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Inhibitory effects of putative peptidic urotensin-II receptor antagonists on urotensin-II-induced contraction of cat isolated respiratory smooth muscle

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Abstract

Urotensin-II is purported to influence pulmonary function by modulating smooth muscle tone/growth. In the present study, Northern blot and reverse transcription polymerase chain reaction (RT-PCR) analysis indicated the presence of UT receptor mRNA in cat trachea, bronchi and lung parenchyma. Urotensin-II contracted cat isolated trachea and bronchi with similar potencies (pEC_{50}s 8.61±0.07–8.81±0.10). Contractile efficacies ranged from 19±9% to 63±11% KCl in the primary and secondary bronchi. The peptidic UT receptor antagonists BIM-23127, SB-710411 and GSK248451 (7.18±0.12, 7.52±0.08 and 9.05±0.16 cat recombinant UT pK_is) inhibited urotensin-II-induced contraction of cat isolated trachea with pK_bs 6.36±0.11, 6.74±0.07 and 9.27±0.12, respectively. As such, feline lung contains significant amounts of UT mRNA and this receptor appears to be functionally coupled to bronchoconstriction (the peptidic tool compound GSK248451 representing a sub-nanomolar inhibitor of such effects). These findings suggest that the cat represents a suitable species for future studies designed to assess the effects of the urotensin-II receptor on pulmonary (patho)physiology.

Keywords: Urotensin-II; GPR14; UT receptor; BIM-23127; SB-710411; GSK248451; Trachea; Bronchus

1. Introduction

The effects of human urotensin-II, an undecapeptide most widely known as the most potent vasoconstrictor identified to date, are mediated by the G-protein-coupled receptor UT (urotensin-II receptor; formerly termed GPR14; Douglas and Ohlstein, 2000). In addition to altering cardiovascular, renal, metabolic and central nervous system function, there is increasing evidence supporting a role for the urotensin-II system in regulating mammalian pulmonary (patho)physiology. Although the relative abundance of urotensin-II mRNA is relatively low in the lung (Ames et al., 1999; Nothacker et al., 1999; Elshourbagy et al., 2002), significant amounts of urotensin-II protein have been observed in this organ (Zhang et al., 2002; Qi et al., 2004). Indeed, the lung appears to be a major source of urotensin-II production in humans since plasma urotensin-II protein levels are markedly elevated in the aortic root as compared to the pulmonary artery (Russell et al., 2003). Further to this, UT receptor protein expression is clearly evident in rat airway smooth muscle cells (where urotensin-II is a potent mitogen; Chen et al., 2004). In addition to being a growth factor, urotensin-II also elicits potent contractile effects in the monkey and human isolated respiratory tract and isolated pulmonary arteries (Douglas et al., 2000; Hay et al., 2000; MacLean et al., 2000). Consistent with these in vitro observations, inhalation of urotensin-II contracted sheep airways in vivo (De Garavilla et al., 2001).

In addition to its ability to regulate smooth muscle tone and growth, recent studies suggest that the urotensin-II system might be involved in the (patho)physiological regulation of pulmonary function. For example, urotensin-

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II protein expression in rat pulmonary arteries is significantly increased following pulmonary hypertension (Qi et al., 2004). In order to further define such a role, the present study evaluated (a) the presence of UT receptor mRNA in cat lung, (b) urotensin-II contraction in the cat isolated respiratory tract as an alternative to studying urotensin-II in the monkey (Hay et al., 2000) and (c) the ability of several putative, tool peptidic UT receptor antagonists (BIM-23127, Herold et al., 2003; SB-710411, Coy et al., 2000; Behm et al., 2002, 2004b; GSK248451, Coy et al., 2003, Behm et al., 2003) to inhibit such a contractile response. The data generated suggest that such agents might be suitable for delineating a role for urotensin-II in preclinical airway disease models.

2. Material and methods

All protocols were approved by the GlaxoSmithKline Animal Care and Use Committee. Procedures were performed in accredited facilities in accordance with institutional guidelines and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources [1996], National Research Council).

2.1. Tissue isolation and mRNA extraction

Following sodium pentobarbital overdose, respiratory tissues (trachea, bronchi and lung parenchyma) were harvested from four adult male cats (4–5 kg; 1–2 years; Liberty Research Inc., Waverly, NY, USA). Tissues were flash frozen in liquid N_2 and homogenized using a mortar and pestle. RNA was extracted using RNAzol (GIBCO BRL, Gaithersburg, MD) followed by RNeasy column purification (Qiagen, Valencia, CA).

2.2. Northern blot analysis

Total RNA (20 μg) was transferred to a nylon membrane (GeneScreen plus, NEN Life Science Products, MA) and probed with cDNA corresponding to the cat full length UT receptor open reading frame (ORF). cDNAs were labeled with [α-^{32}P] 2′-deoxycytidine 5-triphosphate (dCTP) using standard random primed methods (T7 QuickPrime; Pharmacia Biotech, Piscataway, NJ). Membranes were pre-hybridized for 2 h at 42 °C and incubated overnight at 42 °C with 1×10^9 cpm/μg denatured radiolabeled cat UT receptor ORF probe in standard buffer (50% deionized formamide, 1.5 M NaCl, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate). Membranes were washed under conditions of low stringency (three 15 min washes in 1X sodium citrate, 0.1% SDS at 25 °C) followed by a high stringency wash in 0.1X sodium citrate/0.1% SDS for 30 min at 55 °C. Hybridization signals were detected by conventional X-ray autoradiography (Hyperfilm, Amersham Life Science, UK) and quantitated by phosphorimaging (Storm 860, Molecular Dynamics, Sunnyvale, CA).

2.3. Quantitative mRNA analysis

Quantitative real time TaqMan RT-PCR (reverse transcription polymerase chain reaction) was performed on total RNA following treatment with DNase-I as described previously in detail (Douglas et al., 2005). Briefly, cDNAs, generated using oligo (dT_{16})-primed MultiScribe reverse transcriptase (TaqMan, Perkin Elmer, Branchburg, NJ), were amplified using cat UT primer/probe sequences as follows (5′ to 3′): forward CCG AGT TCT CTT CAG; reverse TGC TCA TGA CGG TCA; TaqMan probe TTC CTG ACC ATG CAC GCC [with FAM and TAMRA as the reporter and quencher dyes, respectively]). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeper control. PCR was performed at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s then 60 °C for 1 min.

2.4. Binding of [^{125}I] human urotensin-II to cat tissue membrane preparations

Cat trachea and lung tissues were homogenized using a Tekmar Tissuemizer (Cincinnati, OH) in 10 mM Tris–HCl containing 1 mM EDTA (pH 7.4) and centrifuged at 1,000 ×g for 10 min. The supernatant was centrifuged at 47,000 ×g for 20 min and the resulting pellets were resuspended in 25 mM Tris–HCl containing 5 mM $MgCl_2$ (pH 7.4). This washing procedure was repeated twice. Protein concentration was measured using the Pierce (Rockford, IL) bicinchoninic acid (BCA) method with bovine serum albumin as the standard. Membranes (50–70 μg protein) were incubated for 60 min at 25 °C in 200 μl buffer [25 mM Tri–HCl, pH 7.4, 5 mM $MgCl_2$, 0.20 mg/ml bacitracin and 0.1% bovine serum albumin (BSA)] containing 200 pM [^{125}I] human urotensin-II in the absence or presence of 1 μM unlabeled human urotensin-II. The incubation was terminated by the addition of 2 ml wash buffer (0.9% NaCl, 4 °C) followed by rapid filtration over Whatman GF/C filters (Clifton, NJ) using a cell harvester (Brandel Research and Development Laboratories, Gaithersburg, MD). The filters were washed three times with 4 ml of wash buffer. Non-specific binding was determined in the presence of 1 μM unlabeled human urotensin-II and filter papers were counted in a gamma counter (Packard Instrument Company, Meriden, CT).

2.5. Binding of [^{125}I] human urotensin-II to cat recombinant UT

[^{125}I] human urotensin-II binding assays were performed with cat recombinant UT-transfected HEK-293 cell membranes using a scintillation proximity assay (SPA). Competition binding assays were performed with [^{125}I] human urotensin-II (300 pM) in the presence or absence of unlabeled urotensin-II or peptidic UT receptor antagonists (BIM-23127, SB-710411, GSK248451; 1 pM to 1 μM) in assay buffer (20 mM Tris–HCl [pH 7.4], 5 mM $MgCl_2$ and 0.05% BSA) and cell membranes pre-coupled to wheat germ agglutinin–SPA (WGA–SPA) beads (Amersham, Arlington Heights, IL) at a concentration of 5 μg membrane protein and 0.5 mg of WGA–SPA beads. Non-specific binding was determined using 1 μM unlabeled urotensin-II. Assay plates were sealed, shaken gently for 45 min at room temperature and centrifuged at 2000 ×g for 10 min before counting in a Packard Top Count Scintillation Counter.

2.6. Preparation and utilization of feline isolated respiratory tissue for contraction studies

Following sodium pentobarbital overdose, trachea and bronchi (primary, secondary and tertiary branches) were isolated from adult male cats. Rings, approximately 3 mm in length, were cut from each tissue and suspended in 10 ml organ baths containing Krebs–

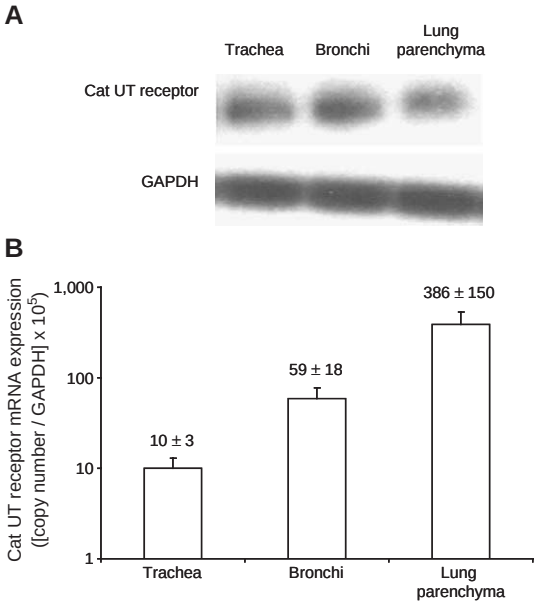

Fig. 1. Cat UT receptor mRNA expression in isolated respiratory tissues was demonstrated by both (A) Northern blot and (B) TaqMan analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeper control.

Henseleit 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. Krebs was maintained at 37 °C and aerated with 95% O_2:5% CO_2 (pH 7.4). Agonist-induced changes in isometric force were measured under 1 g resting tension (0.5 g for secondary and tertiary bronchi) using FT03 force-displacement transducers (Grass Instruments, Quincy, MA) coupled to Model 7D polygraphs. Following a 60 min equilibration period, tissues were exposed to standard concentrations of KCl (60 mM) and carbachol (10 μM) and all subsequent contractile responses were normalized to the maximal contractile response to KCl (responses to 60 mM KCl were typically ~2-fold less efficacious than those seen with 10 μM carbachol). Cumulative concentration–response curves to human urotensin-II (0.1 nM–10 μM) were obtained for each tissue by adding the spasmogen to the tissue bath at half-log increments.

To determine the potency of putative UT receptor antagonists, tissues were pre-treated with vehicle (0.1% DMSO), BIM-23127 (3 μM; Herold et al., 2003), GSK248451 (0.1 μM; Coy et al., 2003; Behm et al., 2003) or SB-710411 (10 μM; Coy et al., 1999; Behm et al., 2002, 2004b) for 30 min prior to generating the cumulative concentration–response curves to human urotensin-II. Each response was allowed to plateau before the addition of subsequent human urotensin-II concentrations (tissues were used to study the effects of a single antagonist only).

2.7. Statistical and data analysis

All values are expressed as mean±S.E.M. and n represents either the number of independent experiments performed in triplicate or the total number of animals from which tissues were isolated. Competition binding curves were analyzed by nonlinear regression (GraphPad Software, San Diego, CA). For isolated vascular tissue studies, concentration–response curves were fitted to a logistic equation as described previously (Douglas et al., 1995). Statistical comparisons were made using either a paired, two-tailed t-test or ANOVA and differences were considered significant when $P \leq 0.05$. Antagonist affinities (pK_bs) were determined using the Schild equation (Jenkinson et al., 1998).

2.8. Drugs and reagents

Human urotensin-II, GSK248451 (4-Cl-cinnamoyl(*trans*)-c[D-Cys-4-pyridylalanine-D-Trp-Orn-Val-Cys]-His-amide) and SB-710411 (Cpa-c[D-Cys-3pyridylalanine-D-Trp-Lys-Val-Cys]-Cpa-amide; Cpa, *p*-chloro-L-phenylalanine) were custom-synthesized by California Peptide Research Inc. (Napa, CA). BIM-23127 was from Bachem (King of Prussia, PA). Monoiodinated human urotensin-II ([^{125}I]-Tyr^9, specific activity 2000 Ci/mmol) was custom synthesized by Amersham (Arlington Heights, IL). Carbachol, indomethacin and norepinephrine were purchased from Sigma (St. Louis, MO) and sodium pentobarbital from Vortech Pharmaceuticals (Dearborn, MI). All other reagents used were of analytical grade.

3. Results

3.1. Cat UT receptor mRNA distribution in respiratory tissues

Qualitative Northern blot analysis revealed the presence of UT receptor mRNA in cat isolated respiratory tissues (Fig. 1A). Quantitative analysis by TaqMan RT-PCR demonstrated that UT receptor mRNA levels were highest in lung parenchyma, where values were 6- and 40-fold greater than those observed in the bronchi and trachea, respectively (Fig. 1B).

3.2. Radioligand binding

To determine the binding characteristics of urotensin-II at the native cat UT receptor, membranes from trachea and lung parenchyma were prepared and [^{125}I] human urotensin-II specific binding was determined in the presence of 1 μM unlabeled urotensin-II. In contrast to the UT mRNA expression level, the presence of specific urotensin-II binding sites was low (1.2±0.1 and 2.6±0.4 fmol/mg, respectively). Binding levels were not sufficient to perform saturation binding studies (i.e. K_D/B_{max} determination).

To determine the binding affinity for urotensin-II and the UT receptor antagonists BIM-23127, SB-710411 and GSK248451, competition binding experiments were performed using recombinant cat UT membranes. Human urotensin-II, BIM-23127, SB-

Table 1
Competition with [^{125}I]human urotensin-II for binding at the cat recombinant UT receptor

UT receptor competing ligand	pK_i	n
Human urotensin-II	9.00±0.11	3
BIM-23127	7.18±0.12	3
SB-710411	7.52±0.08	3
GSK248451	9.05±0.16	4

All values are expressed as mean±S.E.M. where n represents the number of independent experiments performed in triplicate.

Table 2
Contractile effects of human urotensin-II in cat isolated respiratory tissues

Tissue	pEC_{50}	E_{max} (% KCl)	Responders (n)
Trachea	8.78±0.08	36±13	7/8
1° bronchus	8.77±0.18	19±9	7/8
2° bronchus	8.81±0.10	63±11	8/8
3° bronchus	8.61±0.07	54±22	5/8

All values are expressed as mean±S.E.M. where n represents the number of animals from which tissues were isolated. Non-responding tissues were not included in mean±S.E.M. calculations. Statistical comparisons were made using ANOVA analysis and no values were determined to be significantly ($P<0.05$) different.

710411 and GSK248451 each competed for [^{125}I] human urotensin-II binding at the cat recombinant UT receptor. Competition curves for each peptide were monophasic with Hill coefficients approximating unity, consistent with a single site interaction. SB-710411(30 nM K_i) and BIM-23127 (66 nM K_i) inhibited [^{125}I] human urotensin-II binding with affinities 30- and 66-fold less potent than urotensin-II (1 nM K_i), respectively. Strikingly, GSK248451 was extremely potent, displacing [^{125}I] human urotensin-II from cat UT with sub-nanomolar affinity (0.9 nM K_i; Table 1).

3.3. Contractile activity of human urotensin-II in the cat isolated respiratory tract

Tissue viability was confirmed by assessing the ability of isolated respiratory tract to respond to standard concentrations of 60 mM KCl and 10 μM carbachol where responses to the former were typically ~2-fold less efficacious than those observed in the latter.

Human urotensin-II contracted cat isolated trachea and bronchi (primary, secondary and tertiary generations) with similar potencies (~1–2 nM EC_{50}s) and efficacies (E_{max}s from 19 to 63% KCl; Table 2; Fig. 2). The time to onset of the human urotensin-II contraction was similar to those induced by KCl (60 mM) and carbachol (10 μM), requiring ~10 min for each contractile response to plateau. However, whereas the tone induced by KCl and carbachol remained constant (no loss of tone for over 20 min),

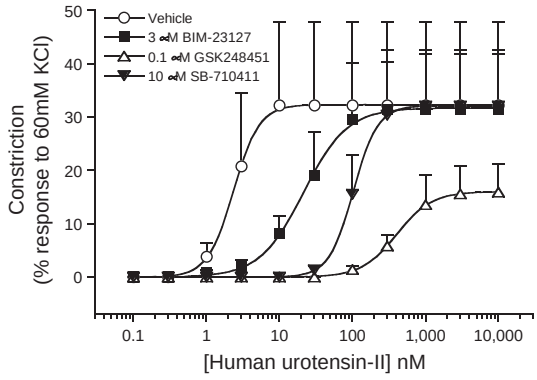

Fig. 3. The contractile effects of human urotensin-II in cat isolated trachea are competitively inhibited by BIM-23127, GSK248451 and SB-710411. Values are mean and vertical bars represent the S.E.M. Curves were derived by fitting to a logistic equation (Douglas et al., 1995).

it was of interest to note that the contractile tone induced by urotensin-II was less sustained (typically <50% tone remained after 20 min). Trachea and primary bronchi from one out of eight animals (12.5%) did not respond to human urotensin-II whereas tertiary bronchi from three out of eight animals did not respond (37.5%).

3.4. UT antagonists inhibit urotensin-II-induced contraction of cat isolated trachea

The contractile effects of human urotensin-II were inhibited by BIM-23127, GSK248451 and SB-710411 in cat isolated trachea in a surmountable manner (Fig. 3; Table 3). GSK248451 was the most potent antagonist studied. Pre-treatment with 0.1 μM GSK248451 caused a parallel, 220-fold rightward shift in the concentration–response curve to human urotensin-II. As such, GSK248451 was a sub-nanomolar functional antagonist (0.5 nM K_b). In contrast, pre-treatment with BIM-23127 (3 μM) or SB-710411 (10 μM) caused only 9- and 60-fold shifts (K_bs of 430 and 180 nM, respectively). The maximal response to human urotensin-II was not significantly suppressed by pretreatment with any of the

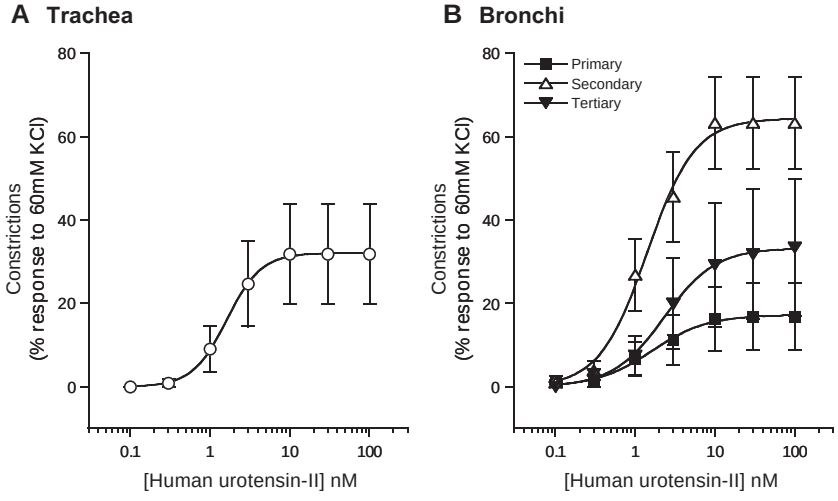

Fig. 2. Human urotensin-II is a potent spasmogen of cat isolated (A) trachea and the (B) primary, secondary and tertiary branches of bronchi. Values are mean and vertical bars represent the S.E.M. Curves were derived by fitting to a logistic equation (Douglas et al., 1995).

Table 3
Effects of peptidic UT receptor antagonists on human urotensin-II-induced contraction of cat isolated trachea

UT receptor antagonist	pK_b	pEC_{50}		E_{max} (% KCl)		n
		Vehicle	Antagonist	Vehicle	Antagonist	
BIM-23127 (3 μM)	6.36±0.11	8.68±0.09	7.78±0.13***	34±12	32±11	6
SB-710411 (10 μM)	6.74±0.07	8.68±0.09	6.93±0.04***	34±12	32±10	6
GSK248451A (0.1 μM)	9.27±0.12	8.62±0.07	6.35±0.13***	32±16	16±5	5

All values are expressed as mean±S.E.M. where n represents the number of animals from which tissues were isolated. Statistical comparisons to vehicle were made using a paired, two-tailed t-test where ***$P<0.001$.

three peptidic moieties, suggestive of competitive modes of antagonism.

4. Discussion

There is a growing body of evidence suggesting a role for the urotensin-II system in the (patho)physiological regulation of pulmonary function. However, urotensin-II-mediated smooth muscle contraction is species-dependent, in both isolated vascular (Douglas et al., 2000; Maguire et al., 2000; MacLean et al., 2000) and respiratory (Hay et al., 2000) tissues. For instance, whereas urotensin-II was devoid of any contractile activity in rodent isolated trachea (Douglas et al., 2000), it potently contracted non-human primate isolated tracheal and bronchial smooth muscle (Hay et al., 2000). Clearly, however, cynomolgus monkeys are of only limited availability to most investigators. Since urotensin-II was recently described as a potent and efficacious vasoconstrictor in the cat (both in vitro and in vivo; Behm et al., 2004a), the present study investigated the utility of this species as an alternative to the monkey for investigating the contractile effects of urotensin-II in isolated airway tissues.

Northern blot and RT-PCR analysis revealed the presence of UT receptor mRNA in cat trachea, bronchi and lung parenchyma. Although specific binding sites in these tissues were low (<3 fmol/mg), the presence of UT in the lung was sufficient to induce a functional response upon urotensin-II exposure. Namely, this cyclic undecapeptide contracted all sections of isolated cat respiratory tract including trachea and bronchi. Contractility was observed throughout the bronchial tree (i.e. in primary, secondary and tertiary bronchi). Urotensin-II was a potent spasmogen (EC_{50}s ~2–3 nM) although contractile efficacy was lower than KCl and carbachol (E_{max}s ranged from 20% to 60% 60 mM KCl). These observations were similar to those made in the monkey, where EC_{50}s and E_{max}s ranged from 0.6 to 3 nM and 10% to 40% 10 μM carbachol (Hay et al., 2000). Inter-animal variation was less evident in the cat as compared to previous observations in the monkey. For instance, whereas the secondary bronchus isolated from all eight cats responded to urotensin-II, there were little or no responses observed in airway smooth muscle isolated from three of seven monkeys studied (Hay et al., 2000). However, anatomical variability was observed in the cat (only five of eight tertiary bronchi responded). In addition, the maximal response to urotensin-II did not increase progressively from trachea to smaller airway regions in the cat as was previously observed in the monkey (Hay et al., 2000).

All three peptidic analogs studied (BIM-23127, SB-710411 and GSK248451) inhibited urotensin-II-induced contraction of cat isolated trachea in a competitive, surmountable fashion (although GSK248451 caused a trend towards suppression of the urotensin-II maximal response, the change was not statistically significant, possibly due to the high variations in maximal contraction) with potencies in correlation with (a) their binding affinities at the cat recombinant UT receptor and (b) their ability to inhibit contraction in the rat isolated aorta (Coy et al., 2000, 2003; Behm et al., 2002, 2003, 2004b; Herold et al., 2003). Most notably, GSK24845 was the most potent analog tested, where both its binding affinity (K_i) at the UT receptor and its inhibitory potency (K_b) in isolated trachea were sub-nanomolar (0.9 and 0.5 nM, respectively). These observations were similar to those made in cat arterial smooth muscle, where GSK248451 inhibited human urotensin-II-induced contraction in the cat femoral artery with a $K_b \sim 1$ nM (Behm et al., 2003), making GSK248451 the most potent competitive, functional UT receptor antagonist identified to date.

In summary, the present study is the first to describe the contractile effects of urotensin-II in respiratory tissues isolated from the cat and that such a response can be inhibited by UT receptor antagonists. As such, the cat might represent a species suitable for further evaluation of the urotensin-II/UT receptor system in the (patho)physiology of mammalian pulmonary function. In addition, GSK248451, a sub-nanomolar functional UT receptor antagonist, should be of utility for further examining the effects of the urotensin-II system in the regulation of pulmonary smooth muscle tone and growth in vivo.

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