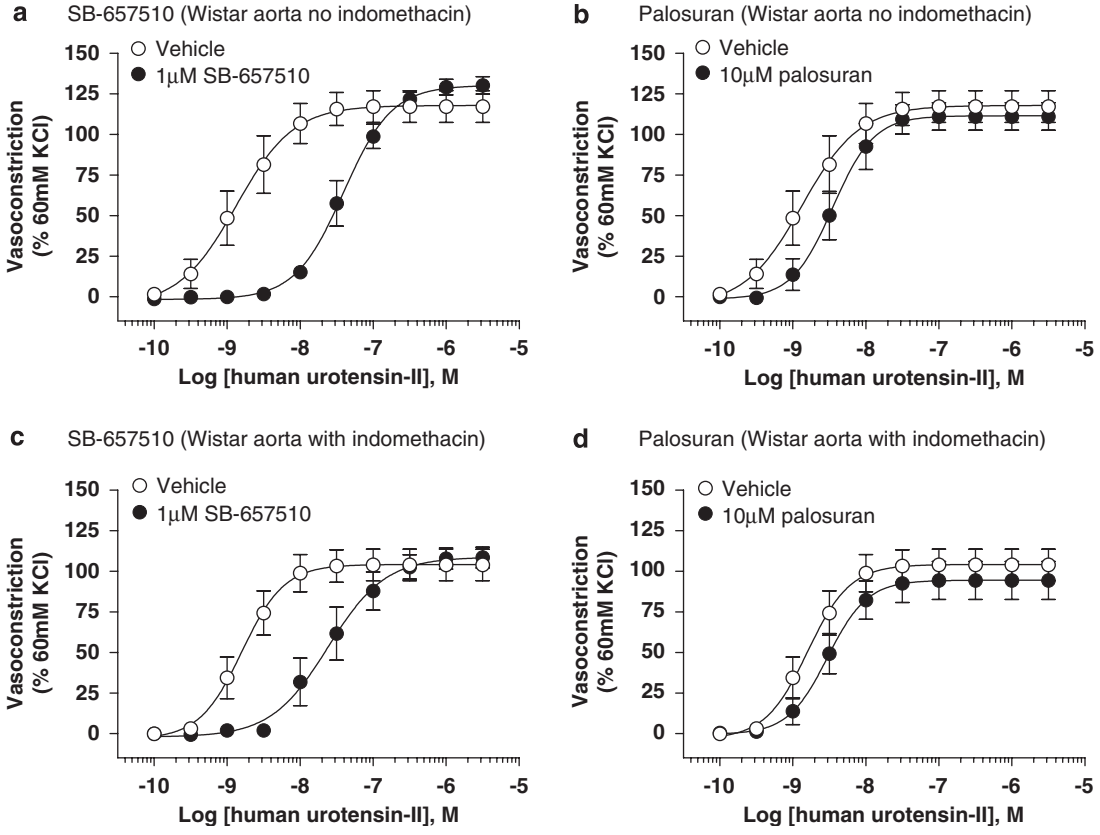

Figure 6 Inhibition of hU-II (human urotensin-II)-induced contraction of Wistar rat isolated aorta by 1 μM SB-657510 in the (**a**) absence and (**c**) presence of 10 μM indomethacin and 10 μM palosuran in the (**b**) absence and (**d**) presence of 10 μM indomethacin. Whereas SB-657510 inhibited hU-II contraction with K_b values of 49–79 nM, palosuran failed to elicit an inhibitory effect ($K_b > 10\,000$ nM; Table 6).

Table 5 Palosuran exhibits poor activity as a UT antagonist in mammalian isolated arteries

	E_{max} (% KCl)		EC_{50} (nM)		Palosuran K_b (nM)	K_b/K_i
	Vehicle-treated control	*Palosuran-treated*	*Vehicle-treated control*	*Palosuran-treated*		
Rat						
Thoracic aorta ($n=5$)	109 ± 8	118 ± 6	4.0 ± 0.6	3.3 ± 0.6	> 10 000	> 10.0
Cat						
Femoral artery ($n=3$)	235 ± 25	202 ± 16	0.5 ± 0.1	1.1 ± 0.2	8541 ± 684	8.5
Thoracic aorta ($n=3$)	160 ± 6	153 ± 15	2.9 ± 0.6	3.6 ± 1.1	> 10 000	> 10.0
Monkey						
Renal artery ($n=4$)	127 ± 33	124 ± 27	1.2 ± 0.4	14.0 ± 5.0	1568 ± 773	392.0
Mesenteric artery ($n=3$)	127 ± 42	143 ± 47	1.2 ± 0.2	7.7 ± 3.0	2760 ± 984	690.0
Human UT transgenic mouse						
Thoracic aorta ($n=4$)	69 ± 4	66 ± 7	2.6 ± 0.6	14.8 ± 0.7***	2,214 ± 637	442.8

All values are expressed as mean ± s.e.mean. Tissues were pretreated for 30 min with 10 μM palosuran prior to generating cumulative concentration–response curves to hU-II (human urotensin-II). K_b/K_i is a ratio comparing functional activity of palosuran in an isolated arterial preparation (K_b above) with the corresponding affinity (K_i, see Table 1) in the UT recombinant binding assay in the same species. Statistical comparisons of E_{max} and EC_{50} values were made using a paired, two-tailed *t*-test where ***$P<0.001$ versus vehicle.

However, this is most likely an overestimate of palosuran–hUT occupancy because it assumes that

(a) Palosuran–plasma protein binding does not restrict access from the plasma to the 'extra-circulatory' site of action (as plasma albumin binding is 96%, the maximum 'free fraction' of palosuran in human plasma could be as little as ~10 nM (4% total drug concentration) resulting in ⩽1% hUT occupancy based on the Langmuir–Clark calculation described above; Sidharta *et al*., 2006).

(b) Palosuran concentration is maintained at a constant 260 nM (C_{max}) throughout the 12-h dosing period. This is clearly an invalid assumption (Sidharta *et al*., 2006) as pharmacokinetic analysis demonstrates that patients

Table 6 SB-657510 is a competitive UT antagonist, whereas palosuran lacks inhibitory activity in the Wistar rat isolated aorta

	E_{max} (% KCl)			EC_{50} (nM)			K_b (nM)	
	Vehicle-treated control	*SB-657510-treated*	*Palosuran-treated*	*Vehicle-treated control*	*SB-657510-treated*	*Palosuran-treated*	*SB-657510-treated*	*Palosuran-treated*
No indomethacin	117 ± 9	130 ± 5	111 ± 8	2.4 ± 0.7	48.3 ± 11.4**	4.9 ± 1.4	49 ± 10	>10 000
Indomethacin 10 μM	104 ± 9	108 ± 6	94 ± 12	2.2 ± 0.5	41.1 ± 12.6**	3.5 ± 0.8	79 ± 14	>10 000

All values are expressed as mean ± s.e.mean. Tissues were pretreated for 30 min with vehicle, 1 μM SB-657510 or 10 μM palosuran prior to generating cumulative concentration–response curves to hU-II (human urotensin-II). Statistical comparisons of E_{max} and EC_{50} values were made using a paired, two-tailed *t*-test where $^{**}P<0.01$ versus vehicle. The presence of indomethacin did not alter the contractile responses to hU-II (E_{max}, EC_{50}) nor the inhibitory effects of the antagonists (K_b; paired, two-tailed *t*-test; $P>0.05$).

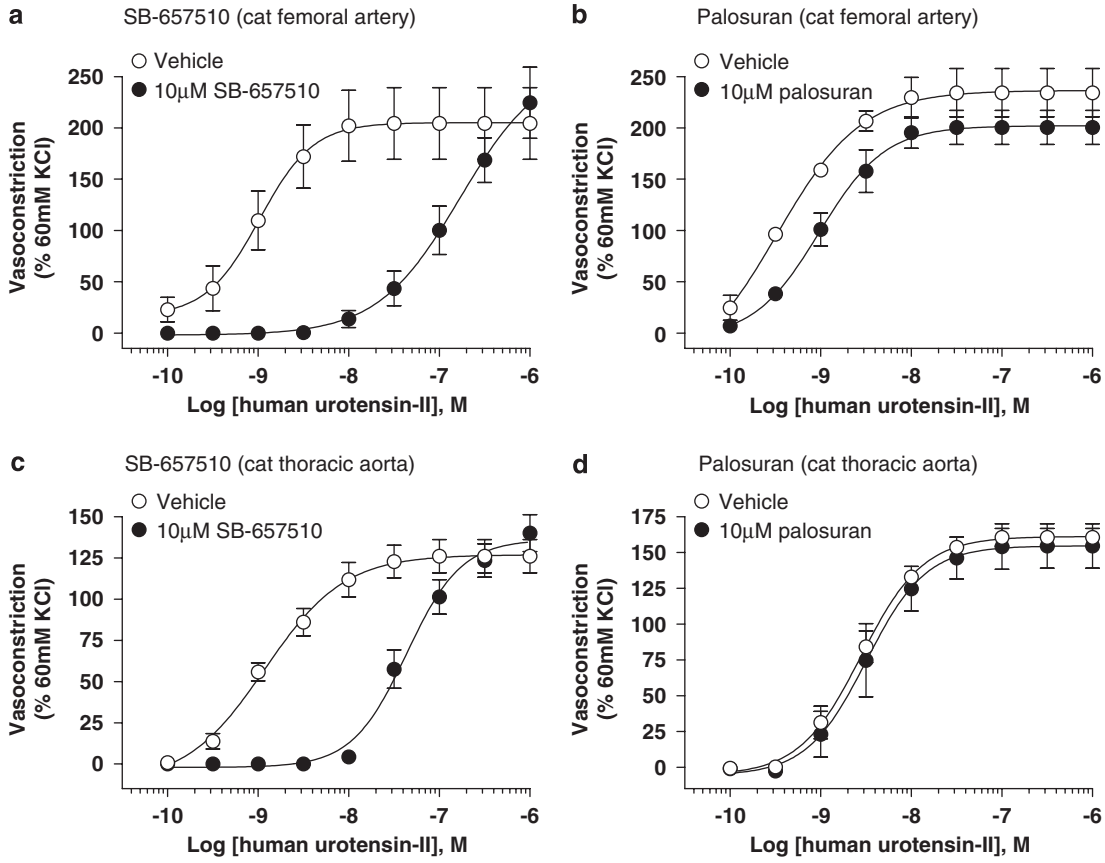

Figure 7 Inhibition of hU-II (human urotensin-II)-induced contraction by 10 μM SB-657510 and 10 μM palosuran in cat isolated femoral artery (**a**, **b**) and thoracic aorta (**c**, **d**). Whereas SB-657510 inhibited hU-II contraction of cat isolated femoral artery and thoracic aorta with K_b values of 106 and 824 nM (Table 4), palosuran was a weak UT receptor functional antagonist in cat isolated arteries (K_b values of 8541 and >10 000 nM, respectively; Table 5).

readily clear palosuran from the plasma (assuming palosuran–hUT dissociation (K_{off}) is not rate limiting; Copeland *et al*., 2006). Palosuran concentration falls rapidly to <50 nM at $t_{12\,h}$ (this would equate to ~2% hUT occupancy or <0.1% if plasma protein binding limits access to the extra-luminal hUT site of action).

(c) No native ligand (such as hU-II, URP, etc.) is present to compete with palosuran for UT occupancy. It is estimated that human plasma U-II-like immunoreactivity is ⩽10 nM (Heller *et al*., 2002; Aiyar *et al*., 2004; Heringlake *et al*., 2004; Lapp *et al*., 2004; Krüger *et al*., 2005; Rdzanek *et al*., 2006), a concentration ~10- to 100-fold above its EC_{50} activity as a vasoconstrictor of human blood vessels (~0.1 nM in small coronary epicardial arteries; Maguire *et al*., 2004). This estimate of 'intrinsic' UT occupancy by the native ligand(s) would equate to occupancy of ~99% total hUT reserve (NB: hU-II is also a 'pseudo-irreversible' ligand; Douglas *et al*., 2004a; Tölle and van der Giet, 2008). At this level of hUT occupancy by hU-II, Schild analysis suggests that significant UT antagonism would only be observed at ~200 μM palosuran, plasma concentrations ~100-fold above the 2 μM K_b determined at hUT in transgenic mice (Bowman and Rand, 1980; Kenakin, 2003). Such concentrations

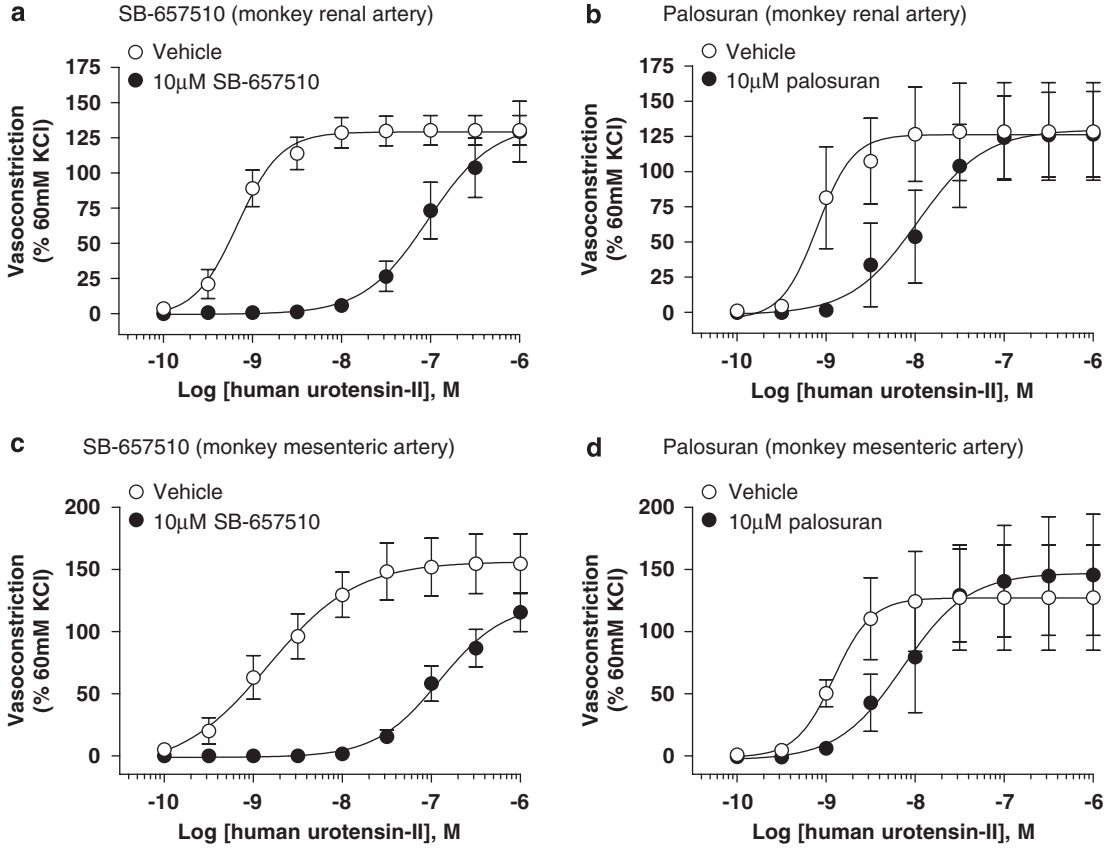

Figure 8 Inhibition of hU-II (human urotensin-II)-induced contraction by 10 μM SB-657510 and 10 μM palosuran in monkey isolated renal artery (**a**, **b**) and superior mesenteric artery (**c**, **d**). SB-657510 inhibited hU-II contraction of monkey isolated arteries (K_b values of 71 and 158 nM; Table 4) with activities similar to binding affinities at the monkey UT receptor (K_i 17 nM; Table 1). In contrast, palosuran was a weak functional antagonist; in monkey isolated artery K_b values (1568 and 2760 nM; Table 5) were 392- to 690-fold lower than monkey UT K_i values (4 nM; Table 1).

are clearly never attained in the clinic using a 125 mg b.i.d. dosage regimen (1000-fold below even at C_{max}; Sidharta *et al*., 2006).

Palosuran is purported to regulate endocrine, angiogenic and, most strikingly, renal (dys)function in the rat both *in vitro* and *in vivo* (Clozel *et al*., 2004, 2006; Egido *et al*., 2005; Marco *et al*., 2005; Albertin *et al*., 2006; Spinazzi *et al*., 2006). Although the current study demonstrates that palosuran is indeed a high-affinity human and primate UT ligand in cell membrane assays (K_i values of 4–5 nM, in accord with Clozel *et al*., 2004), it was inactive at rat, mouse and cat UT membranes (K_i values >1 μM). Such observations mirror those reported previously (IC_{50} values >10 μM in rat UT radioligand and $[Ca^{2+}]_i$ mobilization assays; Clozel *et al*., 2004). These findings are supported further by a lack of activity in the rat isolated aorta ($K_b > 10$ μM in the present study). These observations suggest that the beneficial actions of palosuran in rat renal, endocrine, angiogenic, etc. assays (Clozel *et al*., 2004, 2006; Egido *et al*., 2005; Marco *et al*., 2005; Albertin *et al*., 2006; Spinazzi *et al*., 2006) reflect the use of high ($\geqslant 1$ μM), 'non-selective', palosuran concentrations in these preclinical models, that is, these beneficial actions are unrelated to UT occupancy.

To date, very little is known about the detailed pharmacological characteristics of palosuran (beyond the limited observations reported in the rat aorta using 5-HT, ET-1 and noradrenaline with 10 μM palosuran, a concentration less than twofold above the pD_2 for U-II (6 μM); Clozel *et al*., 2004). Indeed, this is supported, for example, by recent data from Malagon *et al*. (2008) demonstrating that palosuran is a somatostatin receptor agonist (altering $[^3H]$thymidine incorporation and $[Ca^{2+}]_i$ mobilization at recombinant *sst2* and *sst5* subtypes) at low concentrations (10 nM). Indeed, somatostatin agonists have been shown to lower both glucose and albuminuria levels in diabetic mice (Segev *et al*., 2004; Strowski *et al*., 2006). In this context, it is noteworthy that the peptidic UT antagonist SB-710411 does not mimic the actions of palosuran in rat angiogenesis and adrenal cell assays (Albertin *et al*., 2006; Spinazzi *et al*., 2006), even though this moiety is significantly more active at rat UT than palosuran (aorta K_b 716 nM *cf*. >10 μM; Behm *et al*., 2006). Thus, it is prudent to conclude that caution must be exercised when interpreting data generated in preclinical models with palosuran due to (a) poor non-primate activity, (b) limited understanding of selectivity (particularly where responses to palosuran cannot be replicated with alternate UT antagonists such as SB-710411) and (c) the use of high-dosing regimens. It is, therefore, concluded that UT antagonists, such as urantide, GSK248451 and UFP-803, represent more suitable tools than palosuran for investigations performed in non-primate species including the rat

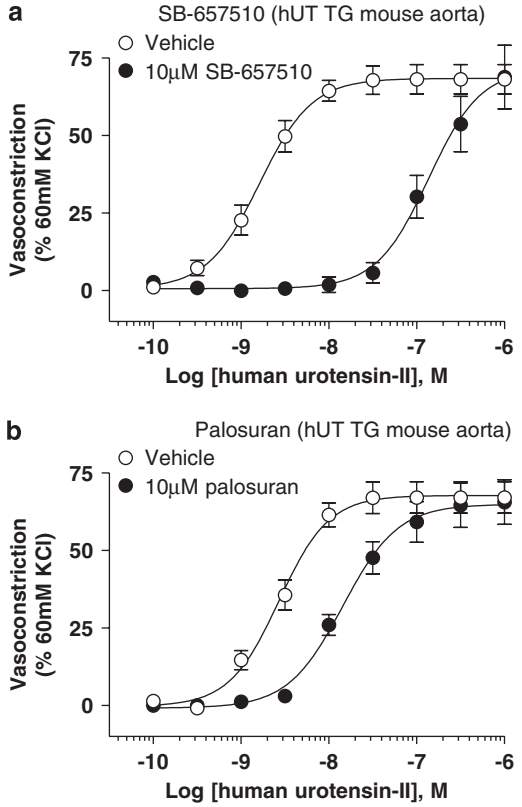

Figure 9 Inhibition of hU-II (human urotensin-II)-induced contraction of hUT TG (human urotensin-II transgenic mice) mouse isolated aorta by (**a**) 10 μM SB-657510 and (**b**) 10 μM palosuran. Whereas SB-657510 inhibited hU-II contraction with a K_b (117 nM; Table 4) similar to its membrane hUT K_i (61 nM; Table 1), palosuran was a weak functional antagonist, inhibited hU-II-induced contraction with a K_b (2214 nM; Table 5) 443-fold lower than its membrane hUT K_i (5 nM; Table 1).

(such moieties are ~300- to 2000-fold more active than palosuran in the rat aortic contraction assay (5–35 nM K_b values); Patacchini *et al*., 2003; Behm *et al*., 2006; Camarda *et al*., 2006).

Concerns regarding palosuran selectivity also apply *in vivo* where similar ~5 μM plasma concentrations (Clozel *et al*., 2004, 2006) were achieved in the rat ischaemia and diabetic nephropathy models. The exposures achieved in both rodent models lie below the rat aorta functional activities described both herein (>10 μM) and by Clozel *et al*. (2004; ~6 μM pD_2 in rat aortae, >10 μM IC_{50} values in rat recombinant UT CHO assays). It is difficult to envision significant, sustained UT receptor occupancy by palosuran under such conditions (yet strikingly, chronic oral palosuran administration effectively normalized both the hyper-triglyceridaemia and hyper-insulinaemia observed in the diabetic rat; Clozel *et al*., 2006). Considering the relative exposure and lack of rat UT affinity, an 'off target' (non-UT) effect(s) is an equally plausible explanation for the profound metabolic effects observed in these nephropathy models.

Such considerations would have been mitigated in part had it been demonstrated that the dosage regimens employed were effective at (selectively) inhibiting the actions of an exogenous U-II challenge *in vivo*. Although it is acknowledged that such 'challenge' studies have limitations (Clozel *et al*., 2004), they would have been illuminating with regard to pharmacokinetic *cf*. pharmacodynamic considerations, for example, the influence of plasma protein binding on palosuran-free fraction and/or function, etc. Indeed, *in vivo* challenge models have been employed successfully for peptidic UT antagonists (GSK248451, U-II-mediated pressor response in the cat; Behm *et al*., 2004; UFP-803, U-II-induced plasma extravasation in the mouse; Camarda *et al*., 2006) and for other peptide hormones (for example, ET_A antagonist Ro 46-2005; Clozel *et al*., 1993a, b). Thus, to date, there are no data that convincingly demonstrate (a) significant and (b) selective *in vivo* UT receptor blockade with palosuran at the dosage regimens employed in these nephropathy models.

In contrast to the present findings (K_b > 10 μM), palosuran was reported to function as an 'insurmountable' inhibitor (suppression of E_{max}) in the Wistar rat isolated aorta (pD_2 ~6 μM; Clozel *et al*., 2004). There is no obvious explanation for the disparity between the original characterization (Clozel *et al*., 2004) and the present investigation where both Wistar and Sprague–Dawley rats were used. Similar protocols were employed by both groups of investigators and the integrity of the palosuran was confirmed in the present study (both physically (NMR and LC-MS detection) and using pharmacological techniques (confirmation of high-affinity binding to primate UT)). Nevertheless, a lack of activity at the rat aorta described herein is in complete agreement with both (a) the rat UT radioligand-binding data (including those described by Clozel *et al*., 2004) and (b) the functional bioassays performed in arteries isolated from other species (poor activity in cat, monkey and transgenic mouse arteries). The disparity is unlikely to reflect hemi-equilibria considerations or allosterism as, where weak hU-II antagonism was observed, inhibition was competitive (dextral, parallel shifts in the U-II response curves in cat, monkey and hUT transgenic mouse arteries; K_b values of ~2–9 μM).

One alternative explanation for the differences in activity in the rat aorta relates to a procedural consideration, namely randomization of rings to treatment (see Methods). Such an action is imperative to minimize 'reactivity bias' (U-II E_{max} declines precipitously as one transitions to distal regions of the thoracic aorta; Itoh *et al*., 1988; Douglas *et al*., 2000; Maguire *et al*., 2000). Failure to randomize paired tissues to treatment could result in an apparent, yet erroneous, treatment-dependent inhibition marked by E_{max} suppression. However, such an argument remains speculative as it us unknown whether or not Clozel *et al*. (2004) performed such tissue randomization in their studies.

Both hU-II (K_i values of ~2–3 nM) and SB-657510 (K_i values of ~46–61 nM) inhibited [^{125}I]hU-II binding to recombinant hUT with identical affinities (<2-fold difference) in both intact cells and membranes. In contrast, palosuran hUT affinity was attenuated 10- to 54-fold when assessed in intact cells (K_i 50–276 nM *cf*. K_i 5 nM in membrane formats). Similar observations were made at native hUT receptor in intact SJRH30 cells. These results are in accord with the loss in affinity originally reported by Clozel *et al*. (2004) in both CHO (recombinant hUT) and TE-671 (native hUT) cells. The mechanism(s) underlying the pronounced

loss in affinity is, to date, unknown but could suggest that palosuran requires access to a distinct (allosteric) binding site, one that is restricted by an intact cell membrane. However, cell permeabilization (0.1–0.5% v v^{-1} saponin; Breivogel *et al*., 2004) did not alter palosuran hUT-binding affinity in whole cells (data not shown). In addition, K_i values for all three ligands in SJRH30 cells were not significantly altered by length of incubation (1–4 h), indicating that equilibrium binding was attained. Thus, a putative 'slow' K_{on} was unlikely to be rate limiting under these conditions.

It should be noted that the present study did not measure the metabolic stability of palosuran (or the generation of putative metabolites) directly within tissues. High-efficiency catabolism of palosuran at the intact cell/smooth muscle site of action (but not in cell membranes) is speculative but could represent a possible explanation for the poor affinity of this moiety in functional bioassays. This seems unlikely, however, as palosuran was clearly soluble under the present experimental conditions ($\sim$5 μM concentration was measured by LC-MS/MS in organ bath studies). In other words, there remained a large, 1000-fold excess concentration of palosuran (*cf*. hUT K_i 5 nM in cell membranes) in the Krebs solution in the isolated blood vessel experiments.

The loss of affinity in whole cell-binding studies is consistent with the observation that palosuran was 65-fold less active at inhibiting $[Ca^{2+}]_i$ mobilization (IC_{50} 323 nM) in recombinant hUT-CHO cells as compared to the membrane-binding affinity (K_i 5 nM). These results are in contrast to previous results reported by Clozel *et al*. (2004) where palosuran exhibited only a fivefold loss in activity at inhibiting $[Ca^{2+}]_i$ mobilization (17 nM IC_{50}) *cf*. membrane-binding affinity (4 nM). Although the reason for such a discrepancy is unclear (essentially identical protocols were employed), it is of interest to note that Clozel *et al*. (2004) demonstrated a 38-fold loss in activity when assessing the inhibitory effects of palosuran on pERK1/2 generation (150 nM IC_{50}), a value consistent with the inhibitory activity of palosuran on $[Ca^{2+}]_i$ mobilization observed in the present study. In contrast to palosuran, SB-657510 inhibited $[Ca^{2+}]_i$ mobilization with an IC_{50} value consistent with membrane-binding data.

Palosuran also lacked appreciable activity at inhibiting hU-II-induced contraction of monkey arteries (K_b values of 1.6–2.8 μM) and hUT transgenic mouse aorta (K_b 2.2 μM), 392- to 690-fold and 443-fold reductions in functional activity. As in the binding assays, a similar loss in activity was not evident with SB-657510 (rat, $<$2-fold difference; cat, 4- to 28-fold difference; monkey, 4- to 9-fold difference; hUT transgenic mouse, $<$2-fold difference). Thus, such phenomena are consistent across both strains and species.

In summary, it is concluded that palosuran is a suboptimal pharmacological tool for use in preclinical (non-primate) studies as (a) it lacks affinity at 'non-human' UT isoforms (mouse, rat and cat), (b) it is subject to 2–3 orders of magnitude loss in affinity/activity in intact cell assays and functional (tissue-based) assays and (c) insufficient information is available regarding the secondary pharmacological properties of this moiety (several actions of palosuran could not be replicated using an alternative peptidic UT antagonist, SB-701411). Finally, such findings provide a potential explanation for the disappointing clinical efficacy reported recently for palosuran in diabetic nephropathy patients (Clozel *et al*., 2006). As such, the (patho)physiological significance of U-II in diabetic nephropathy remains ambiguous.

Conflict of interest

DJB, JJM, JWD, MJN, HEF, CAE, RRH, KDH, SMH, CW and SAD are employees of GlaxoSmithKline.

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