Palosuran: clinical pharmacology of a urotensin-II receptor antagonist in Type 2 Diabetes Mellitus

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Introduction

Type 2 Diabetes Mellitus

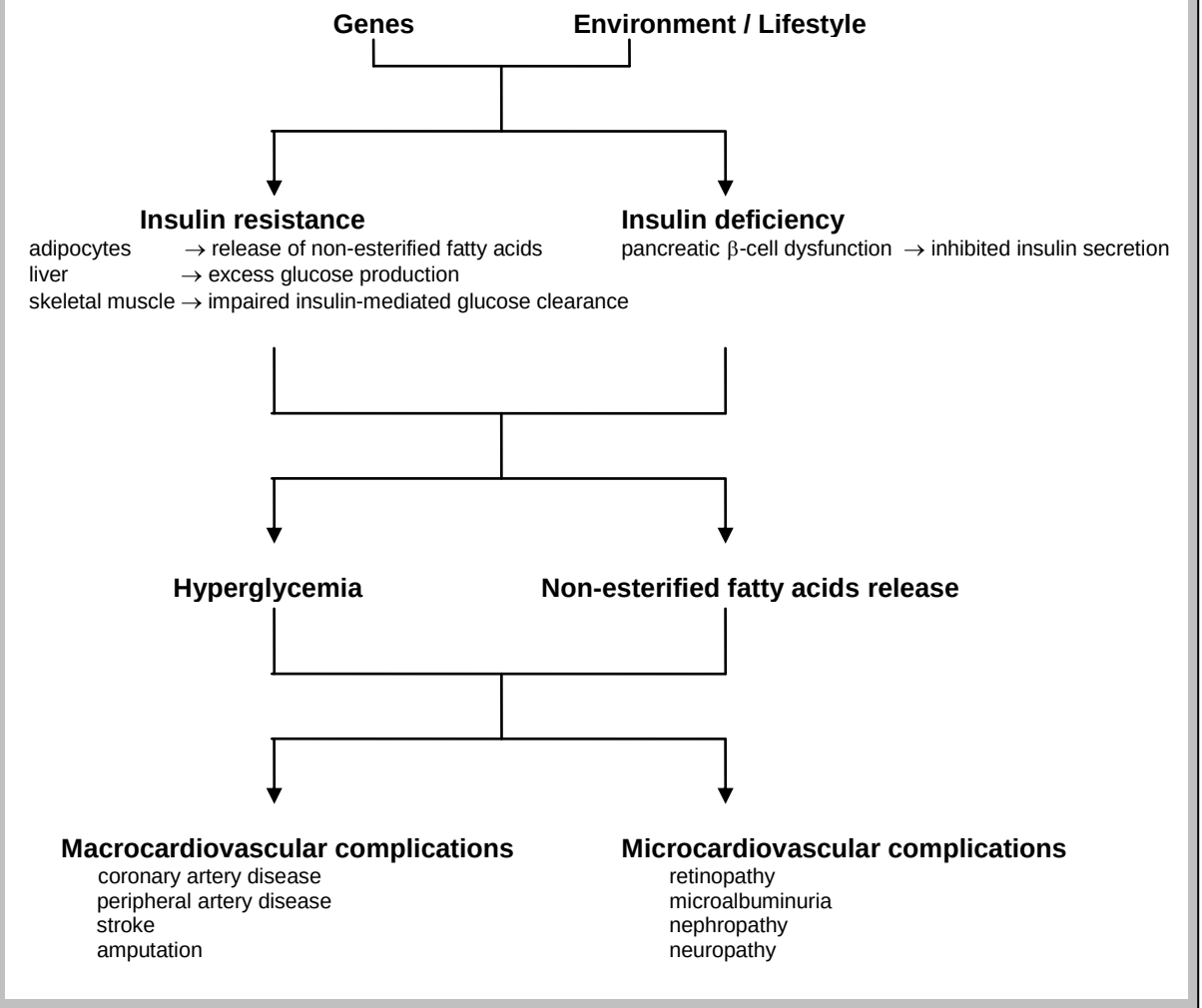
Type 2 Diabetes Mellitus (Type 2 DM) is a growing public health threat demonstrated by a dramatic increase in number of patients in the world. With approximately 194 million diabetic patients in 2003, this number is predicted to increase to 333 million by 2025 [1] due to improved life expectancy, population growth, and progressive urbanization. Type 2 DM increases the risk of hypertension and associated macro- and microcardiovascular diseases, including coronary, cerebrovascular, renal, and peripheral vascular disease [2]. Cardiovascular disease accounts for up to 80% of the deaths in individuals with Type 2 DM. The mortality associated with cardiovascular disease is reported to be 7.5 times greater among persons with Type 2 DM without a previous myocardial infarction than in those without diabetes [3]. Due to the difficult diagnosis of diabetes in early stages, and consequently initiation of proper treatment, the risk of diabetes-related complications is increased. It is estimated that approximately 25% of diabetics in the United States (US) are unaware of their condition [4].

Therefore, diabetes and its associated complications have a significant cost burden on society. For example in the US direct and indirect medical costs (due to work loss, disability, and premature mortality) have been estimated at $176 billion and $64 billion, respectively, for 2012, which is a tremendous increase compared to $116 billion and $58 billion, respectively, for 2007 [4,5]. Diabetic patients spend more time on health care services, thereby increasing healthcare costs [5], while the loss of working days leads to loss of productivity [6,7].

Pathophysiology

The mechanistic background for the disease is an imbalance between increased insulin requirement (insulin resistance) versus insufficient insulin availability (insulin deficiency) resulting in hyperglycemia and increased circulatory fatty acids. Figure 1 illustrates the main pathophysiological factors and consequences of Type 2 DM.

Figure 1 Pathogenesis and cardiovascular complications of Type 2 DM



Biologically, insulin resistance can be defined as diminished tissue response to insulin at one or more sites in the complex pathways of hormone action despite higher than normal plasma insulin levels (also known as compensatory hyperinsulinemia) [8]. Insulin

resistance is strongly associated with obesity and physical inactivity, and several mechanisms mediating this interaction have been identified. A number of circulating hormones, cytokines, and metabolic fuels, such as non-esterified (free) fatty acids (NEFA), originate in the adipocyte and modulate insulin action. Adipocytes can become overly large due to increase of stored triglycerides and as a result become resistant to insulin. Not regulated by insulin, adipocytes will release NEFA and glycerol, both of which will contribute to aggravate insulin resistance in skeletal muscle and liver [9].

Insulin deficiency is characterized by an abnormal insulin secretion pattern due to a pancreatic β-cell defect. Normal β-cell response to glucose is characterized by an early burst of insulin (first phase) release and a second phase characterized by a progressive increase in insulin secretion lasting several hours. The first phase is important as it inhibits the glucose release from the liver, and, thus contributes to the maintenance of normal glucose tolerance. The loss of the first phase insulin secretion can be used as a marker of β-cell dysfunction and can precede and predict overt Type 2 DM [8,10-12]. Potential mechanisms leading to β-cell dysfunction include reversible metabolic abnormalities (glucotoxicity, lipotoxicity), hormonal change (inadequate incretin action, increased glucagon secretion), genetic abnormalities of β-cell proteins, and reduction of β-cell mass (apoptosis) [8].

Treatments

Treatment of Type 2 DM is aimed at increasing *β*-cell function and lowering insulin resistance in order to lower blood glucose levels. The initial therapy is targeted towards improving tissue insulin sensitivity due to its fundamental role in the pathogenesis of Type 2 DM and its relationship to adverse cardiovascular outcomes. This includes life style intervention with modest exercise and weight loss, as well as various oral and s.c.

hypoglycemic agents, used either as mono- or combination therapy. Table 1 provides an overview of treatments available for Type 2 DM. Though most antidiabetic drugs are in general well tolerated, some risks remain. While serious side effects can be monitored and are infrequent, less serious side effects such as weight gain may affect patient compliance [13-15]. Also, while better treatments are available that control glycemic abnormalities and high blood pressure in Type 2 DM, there remains a high rate of cardiovascular and, specifically, renal risk within the diabetic population [16-18]. Therefore, there is a need for better and safer antidiabetic drugs.

New approaches to treatment of Type 2 Diabetes Mellitus

Diabetic nephropathy has become the leading cause of end-stage renal disease in the United States and Europe, accounting for approximately 40% of new cases in the United states [19] and up to 20% in Europe [20]. The earliest clinical manifestation of diabetic nephropathy is the development of low but abnormal levels of albumin in the urine (albuminuria). If not treated, microalbuminuria will progress to proteinuria, which correlates with a decline in renal function [16,21,22].

The underlying mechanism linking albuminuria and chronic renal failure has not been completely elucidated. It has been observed that systemic hypertension accelerates progression of diabetic nephropathy, and lowering blood pressure reduces renal damage. Indeed, reducing blood pressure with renin-angiotensin-aldosterone system blockers (RAAS blockers) has demonstrated protection of patients from cardiovascular and renal events [16,23]. However, these studies also showed that reduction of blood pressure cannot completely account for the therapeutic effect. Most likely albumin directly impacts chronic tubulointerstitial damage by eliciting pro-inflammatory and pro-

fibrotic effects through several pathways [21,24]. One of those pathways may be the urotensin-II (U-II) system.

Table 1 Current diabetic medications and drawbacks

Drug Class	Mechanism of Action	Side Effects
Thiazolidinediones	● Increase in hepatic insulin sensitivity ● Increase in muscle insulin sensitivity ● Suppression of NEFA release ● Fat redistribution (visceral to subcutaneous)	Hepatic injury
Metformine	● Inhibition of glucose production ● Increase in hepatic insulin sensitivity	GI effects Lactic acidosis
α-glucosidase inhibitors	● Inhibition of glucose absorption ● Stimulation of GLP-1 release	Hepatic injury
Sulfonylurea derivatives	● Acute stimulation of insulin release	Hypoglycemia Weight gain
Exogenous insulin	● Inhibition of glucose production ● Increase in muscle insulin sensitivity	Hypoglycemia
Metglinide	● Acute stimulation of insulin release	Hypoglycemia
GLP-1 analogues	● Acute stimulation of insulin release ● Stimulation of insulin biosynthesis ● Inhibition of β-cell apoptosis ● Stimulation of β-cell differentiation	GI effects
Amylin analogues	● Delay of gastric emptying ● Inhibition of glucagon release	GI effects

Objectives of this thesis

The thesis focuses on the urotensin-II (U-II) system and its relevance to Type 2 DM treatment (Part I), the clinical pharmacology of the urotensin-II receptor (UT receptor) antagonist palosuran (Part II) in healthy subjects, and the clinical pharmacology of palosuran in Type 2 DM (Part III). Palosuran is a non-peptide, oral, selective UT receptor antagonist that was the first in its class that was tested in humans. The thesis will discuss the pharmacokinetics and pharmacodynamics in healthy subjects, as well as pharmacokinetic and clinical data in patients, which contributed to the clarification of the (patho)physiological role of U-II.

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Part I: The Urotensin-II system: a new approach to treatment of Type 2 Diabetes Mellitus?

Chapter 1 What is known about Urotensin-II?

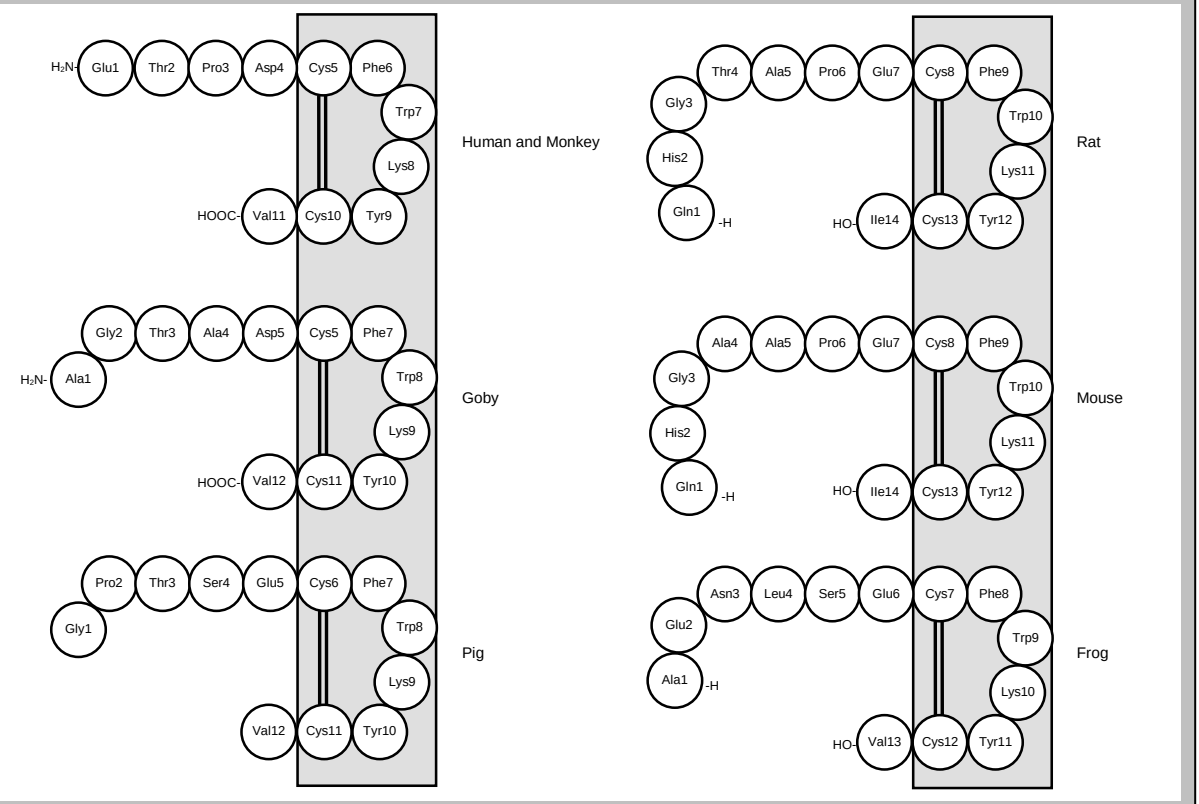
Urotensin-II

The first reports on Urotensin-II (U-II) were published in 1969 by Bern et al., when they identified a urophysial peptide isolated from an extract of goby fish, Gillichthys mirabilis [1]. It was not until the nineties that the function of this peptide was further elucidated. In fish, U-II is involved in cardiovascular regulation, osmoregulation for seawater adaptation, and the regulation of lipid metabolism [2]. For many years considered a potent vasoconstrictor in lower organisms and fish, interest in this system was low until homologs were identified in mammals [3] and Ames et al. cloned a novel human G-protein-coupled receptor for U-II [4]. This human receptor GPR14 was later renamed urotensin (UT) receptor and revived the interest in this field [4-6].

In humans U-II is composed of 11 amino acid residues which is shown in Figure 1 [7,8]. Across species the peptide exhibits a cyclic portion comprising six amino acids linked by cysteine disulfide bridges (Figure 1), indicating that this region is responsible for the biological activity [7-9].

U-II derives from pre-pro U-II, which contains 124-139 amino acid residues. The identity, location, and regulation of urotensin converting enzymes (UCE) which form biologically active human U-II (hU-II) from pro hU-II have only recently been investigated with furin and trypsin able to convert a 25 amino-acid C-terminal fragment of pro hU-II [9]. Furin plays a role in the cleavage of a number of precursor hormones including human pro endothelin-1 and human pro parathyroid hormone, and is characterized by sensitivity to pH, and to the ionic composition within its surrounding milieu. However in assays, in which recombinant furin activity was inhibited by low pH and altered ionic composition of medium, some residual intracellular UCE activity remained, suggesting additional endogenous U-II convertases [9].

Figure 1 Examples of the amino acid sequences of mammalian, amphibian, and fish U-II.



Although the amino acid terminus of U-II differs between species, all isoforms share a conserved cyclic hexapeptide (as marked in the shaded box)

As UCE activity is mainly present in intracellular compartments, similarly to endothelin converting enzyme, processing of the U-II prohormone occurs within cells, with mature peptide secreted from the cells [9].

U-II and UT receptor are strongly expressed in the CNS but also widely expressed throughout peripheral tissues including the heart, vasculature (endothelial and smooth muscle), kidney, liver, adrenal, and other sites. Such distribution suggests that U-II is a potential autonomous regulator of cardiovascular function [10,11]. Furthermore, the presence of circulating U-II in blood indicates that U-II might also be an endocrine factor [3].

Biological response to U-II in the vascular system

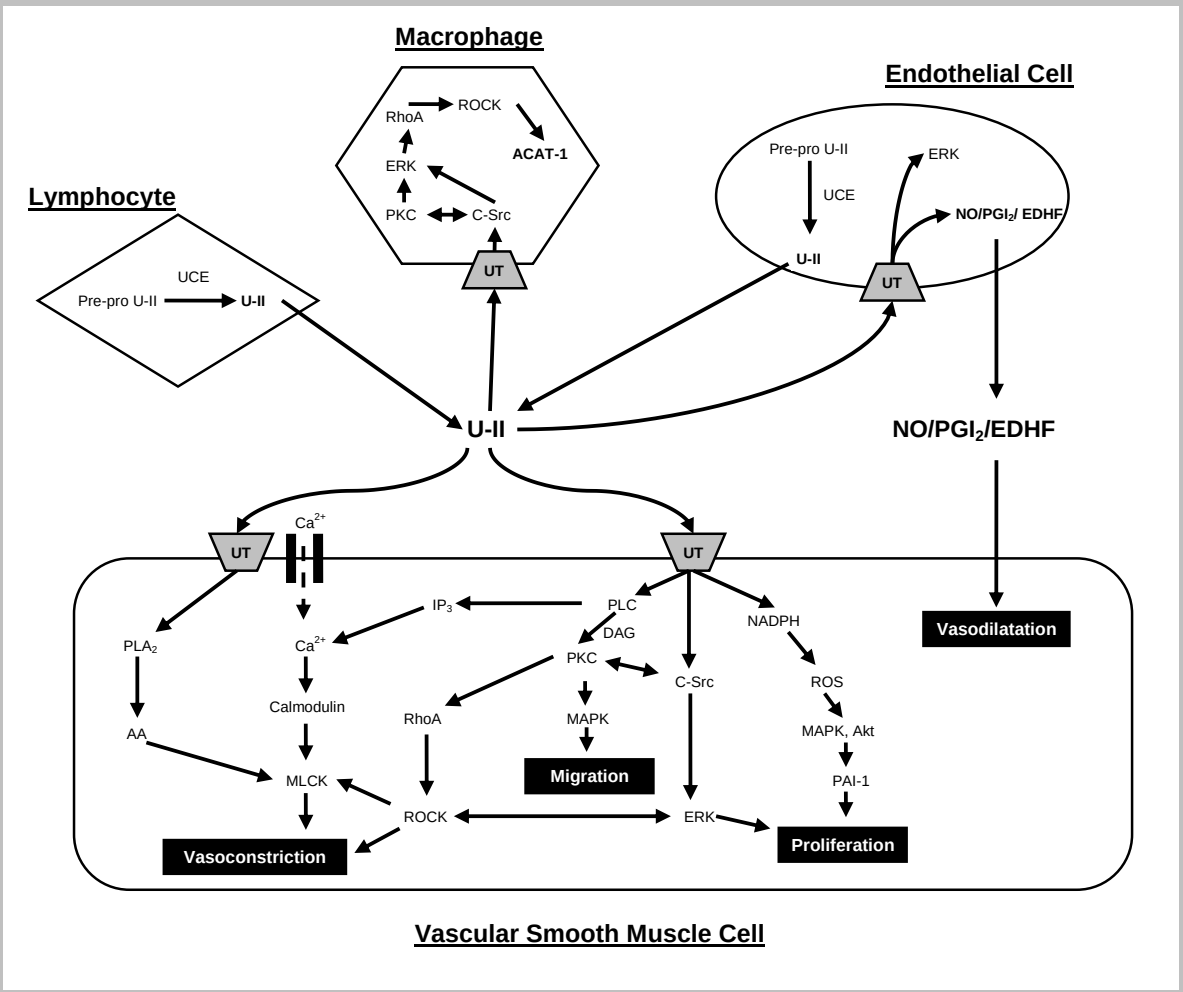
Human U-II induced potent and efficacious contractions of the isolated thoracic aorta of rat, with an order or magnitude larger than other vasoactive peptides such as endothelin-1, noradrenaline, and serotonine [4]. Thus, U-II is the most potent vasoconstrictor identified up to date [4,7]. However, it was observed that response to U-II varies between species, between types of blood vessel, and even between individual vessels of the same type, which is unlike the response of vascular tissues to endothelin-1 and make the role of U-II in vascular systems more difficult to predict [5].

Through binding to the UT receptor, U-II activates the inositol trisphosphate system leading to the release of intracellular calcium and, consequently, vasoconstriction. Additionally, vasoconstriction is mediated by ERK1/2 and RhoA/Rho kinase related pathways [12]. These two pathways are also important in vascular smooth muscle cell proliferation and migration [13]. On the other hand, U-II also demonstrated endothelium-dependent vasodilatory properties through nitric oxide, Prostaglandin I_2, Prostaglandin E_2, and endothelium-derived hyperpolarizing factor (EDHF) release [12]. A schematic overview of the vascular urotensin system is given in Figure 2.

Activity of hU-II *in vitro* and *in vivo*

The effects of U-II have been studied in different mammalian species in a number of *in vitro* and *in vivo* systems. Depending on the species and vessel studies, contrasting responses have been observed **(**Table 1). The most obvious explanation for this variability is that the level of receptor expression is low and possibly absent or below the density required to elicit a response to the peptide. Age might be another contributing factor as it has been observed that the effect of U-II on vascular smooth muscle

Figure 2 Summary of the intracellular pathways of U-II mediated vasoconstriction, proliferation, and migration.



AA = arachidonic acid; ACAT-1 = acyl-coenzyme A:cholesterol O-acyltransferase-1; Akt = serine/threonine protein kinase B; c-Src = Src kinase; Ca^{2+} = calcium; DAG = diacylglycerol; EDHF = endothelium-derived hyperpolarizing factor; ERK = extracellular signal regulated kinase; IP_3 = inositol trisphosphate; MAPK = mitogen-activated protein kinase; MLCK = myosin light chain kinase; NADPH = nicotinamide adenine dinucleotide phosphate-oxidase; NO = nitric oxide; PGI_2 = prostacyclin I_2; PKC = protein kinase C; PLA_2 = phospholipase A_2; PAI-1 = plasminogen activator inhibitor-1; PLC = phospholipase C; ROCK = rho kinase; ROS = reactive oxygen species; U-II = urotensin-II; UCE = urotensin converting enzyme; UT = urotensin receptor.

contraction in aorta diminishes with age in rats [14]. Also, the initial contractility studies and vascular smooth muscle cell proliferation experiments with U-II were performed with rat aorta or cultured rat aortic vascular smooth muscle cells. The contractile responses of the aorta to U-II are atypical as the efficacy of U-II in this vessel is much higher than in many other isolated blood vessels. In these studies, vascular preparations often were stripped of endothelium, which is certainly not physiological. It is therefore possible, that

the vasoconstriction is in part related to the experimental conditions and may not be relevant to in vivo effects [15].

To date, only few studies have investigated the function of hU-II in an *in vivo* setting (Table 2). Also in these studies, the *in vivo* vasocontrictor activity of hU-II in rats, monkeys, and humans was not consistent among studies and seems to be dependent on the animal model used, differences in species, and method of delivery [16]. These differences become very evident in *in vivo* studies in humans. Following infusion of hU-II in the brachial artery, Böhm et al. reported potent, significant reduction in forearm blood flow. However, using a very similar methodology, Wilkinson et al. could not detect any effects of hU-II [17,18]. As these studies were performed in healthy subjects, the true importance of U-II *in vivo* may only be fully evaluated in pathology, for example when endothelial cell function is compromised, in diffuse peripheral arterial disease, or under circumstances in which the UT receptor system is upregulated [9]. This seems to be supported by the findings of Lim et al., who showed a difference in hU-II skin microcirculation response between healthy subjects and patients with chronic heart failure [19].

U-II in cardiovascular disease

Atherosclerosis

In the Apolipoprotein E gene knockout mouse model of atherosclerosis, an increase in UT receptor expression was observed in aortic tissue [37]. A selective induction of UT expression in vascular smooth muscle cells in these mice resulted in far greater aortic lesions when compared to wild-type mice [38]. Although no difference in UT expression has been observed between vascular smooth muscle cells of healthy humans and patients with atherosclerotic coronary arteries, increased expression of UT receptors

has been observed in human abdominal aortic aneurism and carotid atherosclerotic extracts [39,40]. Further, plasma U-II levels are elevated in patients with confirmed atherosclerosis [41]. It has been suggested that U-II is involved in the control of vascular

Table 1 Vascular responses to hU-II in vitro.

Species	Vascular tissue	Response to hU-II	Reference
Mouse	Thoracic and abdominal aorta	Unresponsive	[20,21]
Rat	Thoracic aorta	Vasoconstriction	[4,20,22]
	Femoral, mesenteric, renal, and abdominal aorta	Unresponsive	[4,22]
	Carotid and coronary arteries	Vasoconstriction	[21,22]
Guinea pig	Thoracic aorta	Unresponsive	[23]
Rabbit	Thoracic aorta; coronary artery	Vasoconstriction	[24]
	Pulmonary and ear arteries; ear veins	Unresponsive	[24]
Dog	Coronary artery	Vasoconstriction	[20,21]
	Thoracic aorta	Unresponsive	[20,21]
Pig	Coronary, renal, mammary, and carotid artery; saphenous vein	Unresponsive	[20,21]
Marmoset	Thoracic artery	Vasoconstriction	[20,21,23]
Cynomolgus monkey	Coronary, pulmonary, renal, femoral, mesenteric, internal mammary, basilar arteries; thoracic and abdominal aorta veins	vasoconstriction	[4,20,21,25]
Human	Coronary, radial, and mammary arteries; pulmonary arteries (endothelium removed)	Vasoconstriction	[25-27]
	Vessels (endothelium intact)	Unresponsive	[11]
	Umbilical, facial, epigastric, and saphenous veins (endothelium removed)	Vasoconstriction	[23,27]
	Saphenous veins	Unresponsive	[25]
	Small pulmonary and abdominal adipose tissue arteries	Vasodilation	[28]

Table 2 Vascular responses to hU-II *in vivo*.

Species	Model	Route of administration	Result	Reference
Rat	Anesthesized rat	Bolus i.v.	Vasodepressor response Concomitant tachycardia	[29]
	Conscious rat	Bolus i.v.	Dose-dependent tachycardia Vasodilatation	[30]
Sheep	Conscious ewes	Intracerebroventricular infusion	Increase in adrenocorticotropic hormone and adrenaline levels; increased cardiac output; increased arterial pressure, peripheral vasodilatation; hyperglycemia	[31]
	Conscious ewes	Bolus i.v.	Tachycardia; reduced cardiac stroke volume	[31]
Cynomolgus monkey	Anesthetized monkey	Bolus i.v.	Systemic vasoconstriction; severe myocardial depression;fatal circulatory collapse	[4,32]
Human	Forearm blood flow study in healthy subjects	Local infusion	Dose-dependent reduction in forearm blood flow	[17]
	Forearm blood flow study in healthy subjects	Local infusion	No effects	[18,33]
	Cutaneous microcirculation in healthy subjects and patients with chronic heart failure	Iontophoresis	Healthy subjects: vasodilatation; Patients: constriction of forearm skin microcirculation	[19]
	Cutaneous microcirculation in healthy subjects and patients with essential hypertension	Iontophoresis	Healthy subjects: vasodilatation; Patients: vasodilatation / vasoconstriction	[34]
	Cutaneous microcirculation in healthy subjects and patients with essential hypertension	Iontophoresis	Healthy subjects: vasodilatation; Patients: vasoconstriction	[35]
	Cutaneous microcirculation in healthy subjects and patients with liver cirrhosis	Iontophoresis	Healthy subjects: vasodilatation; Patients: constriction of forearm skin microcirculation	[36]

remodeling by inducing smooth muscle cell proliferation and fibroblast-mediated collagen deposition, which play an important role in the etiology of atherosclerosis [42-44]. In addition, inflammatory compounds such as LPS, TNF-α, and IFN-γ all upregulate UT receptor mRNA expression [45], alluding to the chemotactic and signaling roles that U-II may play in the progression of atherosclerosis.

Congestive heart failure (CHF) and other cardiac diseases

Myocardial remodeling, including hyperthrophy, apoptosis, interstitial fibrosis, and vascular endothelial cell dysfunction are factors that contribute to the pathogenesis and progression of CHF.

U-II expression and U-II plasma levels are increased in many types of cardiac disease. In a rat coronary ligation model of left ventricular myocardial infarction, pre-pro U-II mRNA and expression of UT receptors was elevated in the non-infarct and infarct regions with preferential up-regulation in the right ventricle [9,42,46]. This is in line with the observation of preferential up-regulation of UT receptors in the right ventricle of rats with right heart failure secondary to pulmonary hypertension [47]. Similar observations have been reported in humans; U-II and UT receptor expression were increased in proportion to disease severity in infarct and non-infarct zones of patients with myocardial infarction [42].

Several studies have demonstrated that overexpression of the UT receptor system or stimulation with U-II produced a hypertrophic phenotype in cultured rat neonatal cardiomyocytes [42,48]. A mechanism for U-II mediated hypertrophy may also involve the stimulated release of cytokines from cardiac myocytes. Rat cardiac myoblasts overexpressing UT receptors were incubated with U-II resulting in an increase in interleukin-6 and the development of a hypertrophic phenotype [49].

In myocardial specimens from patients with CHF, immunohistochemical analysis demonstrated strong cardiomyocyte expression of U-II and UT receptors [50]. The presence of U-II in the cardiomyocytes correlated significantly with left ventricular end-diastolic volume and was inversely correlated with ejection fraction. A subsequent study found that U-II plasma levels are also significantly elevated in patients with CHF, and that U-II levels are inversely correlated to ejection fraction [51] or correlated with severity of disease as measured by New York Heart Association functional class [52]. However, there are also study reports in which levels of U-II were not significantly elevated [53].

U-II is also clearly implicated in coronary artery disease [54], left ventricular systolic [52] and diastolic [55] dysfunction and myocardial infarction [32] in humans. Lastly, plasma U-II levels correlate positively with ET-1, adrenomedullin, and N-terminal brain natriuretic peptide [50,56].

Essential Hypertension

The role of the U-II system in the development of essential hypertension is unclear. Indeed, systemic hypertensive responses to U-II were observed in several animal models. However, these effects were not uniform across species [30,57] and were mostly observed in the absence of an intact endothelium. Therefore, the contribution of U-II to hypertension is most likely revealed under conditions of co-existing cardiovascular disease in which endothelial dysfunction is prevalent. In what can be thought of as a cause-and-effect relationship, U-II causes potent vasoconstriction leading to hypertension. Hypertension in turn increased turbulent hemodynamic flow and shear stress on the endothelium leading to endothelial damage and endothelial dysfunction. This endothelial dysfunction then further comprises the arterial system [58].

Increased plasma U-II levels have been observed in spontaneously hypertensive rats [59] as well as in hypertensive patients [60], suggesting an up-regulation.

Pulmonary hypertension

Pulmonary hypertension is a multifactorial disorder characterized by vasoconstriction and pulmonary vascular remodeling [61]. An activated UT receptor system may contribute to the pathogenesis of pulmonary hypertension by remodeling of the pulmonary vasculature. Hypoxia is a well-known cause of pulmonary hypertension. Although one study in chronically hypoxic rats did not observe any increase in plasma U-II levels [47], in another study hypoxia was found to specifically increase U-II in endothelial and smooth muscle cells in the pulmonary arteries of rats [47,62]. The function of U-II in pulmonary hypertension in humans has not been elucidated and data on this topic is not consistent. As previously mentioned, the actions of U-II on the pulmonary circulation are quite variable. Although U-II did not change pulmonary artery perfusion pressure in human isolated perfused lungs, it may be, again, due to masking of the effect by U-II stimulated nitric oxide synthase activity in the endothelium. As endothelial dysfunction is prevalent in patients with pulmonary hypertension [63,64], U-II could still contribute to the pathogenesis of this disease.

Hepatic Disease

It is known that vasoconstrictive substances are important in liver pathologies such as portal hypertension [36,65]. First evidence that U-II may have a pathological role in chronic liver disease has recently been published. In normal rats, continuous infusion of U-II over a time period of 4 weeks induced a significant dose-dependent increase in portal venous pressure. Other effects were an up-regulation in the hepatic transcript for transforming growth factor-β and platelet-derived growth factor-β (both key profibrotic

cytokines) and liver fibrosis as demonstrated by increased hepatic hydroxyproline [36,65]. Liu et al. observed an increase in UT mRNA in liver tissue of cirrhotic patients, when compared to healthy controls. Plasma U-II levels in patients with cirrhosis and portal hypertension were significantly increased and correlated with the extent of portal hypertension [66,67]. Baseline plasma U-II may be used as a predictive marker for determination of survival or disease deterioration [36].

Adrenal Tumors and other Cancer Types

Cancer may be another field in which the U-II system may play a role. U-II and UT receptor mRNA is expressed in several adrenal tumor cell lines (including adrenocortical carcinoma), cervical cancer, and renal carcinoma cells [68,69]. In particular, substantial U-II and UT expression alterations were observed in a number of adrenal cancers [70]. U-II and UT receptor mRNA have both been detected in human lung adenocarcinoma cells. Administration of U-II to nude mice bearing human lung adenocarcinoma cells resulted in a significant increase in tumor volume and tumor weight [71]. In prostate adenocarcinoma cells of cancer patients, UT receptor mRNA was always expressed in hyperplastic tissues and at high intensity in well-differentiated carcinoma. When stimulating the cells with urantide (a U-II agonist) *in vitro* cell motility was decreased and invasion by androgen-dependent LNCaP cells was increased. These findings suggest that U-II may contribute to the pathogenesis of different tumor types by acting as an autocrine/paracrine growth stimulating factor [58,72] and in some cancers may be utilized as a prognostic marker [73].

Protective effects of U-II

An emerging concept proposes that the observed increases in U-II levels in a number of cardiovascular and renal diseases may actually be protective in nature. Although, as

discussed earlier, many reports indicate increased U-II levels in disease when compared to the physiological condition in some individuals a high U-II level appears to correlate with a protection against inflammation, endothelial dysfunction, and cardiovascular adverse events (AEs). For example, when compared to patients with stable coronary artery disease and with healthy subjects, those patients with acute cardiac ischemia displayed lower circulating levels of U-II [74]. There may also be a protective effect from high U-II levels in post-MI patients, as higher levels are associated with a lower risk of AEs [75]. Proposed mechanisms are the effect of U-II on the sympathetic and NO system, as well as a beneficial effect on volume overload and myocardial contractility [76]. Futher studies using UT receptor antagonists or adopting a prospective study design are needed to understand better the functional roles of U-II.

Summary and outlook

Since the discovery of U-II and the UT receptor in humans some 20 years ago much work has been done to further characterize the role and mechanism of action of U-II in a variety of diseases. UT receptor antagonism may become a significant therapy for a number of diseases. Another field of specific interest, which was not discussed in this chapter, is the role of the U-II system in renal diseases (including metabolic syndrome and Type 2 DM). With rising numbers of patients suffering from renal disease, metabolic syndrome, and type 2 DM and current treatments not fully addressing the need of these patients, U-II antagonism might be an interesting new approach to treatment.

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Chapter 2 The role of urotensin in renal disease

Introduction

In the previous chapter U-II and its role in cardiovascular disease were discussed in detail. This chapter will focus on the role of U-II in renal disease which includes metabolic syndrome, Type 2 DM, and end stage renal disease. The metabolic syndrome is a cluster of metabolic abnormalities, including central (abnormal) obesity, raised fasting glucose, raised blood pressure, raised triglycerides, and reduced HDL cholesterol. It is associated with insulin resistance, endothelial dysfunction as well as prothrombotic and proinflammatory status, which are themselves independent risk factors for cardiovascular disease and diabetes [1,2]. Type 2 DM is a metabolic disease characterized by insulin resistance and insulin deficiency caused by a defective pancreatic *β*-cell response to glucose. The physiological and pathological roles of U-II in animals and humans in these diseases will be reviewed as well as the effects of the U-II receptor antagonist palosuran in animal models of renal disease.

Biological response to U-II in the kidney, liver, pancreas, and CNS

In the previous chapter the effect of U-II on vascular tone has been described in detail. Besides the cardiovascular effect, U-II is considered to have other properties that contribute to renal and metabolic disease.

The kidney plays a pivotal role in controlling cardiovascular homeostasis, and influences both cardiac preload (plasma volume) and afterload (peripheral resistance) through regulated natriuresis and diuresis and the control of vasomotor tone. In addition to acting as a potent renal-artery spasmogen, U-II may directly regulate transepithelial transport of electrolytes. While this effect has been observed in fish, it has not yet been fully investigated in other species. However, the finding that, in rats, renal blood flow

and urinary water and Na^+ increased after U-II infusion, indicate a possible role of U-II in Na^+ ion transport in the collecting duct [3].

U-II and UT receptor have been identified in liver and pancreas and may have direct effects on glucose mobilization and insulin secretion by pancreatic β cells. In the central and peripheral nervous system U-II has been associated with increased release of adrenocorticotropic hormone (ACTH) and adrenaline through sympathoadrenal and pituitary-adrenal pathways [4]. ACTH stimulates release of cortisol, which mediates renal vasodilatation [4-6]. Release of ACTH is accompanied by sustained cardiovascular and metabolic changes, including hyperglycemia as a result of cardiac β-adrenoceptor stimulation [7]. The increase in ACTH leads to an increase in insulin secretion. Insulin, besides its metabolic effects, induces endothelium-dependent vasodilatation and increases glucose uptake in peripheral tissues. It is possible that U-II impairs both of these actions and causes insulin resistance similar to endothelin-1 [8]. U-II is associated with an increase in plasma free fatty acids and enhances lipogenesis by increasing glucose-6-phosphate dehydrogenase activity and NADP production. U-II enhances depot lipase activity, which may lead to hyperlipidemia. Further, U-II may contribute to insulin resistance through its inflammatory effects and promotion of endothelial dysfunction [9].

UT receptor mRNA is found in the hypothalamus, which plays a major role in sleep and feeding behavior [10].

Limited information of the effects of U-II on the liver, pancreas, and renal function is available. Also, most studies were performed with U-II of mammal or amphibian nature, making it challenging to interpret the data and evaluate its relevance to humans. An overview of studies performed with hU-II *in vitro* and *in vivo* is shown in Tables 1 and 2.

Table 1 Renovascular responses to hU-II *in vitro.*

Species	Vascular tissue	Response to hU-II	Reference
Rat	Renal arteries	Unresponsive	[3,11,12]
	Small renal arteries	Endothelium-dependent vasodilatation;NO-release from intact endothelium	
Mouse	Renal arteries	Unresponsive	[13]
Dog	Renal arteries	Unresponsive	[13]
Pig	Renal arteries	Unresponsive	[13,14]
Monkey	Renal arteries	Vasoconstriction	[13]

Table 2 Responses to hU-II *in vivo.*

Species	Model	hU-II route of administration	Result	Reference
Rat	Anesthesized rat	Continuous infusion	No effect on mean arterial pressure; dose-dependent increase in renal blood flow, glomerular filtration rate; and urinary water/sodium excretion. All effects blocked by L-NAME	[3]
Man	Cutaneous microcirculation in healthy subjects and patients with DM	Iontophoresis	Healthy subjects: vasodilatation; Patients: constriction of forearm microcirculation	[15]

U-II in renal and metabolic disease

Renal disease

Levels of pre-pro U-II mRNA expression in kidney vary considerably between studies. In rat and mouse kidney hardly any U-II was detected [16], while sheep exhibited renal production of U-II [17]. Also in human kidney the amount of U-II expressed varied [11,18-20]. The cause of disparity in expression levels between studies is unknown, but may be related to differential regulation between subjects.

In humans, U-II was found in distal and proximal convoluted tubules, glomeruli, collecting tubules and collecting ducts, and in endothelial cells in renal arteries [21]. UT mRNA is mainly expressed in the renal cortex [19,22]. In renal biopsy tissue of patients with diabetic nephropathy U-II and UT mRNA were increased by 45 to 2000-fold when compared to normal subjects [23], suggesting a role of U-II in the progression of renal disease. This is in line with data by Totsune et al. who showed that, compared to healthy subjects, circulating levels of U-II-like immunoreactivity were 2- and 3- fold higher in patients with renal dysfunction not on dialysis and patients with renal dysfunction on dialysis, respectively [20]. In this study plasma and urinary U-II levels were also increased in diabetic patients with renal dysfunction when compared to diabetics with normal renal function. In patients with hypertensive renal disease urinary U-II-like immunoreactivity was higher compared to normotensive renal disease patients, which may be the result of hypertensive target organ damage [19].

Children with minimal change nephrotic syndrome (MCNS) showed decreased plasma U-II and increased urinary U-II during relapse. No relationship between U-II and clinical and/or laboratory parameters could be established. Thus, although changes in plasma and urine U-II were observed during relapse, this may be the result of marked

proteinuria rather than reflecting a role in mediating the clinical and laboratory manifestations in children [24].

U-II is altered in patients with end-stage renal disease (ESRD). Interestingly, an inverse correlation to risk was observed. While plasma U-II levels were elevated in ESRD compared to healthy controls, patients had reduced endothelial activation and levels of biomarkers of atherosclerosis were decreased [25,26]. Also, a more favorable echocardiographic profile and a lower overall cardiovascular risk were observed [27]. These findings would point toward a protective role of U-II in some forms of renal disease through interference of U-II with sympathetic and NO systems [28].

Metabolic syndrome and Type 2 DM

In addition to its effect on blood pressure, U-II may contribute to progression of metabolic syndrome and Type 2 DM through other pathways.

A neurohormonal role affecting insulin secretion has been suggested based on several observations. UT is expressed in human liver and pancreas [29,30]. In perfused rat pancreas, infusion of U-II inhibited glucose-induced insulin secretion, not affecting glucagon, somatostatin, and basal insulin secretion [31-33]. Sheep displayed hyperglycemia after infusion of U-II, suggesting a central effect of U-II leading to increases in epinephrine and cortisol levels [4], which trigger increased insulin secretion. U-II and UT receptor in tubular epithelial cells may play a role in activation of vasoactive hormone, injurous cytokines, and extracellular matrix proteins in the diabetic state [34].

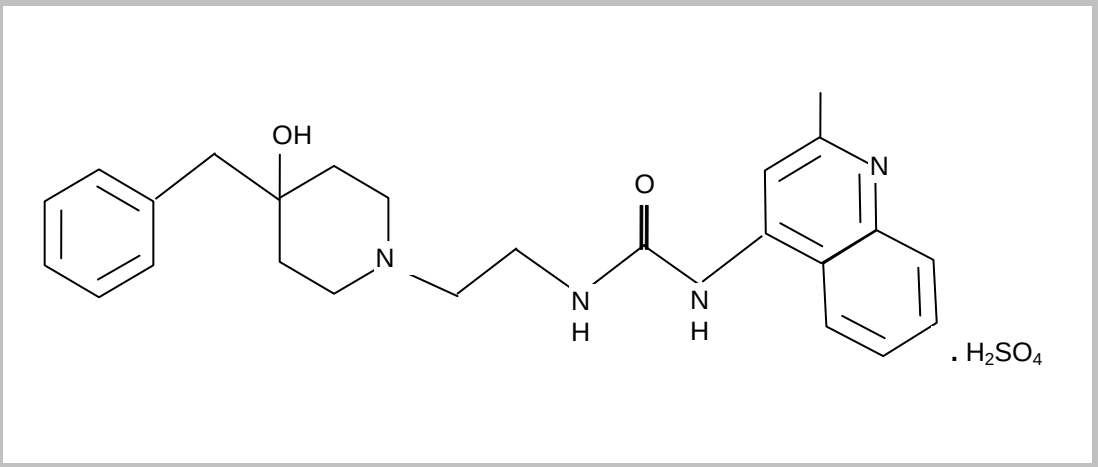
Diabetic mice exhibit higher concentrations of U-II and UT mRNAs in skeletal muscle [35]. In streptozotocin-induced diabetic rats, expression of U-II and UT was significantly upregulated at both mRNA and protein levels in the diabetic kidneys compared with

controls. The upregulated expressions of U-II and UT in the kidney were accompanied by significantly increased renal TGFβ1 expression, renal extracellular matrix (fibronectin and collagen IV) accumulation, and renal dysfunctions [36]. In human diabetic patients plasma levels of U-II are elevated irrespective of the presence or absence of proteinuria. The elevation is independent of fasting plasma glucose or blood glycosylated hemoglobin (HbA_{1C}) level, suggesting that the production or release of U-II is not due to hyperglycemia [9,30,37]. The role of U-II is further suggested based on findings by Ong et al. It was observed that the region 1p36 in human chromosome 1 contained a locus which is associated with a higher susceptibility to developing DM2 in Chinese and Japanese [9]. The exact disease-containing gene in this locus is unknown, but the gene encoding U-II is located at 1p36 and may be one of the candidate genes. Further, the UT receptor has been suggested to play a role in the development of impaired glucose tolerance (IGT), a prediabetic condition. However, these association studies do not prove causation and no data have been generated in other populations, such as Caucasians [9].

The urotensin-II receptor antagonist palosuran

Palosuran (ACT-058362; 1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulfate salt) is a potent and specific antagonist of the human UT receptor (Figure 1). In *in vitro* binding assays, palosuran demonstrated selective binding and competitive mode of antagonism on the human UT receptor [38]. *In vivo*, palosuran prevented the no-reflow phenomenon after renal artery clamping in rats, without a decrease in blood pressure. Subsequent development of acute renal failure and the histological consequences of ischemia could be prevented in this model [38].

Figure 1 Chemical structure of palosuran sulphate salt.



$C_{25}H_{32}N_4O_6S$ (molecular weight = 516.6)

In a rat model of diabetes, palosuran was able to improve the pancreatic and renal function [39]. In this accelerated model, rats were injected with streptozotocin and underwent unilateral nephrectomy, without administration of insulin. The administration of streptozotocin destroyed pancreatic β cells, leading to insulin-sensitive hyperglycemia, and associated complications, including nephropathy [39]. After chronic treatment with palosuran for 25 weeks, more than double the numbers of diabetic rats survived, when compared to untreated rats. During treatment with palosuran for 16 weeks, the drug prevented a further increase in glycemia, as well as an increase in triglycerides and decreased serum cholesterol when compared to untreated diabetic rats. HbA_{1C} concentrations markedly increased in the untreated diabetic rats and slightly but significantly reduced in the diabetic rats treated with palosuran. Histopathology of the pancreas at the end of the 16-week treatment period showed that diabetic rats had smaller and fewer β-cells than non-diabetic rats. While rats treated with palosuran still had a decreased number of β-cells, the cells were larger when compared to untreated diabetic rats. Further, albuminuria and renal damage was assessed in this model. While

albuminuria increased rapidly with time in the untreated diabetic rats, palosuran could attenuate, but not normalize, the albuminuria. Palosuran had no significant effect on renal vascular resistance, but significantly increased renal plasma flow and glomerular filtration rate. It was suggested by the histopathology that palosuran decreased incidence and severity of renal lesions (tubular degeneration/regeneration, tubular vacuolation, glomerulosclerosis) in diabetic rats [39].

Summary and conclusion

Besides a prominent role of U-II as the most potent vasoconstrictor known to date, U-II and its receptor may also play a prominent role in the development of renal and metabolic diseases, such as Type 2 DM. Indeed, in kidney tissue of mice, rats, and humans, U-II and its receptor have been identified and increased levels of U-II and UT receptor have been observed in patients with renal dysfunction and diabetes. Apart from its renovascular effect, it is suggested that U-II has a hypo-osmotic and neurohormonal function, which, when disrupted, can contribute to progression of renal and metabolic disease.

In a rat model of diabetes, treatment with the UT receptor antagonist palosuran improved survival, increased insulin levels and slowed its release in glycemia, HbA_{1C} and serum lipids. Furthermore, palosuran increased renal blood flow and delayed the development of proteinuria and renal damage.

Thus, UT receptor antagonists may constitute a new class of treatments for renal and metabolic diseases. It is therefore of importance to explore the potential of UT receptor antagonists in humans.

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Part II: Clinical Pharmacology of the urotensin-II receptor antagonist palosuran in healthy subjects

Chapter 3 Pharmacokinetics and pharmacodynamics of the urotensin-II receptor antagonist palosuran in healthy male subjects.

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Abstract

Palosuran is a new potent and specific antagonist of the human urotensin II (U-II) receptor (UT receptor). This entry-into-human study evaluated the tolerability and safety, pharmacokinetics, and pharmacodynamics of palosuran in a double-blind, placebo-controlled, single ascending-dose design. Oral doses of 5 to 2000 mg were given to 9 sequential groups of 8 healthy young men (6 on active drug, 2 on placebo) each. At regular intervals, tolerability and safety parameters, and plasma levels of palosuran and U-II were determined. Urine was collected to determine excretion of sodium, potassium, creatinine, and palosuran.

In this study, palosuran was well tolerated. No serious adverse events or dose-related adverse events were reported. No treatment-related pattern was detected for vital signs, clinical laboratory parameters, or electrocardiography parameters. After rapid absorption, palosuran displayed a plasma concentration-time profile characterized by 2 peaks at approximately 1 and 4 hours after drug administration. The apparent terminal elimination half-life was approximately 20 hours. AUC and C_{max} values increased proportionally with doses up to 500 mg. Excretion of unchanged palosuran in urine was limited. No consistent effect was found on any of the pharmacodynamic variables measured.

The results of this entry-into-humans study warrant further investigation of the therapeutic potential of palosuran.

Introduction

Urotensin-II (U-II), a cyclic undecapeptide, was first characterized from the urophysis (terminal organ of the caudal neurosecretory system in teleost fish) in the 1960s [1,2]. Identified to have haemodynamic, gastrointestinal, reproductive, osmoregulatory, and metabolic functions in fish [2-4], the relevance of U-II for human physiology was unknown as it was believed that this peptide was exclusively present in lower organisms. The successive identification of U-II in mammals like rats, pigs, monkeys, and humans [4-6] and its receptor, the orphan G-protein coupled receptor 14, which was renamed as the urotensin-II receptor (UT receptor) [4,7,8] led to a renewed interest in this neurohormonal system. Through binding to the UT receptor, which in humans is mainly found in the heart and arterial vessels, U-II activates the inositol triphosphate system leading to the release of intracellular calcium [3,9]. U-II has been described as the most potent vasoconstrictor to date, being up to 2 orders of magnitude more potent than endothelin-1 (ET-1) [6,8-11]. However, its function as a cardiovascular mediator is not fully understood; the response to U-II varies considerably between species, between vascular beds, and even between individual vessels of the same type [3,4,9,12-14]. Although less present than in the heart and arterial vessels, UT-receptors are also abundantly expressed in the epithelial cells of the renal tubules and, compared to other organs, high concentrations of U-II have been observed in endothelial cells of the renal vasculature. In this context, U-II could play a role in regulating the glomerular filtration rate by paracrine or endocrine action [5,15].

Though U-II is mostly described as a vasoconstrictor, it has also been reported that U-II elicits no or even vasodilating effects [14,16,17]. U-II circulates in the blood of healthy subjects and its concentrations are increased in patients with hypertension, renal

dysfuntion, diabetes, atherosclerosis, and congestive heart failure [18-25]. These observations provide a rationale to study an UT-receptor antagonist in the treatment of these diseases.

Palosuran (ACT-058362; 1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulfate salt) is a non-peptidic, orally active, potent, selective, and competitive antagonist of the human UT receptor [26]. It was synthesized in the course of a chemical optimization effort of UT receptor antagonists identified by random screening of the Actelion compound collection using radioligand binding techniques [26]. In rat models of acute renal failure and diabetes, palosuran significantly improved renal function, decreased the number of tubular and tubulointerstitial lesions, and improved survival [26,27]. This report describes the entry-into-humans study with the first UT-receptor antagonist known to enter clinical trials. We describe the safety and tolerability, pharmacokinetics, and pharmacodynamics of palosuran given as single oral doses in healthy male subjects.

Methods

Study subjects

After written informed consent was obtained, 76 healthy male volunteers participated in this study, which was approved by the Ethics Committee of Baden-Württemberg, Stuttgart, Germany. Subjects (age range 21-50 years) were in good health, were not taking any prescription or nonprescription medication, did not smoke, had a body mass index (BMI) between 20 and 27 kg/m^2, and had values for vital signs, ECG parameters, and clinical laboratory parameters that were either within the normal range or did not deviate to a clinically relevant extent from normal.

Study design

This study was designed as a single-center, double-blind, randomized, placebo-controlled, ascending single-dose study. After screening, 9 successive groups of 8 subjects each received a single oral dose of palosuran. In each group, 6 subjects received a dose of 5, 10, 25, 50, 125, 250, 500, 1000, or 2000 mg palosuran and 2 received matching placebo, all given as capsule formulations. Based on in vivo exposure data in animals a starting dose of 5 mg was chosen, which was 1000- and 2000-fold lower than the no-observed-adverse-effect-level in dogs and rats, respectively. Although there is a marked difference in affinity of palosuran to human versus rat receptors, this dose would still constitute a sufficiently large safety margin. After each dose group, the tolerability and safety was evaluated to decide whether the next higher dose group could proceed.

Safety and tolerability parameters were assessed regularly throughout the study. Subjects were in the clinic from 25 hours before until 36 hours following intake of study drug, during which time blood and urine samples were collected for assessment of pharmacokinetic and pharmacodynamic parameters. In the 5 days preceding the in-clinic period, subjects were requested to refrain from eating foods with particularly high sodium and potassium content. During the in-clinic period, the daily intake of sodium (Na) and potassium (K) was kept at about 120 mEq and 60 mEq, respectively. In addition, the intake of water on days -1 and 1 was standardized. An end-of-study examination was performed three to four days after study drug intake.

Safety and tolerability assessments

All adverse events (AE) that occurred after drug administration and up to the end-of-study examination were recorded together with the seriousness, severity, time of onset,

duration, and relationship to the treatment. A physical examination was performed at screening and at the end-of-study visit. Vital signs (supine and standing diastolic and systolic blood pressure and pulse rate) were measured at screening; 12 and 24 hours before drug intake; immediately prior to and 1, 2, 4, 8, 12, 16, 24, 36 hours after drug administration, and at the end-of-study visit. A 12-lead ECG was recorded at screening; 24 hours before drug intake (day -1); immediately prior to and 1, 4, 8, 24, and 36 hours after drug administration; and at the end-of-study visit. Besides heart rate, QRS, PQ/PR, and QT and QTc intervals were measured. In addition, the ECGs were checked by the investigator and any abnormalities in ECG morphology were recorded. Laboratory test parameters were assessed at screening, 24 hours before drug intake, 24 hours after drug administration (day 2), and at the end-of-study visit.

Pharmacokinetic and pharmacodynamic sampling

For determination of palosuran and U-II, venous blood samples (9 ml) were collected 24, 20, 16, and 12 hours before, immediately prior to and 0.33, 0.67, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 30, and 36 hours after study drug intake in tubes containing EDTA as anticoagulant. Following centrifugation at 1500 g for 10 minutes at 4 ºC, plasma was separated and divided over two tubes (one for palosuran and one for U-II determination) and frozen at -20 ºC until assayed. For determination of palosuran, urinary electrolytes (Na and K), and creatinine, urine was collected on day -1 over 3 intervals of 4 hours, followed by one interval of 12 hours. On day 1, urine was collected during 3 intervals of 4 hours, followed by 2 intervals of 12 hours. From the urine collected during each interval the volume was determined, a sample of 5 ml was taken, and stored at -20 ºC until assayed.

Bioanalytical methods

For determination of palosuran in plasma and urine, to each 100 µl sample 200 µl of a 50/50 mixture of acetonitril/ethanol spiked with a concentration of 80 ng/ml internal standard were added. The samples were vortexed and centrifugated and 50 µl of the supernatant were diluted with 300 µl of water containing 0.3% formic acid. Of this diluted sample 20 µl were transferred to autosampler vials. Plasma and urine concentrations of palosuran were determined using a validated liquid chromatography coupled to tandem mass spectrometry assay operating in the positive ionization detection mode. The limit of quantification (LOQ) was 1.0 ng/ml (between-run coefficients of variation were below 8.0% and 8.7% and intra-day inaccuracies were below 2.5 % and 7.5% for plasma and urine, respectively). The assay was validated in the concentration range 1 - 2000 ng/ml for both plasma and urine. U-II was determined using a RIA method developed in-house at Actelion Pharmaceuticals Ltd. The LOQ was 0.4 - 0.5 pg/ml. Urinary creatinine was determined using an adaptation of the method described by Bartels et al. [28] and urinary Na and K were determined by standard methods of flame photometry using an Eppendorf Flame Photometer, Eppendorf AG, Hamburg, Germany, Model FCM 6341.

Data analysis

Safety and tolerability parameters were analyzed descriptively. Subjects treated with placebo in the different treatment groups were pooled for analysis of safety. Calculation of model-independent pharmacokinetic parameters for palosuran was performed using Professional WinNonlin Version 4.0.1. (Pharsight Corp., Mountain View, California, USA). The maximum observed plasma concentration (C_{max}) and the time to the occurrence of C_{max} (t_{max}) were obtained directly from the plasma concentration-time

curves. The area under the plasma concentration-time curve to the last sample time with a concentration above the LOQ (AUC_{0-t}) was obtained by the linear trapezoidal rule. The area under the plasma concentration-time curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated by combining AUC_{0-t} and AUC_{extra}. The AUC_{extra} represents an extrapolated value obtained by C_t/λ_z, where C_t is the last plasma concentration measured above the LOQ and λ_z is the first order rate constant associated with the terminal log-linear portion of the plasma concentration-time curve. The $t_{1/2}$ was obtained by dividing ln2 by λ_z. From the palosuran urine concentrations, the percentage of total dose excreted in urine and the renal clearance (CL_R) were calculated. CL_R was calculated by dividing the total amount of unchanged drug excreted during 36 hours after study drug intake by AUC_{0-36}. 24-Hour urinary creatinine, and Na and K excretion data corrected for creatinine were analyzed descriptively.

Statistical analysis

Dose-proportionality of palosuran was explored by comparing C_{max} and AUC values, corrected for dose and log transformed, using a power model described by Gough et al. [29]. All analyses were performed first including all doses above 25 mg, and then repeated after sequentially excluding the 2000 and 1000 mg groups. Plasma concentrations of the 5- and 10-mg dose groups could not be determined. In addition, as most of the plasma concentrations of the 25-mg group were close to LOQ, the pharmacokinetics of this group could not be well characterized. Further, dose-normalized values for AUC were plotted and subjected to linear regression.

Results

Four subjects, before taking any study medication, reported nausea and vomiting on day -1. As this could have affected their electrolyte balance, they were taken out of the

study and were replaced. As the subjects were withdrawn before study drug intake, they were not included in the analysis of safety, pharmacokinetics, and pharmacodynamics. All other 72 subjects were compliant with the selection criteria and completed the study according to the protocol. The demographics were similar for all dose groups studied.

No serious adverse events were reported in this study. A summary of the adverse events reported more than once during the study including those AEs judged to be unrelated to study treatment is provided in Table 1. AEs that were reported more than once by the same subject were counted only once in this Table.

Table 1 Summary of AEs reported more than once during the study (treatment emergent and including unrelated) by frequency.

	Treatment									
	5 mg	10 mg	25 mg	50 mg	125 mg	250 mg	500 mg	1000 mg	2000 mg	Placebo
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**18**
Number of subjects with at least 1 AE	**3**	**1**	**1**	**3**	**-**	**2**	**2**	**4**	**4**	**3**
Total number of AEs	**4**	**2**	**1**	**6**	**-**	**2**	**2**	**4**	**4**	**3**
Most common adverse events										
Headache	**1**	**-**	**-**	**1**	**-**	**1**	**1**	**-**	**1**	**3**
Dizziness	**1**	**1**	**1**	**1**	**-**	**-**	**-**	**3**	**-**	**-**
Nausea	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**2**	**-**
Vomiting	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**2**	**-**
Pallor	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**2**	**-**	**-**
Fatigue	**-**	**-**	**-**	**1**	**-**	**-**	**-**	**-**	**1**	**-**
Postural hypotension	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**2**	**-**	**-**

AEs reported more than once by the same subject are counted only once

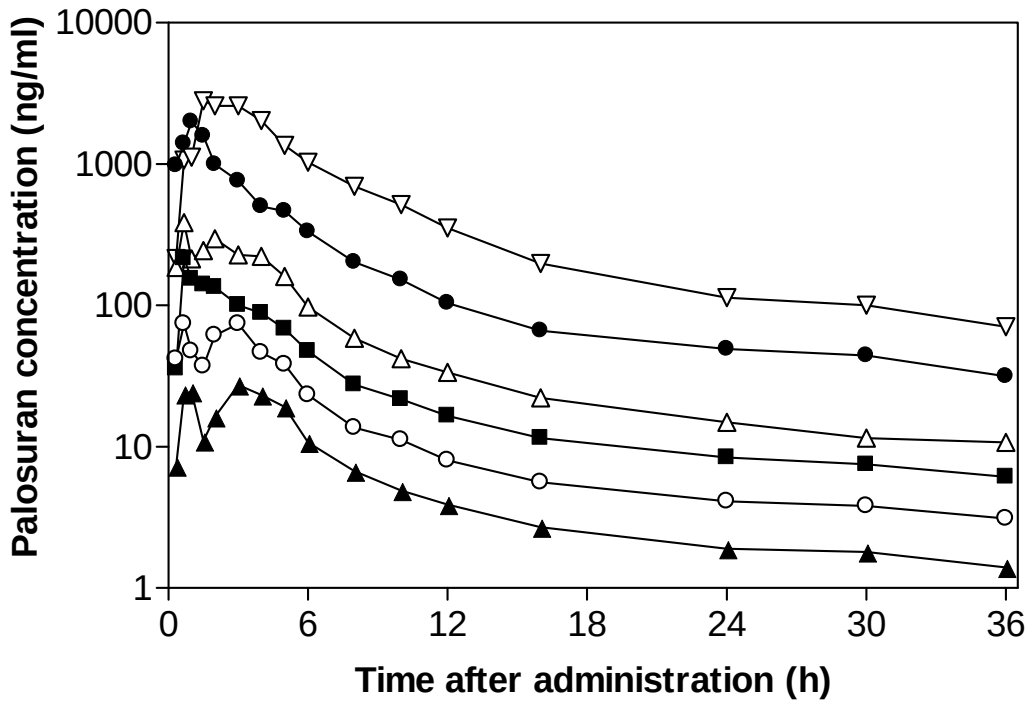
Of the 54 subjects treated with palosuran, 20 reported a total of 51 AEs (17, 20, and 14 were of mild, moderate and severe intensity, respectively). Of the 18 subjects treated with placebo, 3 reported a total of 5 AEs (4 and 1 were of mild and moderate intensity,

respectively). All AEs resolved spontaneously, except for eight subjects, who required treatment for headache with an analgesic (paracetamol), and 1 subject, who was treated for diarrhoea and vomiting. All AEs resolved without sequelae. The most frequently reported AEs were headache, dizziness, and sweating (Table 1). No dose-relationship could be discerned for any AE. No treatment-related pattern could be detected for vital signs, clinical laboratory test parameters, and ECG parameters.

The mean plasma concentration-time curves for palosuran are shown in Figure 1. In the dose groups that received 25 mg or less, most samples were below the LOQ, and were, therefore, not included in the analysis. The plasma concentration-time curve of palosuran was characterized by two absorption peaks at approximately 1 and 4 hours following administration. This double-peak phenomenon was less pronounced in the higher dose groups. The disposition of palosuran was characterized by an apparent elimination half-life of approximately 20 hours. A summary of the pharmacokinetic parameters is presented in Table 2. A graphical presentation of exploration for dose-proportionality of the pharmacokinetics of palosuran is shown in Figure 2. The pharmacokinetics were not dose-proportional over the entire dose range tested, as explored with the power model described by Gough et al. However, results from the statistical analysis showed that up to and including a single dose of 500 mg $AUC_{0-\infty}$ and C_{max} were dose-proportional. Table 3 summarizes the pharmacokinetic parameters of palosuran in urine. Urinary excretion of unchanged palosuran did not exceed 5% of the administered dose. Urinary excretion of unchanged palosuran and CL_R tended to increase with increasing dose.

Most plasma samples had U-II concentrations below or just above the LOQ. No observations were made suggesting that palosuran affected U-II plasma levels. The volume and the creatinine level of the collected urine on day –1 and 1 were similar (data

Figure 1 Mean plasma concentration versus time profiles of palosuran in healthy subjects (n = 6 per group) on a semi-logarithmic scale.



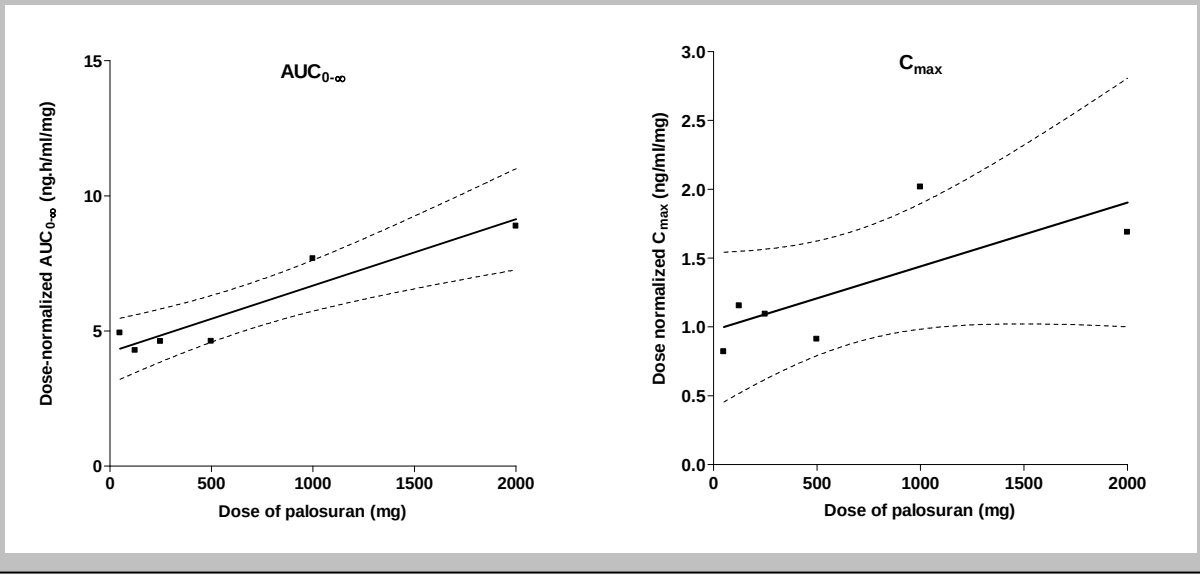
Treatments were a single dose of 50 mg (▲), 125 mg (○), 250 mg (■), 500 mg (△), 1000 mg (●), and 2000 mg (▽).

not shown). In all treatment groups (including placebo), Na and K tended to decrease in the 4-8 hour and 8-12 hour intervals (data not shown) when comparing day 1 to -1. A graphical presentation of the 24-hour excretion data of Na and K after correction for creatinine is shown in Figure 3. No effect of palosuran could be discerned for any of the urinary excretion parameters.

Discussion

The U-II / UT receptor system has only recently been discovered in humans and its role is up to date poorly understood. As increased plasma levels of U-II and/or upregulation of the UT-receptor have been observed in diseases such as hypertension, heart failure,

Figure 2 Dose-normalized values for $AUC_{0-\infty}$ and C_{max} (arithmetic mean) of palosuran and results from linear regression (with 95% CI).



and diabetes, UT-receptor antagonists may have a potential to be beneficial in the treatment of these diseases.

To our knowledge, this is the first study in which a U-II receptor antagonist was administered to human subjects. In this study we observed that palosuran was well tolerated up to and including a single dose of 2000 mg. Further, no treatment-related effects on clinical laboratory and ECG parameters could be discerned. Although U-II is considered to be a potent vasoconstrictor and could play a role in diseases such as hypertension, no effect on vital signs was observed in this group of healthy subjects.

Table 2 **Plasma pharmacokinetic parameters of single-dose palosuran in healthy subjects.**

Treatment	C_{max} (ng/ml)	t_{max} (h)	$AUC_{0-\infty}$ (ng.h/ml)	$t_{1/2}$ (h)
50 mg	40.9 (25.5, 65.5)	2.0 (0.7 - 5.0)	246 (163, 370)	19.7 (10.3, 37.7)
125 mg	144 (79.6, 260)	1.5 (0.3 - 3.0)	534 (308, 927)	22.8 (18.4, 28.2)
250 mg	273 (217, 343)	0.7 (0.7 - 1.5)	1150 (795, 1665)	23.1 (16.3, 32.8)
500 mg	455 (289, 715)	1.5 (0.7 - 4.0)	2306 (1927, 2758)	20.1 (16.9, 23.9)
1000 mg	2015 (1180, 3442)	1.0 (0.3 - 1.0)	7671 (4570, 12876)	20.9 (17.3, 25.2)
2000 mg	3373 (1890, 6018)	3.0 (1.5 - 4.0)	17446 (10403, 29256)	14.5 (10.2, 20.6)

Data are geometric means (95% CI); for t_{max} data are median (range).

Figure 3 **24-hour urinary excretion results (arithmetic mean and SEM) of sodium (left) and potassium (right) on day -1 (□) and 1 (■) (n = 6 for each palosuran dose; n = 18 for placebo).**

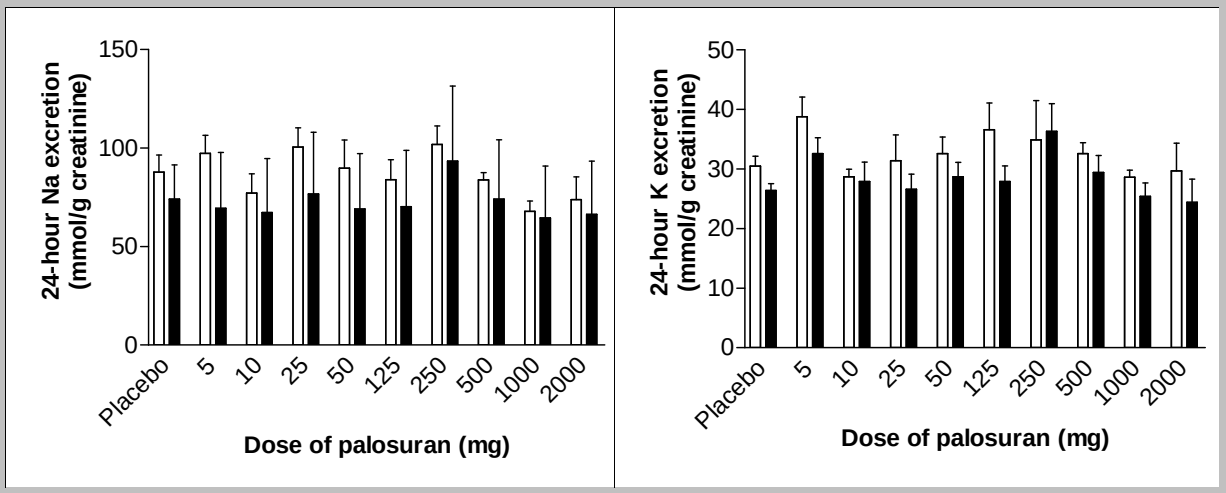


Table 3 Urine pharmacokinetic parameters of single-dose palosuran in healthy subjects.

Treatment	% dose excreted unchanged in urine	CL_R (ml/min)
50 mg	0.85 (0.58, 1.23)	36.1 (19.4, 67.0)
125 mg	1.7 (0.96, 2.9)	79.3 (64.8, 96.9)
250 mg	1.4 (0.91, 2.1)	60.8 (49.0, 75.5)
500 mg	1.7 (1.1, 2.5)	69.5 (52.3, 92.3)
1000 mg	4.1 (2.2, 7.4)	99.6 (88.0, 113)
2000 mg	4.5 (2.7, 7.6)	95.3 (83.1, 109)

Data are geometric means (95% CI).

Indeed, in vitro experiments showed that U-II causes vasoconstriction in isolated coronary arteries and perfused hearts, but the magnitude of the effect considerably differs between species [30,31]. In humans mixed effects have been observed. In a study performed by Wilkinson et al. in which healthy subjects received intra-arterial human U-II, no response in any haemodynamic measure was observed despite a near 30-fold increase in plasma concentrations of U-II [32]. In contrast, Böhm et al. demonstrated in an almost identical setting that U-II evoked potent vasoconstriction [33]. Some reports have suggested that the cardiovascular effects of U-II are mainly centrally induced [31,34,35]. Others have linked the effects of U-II to settings where endothelial cell function is comprised [36], which could be a reason why no effects can be observed in a healthy population. Also, effects have been attributed to direct activation of UT-receptors, especially in circumstances in which the UT-receptor system is upregulated [4,6,37]. Indeed, a difference in density of UT-receptors in the vessels resulted in a difference in efficacy of U-II. Usually the efficacy of U-II is found to be lower than that of other vasoconstrictors such as endothelin-1, angiotensin II, and

noradrenaline. Therefore, it is important to investigate whether U-II contributes to elevated blood pressure, and whether antagonism of the UT-receptor may provide an adequate therapy [36].

Palosuran was rapidly absorbed. The plasma concentration-time curve is characterized by a double-peak phenomenon, which could be related to enterohepatic recycling. The apparent terminal elimination half-life was approximately 20 hours, but in most subjects low palosuran plasma concentrations were measured after 12 hours. Based on this pharmacokinetic profile a twice-daily dosing regimen would be appropriate for further studies. The pharmacokinetics were dose proportional up to and including a single dose of 500 mg. At higher doses a more than dose proportional increase was observed together with an increase in renal excretion of unchanged palosuran, though the latter was still below 5% of the total dose administered. Several studies suggested the ability of U-II to regulate transepithelial transport of ions and water across a variety of osmoregulatory surfaces in teleost fist [38]. A role on the renal physiology is also suggested for mammals based on the detection of mRNA transcripts for U-II and UT receptors in human kidneys [15,39]. As U-II is produced in the kidney [40] it might have a significant contribution to renal disease. It was hypothesized that antagonism of the U-II/UT receptor system by the UT receptor antagonist palosuran could affect the excretion of urinary electrolytes and creatinine extreation, and, for this reason, these variables were assessed in this study to serve as potential pharmacodynamic markers. As it is unknown how the production of U-II is regulated, plasma levels of U-II were measured in this healthy subject to gain more understanding of the effects of antagonism of the U-II system. No pharmacodynamic markers (were identified that could guide dose selection for further clinical studies in patients. Indeed, in patients with diabetes and diabetic nephropathy, increased plasma levels of U-II and increased

expression of UT-receptors have been observed [21,23]. However, no effects on Na, K or creatinine excretion in urine were found in this healthy subjects study. As previously mentioned, the effects of U-II might be dependent on the state of the endothelium of the vessel. In healthy subjects, the endothelium-derived nitric oxide function is intact, which could explain why no effects of antagonism of UT-receptors were seen. Alternatively, the treatment duration, i.e., a single dose, might have been too short to observe an effect. Clozel et al. have shown in pathological rat models that palosuran improved blood flow after renal ischaemia, preserved renal function in renal failure models, and increased survival in diabetic models, without having an effect on blood pressure [27]. The improved survival results from a multitude of factors attributed to palosuran treatment. Palosuran increased insulin and slowed the increase in glycemia, glycosylated hemoglobin, and serum lipids. Furthermore, palosuran increased renal blood flow and delayed the development of proteinuria and renal damage.[27] As in healthy subjects glucose production is well regulated, the use of pharmacodynamic parameters from the rat model was limited.

More insight in the function of U-II in patients with diabetes and renal impairment is needed. Thus far, due to the lack of UT-receptor antagonists, the role of the U-II/UT receptor system in disease has not been fully characterized [30]. With the development of the selective, potent, U-II receptor antagonist palosuran, it will be possible to investigate the role of U-II in disease more clearly.

In conclusion, though its clinical usefulness has not yet been established in humans, the tolerability and pharmacokinetic profile of palosuran warrant further studies in patients to further investigate and understand the potential role of UT receptor antagonists in different diseases.

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Chapter 4 Multiple-dose pharmacokinetics, pharmacodynamics, tolerability, and safety of the urotensin-II receptor antagonist palosuran in healthy male subjects

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Submitted

Abstract

Purpose: To investigate the multiple-dose pharmacokinetics, pharmacodynamics, tolerability, and safety of palosuran, a selective, potent antagonist of the human urotensin-II receptor.

Methods: This was a double-blind, randomized, placebo-controlled study. Three sequential groups were treated with 25, 125, and 500 mg palosuran b.i.d. or placebo for 6.5 days.

Results: The plasma concentration-time profile was characterized by rapid absorption and peaks at 1 and 4 h after drug administration. Steady-state conditions were reached after 4 to 5 days of dosing. The apparent terminal half-life was approximately 25 h. The accumulation factor was approximately 2.5. With increasing dose, a more than dose proportional increase in AUC_{τ} and C_{max} was observed. Urinary excretion of unchanged palosuran was less than 3% of the administered dose. No consistent effect was found on any of the investigated pharmacodynamic parameters. Palosuran was well tolerated in multiple doses up to 500 mg b.i.d.

Conclusion: Palosuran has a favorable pharmacokinetic, tolerability, and safety profile that warrants further investigations in humans.

Introduction

The discovery of urotensin-II (U-II), a cyclic undecapeptide, in the urophysis (terminal organ of the caudal neurosecretory system) in teleost fish dates from the 1960s [1,2]. Initially believed to be only present in lower organisms, the subsequent identification in mammals of U-II and the U-II receptor (UT receptor, originally named the G-protein coupled receptor 14) [3-5], led to a renewed interest in this neurohormonal system [5-7]. U-II has been described as a vasoconstrictor, with a potency even larger than that of endothelin-1 (ET-1) [4,8,9]. Currently, the precise function of U-II is not fully understood, and responses to U-II are found to vary between species, vascular beds, as well as individual vessels of the same type [5,8,10-14]. Several reports have shown that there are differences in U-II plasma levels or UT-receptor expression between healthy subjects and patients with hypertension, renal dysfunction, diabetes, atherosclerosis, or congestive heart failure [15-22], suggesting that elevated plasma levels of U-II are associated with a detrimental effect in such diseases. On the basis of these findings, the development of an antagonist of the UT receptor to block such effects would possibly present a new approach.

Palosuran (ACT-058362; 1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulfate salt) is a non-peptidic, orally active, potent, selective, and competitive antagonist of the human UT receptor [23]. In rat models of acute renal failure and diabetes, palosuran significantly improved renal function, decreased the number of tubular and tubulointerstitial lesions, and improved survival [23,24]. In the entry-into-humans study, it was shown that palosuran exhibited a good safety profile and was well tolerated up to a single dose of 2000 mg (no higher dose was tested)

[25]. Excretion of palosuran in urine was found to be limited [25]. In addition, no pharmacodynamic markers (plasma levels of U-II, urinary excretion parameters) could be identified after single dosing that could guide further dosing considerations [25]. In this report, we describe the pharmacokinetics, pharmacodynamics, tolerability, and safety of palosuran administered as multiple doses to healthy male subjects.

Methods

Study subjects

After approval of the study by the Ethics Committee of Baden-Württemberg, Stuttgart, Germany, 24 healthy male subjects were recruited. All subjects (age range 22-48 years) signed the informed consent and completed the study. At screening, the subjects were in good health, did not take any prescription or nonprescription medication, did not smoke, had a body mass index (BMI) between 19.6 and 28.0 kg/m^2, and had values for vital signs (heart rate [HR], systolic blood pressure [SBP], and diastolic blood pressure [DBP]), ECG, and clinical laboratory parameters either within the normal range or not deviating to a clinically relevant extent from normal.

Study design

This study was designed as a single-center, double-blind, randomized, placebo-controlled, ascending multiple-dose study. After screening, 3 successive groups of 8 subjects (6 on palosuran and 2 on matching placebo) each received multiple doses of palosuran b.i.d. for 6.5 days. A relatively wide dose range of 25, 125, and 500 mg palosuran, administered orally in capsules, was chosen to enable the initiation of clinical studies in patients at varying doses. Based on the pharmacokinetic profile of

palosuran in the entry-into-humans study, b.i.d. dosing was considered to be the most appropriate dosing regimen [25].

After each dose group, the tolerability and safety was evaluated to decide whether the next higher dose group could start. Tolerability and safety parameters were assessed regularly throughout the study. Subjects were in the clinic from approximately 24 h before the first drug intake until 36 h after the last study drug intake, during which time blood and urine samples were collected for assessment of pharmacokinetic and pharmacodynamic parameters. An end-of-study examination was performed 2 to 4 days after last study drug intake. In the 5 days preceding the in-clinic period, subjects were requested to refrain from eating foods with particularly high sodium and potassium content. During the in-clinic period, the daily intake of sodium (Na) and potassium (K) was kept at about 120 mEq and 60 mEq, respectively. The timing and composition of meals was standardized for all subjects throughout the in-clinic period. During the days of pharmacokinetic profiling, i.e., Days 1 and 7, the meals were identical for all subjects. Products containing grapefruit were forbidden from screening until the end-of-study examination.

Pharmacokinetic and pharmacodynamic sampling

For determination of palosuran and U-II, venous blood samples (9 ml) were collected at 0.33, 0.67, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 h after the morning study drug intake on Day 1 and 7 and immediately prior to each study drug intake from Days 1 to 7. Samples were collected in tubes containing EDTA as anticoagulant. Following centrifugation at 1500 g for 10 min at 4 ºC, plasma was separated and divided over two tubes (one for palosuran and one for U-II determination) and frozen at -20 ºC until

assayed. For determination of palosuran, urinary electrolytes (Na and K), and creatinine, urine was collected on Day 1 and Day 7 over 3 intervals of 4 h. Of the urine collected during each interval the volume was determined, a sample of 5 ml was taken, and stored at -20 ºC until assayed.

Bioanalytical methods

For determination of palosuran in plasma and urine, to each 100 µl sample 200 µl of a 50/50 mixture of acetonitril/ethanol spiked with a concentration of 80 ng/ml internal standard (deuterated analog) were added. The samples were vortexed and centrifugated and 50 µl of the supernatant were diluted with 300 µl of water containing 0.3% formic acid. Of this diluted sample 20 µl were transferred to autosampler vials. Plasma and urine concentrations of palosuran were determined using a validated liquid chromatography coupled to tandem mass spectrometry assay operating in the positive ionization detection mode. The limit of quantification (LOQ) was 1.0 ng/ml and between-run coefficients of variation were below 12.0% and 10.0% with intra-day inaccuracies below 6.0 % and 2.5% for plasma and urine, respectively. The assay was validated in the concentration range 1 - 2000 ng/ml for both plasma and urine. U-II plasma concentrations were investigated in the 500 mg dose group using a RIA method developed in-house at Actelion Pharmaceuticals Ltd. The LOQ was 0.6 pg/ml. Urinary creatinine was determined using an adaptation of the method described by Bartels et al. [26] and urinary Na and K were determined by standard methods of flame photometry using an Eppendorf Flame Photometer (Eppendorf AG, Hamburg, Germany, Model FCM 6341).

Tolerability and safety assessments

All adverse events (AE) that occurred after drug administration and up to the end-of-study examination were recorded together with the seriousness, severity, time of onset, duration, and relationship to the treatment. A physical examination was performed at screening and at the end-of-study visit. Vital signs (supine and standing SBP, DBP, and HR) were measured at screening; 24 h before drug intake; immediately prior to and 1, 2, 4, 8, 12 h after morning drug administration on Day 1, 4 h after evening administration on Day 1; immediately before and 4 h after each dose administration on Days 2-6; immediately prior to and 1, 2, 4, 8, 12, 24, 36 h after morning drug administration on Day 7; and at the end-of-study visit. A 12-lead ECG was recorded at screening; immediately prior to and 1, 4, 8, 12 h after morning drug administration on Day 1; immediately before and 4 h after each dose administration on Days 3 and 5, immediately prior to and 1, 4, 8, 12, 24 h after morning drug administration on Day 7; and at the end-of-study visit. Besides heart rate, QRS, PQ/PR, QT, and QTc intervals were measured. Laboratory test parameters were assessed at screening, 24 h before drug intake, immediately prior to the morning dose administration of day 4, 24 h after the morning drug administration on Day 7, and at the end-of-study visit.

Data analysis

Tolerability and safety parameters were analyzed descriptively. For this, subjects treated with placebo in the different treatment groups were pooled. Calculation of model-independent pharmacokinetic parameters of palosuran was performed using Professional WinNonlin Version 4.0.1. (Pharsight Corp., Mountain View, California,

USA). The maximum observed plasma concentration (C_{max}) and the time to the occurrence of C_{max} (t_{max}) were obtained directly from the plasma concentration-time curves. The area under the plasma concentration-time curve during one dosing interval (AUC_T) was calculated according to the linear trapezoidal rule using the measured concentration-time values above the LOQ during one dosing interval. The $t_{1/2}$ was obtained by dividing ln2 by λ_z. Trough levels of palosuran were used to assess the attainment of steady-state conditions. The accumulation index was calculated by dividing AUC_T on Day 7 by AUC_T on Day 1. From the palosuran urine concentrations, the percentage of total dose excreted unchanged in urine and the renal clearance (CL_R) were calculated. CL_R was calculated by dividing the total quantity of unchanged drug excreted during 12 h after study drug intake by AUC_T. 12-Hour urinary creatinine, and Na and K excretion data, corrected for creatinine to reduce the variability introduced by potential incomplete sampling, were analyzed descriptively.

Statistical analysis

Dose-proportionality of palosuran pharmacokinetics was explored by comparing C_{max} and AUC values, corrected for dose and log transformed, using a power model described by Gough et al. [27]. Further, dose-normalized values for AUC were plotted and subjected to linear regression. Attainment of steady-state conditions was performed using graphical depictions.

Results

All 24 subjects were compliant with the selection criteria and completed the study according to the protocol. The demographics were similar for all dose groups studied.

No serious AEs were reported in this study. A summary of the AEs reported more than once during the study including those AEs judged to be unrelated to study treatment is provided in Table 1. AEs that were reported more than once by the same subject were counted only once in this Table. Of the 18 subjects treated with palosuran, 9 reported a total of 16 AEs. Of the 6 subjects treated with placebo, 1 reported a case of mild pruritis. Except for 1 case of moderate stye, which was treated with topical dexamethasone, dexpanthenol, and gentamicin preparations, all AEs reported during treatment with palosuran were of mild intensity. All AEs resolved without sequelae. The reporting of AEs was not concentrated on certain days of the study but appeared to be evenly distributed (data not shown). Abdominal distension, diarrhea, fatigue, loose stools, and procedural site reaction were reported by several subjects whereas all other AEs occurred only once. The total number of AEs increased with dose but no dose-relationship could be detected for any AE.

No treatment-related pattern was detected to suggest an effect of palosuran on vital signs, clinical chemistry (including liver enzymes) or urinalysis parameters. No treatment-emergent ECG abnormalities were identified or reported by the investigator. There were no clinically significant changes in mean PQ, QT, and QTc intervals.

The mean plasma concentration-time profile of palosuran, after administration of 125 mg palosuran b.i.d., is shown in Figure 1. The mean plasma concentration-time profiles on Day 7 of palosuran for the different doses are shown in Figure 2. Palosuran was rapidly absorbed after dosing. Both after single- and multiple-dose administration, the plasma concentration-time profiles were characterized by two peaks at approximately 1 and 4 h after drug administration.

Table 1 Summary of AEs reported more than once during the study (treatment-emergent and including unrelated) by frequency.

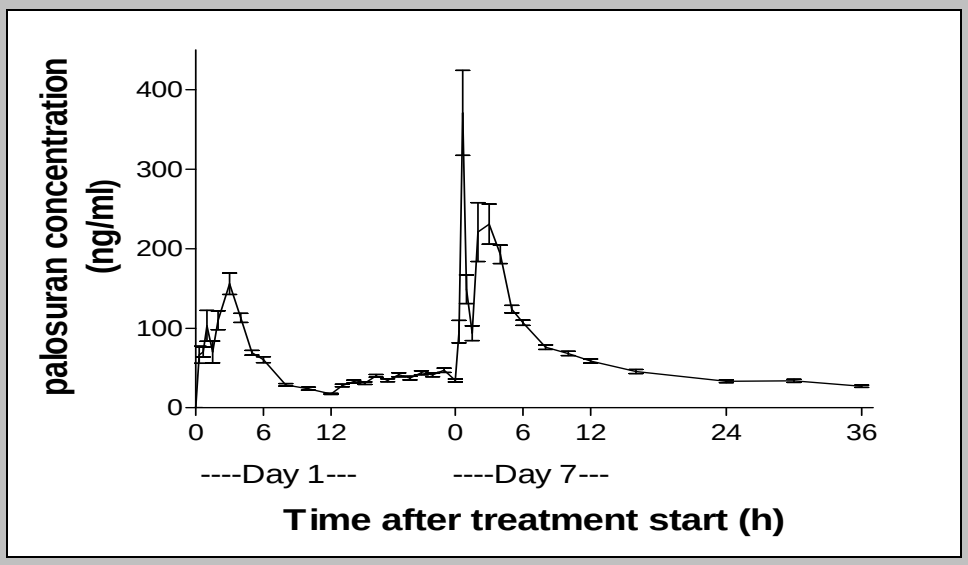
	Treatment		Treatment	
	Placebo	25 mg	125 mg	500 mg
	N=6	N=6	N=6	N=6
Adverse event	**No.**	**No.**	**No.**	**No.**
Total subjects with at least one AE	1	2	3	4
Total number of AEs	1	2	5	9
Abdominal distension	-	-	1	1
Diarrhea	-	-	-	2
Fatigue	-	1	-	1
Loose stools	-	-	1	1
Procedural site reaction	-	-	2	-
Abdominal pain	-	-	1	-
Abdominal pain upper	-	-	-	1
Dyspepsia	-	-	-	1
Headache	-	-	-	1
Nausea	-	-	-	1
Pruritus	1	-	-	-
Stye	-	1	-	-

AEs reported more than once by the same subject are counted only once

The disposition of palosuran on Day 7 was characterized by a biphasic elimination with an apparent elimination half-life that varied from 21.1 h to 29.9 h in the different dose groups. A summary of the pharmacokinetic parameters is presented in Table 2. Visual inspection of the trough concentrations indicated that steady-state conditions were reached after 4 to 5 days of dosing (Figure 1). The b.i.d. dosing regimen chosen in this study led to consistently greater AUC_{τ} values on Day 7 when compared to Day 1 (Table 2). The accumulation index varied from 1.9 to 2.7 between the different

doses. A graphical presentation of exploration for dose-proportionality of the pharmacokinetics of palosuran on Day 7 is shown in Figure 3. The results of the power model indicated that the pharmacokinetics were not dose-proportional over the entire dose range tested. The estimate for β [and 95 confidence limits] was 1.6 [1.2 ; 1.7] and 1.6 [1.4 ; 1.7] for C_{max} and AUC_{τ}, respectively, which indicated that with increasing dose a more than dose-proportional increase in C_{max} and AUC_{τ} occurred. Table 3 summarizes the pharmacokinetic parameters of palosuran in urine. Urinary excretion of unchanged palosuran did not exceed 3% of the dose administered. The % of dose excreted increased with multiple dosing and with dose, whereas the renal clearance of palosuran did not change.

Figure 1 **Mean plasma concentration versus time profile of 125 mg palosuran b.i.d. during 6.5 days in healthy subjects (mean ± SD, n=6).**

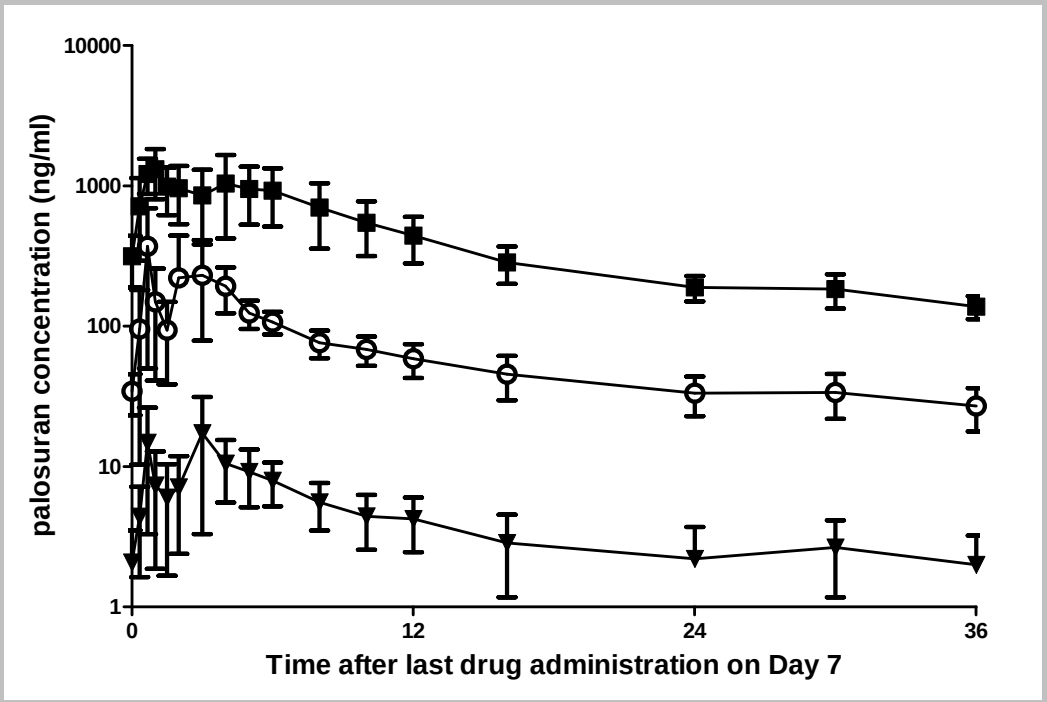


On Day 1 and 7 a full profile is shown; for Day 2-6 only the trough plasma concentrations are displayed.

The concentration of U-II was below the LOQ or just above in all samples of the 500 mg dose group. There was no indication that palosuran affected plasma U-II levels at this dose level and, therefore, the samples of lower dose groups were not analyzed. Due to the limited data available, no pharmacodynamic analysis could be performed.

Total 12-hour urine volume and creatinine excreted were similar on Days 1 and 7 (data not shown). There was a trend for an increased excretion of sodium on day 7 when compared to day 1 whereas potassium excretion was unchanged as shown in Figure 4. This, however, also occurred in the placebo group. No effect of palosuran on any of the urinary excretion parameters could be discerned.

Figure 2 **Mean plasma concentration versus time profiles of palosuran in healthy subjects after 6.5 days of treatment (mean ± SD, n=6 per group) on a semi-logarithmic scale.**



Treatments were 25 mg b.i.d. (▼), 125 mg b.i.d. (O), and 500 mg b.i.d. (■).

Discussion

While the UT system is one of the oldest conserved mechanisms involved in cardiovascular homeostasis over different species, its precise role in humans remains unclear. In recent years, agonists and antagonists of the UT receptor have been developed to attempt to clarify its usefulness in diseases such as diabetes, chronic heart failure, and renal failure [28-32].

Palosuran is a selective, non-peptidic, orally active, potent, and competitive antagonist of the UT receptor, and is the first UT receptor antagonist studied in human subjects. In this study we confirmed the favorable tolerability profile of palosuran up to and Including a dose of 500 mg b.i.d. for 6.5 days.

Figure 3 Dose-normalized values for AUC_T and C_{max} (arithmetic mean) of palosuran on Day 7 and results from linear regression (with 95% CI).

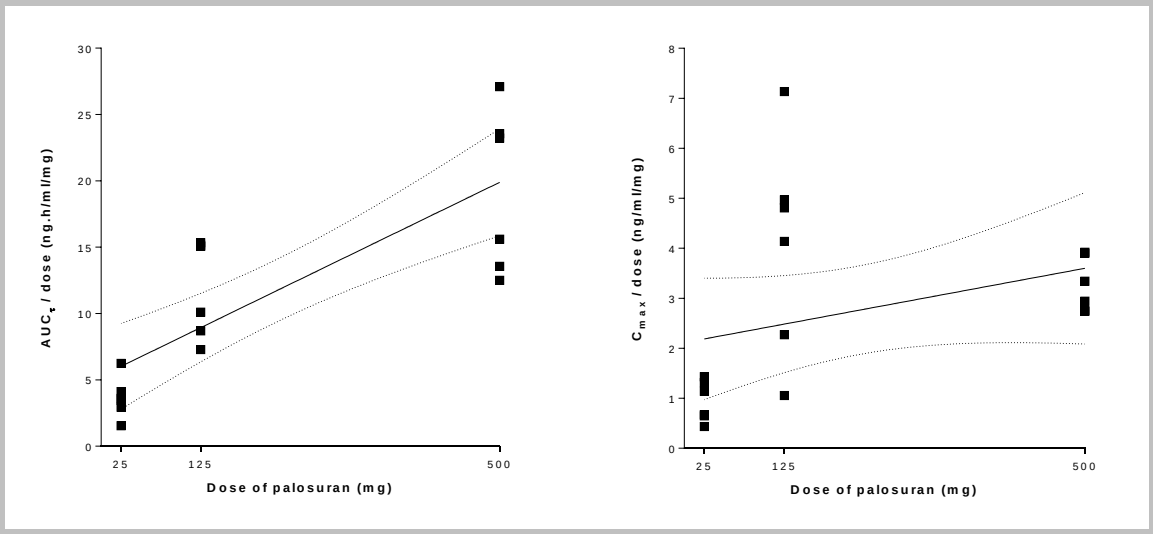
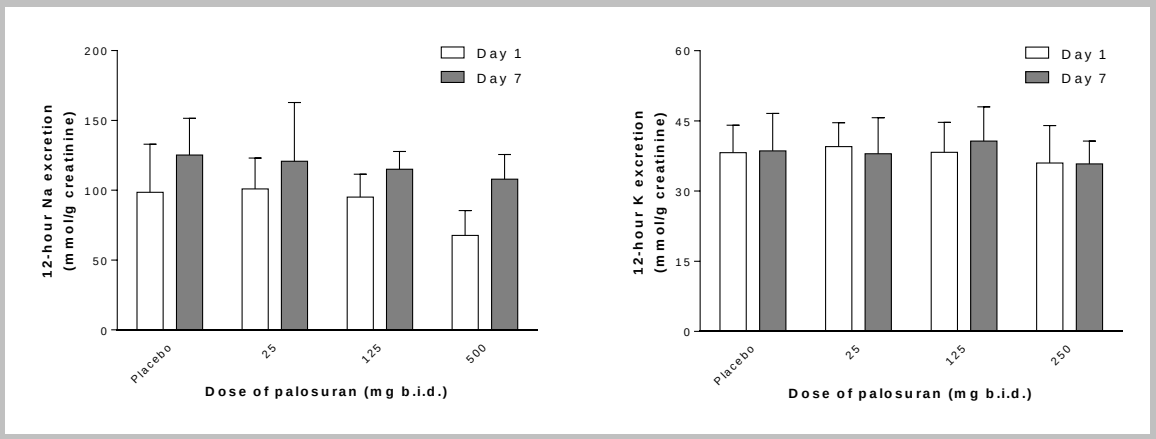


Figure 4 **12-hour urinary excretion results (arithmetic mean and SD) of sodium (left) and potassium (right) (n = 6 for each palosuran dose; n = 6 for placebo).**



Day 1 (□) and 7 ( )

No SAEs were reported in this study. AEs were of mild to moderate intensity and resolved without sequelae. Further, no treatment-related effects on clinical laboratory and ECG parameters could be discerned.

Though U-II is believed to play a role in hypertension [15], no effect on vital signs was observed in this healthy population. Possible explanations for the absence of any effect on vital signs could be a) the difference between species in function of U-II and the UT receptor [33,34], b) the highly variable response to U-II in humans [12,35-37], or c) the suggestion that the effect of U-II is associated with settings in which endothelial cell function is compromised and that, therefore, antagonism of the UT receptor would not elicit any changes in a healthy subject population [38].

Table 2 Plasma pharmacokinetic parameters of palosuran in healthy subjects after administration of multiple doses of 25, 125, or 500 mg b.i.d. for 6.5 days (n=6 per dose level).

Treatment	Day	C_{max} (ng/ml)	t_{max} (h)	AUC_{τ} (ng.h/ml)	$t_{1/2}$ (h)	Accumulation Index
25 mg bid	1	11.5	2	34.2		
		(5.9, 22.3)	(0.67 - 4.0)	(11.1, 105)		
	7	21.5	3	84.1	21.1	2.5
		(13.1, 35.4)	(0.67 - 4.0)	(51.9, 136)	(9.1, 49.3)	(1.2, 5.0)
125 mg bid	1	196	3	735		
		(132, 290)	(1.0 - 4.0)	(547, 987)		
	7	431	1.3	1429	29.9	1.9
		(209, 890)	(0.67 - 4.0)	(1014, 2015)	(21.2, 42.1)	(1.4, 2.8)
500 mg bid	1	1069	1	3350		
		(676, 1689)	(0.67 - 3.0)	(2266, 4953)		
	7	1615	1.5	9216	23.2	2.7
		(1359, 1919)	(0.67 - 6.0)	(6540, 12987)	(20.0, 26.9)	(2.0, 3.7)

Data are geometric means (and 95% CI) or for t_{max} the median (and range).

Table 3 Urine pharmacokinetic parameters of palosuran in healthy subjects after administration of multiple doses of 25, 125, or 500 mg b.i.d. for 6.5 days (n=6 per dose level).

Treatment	% dose excreted in urine during 12 hours	CL_R (ml/min)
25 mg bid		
Day 1	0.45 (0.24, 0.83)	54.6 (12.9, 231)
Day 7	0.56 (0.27, 1.2)	27.6 (16.6, 46.0)
125 mg bid		
Day 1	0.85 (0.50, 1.4)	24.2 (14.6, 39.9)
Day 7	2.3 (1.6, 3.5)	34.0 (29.2, 39.7)
500 mg bid		
Day 1	0.98 (0.69, 1.4)	24.3 (15.8, 37.5)
Day 7	2.6 (1.2, 5.5)	23.4 (12.6, 43.1)

Data are geometric means (and 95% CI)

Palosuran was rapidly absorbed with double peaks at 1 and 4 h after administration. The underlying mechanism of the double-peak phenomenon is unknown and may be the outcome of a plethora of factors such as solubility limiting absorption due to physicochemical or formulation factors, complexation, enterohepatic recycling, site-specific absorption, gastric emptying, and intestinal transit time [39]. As palosuran has a good solubility in water [23] it is not expected that solubility limiting absorption contributes significantly to the double-peak phenomenon. Palosuran was not fully administered in the fasted state, i.e., the evening dose was given several hours after a light meal, unlike the morning drug administration that was performed after an overnight fast. However, the double peak was also observed after the morning dose, and

therefore it is unlikely that absorption of palosuran is affected by differences in gastric motility, or bile salt micellular complexation in the small intestine due to food. More clinical data are needed to further characterize the mechanism behind the double absorption peaks of palosuran.

Palosuran was eliminated in a biphasic way, with an apparent elimination half-life of approximately 25 h. As after 12 h palosuran plasma concentrations were low, a twice daily dosing regimen was considered appropriate for future studies. Using the b.i.d. dosing regimen, an accumulation index of approximately 2.5 was observed on Day 7. Steady-state conditions were attained after 4 days. Interestingly, at doses of 25 and 125 mg b.i.d., evening trough concentrations were consistently higher than morning trough concentrations, though not clinically relevant due to the good tolerability profile of palosuran (data not shown). This phenomenon could be an example of chronopharmacokinetics, i.e., a difference in the disposition of palosuran during the night and the day [40-42]. An alternative explanation could be the intake of food. It is hypothesized that food reduces the bioavailability of palosuran. Indeed, in this study it was observed that evening trough concentrations on Day 7 were notably higher than on Day 6. The only striking difference between these days was a difference in the total daily amount of food given to the subjects, as subjects did not receive breakfast on Day 7. Further efforts should be made to investigate the effects of food on the pharmaockinetics of palosuran in a dedicated study.

Although the pharmacokinetics of palosuran were more than dose-proportional over the dose range tested, the wide dosing range tested in this study, the excellent tolerability of palosuran, and the limited increase (i.e., 2 to 3 fold), provide sufficient margins for further investigation of palosuran in clinical trials. Only a small amount of palosuran was

excreted unchanged in urine and renal excretion was limited. Thus, further studies with radio-labeled palosuran are recommended to further clarify the metabolism of the drug. After single-dose administration no pharmacodynamic markers could be identified that could guide dose selection for further studies [25] which was, in part, attributed to the short duration of treatment. However, in this study, after multiple-dose treatment with palosuran, we did not detect an effect on plasma U-II levels and urinary excretion parameters. Several reports in animals suggest that U-II plays an important role in the occurrence of renal fibrosis and dysfunction [43,44] which is substantiated by the fact that U-II is produced in the kidney [45]. As the (patho)physiological pathway of U-II has not been elucidated in patients or healthy subjects, differences in physiology could explain the absence of effect in healthy subjects in this study.

In conclusion, the results of the study indicate that the U-II receptor antagonist palosuran is a well-tolerated drug with a pharmacokinetic profile that supports twice daily dosing. No pharmacodynamic markers could be identified in healthy human subjects, which might be attributed to a difference in function of U-II and its receptor between healthy subjects and patients. Thus, in order to further elucidate the physiological or pathophysiological mechanisms of U-II in disease, more investigations are needed in patients.

Statement of competing interest

Patricia Sidharta and Jasper Dingemanse are full-time employees of Actelion Pharmaceuticals Ltd.

Paul van Giersbergen was a full-time employee of Actelion Pharmaceuticals Ltd at the time of the study.

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Chapter 5 Investigation of the effect of food on the pharmacokinetics of palosuran

Introduction

During drug development it is important to understand the impact of food on the pharmacokinetic properties of a drug that will be orally administered. Food can change the bioavailability of a drug by impacting different mechanisms including gastric emptying, bile flow, gastrointestinal pH, alteration of the luminal metabolism of the drug [1]. Further, food can physically or chemically interact with a substance as well as have an impact on enzymes involved in metabolism [2]. As the majority of drugs will be taken chronically, a clear dosing recommendation with regard to food intake is imperative. As meals with a high total calorie and fat content have a larger effect on the bioavailability of a drug, the effect of such a meal on the pharmacokinetics of palosuran was investigated in a clinical study.

Methods

Study subjects

Eight healthy male subjects were recruited into this study, after providing informed consent. The study was approved by the Ethics Committee of Hamburg, Germany. Subjects had to be between 20-50 years of age, healthy as confirmed by a medical examination and non-smokers. The body mass index (BMI) had to be between 18 and 28 kg/m^2 and values for vital signs, ECG parameters, and clinical laboratory had to be either within the normal range or not deviating from normal to a clinically relevant extent.

Study design

The study was conducted as a single-center, open-label, randomized, 2-period crossover study. After screening all subjects received the following treatments: A) palosuran as a single dose of 125 mg in the fasted state; B) palosuran as a single dose

of 125 mg after the intake of a high fat, high calorie breakfast. The breakfast contained approximately 1000 calories, i.e., approximately 150, 250, and 600 calories from protein, carbohydrate, and fat, respectively, and followed the recommendations given by the FDA [1]. Treatments were separated by a washout of 1 week. The dose of 125 mg was used as this was the highest dose selected for clinical development. Safety and tolerability parameters were assessed at regular time points throughout the study. Subjects were in the clinic from 12 h before until 24 h after drug intake, during which time blood samples were collected for pharmacokinetic assessments. Subjects had to return to the clinic 36 h after drug intake for a last blood sample collection. An end-of-study (EOS) examination was performed 2 days after study drug intake.

Safety and tolerability assessments

All adverse events (AE) that occurred after drug administration and up to the end-of-study examination were recorded together with the seriousness, severity, time of onset, duration, and relationship to the treatment. A physical examination was performed at screening and at the EOS. Vital signs (supine and standing diastolic and systolic blood pressure and pulse rate) and ECG (heart rate, QRS, PQ/PR, and QT and QTc intervals) parameters were measured at screening; immediately prior to and 1, 4, 12, and 24 h after drug intake; and at EOS. Clinical laboratory test parameters were assessed at screening and EOS.

Pharmacokinetic sampling

Blood samples for palosuran determination were taken immediately prior to and 0.08, 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 16, 24, and 36 h after study drug intake in tubes containing EDTA as anticoagulant. Following centrifugation at 1500 g for 10 min at 4 ºC, plasma was separated and stored at -20 ºC until assayed.

Bioanalytical method

For the determination of palosuran in plasma, a validated liquid chromatography coupled to tandem mass spectrometry assay was used as described by Sidharta et. al. [3]. The limit of quantification (LOQ) was 1.0 ng/ml with between-run and intra-day coefficient of variation below 7% and 3%, respectively.

Data analysis

Safety and tolerability parameters were analyzed descriptively. Calculation of model-independent pharmacokinetic parameters of palosuran was performed using Professional WinNonlin Version 4.0.1. (Pharsight Corp., Mountain View, California, USA). The maximum observed plasma concentration (C_{max}) and the time to the occurrence of C_{max} (t_{max}) were obtained directly from the plasma concentration-time curves. The area under the plasma concentration-time curve from zero to infinity ($AUC_{0-\infty}$) and the half-life ($t_{1/2}$) were derived by non-compartmental analysis of the plasma concentration-time profiles. C_{max} and $AUC_{0-\infty}$ were assumed to be non- normally distributed [4].

Statistical analysis

To explore differences between treatment A (fasted) and B (fed) in the pharmacokinetics of palosuran, log-transformed $AUC_{0-\infty}$ and C_{max} values, and untransformed $t_{1/2}$ values were compared with ANOVA using treatment, period, sequence, and subject (sequence) as factors.

Results

Palosuran was well tolerated in this study; no serious AEs or AEs leading to study discontinuation were reported. Of the 8 subjects treated, 3 subjects reported 5 AEs. Mild to moderate headache was the only reported AE; 2 occurred during treatment A and 3 during treatment B. All reported cases of headache resolved spontaneously, without need for concomitant treatment, and resolved without sequelae. Few subjects had isolated changes outside of the normal range for clinical laboratory parameters that were not related to AEs or that showed a treatment-related pattern. Small decreases between EOS and baseline were observed in hemoglobin, hematocrit, and erythrocytes (Table 1). Four subjects presented with a blood pressure value (SBP or DBP) outside of the upper limit of normal at several time points, but no treatment-related pattern could be identified. No clinically relevant changes in ECG parameters were observed in this study. In all subjects ECG abnormalities were reported that were either present at baseline, or observed incidentally. These abnormalities were not related to AEs and no pattern was detected that suggested a drug effect.

The mean plasma concentration-time curves of palosuran in the fasted and fed state are shown in Figure 1. The plasma concentration-time profiles of palosuran were characterized by two peaks at approximately 1 and 5 h without breakfast and at 2 and 5 h with breakfast (Figure 1). The first peak tended to be less pronounced after breakfast. In the fasted condition and after breakfast, maximum plasma concentrations were reached after 1.8 h and 5 h, respectively. Maximum plasma concentrations and overall exposure to palosuran were slightly lower (28 and 26% reduction, respectively) after breakfast, but no statistically significant effect was detected. The terminal elimination half-life was similar without or with breakfast (16.7 h and 19.2 h, respectively). The

pharmacokinetic parameters of palosuran in the fasted and fed condition are summarized in Table 2.

The result of the test for carry over effect was borderline statistically significant (p=0.05) when comparing log transformed $AUC_{0-\infty}$ values of treatment A and B, which was probably caused by the data of one subject that were different from the other 7 subjects. Exploratory ad hoc analysis of the data excluding subject 7 indicated a small statistically significant effect of treatment without carry over effect (p=0.11). In this analysis, exposure to palosuran expressed as $AUC_{0-\infty}$ was reduced by 31% (p=0.04) after breakfast.

Table 1 Summary of hemoglobin, hematocrit, and erythrocytes values during the study.

Laboratory parameter	N	Visit	Absolute value		Change from baseline	
			Median	SD	Median	SD
Hemolobin (g/dl)	8	Scr	15.6	1.3		
		EOT	15.1	1.3	-1.0	0.3
Hematocrit	8	Scr	0.45	0.03		
		EOT	0.43	0.03	-0.02	0.01
Erythrocytes (10^{12}/L)	8	Scr	4.97	0.47		
		EOT	4.76	0.43	-0.25	0.11

Discussion

The objectives of this study were to assess the effect of food on the safety, tolerability, and pharmacokinetics of a single 125 mg dose of palosuran. The results of this study indicate that palosuran was well tolerated. No treatment-related patterns could be detected for any safety or tolerability variable. The most notable observations were the

decreases in hemoglobin, hematocrit, and erythrocytes that were most likely caused by the intense blood sampling performed in this study.

Figure 1 **Mean plasma concentration (±SD) versus time profiles of palosuran in healthy subjects (n = 8) in the fasted and fed state on linear scale.**

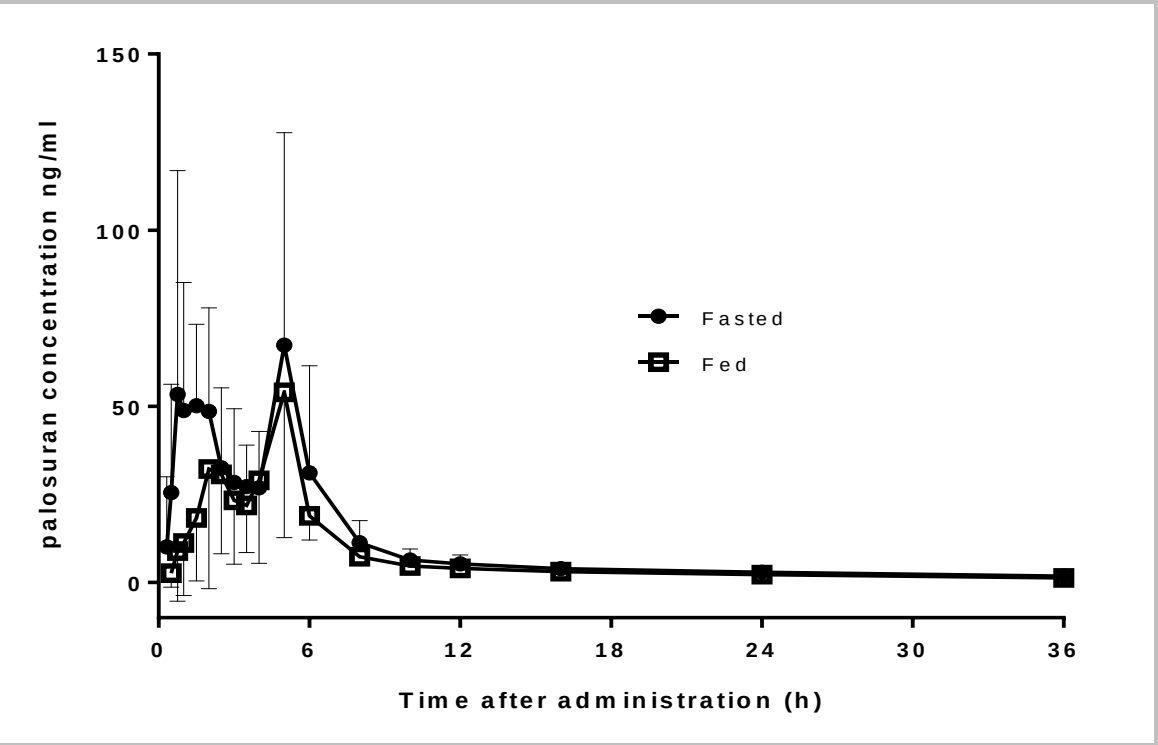

Table 2 **Plasma pharmacokinetic parameters of palosuran in healthy subjects in the fasted and fed state.**

Treatment	N	C_{max} (ng/ml)	t_{max} (h)	$AUC_{0-\infty}$ (ng.h/ml)	$t_{1/2}$ (h)
A	8	92.3 (59.7, 143)	1.8 (0.75-5.0)	392 (244, 629)	16.7 (12.1, 22.9)
B	8	66.9 (43.6, 103)	5.0 (2.0-6.0)	289 (213, 393)	19.2 (15.9, 23.3)

Data are geometric means (95% CI); for t_{max} data are median (range).

The only AE reported in this study was mild to moderate headache, which resolved spontaneously without need for concomitant medication. The good safety and tolerability profile of palosuran is consistent with other results reported for palosuran [3,5].

The pharmacokinetic properties of palosuran in the fasted condition were also similar to those observed in other studies [3,5]. When given with food, a small decrease of 31% in $AUC_{0-\infty}$ was observed, mainly due to a less pronounced first peak in palosuran concentration. However, given the variability in palosuran concentrations, this difference was not considered to be clinically relevant.

Overall, the results of this study indicate that food does not affect the safety, tolerability, and pharmacokinetics of palosuran. Therefore, further clinical studies of palosuran can be performed without specific instructions regarding food intake.

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Part III: Clinical pharmacology of the urotensin-II receptor antagonist palosuran in Type 2 Diabetes Mellitus

Chapter 6 Pharmacodynamics and pharmacokinetics of the urotensin-II receptor antagonist palosuran in macroalbuminuric, diabetic patients.

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Abstract

Objective: In patients with renal disease, increased urotensin-II plasma levels have been observed. We have investigated whether palosuran, a potent, selective, and competitive antagonist of the urotensin-II receptor has effects in patients who are prone to develop renal disease.

Methods: Macroalbuminuric, diabetic patients, categorized by renal function, were treated with oral doses of 125 mg palosuran b.i.d. for 13.5 days on top of treatment with either an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker. The 24-hour urinary albumin excretion rate was determined twice at baseline and after 13.5 days of treatment. Plasma concentrations of palosuran were determined for 12 hours after first and last drug intake. Renal hemodynamics were measured before and after 12.5 days of treatment. Tolerability and safety parameters were monitored.

Results: An overall clinically significant reduction of 24.3% (geometric mean, 95% CI: 4.1 - 45.0) in 24-hour urinary albumin excretion rate was observed (P = 0.014). No effect was observed on renal hemodynamic parameters. Palosuran was rapidly absorbed with maximum plasma concentrations at 1 hour after drug administration. The accumulation factor was 1.7 (geometric mean, 95% CI: 1.3 - 2.1). Palosuran was well tolerated.

Conclusions: The good tolerability profile and decrease of 24-hour urinary albumin excretion rate may benefit diabetic patients with renal failure with regard to their disease progression. Larger placebo-controlled trials in this patient population are needed to investigate whether urotensin-II receptor antagonists, given as mono- or combination therapy, may improve the current treatment of diabetic nephropathy.

Introduction

Chronic renal failure is the consequence of a progressive loss of different functions of the kidney. If not treated, chronic renal failure may progress to end-stage renal disease, a serious condition that can only be treated with dialysis or renal transplantation. Diabetes has become the leading cause of end-stage renal disease in many parts of the world [1,2], partly due to the fact that prevalence of type 2 diabetes is increasing and that these patients live longer [3]. The earliest clinical evidence of nephropathy is the appearance of low but abnormal albumin levels in urine (≥ 30 mg/day), referred to as microalbuminuria [4-6]. Without specific intervention, 20 to 40% of the patients with type 2 diabetic nephropathy with microalbuminuria progress to overt diabetic nephropathy, a condition associated with macroalbuminuria (≥ 300 mg/day). Within 20 years of onset of overt nephropathy, 20% of these patients will have progressed to end-stage renal disease [7]. To date, treatments to delay disease progression are mainly aimed at controlling systemic blood pressure and albuminuria [8-10]. Angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, two drug classes inhibiting the renin-angiotensin-aldosterone system, not only decrease blood pressure but also reduce albuminuria. In addition, they have been shown to significantly decrease morbidity and mortality by reducing combined clinical endpoints (such as doubling of serum creatinine, end-stage renal disease or death) and cardiovascular events [11-16]. Although these findings are very promising for the treatment of patients with diabetic nephropathy, there is still a significant unmet medical need for new drugs that demonstrate an additional benefit on morbidity and mortality on top of background treatment with an inhibitor of the renin-angiotensin-aldosterone system.

Urotensin-II is a cyclic peptide described as one of the most potent vasoconstrictors known, though the magnitude of its effect is highly dependent on the species and anatomical source of the vessel [17-20]. Increased plasma urotensin-II concentrations have been observed in patients with diabetes, chronic heart failure, and kidney diseases [17,21-24]. Therefore, urotensin-II receptor antagonists may have therapeutic applications in these diseases [25-27]. Palosuran (ACT-058362; 1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl)-urea sulfate salt) is a non-peptidic, orally active, potent, selective, and competitive antagonist of the human urotensin-II receptor [28]. In rat models of renal ischemia, palosuran was effective in both preventing post-ischemic renal vasoconstriction and in reducing post-ischemic acute renal failure. As the effects on renal blood flow were not accompanied by any systemic vasodilation, it is suggested that palosuran may have a selective renal vasodilating effect. Subsequently, palosuran prevented the development of acute renal failure and the histological consequences of ischemia [28]. In a rat model of diabetes, palosuran increased renal blood flow and delayed the development of proteinuria and renal damage [29].

In the phase I program, palosuran was well tolerated by healthy male subjects. Its plasma concentration-time profile (both after single- and multiple-dosing) could be characterized by two peaks at approximately 1 and 4 hours after drug administration. The apparent elimination half-life was approximately 25 hours. Steady-state conditions were reached after 4 to 5 days of dosing and an accumulation of approximately 2.5 was observed. Less than 3% of the administered dose was excreted as unchanged drug in the urine. *In vitro* studies showed high plasma albumin binding of palosuran and no inhibition of cytochrome P450 enzymes or induction of CYP3A4 [30,31]. Further, it was suggested from *in vivo* studies in rats that biliary excretion is the major elimination route

of palosuran. In the absence of a pharmacodynamic marker, in healthy subjects, the effective daily dose in patients was estimated to be at 50 mg at the maximum. This was based on *in vivo* exposure in animal models and the large difference in affinity to human versus rat receptors [28]. In the context of this proof-of-concept study exploring only one dosing regimen, a dose of 125 mg b.i.d. palosuran was selected, which in healthy subjects had shown a high but well-tolerated exposure [30,31]. The pharmacokinetic profile in healthy subjects showed a pronounced distribution with a rapid and slower phase of disposition resulting in relatively low plasma concentrations of palosuran twelve hours post dose [30]. Based on these observations, a twice-daily dose regimen was chosen for this study.

This report describes the first clinical study in patients with a urotensin-II receptor antagonist. We investigated the effects of multiple-dose palosuran on 24-hour urinary albumin excretion rate, renal hemodynamics, and renal function in patients with different degrees of renal function as well as the effect of renal impairment on the single- and multiple-dose pharmacokinetics of palosuran, and tolerability and safety in this population.

Methods

Study population

Study participants were hypertensive patients of both sexes with type 2 diabetic nephropathy, between 30 and 75 years old, who were on stable treatment with either an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker at least three months prior to start of the study. Arterial hypertension was defined as supine systolic and diastolic blood pressure above 135 mmHg and/or 85 mmHg (limits included), respectively. Type 2 diabetic nephropathy was defined as a medical history of

type 2 diabetes mellitus with an average 24-hour urinary albumin excretion rate between 300 mg and 3000 mg (limits included), measured twice during a two-week screening period with a coefficient of variation smaller than 30%. Patients were categorized in two groups; one with normal to mildly impaired renal function and one with moderately to severely impaired renal function. Renal function was defined as a (body surface area) corrected creatinine clearance of > 50 ml/min/1.73m^2 for normal to mildly impaired and a (body surface area) corrected creatinine clearance of ≤ 50 ml/min/1.73m^2 for moderately impaired patients during the two-week screening period. Patients were excluded if they had severe concomitant diseases (e.g., unstable angina, severe heart failure, ventricular arrhythmias), or, specifically, clinical evidence of renal artery stenosis or nephrotic syndrome. Signed and dated written informed consent was obtained from all patients.

Study design

The protocol for this study was approved by the Ethics Committee of each of the three participating centers (Ethics Committee of Brandenburg, Cottbus, Germany; Ethics Committee of Baden-Württemberg, Stuttgart, Germany; and Ethics Committee of Schleswig-Holstein, Bad Segeberg, Germany). This study was designed as a three-center, open-label, multiple-dose study in two groups of patients with different disease severity. After a screening period of about two weeks, during which the 24-hour urinary albumin excretion rate and the creatinine clearance were determined twice, 9 patients were included in the group with normal to mild renal impairment and 10 patients in the group with moderate to severe renal impairment. Patients were treated with 125 mg of palosuran twice daily for a period of 13.5 days, a dose regimen chosen based on the pharmacokinetic profile in healthy subjects [30,31]. For pharmacokinetic assessments,

blood sampling and urine collection was performed on Day 1 and Day 14 for a time period of 12 hours. In addition, on Day 14, urine was collected for subsequent 12 hours for determination of the 24-hour urinary albumin excretion rate. For renal hemodynamic assessments, on the day before the first study drug intake (Day -1) and after 12.5 days of treatment, glomerular filtration rate (GFR) and renal blood flow (RBF) were determined using inulin and p-aminohippuric acid clearance techniques, respectively [32,33]. The renal filtration fraction (FF), which represents the fraction of renal plasma flow filtered by the glomerulus, was calculated subsequently. Safety and tolerability parameters were assessed regularly throughout the study.

Palosuran pharmacokinetics and 24-hour urinary albumin excretion rate

Venous blood samples (4 ml) were collected immediately prior to study drug intake and 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, and 12 h after drug intake on Day 1 and Day 14. Blood was collected into tubes containing EDTA and immediately centrifuged at 1500 *g* for 10 minutes at 4 ºC. Plasma was separated and frozen at -20 ºC until assayed. On Day 1 and 14, urine was collected for 12 hours after morning study drug administration. From the collected 12-h urine, two aliquots of 5 ml were taken and stored at -20 ºC until assayed. Plasma and urine concentrations of palosuran were determined using a validated liquid chromatography assay coupled to tandem mass spectrometry operating in the positive ionization detection mode. The limit of quantification was 1.0 ng/ml (between-run coefficients of variation below 7.4% and 4.2% for plasma and urine, respectively). On Day 14, urine was collected for subsequent 12 hours. From the pooled 24-hour urine, two aliquots of 5 ml were taken and 24-hour urinary albumin excretion rate was determined.

Renal hemodynamics

For determination of GFR and RBF a loading dose of inulin (Inutest®; Fresenius Kabi, Linz, Austria) and p-aminohippuric acid (Clinalfa®; Merck Biosciences AG, Läufelingen, Switzerland) was administered on the basis of the body weight of the patient, followed by a maintenance infusion to keep inulin and p-aminohippuric acid at steady state. The infusion regimen was based on the creatinine clearance determined during the screening phase. Three venous blood samples of 1.2 ml each were taken at 1, 1.5 and 2 hours after infusion start (i.e., when inulin and p-aminohippuric acid were in steady state). The infusion was started 2 hours after the (theoretical) morning drug intake to assure that the measurement was performed around the t_{max} of palosuran. Blood was collected into tubes containing EDTA and immediately centrifuged at 1500 *g* for 10 minutes at 4 ºC. Plasma was separated and frozen at -20 ºC until assayed. From the infusion solution a sample of 2 ml was taken and frozen at -20 ºC for inulin and PAH determination. Inulin concentrations of the plasma and infusion samples were determined using an enzymatic assay based on the method of Kuehnle et al. [34]. The limit of quantification was 5 µg/ml (between-run coefficients of variation below 9.2%). p-Aminohippuric acid concentrations were determined using a high performance liquid chromatography assay with ultraviolet detection that was adapted from the method of Marsilio et al. [35]. The limit of quantification was 1.2 µg/ml (between-run coefficients of variation below 16.0%).

Tolerability and safety assessments

Tolerability and safety were evaluated using spontaneously reported adverse events, physical examination, measurements of vital signs (supine diastolic and systolic blood

pressure, and pulse rate), ECG and laboratory test parameters (including fructosamine, Hb A_{1C}, insulin, and hematocrit), performed before, during and after the study.

Data analysis

GFR is identical to inulin clearance, which was determined by the ratio of the infusion rate of inulin (accurately assessed) and its steady-state plasma concentration. The latter was given by the mean of 3 separate measurements. The RBF was calculated as RBF = RPF / (1- hematocrit), where RPF represents the renal plasma flow. The hematocrit was taken from the clinical laboratory report obtained closest to the assessment day.

RPF was calculated by the ratio of the infusion rate of PAH (accurately assessed) and its steady-state plasma concentration. The latter was given by the mean of 3 separate measurements. The filtration fraction (FF) was calculated as FF = GFR / RPF.

Calculation of model-independent pharmacokinetic parameters for palosuran was performed using Professional WinNonlin Version 4.0.1. [36]. The maximum plasma concentration (C_{max}) and the time of its occurrence (t_{max}) were obtained from individual data. The area under the plasma concentration versus time curve during one dosing interval of 12 h (AUC_T) was calculated using the linear trapezoidal rule. The accumulation index was defined as AUC_T Day 14/ AUC_T Day 1 [37].

From the urine concentrations of palosuran, the renal clearance (CL_R) and % of total dose excreted as unchanged drug were calculated.

Statistical analysis

To explore differences between the mean of the screening values and the 24-hour urinary albumin excretion rate after 13.5 days of treatment, logarithmically transformed 24-hour urinary albumin excretion rate values were compared using the Wilcoxon

Paired Signed Rank test (2-sided, α=5%). It was chosen to logarithmically transform the 24-hour urinary albumin excretion rate, as these values are not normally distributed due to the nature of the patient population.

To explore differences within each renal function group on the renal hemodynamic parameters GFR, RBF, and FF, untransformed values of Day 1 and 13 were compared using the Wilcoxon Paired Signed Rank test (2-sided, α=5%).

To explore differences between the two groups, untransformed changes from baseline of GFR, RBF, and FF were compared using the Signed Rank Wilcoxon test (2-sided, α=5%).

To confirm that the degree of renal function was different between the two groups at baseline, untransformed GFR, RBF, and FF values on Day -1 were compared using the Signed Rank Wilcoxon test (2-sided, α=5%).

Comparisons of pharmacokinetic parameters between the two renal function groups were performed using a two-sample t-test (2-sided, α=5%) for log-transformed AUC_τ and C_{max} and the Signed Rank Wilcoxon test (2-sided, α=5%) for untransformed t_{max} and accumulation index. To explore differences within each renal function group log-transformed AUC_τ and C_{max} and untransformed t_{max} values of Day 1 and 14 were compared using the Wilcoxon Paired Signed Rank test (2-sided, α=5%).

Results

One patient in the group with moderately impaired renal function withdrew consent on Day 6 of the study. This patient was, therefore, only analyzed for safety. The other 18 patients completed the study according to the protocol, although another patient in the group with moderately impaired renal function was not included in the pharmacokinetic

and renal hemodynamic analyses, due to missing samples. Table 1 summarizes the demographic characteristics of those patients who received treatment.

Table 2 summarizes the 24-hour urinary albumin excretion rate values categorized by renal function group at baseline and after 13.5 days of treatment. Individual changes from baseline after 13.5 days of treatment in 24-hour urinary albumin excretion rate categorized by renal function are presented in Figure 1. Comparison of the geometric means between Day 14 and baseline revealed a statistically significant decrease of 26.2% (geometric mean, 95% CI: 5.0 – 46.3) in the group with normal to mild renal impairment (P = 0.027). In the group with moderate to severe renal impairment a decrease of 22.3% (geometric mean, 95% CI: -11.6 – 45.0) was observed, however, this finding was not statistically significant (P = 0.250). The overall change in geometric means of 24-hour urinary albumin excretion rate from baseline to Day 14 was a decrease of 24.3% (geometric mean, 95% CI: 4.1 – 45.0) with P = 0.014.

Table 3 summarizes the renal hemodynamic parameters of the two renal function groups. Analysis of the GFR data plotted versus the corrected CrCl data by means of linear regression demonstrated a good correlation between these two parameters (r = 0.90). At baseline (Day -1) GFR and RBF were statistically significantly lower in the group with moderate to severe renal impairment. No statistically significant difference was observed in FF. Results from the statistical analyses showed no statistically significant change from Day -1 to Day 13 in GFR and FF for both renal function groups. For RBF a statistically significant decrease of 7.7% was observed in the group with moderate renal impairment (P = 0.023), however, this change is deemed not to be clinically relevant. No statistically significant differences were observed when comparing

the changes from baseline in GFR, RBF, and FF between the two renal function groups (*P* > 0.050).

Table 1 Demographic data summary.

	Corrected creatinine clearance > 50 ml/min/1.73m^2 (n=9)	Corrected creatinine clearance <= 50 ml/min/1.73m^2 (n=10)
Sex [n (%)]		
Males	8 (88.9)	9 (90.0)
Females	1 (11.1)	1 (10.0)
Age (y) [mean (SD)]	65.2 (8.5)	62.7 (10.2)
Weight (kg) [mean (SD)]	94.1 (27.8)	88.9 (14.4)
Height (cm) [mean (SD)]	175.4 (8.0)	173.7 (7.9)
BMI (kg/m^2) [mean (SD)]	30.3 (7.7)	29.4 (4.0)
Caucasian/white race	100%	100%
Antihypertensive treatment [n (%)]		
ARB	1 (11.1)	3 (30.0)
ACEI	8 (88.9)	7 (70.0)
Antidiabetic treatment [n (%)]		
No therapy		3 (30.0)
Monotherapy		
Insulin	4 (44.4)	4 (40.0)
Sulphonylureas	2 (22.2)	1 (10.0)
Meglitinides		1 (10.0)
Combination therapy		
Insulin + metformin	1 (11.1)	
Insulin + α-glucosidase inhibitor		1 (10.0)
Metformin + meglitinide	1 (11.1)	
Metformin + sulphonylureas	1 (11.1)	
Hemoglobin A_{1C}	6.98 (0.79)	7.10 (0.89)
Serum creatinine (μmol/l) [mean (SD)]	103 (26)	245 (77)
Corrected creatinine clearance (ml/min/1.73m^2) [mean (SD)]	89.7 (35.7)	30.4 (7.9)

BMI = body mass index; ARB = angiotensin receptor blocker; ACEI = angtiotensin-converting enzyme inhibitor.

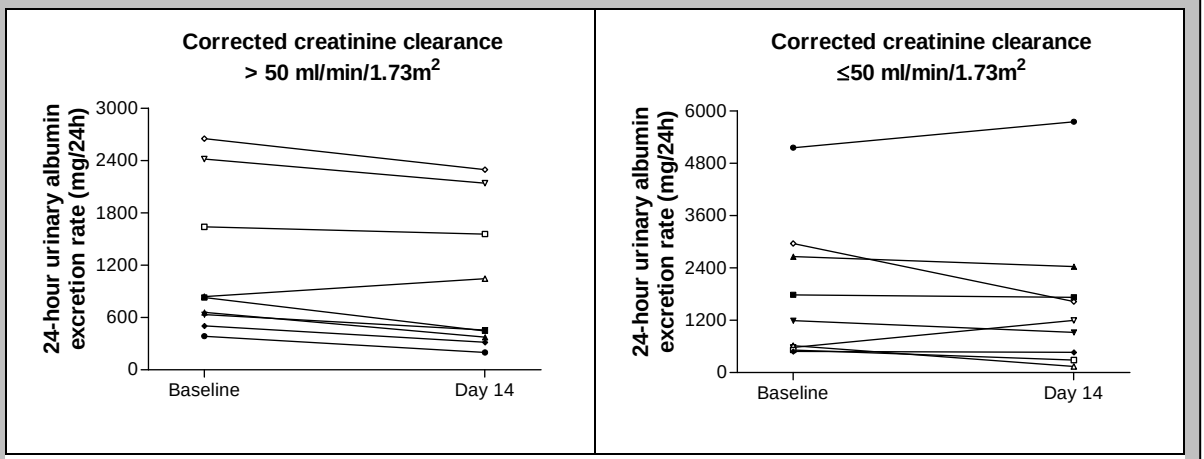
The mean plasma concentration-time profiles of palosuran for the two renal function groups are shown in Figure 2. The plasma concentration-time profiles after a single oral dose of palosuran and after 13.5 days of treatment were similar in both groups and could be characterized by rapid absorption with a peak at approximately 1 hour. Some patients showed a second peak at 2 hours, but this double-peak phenomenon was less frequently observed on Day 14. No statistically significant differences between the two groups were detected for any plasma pharmacokinetic parameter (*P* > 0.050), nor was any statistically significant change detected from Day 1 to Day 14 within each renal function group (*P* > 0.050).

Table 2 24-hour urinary albumin excretion rate values by renal function group and for the groups combined.

	N	24-hour urinary albumin excretion rate (mg/24h)	P value*
Corrected creatinine clearance > 50 ml/min/1.73m^2			
Baseline	9	944 (506 - 2416)	
Day 14	9	696 (317 - 2141)	0.027
Corrected creatinine clearance ≤ 50 ml/min/1.73m^2			
Baseline	9	1255 (518 - 2961)	
Day 14	9	975 (285 - 2430)	0.250
All patients			
Baseline	18	1088 (576 - 2416)	
Day 14	18	824 (374 - 1727)	0.014

BMI = body mass index; ARB = angiotensin receptor blocker; ACEI = angtiotensin-converting enzyme inhibitor.

Figure 1 Individual 24-hour urinary albumin excretion rate values at baseline and after 14 days of treatment with 125 mg palosuran b.i.d.



P = 0.027 when comparing Day 14 to baseline for the group with corrected creatinine clearance > 50 ml/min/1.73 m^2. P = 0.250 when comparing Day 14 to baseline for the group with corrected creatinine clearance ≤ 50 ml/min/1.73 m^2.

Table 3 Renal hemodynamic parameters in patients grouped by renal function.

	N	GFR (ml/min)	RBF (ml/min)	FF
Corrected creatinine clearance > 50 ml/min/1.73m^2				
Day -1	9	82.6 (63.3 - 108)	767 (566 - 1040)	0.19 (0.16 - 0.22)
Day 13	9	91.8 (67.5 - 125)	698 (477 - 1022)	0.21 (0.18 - 0.25)
Corrected creatinine clearance <= 50 ml/min/1.73m^2				
Day -1	8	30.7* (20.3 - 46.6)	271* (191 - 385)	0.18 (0.13 - 0.25)
Day 13	8	42.2 (29.7 - 60.0)	250$^{\#}$ (181 - 344)	0.26 (0.19 - 0.37)

Data are geometric means (and 95% CI).
GFR = Glomerular filtration rate; RBF = renal blood flow; FF = filtration fraction.
*P < 0.050 vs. Day - 1 of the group with a corrected creatinine clearance >50 ml/min/1.73m^2.
$^{\#}P$ = 0.023 versus Day -1.

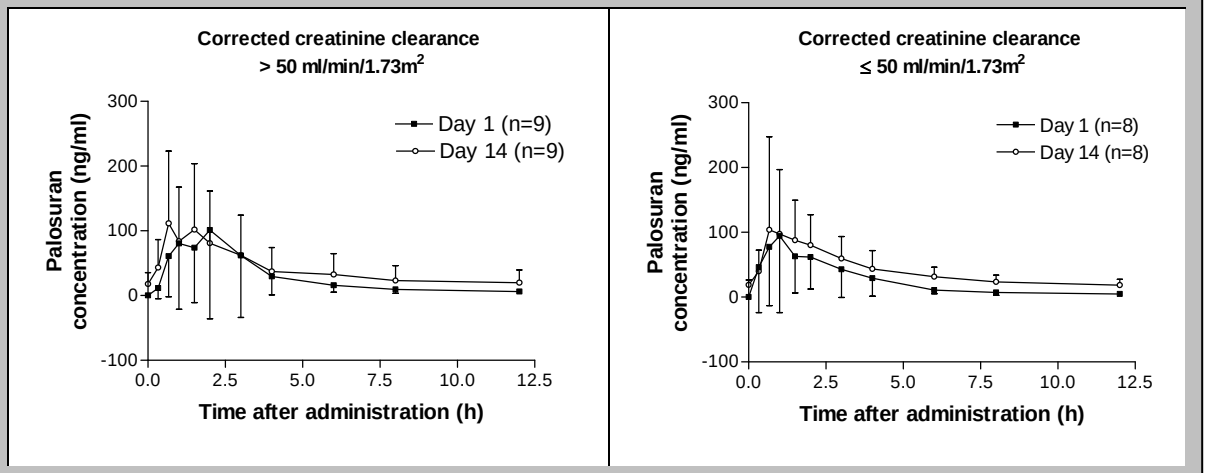
A summary of the plasma pharmacokinetic parameters is shown in Table 4. The accumulation index for the group with normal or mild renal impairment was 1.5 (geometric mean, 95% CI: 1.2 - 2.0) and for the group with moderate to severe renal impairment 1.8 (geometric mean, 95% CI: 1.2 - 2.7) (P > 0.050).

A summary of the urine pharmacokinetic parameters is shown in Table 5. In the group with normal to mildly impaired and that with moderately to severely impaired renal function, on average less than 1% and 0.7% of the administered dose, respectively, was excreted unchanged in urine. The renal clearance of palosuran was higher in the group with normal to mildly compared to that with moderately to severely impaired renal function (48.4 ml/min vs. 28.6 ml/min and 50.3 ml/min vs. 32.2 ml/min on Day 1 and Day 14, respectively).

No serious adverse events or adverse events that led to premature withdrawal from the study were reported. Of the 19 patients treated with palosuran, 17 reported a total of 43 adverse events. The most frequently reported adverse event was “feeling hot”, which,

with regard to time and duration of the inulin/PAH infusion, is most likely related to the infusion of inulin/p-aminohippuric acid, though a relation between study drug and adverse event cannot be completely excluded. In addition, only headache, fatigue, nasopharyngitis, and vertigo were reported more than once.

Figure 2 **Arithmetic mean plasma concentration (SD) versus time profiles (0-12 hours) of palosuran in patients categorized by renal function group (linear scale).**



No statistically significant differences were observed when comparing the group with a corrected creatinine clearance >50 ml/min/1.73m^2 versus ≤ 50 ml/min/1.73m^2 and when comparing Day 14 to Day 1 within each renal function group.

A summary of the most frequently reported adverse events including those adverse events judged to be unrelated to study treatment is provided in Table 6. Most adverse events were of mild to moderate intensity and all adverse events resolved without sequelae. No effects of palosuran on hematology and biochemistry parameters, vital signs (including blood pressure), physical examination, or ECG parameters could be detected.

Discussion

To the best of our knowledge, this study is the first in which a urotensin-II antagonist has been administered to patients who are prone to cardiovascular disease. While the peptide urotensin-II, an endogenous agonist of the urotensin-II receptor has been

Table 4 Plasma pharmacokinetic parameters of palosuran in patients grouped by renal function (125 mg palosuran twice daily for 13.5 days).

	N	C_{max} (ng/ml)	t_{max} (h)	AUCτ (ng.h/ml)	Accumulation index
Corrected creatinine clearance > 50 ml/min/1.73m^2					
Day 1	9	139 (76.5 - 254)	1.0 (0.67-4.0)	283 (171 - 470)	
Day 14	9	135 (70.6 - 259)	1.5 (0.67-4.0)	433 (281 - 668)	1.5 (1.2 - 2.0)
Corrected creatinine clearance ≤ 50 ml/min/1.73m^2					
Day 1	8	109 (44.1 - 271)	1.3 (0.55-4.0)	235 (118 - 470)	
Day 14	8	107 (46.8 - 243)	1.5 (0.67-3.0)	429 (261 - 703)	1.8 (1.2 - 2.7)

Data are geometric means (and 95% CI); for tmax data are median (range).
No statistically significant differences were observed either within or between the 2 renal function groups.

known for over four decades, the urotensin-II receptor itself has only recently been characterized and its function is still not fully understood [19]. Urotensin-II receptors are predominantly present in human heart and arterial vessels, suggesting urotensin-II, which is shown to be a more potent vasoconstrictor than endothelin-1, to be of importance as a cardiovascular mediator [19-23]. Indeed, in patients with renal failure increased plasma urotensin-II levels (2-3 fold greater than control) have been observed, suggesting that a urotensin-II receptor antagonist could have a therapeutic role in such patients [20-26].

Palosuran is a non-peptidic, specific antagonist of the urotensin-II receptor and has been studied in preclinical disease models and healthy human subjects [28-31]. In rat

Table 5 Urine pharmacokinetic parameters of palosuran in patients grouped by renal function (125 mg palosuran twice daily for 13.5 days).

	N	% dose excreted unchanged in urine	CL_R (ml/min)
Corrected creatinine clearance > 50 ml/min/1.73m^2			
Day 1	9	0.66 (0.35 - 1.3)	48.4 (35.7- 65.5)
Day 14	9	1.0 (0.60 - 1.8)	50.3 (35.4 - 71.5)
Corrected creatinine clearance $\leq$ 50 ml/min/1.73m^2			
Day 1	8	0.32 (0.14 - 0.72)	28.6 (20.7 - 39.5)
Day 14	8	0.66 (0.35 - 1.2)	32.2 (22.0 - 47.0)

Data are geometric means (and 95% CI).

Table 6 Summary of adverse events reported more than once during study (treatment-emergent and unrelated to treatment) by frequency.

System Organ Class/ Preferred Term	Number of events		
	Corrected creatinine clearance > 50 ml/min/1.73m^2 (n=9)	Corrected creatinine clearance <= 50 ml/min/1.73m^2 (n=10)	Total (n=19)
Feeling hot	5	5	10
Headache	4	3	7
Fatigue	1	1	2
Nasopharyngitis	-	2	2
Vertigo	1	1	2

models of acute renal failure and diabetes, palosuran significantly improved renal function, decreased the number of tubular and tubulointerstitial lesions, and improved survival [28,29]. Preclinical data also suggested that palosuran exhibited selective renal vasodilating effects [28], indicating that compared to angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, which induce systemic vasodilation, palosuran has a completely different mode of action. In healthy male subjects, good tolerability and convenient pharmacokinetic properties were observed, permitting us to investigate palosuran in diabetic patients [30,31].

The most interesting observation of this study was that after 13.5 days of treatment, the 24-hour urinary albumin excretion rate had, compared to baseline, statistically significantly decreased with 26.2% (geometric mean, 95% CI: 5.0 – 46.3, *P* = 0.027) in the group with normal to mildly impaired renal function. A decrease of 22.3% (geometric mean, 95% CI: - 11.6 - 45.0) was observed in the group with moderately to severely impaired renal function, although this difference did not reach statistical significance (*P* = 0.250).

Although the relationship between 24-hour urinary albumin excretion rate and effect on renal function has not been completely elucidated, 24-hour urinary albumin excretion rate is accepted as a clinical marker for cardiorenal disease [38-40]. Therefore, a decrease of the magnitude found in this study would be clinically significant and beneficial with regard to the patient's disease progression [38]. As this study was not powered to prove statistical significance on 24-hour urinary albumin excretion rate, the overall decrease of 24.3% (geometric mean, 95% CI: 4.1 – 45.0, *P* = 0.014) is a strong indication of a drug-related effect. The trial ended with this 24-urinary albumin excretion

rate assessment, hence, no data is available on the reversibility of the reduction of albuminuria.

The corrected creatinine clearance is used as an estimate of the GFR, whereas the GFR determined by inulin infusion is the standard to assess kidney function. Statistical analysis of GFR and RBF at baseline confirmed that the groups studied were significantly different regarding these parameters. However, no clinically relevant changes in the renal function parameters GFR, RBF, and FF were observed between baseline and after 12.5 days of treatment with palosuran. Importantly, the renal hemodynamic assessments were performed 2 hours after morning study drug intake to ensure sufficient exposure to palosuran. Review of the individual plasma concentration-time profiles showed that all patients were exposed to palosuran at the time of the renal hemodynamic assessments. As known from studies performed by Björck et al. [41] and Buter et al. [42], effects on renal hemodynamic parameters can already be seen after nine days of treatment with angiotensin-converting enzyme inhibitors and after three days of treatment with angiotensin receptor blockers. Therefore, the treatment period of two weeks with palosuran should have been sufficient to observe any effects, unless urotensin-II receptor antagonists are associated with an unusually slow onset of action. One of the reasons, why treatment with palosuran did not significantly influence renal function parameters may be the fact that all patients included in the current study were treated either with an angiotensin-converting enzyme inhibitor or angiotensin receptor blocker. As both of these drug classes are associated with changes in renal hemodynamics in diabetic patients [41,42], such effects associated with palosuran may have been masked. Due to the proven efficacy of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers in reducing macroalbuminuria in diabetic patients, for ethical reasons, patients in this trial were not discontinued from their normal

treatment. However, it may be worthwhile to perform a trial in a small number of drug-naive patients to investigate effects of palosuran *per se* on renal hemodynamics and on the 24-hour urinary albumin excretion rate.

The mechanism behind the lowering of 24-hour urinary albumin excretion rate remains unknown, but appears to be independent of changes in renal hemodynamics as no significant effects of palosuran on RBF and GFR could be observed. This is in contrast to the study performed by Buter et al. [42] in which consistent effects on urinary albumin excretion rate and renal hemodynamics could be observed after 3 days of treatment with an angiotensin receptor blocker in patients with type 1 diabetes mellitus. The increased urinary excretion of albumin is a marker of glomerular damage. In addition, albumin acts directly or indirectly on tubular cells and elicits a tubular cell response which promotes tubulointerstitial inflammation. The infiltrating interstitial macrophages and T-lymphocytes are considered to play an important role in the progression of glomerulosclerosis [43]. Numerous recent studies indicate that the accumulation of albumin in proximal tubules promotes tubulointerstitial inflammation resulting in endothelial dysfunction [36,40,44,45]. Therefore, palosuran may have a direct, so far not specified action on the tightness of the renal endothelia in patients with type 2 diabetes mellitus and macroalbuminuria.

This study showed that the plasma concentration-time profiles of palosuran in patients with different degrees of renal function are similar. Although the exposure to palosuran in the group with moderate to severe renal impairment was slightly lower than in the group with normal to mildly impaired renal function, this difference was negligible. Therefore, the plasma concentration-time profile in patients can be characterized by a rapid absorption with a peak at 1 hour after study drug intake, accumulation of 1.7-fold

(geometric mean, 95% CI: 1.3 - 2.1), and limited renal excretion of unchanged drug (< 1%). In healthy human subjects, a second peak of palosuran at around 4 hours had been observed[30,31], which was not present in the patient population. As palosuran is probably mainly metabolized through the liver and excreted into the bile, no major difference in renal excretion was observed between healthy subjects and patients with renal impairment. Interestingly, exposure to palosuran (in terms of C_{max} and AUC_{τ}), in comparison with healthy subjects was lower in patients, though the reason is yet unknown. As the dose given to patients (i.e., 125 mg b.i.d.) was based on the pharmacokinetics of palosuran in healthy subjects, dose adjustment might be required in patients. It could be hypothesized that the exposure in patients was not sufficient to elicit an effect on renal hemodynamics, even though an effect on albuminuria was observed. Palosuran is highly bound (96%, unpublished data) to plasma albumin in humans. Several co-medications administered in this patient population (e.g., the angiotensin receptor blocker losartan and the antidiabetic drugs tolbutamide and repaglinide) are known to have high plasma albumin binding as well [46-48] and, therefore, possible drug-drug interactions due to changes in pharmacokinetic parameters could theoretically occur. The clinical relevance of changes in plasma protein binding has been discussed by Benet and Hoener [49]. As palosuran is administered orally, mainly excreted through the liver, and it is expected that the therapeutic window is large, we conclude that the occurrence of drug-drug interactions is unlikely and, therefore, do not expect that dosing regimens should be adapted for an altered unbound fraction. Larger studies with different doses or dosing regimens should provide more insight in the pharmacokinetics of palosuran in different patient groups.

Palosuran was very well tolerated. No serious adverse events or adverse events that led to premature withdrawal from the study were reported. The most frequently reported

adverse events were “feeling hot” and headache, of which the first is probably related to the infusion of inulin and p-aminohippuric acid as this was not observed in healthy subjects [30,31]. No clinically relevant changes in ECG, or clinical laboratory test parameters were observed in this study, in line with the phase I observations. Also in line with preclinical results, no effects on vital signs were observed in these patients. Although the effect on blood pressure with monotreatment of palosuran in patients has not been studied yet, it appears that palosuran indeed lowers the 24-hour urinary albumin excretion rate through a different mechanism than the current therapies (angiotensin-converting enzyme inhibitors or angiotensin receptor blockers).

In conclusion, palosuran on top of treatment with an angiotensin-converting enzyme inhibitor or angiotensin receptor blocker decreases albuminuria in macroalbuminuric patients with type 2 diabetes mellitus without influencing renal or systemic hemodynamics. The observed reduction in 24-hour urinary albumin excretion rate can most likely be explained by renal and not by systemic actions, although in this study no change in renal hemodynamics was observed. Since the magnitude of this effect appears to be clinically relevant even in patients treated with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, larger and placebo-controlled studies with palosuran in this patient population should be initiated to investigate whether or not urotensin-II receptor antagonists could be utilized, as a combination or mono therapy, in the treatment of diabetic nephropathy.

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Chapter 7 Effect of the urotensin-II receptor antagonist palosuran on secretion of and sensitivity to insulin in patients with type 2 diabetes mellitus.

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What is already known about this subject?

- Urotensin-II (U-II) is one of the most potent vasoconstrictors identified thus far. Though differences in both U-II blood levels and U-II receptor (UT-receptor) expression have been observed in patients with cardiovascular and cardiorenal disease, the precise function in humans has not been elucidated.
- U-II and its receptor have been reported to be involved in glucose metabolism and insulin resistance, which can lead to the development of type 2 diabetes mellitus.
- In rat models of diabetes, palosuran, a selective, potent antagonist of the human UT-receptor improved several disease markers.

What this study adds

- In this study in diabetic patients, the effects of palosuran on insulin secretion and sensitivity were investigated using a hyperglycemic glucose clamp and a meal tolerance test and daily glucose levels were also studied. Although no obvious beneficial effect of palosuran in this patient population was observed, the study contributes to providing more insight in the human U-II/UT receptor system.

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Abstract

Aim: To investigate the effects of palosuran, a non-peptidic, potent, and selective antagonist of the urotensin-II receptor on insulin and glucose regulation in 20 diet-treated patients with type 2 diabetes mellitus in a double-blind, placebo-controlled, randomized, crossover, proof-of-concept study.

Methods: After 4 weeks of oral treatment with 125 mg palosuran or placebo b.i.d., effects on insulin secretion and sensitivity and blood glucose levels were assessed by means of a hyperglycemic glucose-clamp, meal tolerance test, Homeostasis Model Assessment-Insulin Resistance (HOMA-IR) score, and daily self monitoring of blood glucose (SMBG). Plasma concentrations of palosuran were determined for 12 hours on the last day of intake.

Results: Palosuran did not affect second-phase insulin response (primary endpoint) during the hyperglycemic glucose-clamp in comparison to placebo (paired difference of -1.8 µU/ml with 95% confidence interval of -7.8, +4.2). Likewise, no effects of palosuran were detected on the first-phase insulin response, as on insulin secretion and blood glucose levels during the meal tolerance test or on HOMA-IR score. No clinically significant effects on daily blood glucose profiles were observed during the study. Geometric mean $C_{max,}$ and AUC_{τ} (95% confidence interval) and median t_{max} (range) in this patient population were 180 (125, 260) ng/ml, 581 (422, 800) ng.h/ml, and 3.0 (0.67, 4.3) h, respectively.

Conclusions: The results of this study indicate that antagonism of the U-II system does not influence insulin secretion or sensitivity or daily blood glucose levels in diet-treated patients with type 2 diabetes.

Introduction

The prevalence of type 2 diabetes mellitus has increased dramatically in the last decades, being the seventh leading cause of death in the United States, and affecting the quality of life in a majority of the patients [1,2]. Important characteristics of type 2 diabetes are impaired insulin action (insulin resistance) and an altered endogenous insulin secretion pattern caused by a defective pancreatic *β*-cell response to glucose (insulin deficiency), of which the latter is predictive of overt type 2 diabetes mellitus [3-5]. The gold standard for estimation of *β*-cell secretory capacity is the hyperglycemic glucose-clamp [6]. Using this clamp technique, the *β*-cell response is evaluated by exposure to the insulin secretagogue glucose. When measured in healthy subjects, the *β*-cell response to glucose is biphasic in nature with an early burst of insulin release within the initial 10 minutes after an increase in blood glucose levels and a second phase characterized by a progressive increase in insulin secretion lasting several hours [7]. As glucose tolerance progresses to full-blown diabetes, the first-phase insulin response diminishes [7-9].

Treatment of patients in early stages of type 2 diabetes is aimed at increasing *β*-cell function and lowering insulin resistance in order to lower blood glucose levels. Though an array of antidiabetic drugs is currently available, treatment can be associated with several acute and long-term side effects ranging from profound hypoglycemia to congestive heart failure [10-12]. Therefore, there is still an unmet need for better and safer antidiabetic drugs.

Urotensin-II (U-II) is a cyclic peptide described as one of the most potent vasoconstrictors known, though the magnitude of its effect is highly dependent on the species and anatomical source of the vessel [13]. In the isolated perfused rat pancreas,

U-II blocked the release of insulin in response to glucose and arginine by acting directly on the β-cells [14]. Increased plasma U-II concentrations have been observed in patients with diabetes, chronic heart failure, and kidney diseases [15-17]. Further, a correlation between polymorphism of the gene encoding U-II and glucose intolerance has been identified in Japanese patients with diabetes mellitus [18,19]. Therefore, Urotensin-II receptor (UT receptor) antagonists may have a therapeutic potential in patients with diabetes, as well as in patients with diabetic nephropathy [16,17,20]. Palosuran is a non-peptidic, orally active, potent antagonist of the human UT receptor [21]. In rat models of renal ischemia, palosuran was effective in both preventing the post ischemic renal vasoconstriction and in reducing the post ischemic acute renal failure [21]. Subsequently, palosuran prevented the development of acute renal failure and the histological consequences of ischemia [21]. In a rat model of diabetes, palosuran increased renal blood flow, delayed the development of proteinuria and renal damage and improved survival [22]. In addition, palosuran had significant beneficial effects on glycemia, serum cholesterol, triglycerides, and HbA_{1C}. In this rat model, administration of palosuran increased insulin concentrations moderately, but significantly [22].

In the palosuran phase I development program, conducted in healthy male subjects, no remarkable safety findings were detected following administration of palosuran up to high doses (2000 mg as a single dose and 500 mg b.i.d.). The plasma-concentration profile revealed a rapid and slower phase of elimination, resulting in low plasma concentrations of palosuran 12 hours post dose. These findings indicated that twice-daily dosing would be the most appropriate dosing regimen. Using this dosing regimen the accumulation index was approximately 2.5 [23,24]. Urinary excretion of unchanged drug in healthy subjects was less than 3% of the administered dose and *in vitro* studies showed no indications of cytochrome P450 inhibition [21,23,24].

Based on the observations of elevated U-II levels in patients with diabetes and the beneficial effect of palosuran in rat models of diabetes, we investigated the effects of multiple-dose palosuran treatment on insulin secretion and sensitivity and daily glucose levels, as well as the pharmacokinetics and safety and tolerability of palosuran, in patients with type 2 diabetes mellitus. The primary objective of this study was to investigate the second-phase insulin response during a hyperglycemic glucose-clamp to palosuran in comparison to placebo. Although the first-phase insulin response provides useful insights into the pathophysiology of type 2 diabetes, it may only reflect limited aspects of the complex process of insulin secretion, may be relatively insensitive to subtle change in function. In order to investigate the effects of palosuran *per se*, a diet-treated patient population was selected. A dose regimen of 125 mg b.i.d. was selected for this proof-of-concept study, as in a previous pilot study in patients with diabetic nephropathy a decrease in 24-hour albumin excretion rate, an accepted clinical marker for cardiorenal disease, was demonstrated, and, had been well tolerated in both healthy subjects and patients [23-25].

Methods

Study population

Study participants were patients of either sex, between 30 and 65 years of age, with type 2 diabetes mellitus treated by diet only, fasting blood glucose level between 110 and 180 mg/dl (6.1 and 10.0 mmol/l, limits included), and stable HbA_{1C} below 8.5%.

Patients were excluded if they were women of childbearing potential, were treated with an antidiabetic drug in the two months before screening, if they had severe concomitant diseases, or, more specifically, had evidence of diabetes complications.

Study design

The protocol for the study was approved by the Ethics Committee of the Ärztekammer Nordrhein, Düsseldorf, Germany. The study was conducted as a mono-center, double-blind, placebo-controlled, randomized, two-way crossover study. In random order, patients were treated with 125 mg palosuran or placebo twice daily for a four week treatment period and, after a 4-week wash-out period, switched to the alternative treatment. Every treatment period started with an assessment of baseline and safety parameters. After 15 days of treatment a check-up visit was performed, which consisted of safety, blood glucose, and insulin assessments. After 28 and 29 days of treatment a meal tolerance test and a hyperglycemic glucose-clamp were performed, respectively. In addition, samples for pharmacokinetic evaluations were taken during a 12-hour interval on the day of the hyperglycemic glucose-clamp. Patients were requested to practice self-monitoring of blood glucose (SMBG) during the whole course of each treatment period. At the end of each treatment period, safety parameters were assessed and, finally, patients were followed-up for 4 weeks after the last study drug intake. Patients were in the clinical research institute on the first day, for the check-up visit on day 15, and during the meal tolerance test and hyperglycemic glucose-clamp assessments on days 28 and 29, respectively. At each visit, capsules with the study drug were counted to check the compliance. In addition, patients were requested to record the drug intake times at home in a patient diary.

Hyperglycemic glucose clamp

The hyperglycemic glucose-clamp was performed under fasted conditions. Patients were in supine position with catheters inserted into antecubital veins for infusion of 20% glucose solution and drawing of blood samples. For continuous measurement of

arterialized venous blood glucose by means of an artificial pancreas (Biostator, mtb Medizintechnik, Ulm, Germany), another catheter was inserted into a dorsal hand vein. Arterialization of venous blood was achieved by using the heated hand technique with the hand placed in a box in which the air was warmed to approximately 55°C. One hour after study drug intake the hyperglycemic glucose-clamp procedure was started which consisted of administration of an intravenous loading dose of 20% glucose solution (Glucose 20 pfrimmer, Baxter Germany GmbH, Unterschleissheim, Germany) adjusted to the patient's bodyweight (150 mg/kg), followed by a 20% glucose solution infusion regulated by means of the Biostator in order to increase the patient's blood glucose to the target level of 240 mg/dl. Subsequently to the loading dose the blood glucose was maintained for 120 minutes at that target level by a variable infusion of 20% glucose solution applied by the Biostator. For the determination of the baseline, three samples were taken at 10-minute intervals before the start of the clamp procedures. During the entire course of the hyperglycemic glucose-clamp patient's blood glucose levels and glucose infusion rates (GIR) were recorded by the Biostator on a minute-to-minute basis. Blood samples for serum insulin measurements were drawn every 2 minutes for the first 10 minutes and thereafter every 10 minutes using a local laboratory method.

Meal tolerance test

The meal tolerance test was performed one hour after study drug administration. After an overnight fasting period, patients consumed a standardized breakfast containing approximately 618 kcal, which was composed of 65% carbohydrates, 17% proteins, and 18% lipids. Before breakfast intake, 4 blood samples were collected for determination of baseline blood glucose and serum insulin levels. After breakfast intake, blood samples for determination of blood glucose and serum insulin levels were collected at 15-minute

intervals over a period of 4 hours. Blood glucose was measured using a laboratory device based on a glucose oxidase method (Super GL analyzer, Hitado, Moehnesee-Dellecke, Germany). Serum insulin levels were measured by the local laboratory using a radio immuno assay.

Additional metabolic assessments

For calculation of the HOMA insulin resistance score (HOMA-IR score), which is a measure of insulin sensitivity, fasted venous blood glucose and serum insulin levels were determined at baseline (Visit 1) and after 15, 28, and 29 days of treatment (Visit 2, 3, and 4, respectively) under fasted conditions.

When not in clinic, for evaluation of daily blood glucose levels, patients performed SMBG by measurement of pre-meal blood glucose values three times a day during the whole course of each treatment period using a point-of-care blood glucose meter (Glucometer, Bayer Leverkusen, Germany).

Pharmacokinetic sampling

Venous blood samples were collected immediately prior to study drug intake and 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, and 12 h thereafter on Day 29. Plasma was separated and frozen at -20 ºC until assayed. Plasma concentrations of palosuran were determined using a validated liquid chromatography assay coupled to tandem mass spectrometry operating in the positive ionization detection mode. The limit of quantification was 1.0 ng/ml (between-run coefficient of variation below 9.3%).

Safety and tolerability assessments

Tolerability and safety were evaluated using spontaneously reported adverse events (AEs), physical examination, measurements of vital signs (supine systolic and diastolic

blood pressure, and pulse rate), ECG and laboratory test parameters (including fructosamine, HbA_{1C}, and insulin), performed before, during, and after the study. For safety reasons, blood glucose was measured for 4 hours on the first day of each treatment period.

Data analysis

All calculations of the area under the concentration versus time curve (AUC), which is a measure to express overall drug effect, were performed using the linear trapezoidal method.

From the hyperglycemic glucose-clamp, the first- and the second-phase insulin response, the AUC of serum insulin levels during the clamp, and insulin sensitivity as assessed as total of glucose consumption were derived. Second-phase insulin secretion, the primary variable in this study, was calculated as the difference from baseline (defined as the mean of pre-infusion values) in the incremental insulin response during the last hour of the hyperglycemic glucose-clamp (expressed as absolute and % change). Likewise, total serum insulin AUCs during the hyperglycemic glucose-clamp were also baseline corrected. The occurrence of a first-phase insulin response was assessed before unblinding of the study. Glucose consumption during the hyperglycemic glucose-clamp was calculated by plotting the glucose infusion rate (GIR) per kilogram bodyweight versus time and determining the AUC.

From the meal tolerance test results, blood glucose and insulin levels were assessed by calculating the baseline-corrected (defined as the mean of the values before breakfast intake) AUC of blood glucose and serum insulin, respectively. HOMA-IR was calculated as fasting serum insulin level [µU/ml]*fasting blood glucose [mmol/l])/22.5 [26,27].

From the SMBG results, the AUC of the fasted blood glucose values during the study was calculated, standardized for 27 days.

Calculation of model-independent pharmacokinetic parameters for palosuran was performed using Professional WinNonlin Version 4.0.1. (Pharsight Corporation, Mountain View, USA). The maximum plasma concentration (C_{max}) and the time of its occurrence (t_{max}) were obtained from individual data. The area under the plasma concentration versus time curve during one dosing interval of 12 h (AUC_{τ}) was calculated.

Statistical analysis

The sample size of 20 patients was calculated on the basis of detecting a change of 30% in mean incremental second-phase insulin response using the Wilcoxon signed-rank test with a 2-sided Type I error of 5% and a power of 90%.

To explore differences between treatments on the hyperglycemic glucose-clamp, meal tolerance test, and additional blood glucose parameters, the two-sided Wilcoxon signed-rank test was used. All variables other than the second-phase insulin response were statistically analyzed in an exploratory fashion and, therefore, no correction for multiple testing was performed.

Results

Twenty-one patients were included in the study, received treatment with study drug and of these patients, twenty completed the study according to the protocol. One patient was withdrawn from the study due to an adverse event and was, therefore, only analyzed for safety (more details are provided below). Demographic characteristics of the per-protocol group at screening are summarized in Table 1.

Table 1 Demographic data summary.

Per protocol population	N = 20
Male / Female [n (%)]	16 (80.0) / 4 (20.0)
Age* [years (SD)]	53.7 (7.5)
Body weight* [kg (SD)]	88.8 (11.1)
BMI [n (%)]	
Normal (18.5 - 24.9 kg/m^2)	2 (10.0)
Overweight (25.0 - 29.9 kg/m^2)	11 (55.0)
Obese ($\geq$ 30.0 kg/m^2)	7 (35.0)
Waist-to-hip ratio* (SD)	1.0 (0.1)
HbA_{1C}* [% (SD)]	6.4 (0.8)

Data are expressed as arithmic means.

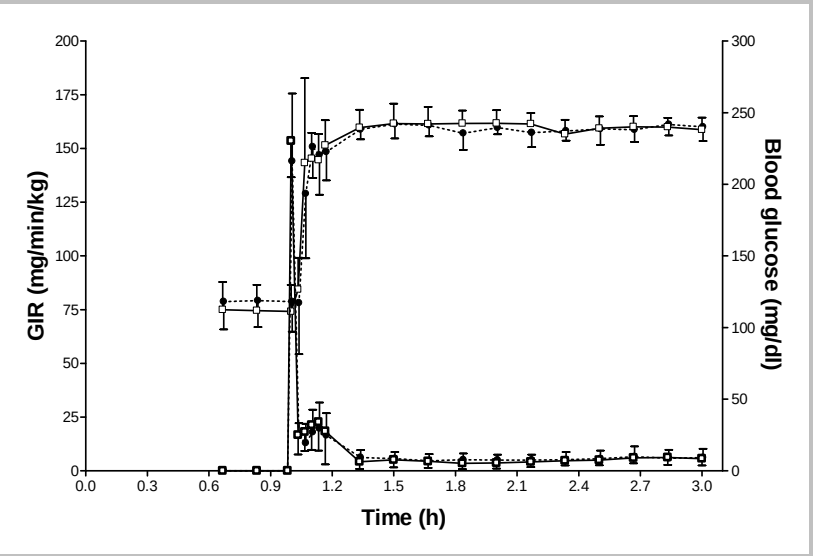
During the hyperglycemic glucose-clamps the blood glucose target of 240 mg/dl was rapidly reached and maintained throughout the glucose-clamp assessment (Figure 1).

The mean insulin concentration-time profiles during the hyperglycemic glucose-clamp for each treatment are displayed in Figure 2, showing no difference for palosuran versus placebo. The second-phase insulin response of each treatment and the difference between treatments during the glucose-clamp are presented in Table 2.

Treatment with palosuran did not affect the second-phase insulin response (primary endpoint). In addition, the insulin levels and exposure to insulin expressed in AUC (Table 2) during the glucose-clamp were comparable between treatment with palosuran and placebo.

A first-phase insulin response was identified in only 8 patients (40% of the per-protocol population).

Figure 1 **Arithmetic mean (± SD) of glucose infusion rate per kilogram bodyweight (lower) and blood glucose (upper) vs. time during the hyperglycemic glucose-clamp test (n = 20).**

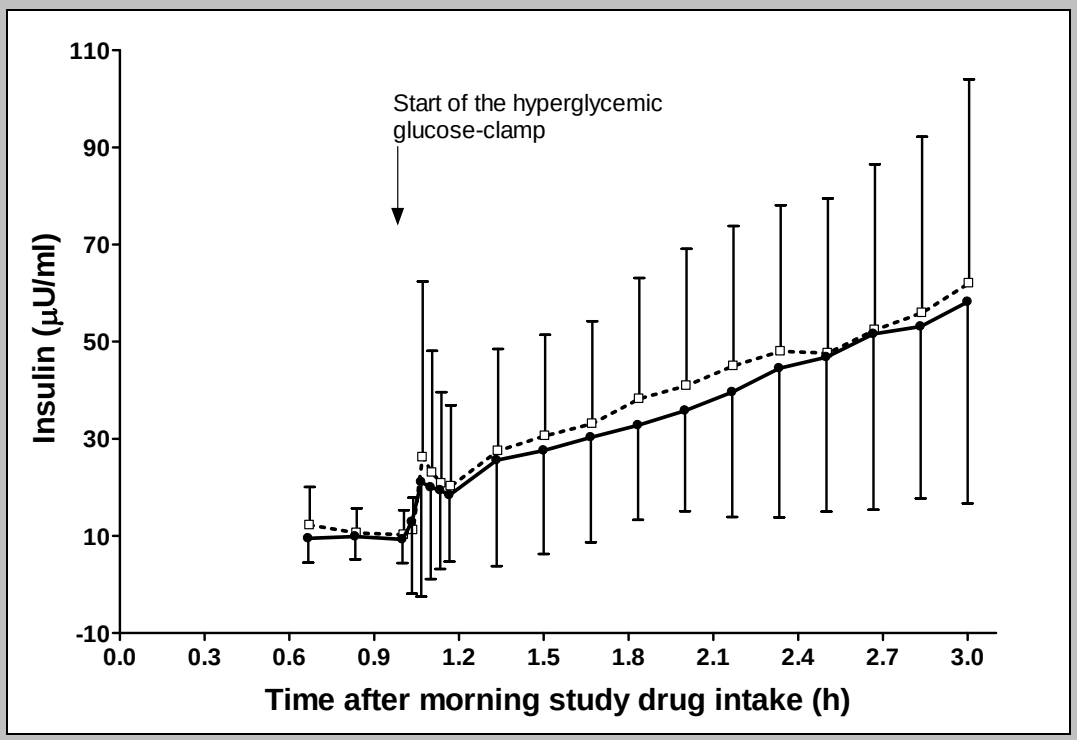


Palosuran (—□—); placebo (▪▪■▪▪).

Palosuran showed no effect on any of the secondary efficacy parameters, i.e., glucose consumption during the hyperglycemic glucose-clamp (Figure 1 and Table 3), meal tolerance test parameters (Figure 3), changes in insulin sensitivity measured by HOMA-IR score (Table 4) and AUC of the SMBG (Table 3). No effects on fructosamine levels were detected during this study.

The mean plasma concentration-time profile of palosuran in diet-treated patients with type 2 diabetes mellitus in this study is shown in Figure 4. Geometric mean $C_{max,}$ and AUC_{τ} (95% confidence interval) and median t_{max} (range) values in this patient population were 180 (125, 260) ng/ml, 581 (422, 800) ng.h/ml, and 3.0 (0.67, 4.3) h, respectively. The plasma concentration-time profile in this patient population can be characterized by rapid absorption with a peak at approximately 1 hour. Some patients showed a second peak between 3 and 4 hours after drug administration.

Figure 2 Arithmetic mean (± SD) concentration-time course of insulin during the hyperglycemic glucose-clamp test (n = 20).



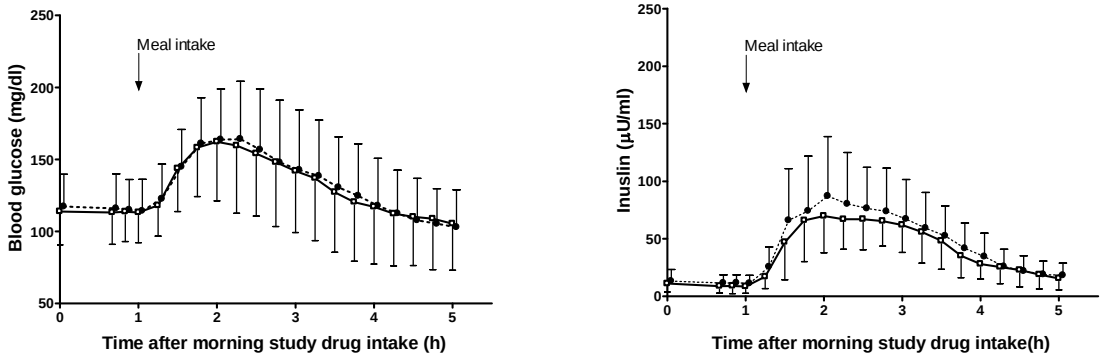
Palosuran (—□—); placebo (▪▪■▪▪).

Table 2 Insulin response during the hyperglycaemic glucose clamp.

Treatment	Second-phase insulin response [µU/ml (95%CI)]	Second-phase insulin response [% change (95%CI)]	Total AUC of insulin [µU.min/ml (95%CI)]
Palosuran	37.5 (24.3, 50.7)	400 (260, 541)	3284 (2076, 4492)
Placebo	39.3 (25.7, 52.9)	372 (256, 489)	3472 (2225, 47120)
Differences between palosuran and placebo	-1.8 (-7.8, +4.2)	28.1 (-59.6, +116)	-188 (-760, +383)

Data are expressed as arithmetic means. No statistically significant difference was observed between palosuran and placebo.

Figure 3 **Arithmetic mean (± SD) concentration-time courses of blood glucose (left) and insulin (right) during the meal tolerance test (n = 20).**



Palosuran (—□—); placebo (▪▪■▪▪).

Table 3 **Results of the hyperglycaemic glucose clamp, the meal tolerance test, and self-monitoring of blood glucose.**

Treatment	Glucose consumption during the hyperglycemic glucose-clamp [mg/kg (95%CI)]	Blood glucose levels in meal tolerance test [mg.min/dl (95%CI)]	Insulin secretion in meal tolerance test [µU.min/ml (95%CI)]	AUC of fasted, pre-meal blood glucose recorded by patients during the study [mg.day/dl (95%CI)]
Palosuran	832 (744, 920)	4693 (2896, 6490)	8363 (6936, 9790)	3450 (3212, 3688)
Placebo	862 (738, 986)	4511 (2838, 6184)	9478 (7381, 11576)	3505 (3195, 3816)
Difference between palosuran and placebo	-29.5 (-131, +71.6)	182 (-1129, +1493)	-1115 (-2456. +226)	-55.8 (-184, +72.9)

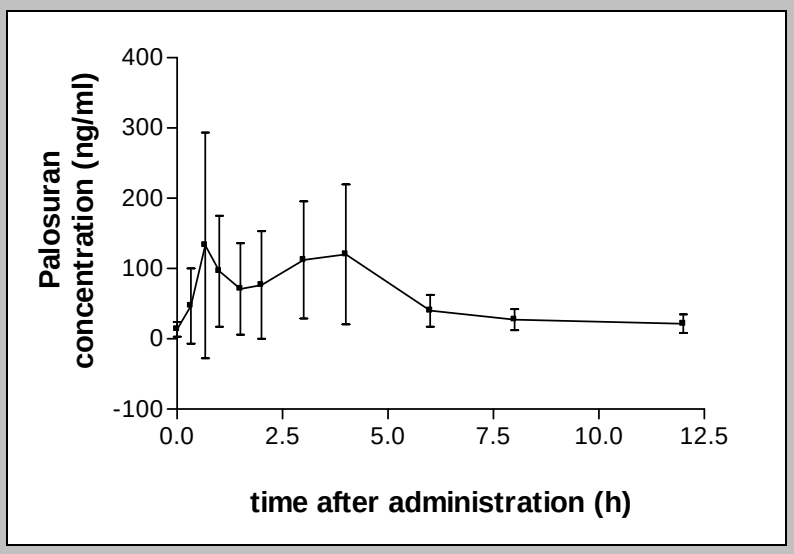
Data are expressed as arithmetic means. No statistically significant differences were observed between palosuran and placebo.

Table 3 **Insulin sensitivity expressed as homeostatis model assessment-insulin resistance score.**

Day	Palosuran [µU.ml-1.mM (SD)]	Placebo [µU.ml-1.mM (SD)]
1	2.3 (1.5)	4.4 (5.2)
15	4.0 (4.8)	4.1 (4.2)
28	3.0 (1.6)	3.7 (2.6)
29	2.8 (1.4)	4.5 (4.1)

Data are expressed as arithmetic means.

Figure 4 **Arithmetic mean plasma concentration (SD) versus time profile (0 - 12 hours) of palosuran in patients with diet-treated type 2 diabetes mellitus (n = 20) after 4 weeks of treatment with palosuran 125 mg b.i.d.**



Of the 21 patients, while treated with palosuran, 9 reported a total of 12 AEs. One of the patients was withdrawn from the study on Day 17 of the first treatment period due to a severe case of collapse. Though a relationship of this AE to palosuran cannot be excluded, the patient reported to have had previous experience of collapses before start of the study. Of the 20 remaining patients, while treated with placebo, 11 reported a

total of 17 AEs. Mild to moderate headache was the most commonly reported AE, all other AEs were mostly reported once or twice and were of mild to moderate intensity. Except for one mild case of aptyalism ("dry mouth") at night, all AEs resolved without sequelae. No effects of palosuran on hematology and biochemistry parameters, urinalysis, vital signs, body weight, physical examination, or ECG parameters could be detected.

Discussion

In animal models of diabetes, palosuran, a potent and selective UT-receptor antagonist, significantly improved survival of the rats and reduced hyperglycemia, serum cholesterol, triglyceride, and HbA_{1C} levels compared to untreated rats [22]. However, in humans the function of the U-II system has not yet been elucidated and is complicated by the fact that effects of U-II differ between species and vascular beds [13,28,29]. On the basis of the observation of high U-II levels in patients with diabetes mellitus, we investigated for the first time the effects of the U-II receptor antagonist palosuran in this patient population.

Our main focus in this study was to investigate the effects of palosuran on insulin secretion and sensitivity. Therefore, the change in second-phase insulin response during the hyperglycemic glucose-clamp was chosen as the primary efficacy endpoint. The hyperglycemic glucose-clamp is considered as gold standard to evaluate *β*-cell function [6,30]. Although a more laborious procedure, this method shows good reproducibility with within-subject variations below 10%, can focus on specific aspects of insulin action by adapting the concentrations of glucose and insulin, can be combined with various other techniques (e.g., tracers), and is not influenced by factors such as gut hormones and neural stimulation. A drawback of this method is that the glucose level is

raised to a higher level than that found in normal physiology and that it requires specialized equipment and personnel [31,32]. In this study we observed that palosuran did not affect the second-phase insulin response, neither in terms of absolute nor as percentage difference from baseline. Also no effect on the first-phase insulin response was detected, although only a part of the patients (40%) had a pronounced first-phase rise in insulin. In addition, no effects on the glucose infusion rate, or total AUC of insulin were detected during treatment with palosuran.

To explore the effects of palosuran under more physiological conditions, we also assessed β-cell secretory capacity with a meal tolerance test, in which glucose and insulin levels were measured after the administration of a standardized meal. In line with the findings of the hyperglycemic glucose-clamp, palosuran did not affect the insulin or glucose levels during the meal tolerance test. Interestingly, when comparing the insulin response, insulin levels during the meal tolerance test were in general higher than during the glucose-clamp, whereas in some patients the insulin response was marginal. This last observation indicates that factors other than the intravenous glucose stimulus alone influence insulin release from pancreatic β-cells after a meal. These factors could be the incretin peptide hormones such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like-peptide-1 (GLP-1). These hormones are released in the intestine only in response to nutrient ingestion and stimulation of the gastrointestinal tract, hence the additional effect on insulin secretion is not observed during the hyperglycemic glucose-clamp [33-35]. These results suggest that although the hyperglycemic glucose-clamp is a reliable, reproducible method to assess β-cell function, the meal tolerance test provides useful additional insight into the normal physiology of diabetic patients.

We further investigated insulin sensitivity, assessed both by glucose consumption during the hyperglycemic glucose clamp and HOMA-IR score and glucose levels during 28 days of treatment. Though the euglycemic glucose-clamp is the gold standard to assess insulin resistance, HOMA-IR scores are highly correlated with euglycemic glucose-clamp results [36]. No differences were observed between palosuran and placebo. It is noted that there was a difference between values at baseline and at end of treatment between palosuran and placebo (Table 4). This might be due to variation in HOMA, which is dependent on the number of fasting samples obtained and type of insulin assay used. Evaluation of the data using a log transformation approach as suggested by Muniyappa et al. shows that, the differences observed in this study are of no relevance [27]

The mean plasma concentration-time profile of palosuran in diet-treated patients with type 2 diabetes mellitus was similar to that of healthy subjects [24]. However, when reviewing the individual profiles it was observed that only few patients had the typical second peak that was observed in healthy subjects. The plasma concentration-time profile in patients showed a peak at approximately 1 hour after study drug administration, ensuring sufficient exposure to palosuran at the time of the hyperglycemic glucose-clamp and meal tolerance test. It was observed that the exposure to palosuran in this patient group was quite variable. Compared to a historical group of healthy subjects the C_{max} and AUC_{τ} of this patient population showed an overall decrease in exposure of approximately 50% [24].

Palosuran was well tolerated, in line with previous observations in healthy subjects and in a pilot study in patients with diabetic nephropathy [23-25]. No serious adverse events were reported. The most frequently reported AE was headache. The incidence of AEs

reported during treatment with palosuran was similar compared to placebo. No clinically relevant changes in vital signs, ECG, and clinical laboratory parameters were observed. For HbA_{1C} as a marker of glycemia this was expected as changes can only be seen after several months of treatment [37,38]. However, as fructosamine is a marker of blood glucose levels in the preceding 2-3 weeks, it was confirmed that palosuran does not influence glycemia [37,38].

From the results of this study we can conclude that neither under hyperglycemic glucose-clamp conditions nor during a more physiological meal test approach, palosuran had an influence on insulin secretion in diet-treated patients with type 2 diabetes. Also, no signs on insulin sensitivity or glucose levels during the treatment period of 28 days could be observed.

Due to the absence of any effects on insulin and glucose regulation parameters, it could be hypothesized that the exposure to palosuran was too low to invoke an effect and that dose adjustment would have been required in this patient group. However, in a study with type 2 diabetic nephropathy patients exposure to palosuran was comparable to our patient group, whereas in that study a significant decrease in 24 h-urinary albumin excretion rate was observed [25]. Furthermore, Clozel et al. showed a significant decrease in U-II induced contraction at 1 µM palosuran in a rat assay that assessed the functional selectivity of palosuran for the UT receptor [21]. Taking into account the 10−fold difference in inhibitory potency on the human compared to the rat UT receptor, this would translate in a concentration of approximately 50 ng/ml, which is well covered by the concentrations found in patients in this study. Therefore, we believe that a dose of 125 mg palosuran b.i.d. was sufficient to investigate the objectives of this study.

Another factor could be that the secretory capacity of insulin in the study population was too low. Indeed, only 40% of the included patients had a first-phase insulin response. However, this observation is in line with the current knowledge of insulin secretion in type 2 diabetes mellitus patients; a reduction or loss of the first-phase insulin response is one of the first characteristics of the disease [7,9,39] and does not necessarily impair the second-phase insulin response, which was the primary efficacy endpoint. Additionally, in a study performed by Rizzo et al., an effect of repaglinide on insulin secretion in a hyperglycemic glucose-clamp could be demonstrated in a similar patient population group, with similar serum insulin levels [40].

In diabetic rats, palosuran has been demonstrated to have acute and chronic effects on glucose control. Acutely, palosuran decreased serum glucose and increased insulin in response to a glucose load, suggesting that palosuran has a direct effect on release and/or production of insulin in diabetic rats [22]. Palosuran is a selective antagonist of the human UT-receptor with an *in vitro activity* about 300-fold greater than on the rat receptor. As our data show that in humans, antagonism of the U-II receptor does not affect insulin secretion, this finding suggests that the U-II system has a different function in humans. In animals, in addition to the direct effect on insulin secretion, palosuran could be beneficial by attenuating the increased sympathetic activity of diabetes. Data in animals have shown that exogenous U-II increases sympathetic activity by increasing circulating catecholamines and cortisol and stimulating fatty acid release [41,42]. It would be of interest to investigate whether U-II in man also has an effect on sympathetic activity, and whether antagonism of the UT-receptor would be beneficial in patients in whom sympathetic activity is increased.

In the present study plasma U-II concentrations were not determined. Therefore, it could be that the study participants did not have elevated U-II levels and, thus, antagonism of the UT-receptor would not elicit an effect. Although possible, we believe that this is unlikely given the fact that a clear relationship between diabetic state and elevated U-II levels has been observed [43,44].

In conclusion, although there are strong indications that the U-II system is involved in the pathogenesis of type 2 diabetes mellitus, and beneficial effects of palosuran have been observed in rat models, palosuran did not show any effects on insulin and glucose regulation (measured with a hyperglycemic glucose-clamp and meal tolerance test) in a diet-treated patient population. Also in terms of insulin sensitivity, as assessed with HOMA-IR score, no differences between palosuran and placebo were observed. Palosuran does not appear to represent a new treatment strategy in diet-treated patients with type 2 diabetes mellitus. More research is needed in order to understand the role of urotensin-II and its receptor in diabetes mellitus and to unravel the apparent discrepancy between animal and human observations.

Statement of competing interest

Ms Sidharta, Ms Chiossi, and Dr Dingemanse, are full-time employees of Actelion Pharmaceuticals Ltd. Drs Rave and Heise are investigators of the clinical trial and received financial compensation of the clinical costs associated with conducting the study. Prof. Krähenbühl has no conflict of interest with respect to the manuscript.

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General Discussion

Urotensin: functional or fishy?

Research on the function of Urotensin-II (U-II) and its receptor (UT receptor) has taken a flight after its discovery in humans in the late nineties. First thought to be a peptide purely existent in lower animal species, predominantly fish, since then the peptide has been identified in various mammalian species. With U-II and the UT receptor expressed in various tissues (e.g., heart, lung, kidney, spinal cord) and the finding that U-II is a more potent vasoconstrictor than endothelin-1, the U-II system as a target for various diseases holds a promise that has not been fulfilled so far.

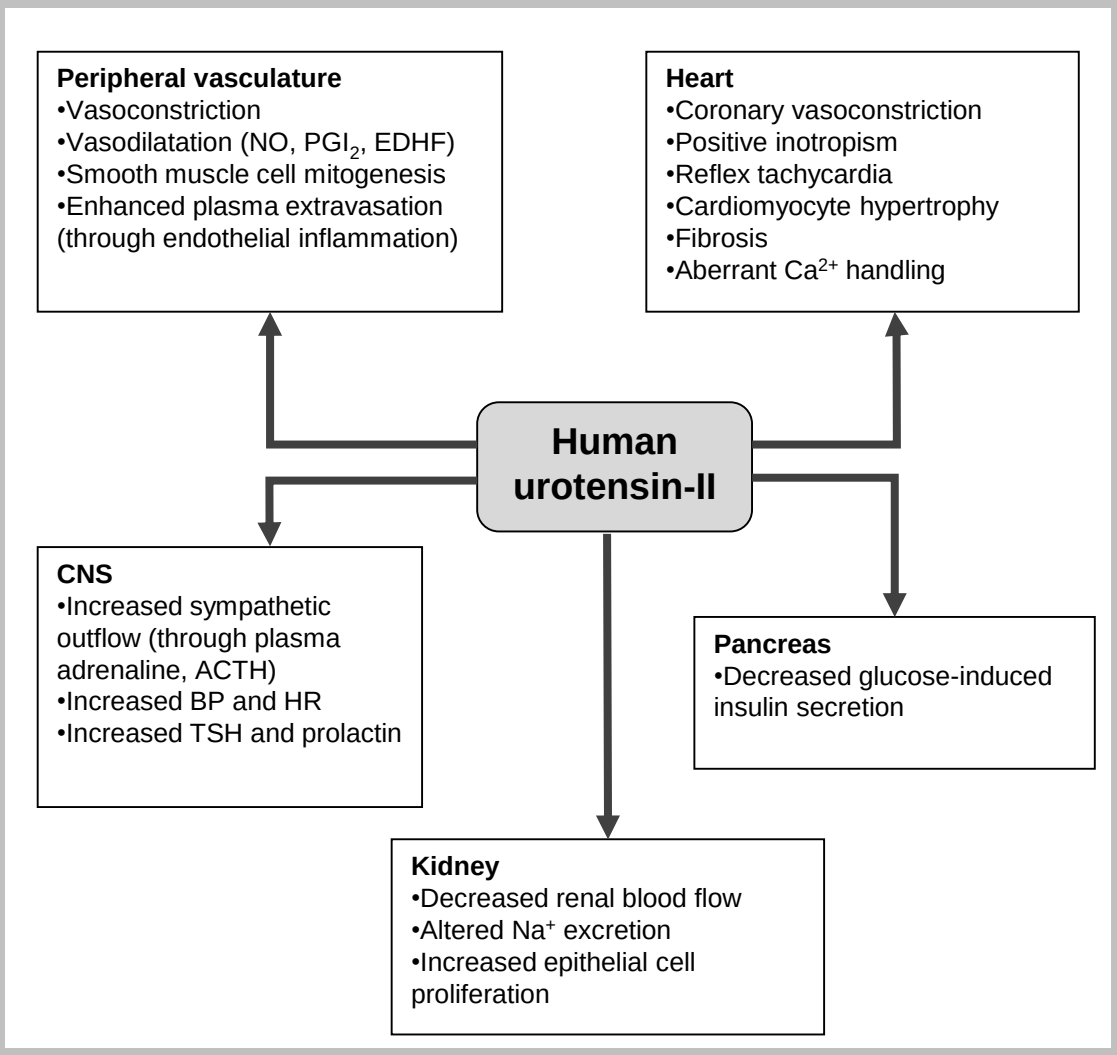
Besides its effect on blood vessels, U-II has other regulatory effects as discussed in Chapters 1 and 2 and summarized in Figure 1. Several functions of U-II are conserved across species, although conflicting responses to U-II also have been reported. Unlike endothelin-1, the response to U-II appears to be dependent on many factors such as the source of U-II used and the species investigated, as well as the vascular bed and vessel types studied. Partly due to this, it remains unclear whether U-II has a detrimental or protective function. It has been suggested that elevated U-II plasma levels could serve as a marker for disease progression. However, in recent studies, high levels of U-II could also be correlated to protective effects (Chapter 1).

What is the potential of the selective urotensin-II receptor antagonist palosuran in the treatment of metabolic and renal disease?

Numerous studies have suggested a role of U-II in glucose regulation through actions on pancreatic cells and stimulation of the central nervous system (Chapter 1) as well as controlling cardiovascular homeostasis through the kidney (Chapter 2).

Thus, a disruption of the U-II system could contribute to metabolic and renal disease such as Type 2 diabetes mellitus (Type 2 DM) and diabetic nephropathy.

Figure 1 Biological actions of human Urotensin-II on major organ systems.



ACTH = adrenocorticotropic hormone; BP = blood pressure; CNS = central nervous system; EDHF = endothelial derived hyperpolarization factor; HR = heart rate; NO = nitric oxide; PGI_2 = prostacyclin; TSH = thyroid-stimulating hormone

With a rapidly increasing number of patients worldwide suffering from Type 2 DM, the increased risk of concomitant cardiovascular disease, and the drawbacks of current diabetic medications, there is a need for an effective and safer treatment (Introduction).

Palosuran, a non-peptide, orally active, selective, potent, and competitive antagonist of the U-II receptor (UT receptor), developed by Actelion Pharmaceuticals Ltd, proved in rat models of diabetes to improve pancreatic and renal function and increase survival (Chapter 2).

In healthy subjects, palosuran was well tolerated after single- and multiple-dose administration over a wide dose range. The pharmacokinetics were indicative of a twice daily dosing regimen. Two absorption peaks could be detected at approximately 1 and 4 h after drug administration. Following this, elimination was biphasic with a faster and slower elimination phase resulting in low plasma concentrations at 12 h after drug administration (Chapter 3 and 4). The intake of food had a minor effect on drug exposure expressed in area under the curve (AUC), but is not considered to be of clinical relevance. However, the double-peak phenomenon as well as differences in morning and evening trough concentrations may be, in part, caused by the intake of food (Chapter 5). With increasing dose, a more than dose-proportional increase in palosuran exposure (expressed as maximum concentration and AUC) occurred. Although no treatment- or dose-related patterns in adverse events, vital signs, clinical laboratory, or ECG parameters were observed, a dose of 125 mg b.i.d. was selected for further clinical development to allow for an additional margin of safety. In addition, no pharmacodynamic markers could be identified in healthy subjects that could guide dose selection for patient studies (Chapter 3 and 4).

In an exploratory pharmacodynamic study in hypertensive patients with diabetic nephropathy it was shown that 125 mg palosuran, given b.i.d. for 13.5 days on top of existing blood pressure lowering medications, decreased 24-h urinary albumin excretion rate (24-h UAER), a clinical marker for cardiorenal disease (Chapter 6). Surprisingly, in this study, no effects on other renal hemodynamic parameters (i.e., glomerular filtration

rate, renal blood flow, filtration fraction) were observed, which hampers the understanding of the mechanism underlying the reduction of 24-h UAER. It is thought that concomitant medications may have masked certain effects of palosuran on renal hemodynamic parameters (Chapter 6) but further studies are needed to validate the effects seen in this trial. Up to date, only one other study has been conducted in an attempt to confirm these study results, without success [1]. The study of Vogt et al. investigated the effect of palosuran on 24-h UAER and systemic blood pressure in 62 patients with diabetic nephropathy, using a multi-center, randomized, double-blind, placebo-controlled, 2-period crossover design. Additionally, renal hemodynamic parameters were measured in a sub-study. In this study, it was shown that 4 week treatment with 125 mg palosuran b.i.d. did not show any effect on 24-h UAER or blood pressure. In the sub-study no effects on renal hemodynamic parameters were observed. Whether the observations were due to insufficient exposure to palosuran, masking of effects by concomitant medication, or other factors, deserves to be further investigated [1].

Palosuran was also investigated in a single-center, randomized, double-blind, placebo-controlled, 2-period crossover design study in twenty patients with Type 2 DM (Chapter 7). The aim of this proof-of-concept study was to evaluate the effect of a 4-week treatment with 125 mg palosuran b.i.d. on blood glucose and insulin levels after hyperglycemic glucose-clamp and a meal tolerance test. In the study, no clinically relevant effects of palosuran on any parameters were observed, indicating that palosuran may not be beneficial in treatment of Type 2 DM (Chapter 7).

Future perspectives and conclusions

The results described in this thesis show that palosuran, an orally active, selective, UT receptor antagonist has no clinically relevant effects in patients with diabetic nephropathy and Type 2 DM. The drug was well tolerated at a total daily dose of 250 mg for a period of 4 weeks. Given the discrepancy between the results of preclinical and clinical studies, several questions remain deserving further investigation.

I. Was the exposure sufficient in clinical studies?

Clinical research was limited to 4 weeks due to the available toxicology data at the time. Although it cannot be excluded that a longer duration of treatment would have resulted in a better effect, in the absence of any trends in the double-blind diabetic nephropathy and Type 2 DM studies, it is not considered to be a relevant study limitation. From pre-clinical experiments it was estimated that a clinically relevant dose would be between 50 and 125 mg palosuran [2]. It is known that direct translations from animal models to humans may not be reliable, specifically in the absence of a robust pharmacodynamic marker. With a number of tools available to predict the efficacious dose better [3], further research is needed to identify pharmacodynamic markers that could be utilized in animals as well in humans.

II. Was the choice of Type 2 DM an appropriate target population for a proof-of-concept study?

Diabetes is a complex metabolic disease that undergoes several stages that cannot be clearly distinguished [4]. In practice, the progression from pre-diabetes (also known as impaired glucose or impaired insulin tolerance) to Type 2 DM is a gray area for which the diagnostic criteria and classification have been frequently revised in the past 50 years [5-7]. In fact, the hyperglycemic clamp data in subjects in the Type 2 DM study

demonstrated that there is no 'typical' profile for Type 2 DM patients [8]. It may be that the U-II mechanism is not relevant at all stages of diabetes, thus the choice of a broad target population may have masked effects of palosuran. Clarifying the exact role of U-II and a choice for a sub-population of Type 2 DM patients by setting boundaries for blood glucose, HbA_{1C} or limiting the time since diagnosis may provide further insight into the utility of UT receptor antagonists in metabolic disease.

III. What is the role of U-II-related peptide (URP) in the U-II system?

While not discussed in detail in this thesis, after discovery of U-II a peptide analog termed U-II-related peptide (URP) was found first in rat brain [9-11], and later in other mammalian species [12]. While the function of URP appears largely the same as that of U-II, distinct differences in biological effects have been observed suggesting that they could also have different pathophysiological roles in disease [12]. Palosuran may have not been sufficiently selective, which may have contributed to the observed lack of effect in the clinical studies.

IV. Are there more promising indications for UT receptor antagonists?

Besides the renal and metabolic system, the UT receptor is widely expressed in the cardiovascular, pulmonary, and central nervous system (Chapter 1), which indicates that UT receptor antagonism can be useful in other indications than Type 2 DM and diabetic nephropathy. An upcoming indication of interest is pulmonary arterial hypertension (PAH). PAH is a disease that is associated with structural changes in both the pulmonary vasculature and the right heart ventricle. Changes involve three combined elements: vasoconstriction, vascular-wall remodeling, and thrombosis *in situ* [13]. Current therapies have targeted three major pathways (i.e., the prostacyclin pathway; the endothelin pathway; and the nitric oxide pathway) [14]. U-II is much more

potent than endothelin-1 [15] and UT receptor antagonists are believed to have vascular-wall remodeling properties through effects on the nitric oxide and endothelin pathways [16,17]. Indeed, recent data in animal models suggested that palosuran could provide benefit in the of treatment PAH as well as scleroderma, another disease associated with vascular alterations [18,19]. Last but not least *in vitro* data shows that U-II is expressed in a number of tumor tissues including adrenocortical tumors and neuroblastomas [20-23], which broadens the potential use of UT receptor antagonists.

In conclusion, while studies performed with palosuran have not shown efficacy in subjects with diabetic nephropathy and Type 2 DM, there is enough reason to believe that UT receptor antagonists can have therapeutic value in various other cardiovascular, pulmonary, renal, and oncologic indications. More insight in the U-II mechanism as well as clinical data is needed.

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Curriculum Vitae

Patricia N. Sidharta

Professional Experiences

2008 – present **Senior Clinical Pharmacologist Actelion Pharmaceuticals Ltd, Allschwil, Switzerland.**

- Managing clinical pharmacologists.
- Providing strategic input in drug development of several compounds in terms of pharmacokinetics/pharmacodynamics
- Representing clinical pharmacology at FDA and other health authorities
- Provide clinical pharmacology expertise within Strategy Portfolio Board, Life Cycle Team, Clinical Team and Clinical Trial Team (when appropriate)Design, coordination, evaluation, and reporting of clinical pharmacology studies during Phases I-IIa of drug development.

2003 – 2008 **Clinical pharmacologist, Actelion Pharmaceuticals Ltd, Allschwil, Switzerland.**

- Design, coordination, evaluation, and reporting of clinical pharmacology studies during Phases I-IIa of drug development.
- Member of several working groups focusing on standardization of processes within the company.

2002 – 2003 **Clinical scientist / project leader, Centre for Human Drug Research, Leiden, the Netherlands.**

- Focusing on CNS studies
- Advice in design, coordination, evaluation, and reporting of clinical pharmacology studies (single- and multicenter) in collaboration with multinational drug companies, Phase I of drug development.

2002 **Intern department of Pharmaceutics, Organon N.V., Oss, the Netherlands.**

- Title of research: "*Development of an universal formulation for use in toxicological studies through clinical phase I –III"*, (Supervisor: Prof. Dr. H. Vromans)

1996 – 2001 **Pharmacist (under supervision) / Pharmaceutical technician, the Netherlands**

- During college education worked at several pharmacies (Apotheek Westwijk, Vlaardingen, Apotheek Backer, Vlaardingen, Bohemenwijk Apotheek, the Hague).
- Preparation and dispensing of drugs.
- Provide information to patients, perform therapeutic drug monitoring and set up systems to improve the information flow.
- Set up systems to improve therapeutic drug monitoring/surveillance of patients.
- Improve logistics of the pharmacy (distribution of drugs, task schedules of the pharmacy team).

Education

2009 –2010 **Actelion Management Course**

2007 **Certification Pharmaceutical Professional: European Center of Pharmaceutical Medicine (ECPM) course, ECPM, Basel, Switzerland.**

1995 – 2002 **Pharmacy, Faculty of Pharmacy, University of Utrecht, the Netherlands.**

Additional Qualifications

- Certified typist (310 touches a minute)
- Computer skills: Windows, Microsoft Office, WinNonlin, Endnote, SlideWrite, MWPharm, Graphpad, NitroPro, Adobe Acrobat, Adobe Illustrator, Photoshop, Google Chrome, and Internet Explorer.
- Native Dutch speaker.
- Fluent speaker of the German and English language.
- Passive speaker of the Indonesian and French language
- Beginner level Spanish

Publications Manuscripts

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