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RESEARCH PAPER

The adenosine A_1 receptor antagonist SLV320 reduces myocardial fibrosis in rats with 5/6 nephrectomy without affecting blood pressure

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Background and purpose: Myocardial fibrosis is an unwanted effect associated with chronic renal failure. The adenosine system is involved in cardiac and renal function. Therefore, we investigated the novel selective adenosine A_1 receptor antagonist SLV320 focusing on its potential in preventing cardiomyopathy in rats with 5/6 nephrectomy.
Experimental approach: Male Sprague-Dawley rats were allocated to 4 groups of 12 rats each: 5/6 nephrectomy (5/6 NX), 5/6 NX plus SLV320 (10 mg kg^{-1} d^{-1} mixed with food), sham and sham plus SLV320. Study duration was 12 weeks, blood pressure was assessed repeatedly. At study end kidney function was assessed, blood samples and hearts were taken for histology/immunohistochemistry. Pharmacological properties of SLV320 were assessed using receptor binding and enzyme assays and in vivo.
Key results: SLV320 is a selective and potent adenosine A_1 antagonist *in vitro* (Ki = 1 nM) with a selectivity factor of at least 200 versus other adenosine receptor subtypes. Functional A_1 antagonism was demonstrated *in vivo*. In rats with 5/6 NX SLV320 significantly decreased albuminuria by about 50%, but did not alter glomerular filtration rate (GFR). SLV320 normalized cardiac collagen I + III contents in 5/6 NX rats. SLV320 prevented nephrectomy-dependent rise in plasma levels of creatinine kinase (CK), ALT and AST. Blood pressure did not differ between study groups.
Conclusion: SLV320 suppresses cardiac fibrosis and attenuates albuminuria without affecting blood pressure in rats with 5/6 nephrectomy, indicating that selective A_1 receptor antagonists may be beneficial in uraemic cardiomyopathy.
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Keywords: chronic renal failure; myocardial fibrosis; adenosine receptor

Abbreviations: ALT, alanine lactate transaminase (or alanine aminotransferase/glutamic pyruvic transaminase); AST, alanine succinate transaminase (or aspartate aminotransferase/glutamic oxalacetic transaminase); CK, creatine kinase; CRF, chronic renal failure; CRP, C-reactive protein; GFR, glomerular filtration rate; PDE, phosphodiesterase

Introduction

Among patients with chronic renal failure (CRF), cardiovascular diseases such as coronary disease, heart failure, peripheral vascular disease and cerebrovascular disease have a high prevalence; a study in the United States with patients at the initiation of dialysis showed a prevalence of 52% (Foley *et al*., 2003). One of the most detrimental cardiac alterations in CRF is myocardial fibrosis: it impairs cardiac output by causing ventricular stiffening, and promotes arrhythmias by creating electrical heterogeneity (Guerin *et al*., 2004). Animal data show that uraemia enhances cardiac interstitial proliferation and growth factor expression independently from blood pressure or left ventricular hypertrophy (Amann *et al*., 1998b). This is in line with human post-mortem data showing in uraemic patients an increased cardiac interstitial volume density when compared to patients with essential hypertension (Amann *et al*., 1998a). Given the poor clinical outcome in patients with CRF due to cardiac target organ damage, new therapeutic approaches are needed.

The adenosine system is involved in several key functions of both kidney and heart. Adenosine acts via four different receptors: A_1, A_{2A}, A_{2B} and A_3 (Linden, 2001). In the kidney, adenosine plays a key role in the tubuloglomerular feedback mechanism, and thus exerts an inhibitory action on

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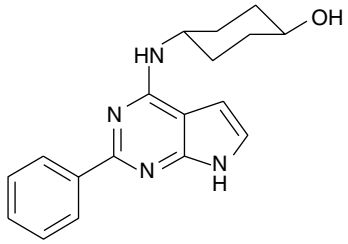

Figure 1 Chemical structure of SLV320.

glomerular haemodynamics and glomerular filtration rate (GFR) via A_1 receptors (Ren *et al*., 2001; Sun *et al*., 2001). In addition, adenosine has anti-natriuretic (and anti-diuretic) effects, through activation of tubular A_1 receptors, which promote sodium resorption (Yamagata *et al*., 1994; Wilcox *et al*., 1999; Bak and Thomsen, 2004). In the heart, A_1 receptor activation may be disadvantageous in ischaemia/reperfusion injury, for instance, through promotion of neutrophil chemoattraction and adhesion (Raschke and Becker, 1995; Becker *et al*., 1996; Forman *et al*., 2000), whereas A_2 and/or A_3 activation is protective in this setting.

In patients with congestive heart failure, A_1 receptor antagonists exhibit the intriguing features of increased diuresis and GFR, whereas the loop diuretic furosemide increases diuresis at the expense of a decreased GFR (Gottlieb *et al*., 2002). Moreover, animal studies show that A_1 receptor antagonists display renoprotective properties in the setting of radiographic contrast medium-induced nephropathy (Arakawa *et al*., 1996), as well as in cisplatin-induced renal failure (Nagashima *et al*., 1995).

Solvay Pharmaceuticals is developing the selective adenosine A_1 receptor antagonist SLV320, a pyrrolo-pyrimidine derivative (Figure 1). In this study, we describe some *in vitro* and *in vivo* properties of this novel compound with special attention to the potential of SLV320 in preventing uraemic cardiomyopathy in a rat model of CRF.

Materials and methods

Receptor binding and enzyme assays

The receptor binding affinities as well as enzyme inhibitory properties of SLV320 were evaluated in a series of 94 receptors and 6 phosphodiesterases (PDE1–PDE6) by Cerep (Celle L'evescault, France). Compounds were dissolved in dimethylsulphoxide (DMSO) (10 mM) and diluted in assay buffer to test concentration. The highest concentration tested for primes was 10 μM in receptor binding and 100 μM for enzyme assays. If the ligand displacement or enzyme inhibition by SLV320 was $>50\%$ at these concentrations, further testing was carried out in a 3 log concentration range around a predetermined IC_{50} for the respective assay: for example 10, 1, 0.1 and 0.01 μM for IC_{50} of 0.3 μM and 300, 30, 3 and 0.3 nM for one with IC_{50} of 10 nM. All determinations were performed as duplicates.

In brief, receptor binding assays were conducted as follows: following incubation of compound with the receptor preparation and the ligand at the appropriate time and temperature, the receptor preparations were rapidly filtered under vacuum through glass fibre filters; the filters were washed extensively with an ice-cold buffer using a harvester. Bound radioactivity was measured by scintillation counting using a liquid scintillation cocktail.

Enzyme assays were carried out as follows: following incubation of compound with the enzyme preparation and the substrate at the appropriate time and temperature, radioactivity of the enzyme product was measured by scintillation counting using a liquid scintillation cocktail.

Results were expressed as percentage of control values (enzyme assay) or for receptor binding assays as percentage of total ligand binding and that of nonspecific binding per concentration of compound tested (duplicates). From the concentration–displacement curves, IC_{50} values were determined by nonlinear regression analysis using Hill equation curve fitting. For receptor binding assays, the inhibition constants (K_i) were calculated from the Cheng–Prusoff equation $K_i = IC_{50}(1 + L/K_d)^{-1}$, where L is the concentration of radioligand in the assay and K_d the affinity of the radioligand for the receptor. The results were expressed as mean pK_i values $\pm$ s.d. of at least two separate experiments; outliers (results outside ± 1 s.e.m.) and discrepancies were excluded. Where no significant affinity was found at concentrations of 10 μM and higher, compounds were concluded to be inactive as denoted by pK_i of <5.0.

Animal procedures

Animal housing and experimental procedures conformed to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS123) and the German law on animal welfare. They were approved by the relevant local authority.

Haemodynamic effects of SLV320

Male CD rats (220–290 g body weight, Charles River, Wiga, Wilmington, MA, USA) were kept for 1 week for acclimatization with free access to a standard chow diet and water. Before the experiments, they were fasted overnight and anaesthetized with 70 mg kg^{-1} (Trapanal, sodium thiopental, Byk Gulden, Konstanz, Germany) intraperitoneally. The trachea and one carotid artery and jugular vein were cannulated. The arterial catheter was connected to a pressure transducer. Needle electrodes were fixed to the right forelimb and left hindlimb and the sternal region for ECG recording. After a 15 min equilibrium period, adenosine (100 μg kg^{-1} in physiological saline; solution prepared by dilution from a DMSO stock solution; final DMSO concentration: 1%) was applied by intravenous (i.v.) bolus injection. The adenosine application was repeated in 15 min intervals throughout the experiments (total duration: 2 h). Each adenosine application produced a transient (<15 s), fully reversible drop in heart rate. Ten minutes after the second and any further adenosine application, cumulative doses of SLV320 (0.05, 0.1, 0.2, 0.5, 1, 2 and 5 mg kg^{-1}, in 1 ml kg^{-1} for each dose) were given by i.v. infusions over 1 min periods (in other words, every SLV320 dose was administered 5 min before the following adenosine challenge). In a separate set of experiments, cumulative application of the vehicle used to dissolve

SLV320 (50% polyethylene glycol 400, 1 ml kg^{-1}) was found to have no effect on the bradycardia after adenosine. Likewise, for oral testing of SLV320, cumulative doses of SLV320 (0.1, 0.3, 1, 3 and 10 mg kg^{-1}, in 1 ml kg^{-1} for each dose) were administered via a gastric probe (every SLV320 dose being administered 10 min before the adenosine challenge).

Heart rate and blood pressure were measured every 10 s throughout the study (and every 2 s for 1 min following each adenosine challenge); heart rate values were averaged over 1 min preceding and over 1 min following each adenosine challenge. The heart rate reduction by adenosine after each SLV320 dose was expressed as percentage of the mean predrug rate reduction (that is the average value of the response measured in the first two adenosine challenges).

5/6 nephrectomy and uraemic cardiomyopathy

Animal model. To carry out 5/6 nephrectomy, rats were anaesthetized with xylazine (2 mg kg^{-1}, Rompun, Bayer, Leverkusen, Germany) and ketamine (40 mg kg^{-1}, Ketavet, Pharmacia & Upjohn, Erlangen, Germany) and placed on a heated table to maintain normal body temperature. The right kidney was exposed via flank incision and removed. After a 2-week recovery period, the left kidney was exposed accordingly and 2/3 was surgically removed. The sham operated animals underwent the same operations, but kidneys were only mobilized instead of removed.

Study design. Male Sprague–Dawley rats, 5-week-old, weighing 120–140 g, were randomly allocated to four groups: 5/6 nephrectomy (5/6 NX, $n=12$), 5/6 nephrectomy plus treatment with SLV320 (5/6 NX + SLV320, 10 mg kg^{-1} day formulated as food pellets; $n=12$), sham operation (Sham, $n=12$) and sham operation plus treatment with SLV320 (Sham + SLV320, $n=12$). All animals received a commercial diet (Altromin; Altromin Co., Lage, Germany) and water *ad libitum* during the study period. On the day following 5/6 nephrectomy, substance administration was begun; the duration of the treatment period was 12 weeks. The animals were weighed every second week; systolic blood pressure was assessed via the tail-cuff method once before 5/6 nephrectomy and start of treatment, and during weeks 1, 4, 9 and 12. The animals were placed in metabolic cages to obtain 24 h urine samples at study end (week 12); at the same time, blood was taken from retro-orbital veins for the purpose of measuring plasma creatinine levels and to calculate creatinine clearance using a standard formula. The animals were then killed, blood samples were taken to assess plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), C-reactive protein (CRP), creatinine, albumin, glucose, as described before (Haffner *et al*., 2005). The hearts and kidneys were removed for histological studies and organ weights were measured.

Histological studies

Tissue samples were all embedded in paraffin, cut into 3 μm sections and subjected to Sirius Red and Elastica-van Gieson staining. Quantitative stereology (that is intima/media and lumen area of the arteries) was analysed using a computer-aided image analysis system as described previously (Hocher *et al*., 1999). Cardiac morphology (perivascular fibrosis and media/lumen ratio of blood vessels) was measured as described recently (Hocher *et al*., 2000; Haffner *et al*., 2005).

In brief, the media/lumen ratio was evaluated after Elastica-van Gieson staining by means of computer-aided histomorphometry: microscopic pictures of cardiac arteries were transferred to a PowerMAC via Hitachi-CCD-camera. We measured the area of the media and the lumen of intracardial arteries using the ImageJ program (shareware from the NIH); afterwards, media/lumen ratio was calculated serving as a marker for arterial wall thickening. Perivascular fibrosis was evaluated after Sirius-Red staining using a semiquantitative score by two independent investigators unaware of the groups to which the animals belonged. Interstitial fibrosis of the kidney was quantified using the ImageJ program on kidney sections after Sirius-Red staining.

Immunohistochemistry

Matrix protein expression (collagen I, collagen III, laminin and fibronectin) was analysed using immunohistochemical staining methods. For antibody incubation, 5 μm thick cryostat sections were mounted on poly-L-lysine coated glass slides. Polyclonal rabbit antibodies against collagen type I and III as well as fibronectin and laminin were used. Detection of the bound antibodies was performed using a biotinylated second antibody and streptavidin Texas red according to the manufacturer's instructions (Amersham Buchler, Braunschweig, Germany). Control experiments were performed omitting the first antibody and using phosphate-buffered saline instead. Matrix protein expression (matrix protein-positive area in relationship to total area of interest) was quantitatively analysed using a computer-aided image analysis system (see above).

Data analysis

For the experiments measuring haemodynamic effects, the statistical significance of differences between values measured after each dose of SLV320 and the predrug (that is baseline) values was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post-test (using Prism 4.0, GraphPad Software Inc., San Diego, CA, USA).

Otherwise, we applied the Kruskal–Wallis test to detect any significant differences between the four groups; the Mann–Whitney test was used to detect significant differences between two groups of interest. Results (given as mean $\pm$ s.e.m.) were considered significantly different when the probability error (P) was less than 0.05.

Chemicals

Unless otherwise stated all reagents were of analytical grade and were purchased from Sigma (Seelze, Germany), Merck (Darmstadt, Germany) and Roth (Karlsruhe, Germany).

The compound SLV320, a pyrrolo-pyrimidine derivative (for chemical structure, see Figure 1), is a selective A_1

Table 1a Effects of SLV320 in receptor binding and enzyme assays

Assay	*Species*	*Ligand*	*Tissue*	*SLV320 affinity*[a]
Adenosine A_1	Human	^{3}H-DPCPX	CHO cells	9.0±0.3
Adenosine A_1	Rat	^{3}H-CCPA	Cerebral cortex	8.6[b]
PDE4 rolipram binding	Mouse	^{3}H-8-OH-DPAT	Total brain	7.1±0.1
Adenosine A_3	Human	^{3}H-AB-MECA	HEK293 cells	6.7±0.2
Adenosine A_{2A}	Human	^{3}H-CGS21680	HEK293 cells	6.4±0.2
Adenosine A_{2B}	Rat	^{3}H-CGS21680	Striatum	6.3[b]
Adenosine A_{2B}	Human	^{3}H-DPCPX	HEK293 cells	5.4±0.1
PDE4 enzyme	Human	^{3}H-cAMP	U-937 cells	6.0±0.1
PDE6 enzyme	Bovine	^{3}H-cGMP	Retina	4.6±0.3
PDE1 enzyme	Bovine	^{3}H-cAMP	Brain	4.2±0.4
PDE2 enzyme	Human	^{3}H-cAMP	U-937 cells	4.2±0.3
PDE3 enzyme	Human	^{3}H-cAMP	Platelets	<4.0
PDE5 enzyme	Human	^{3}H-cGMP	Platelets	<4.0

Abbreviations: AMP, adenosine monophosphate; CHO, Chinese hamster ovary. GMP, guanosine monophosphate.
[a]Results are expressed as pK_i for radioligand affinity assays, and as pIC_{50} for enzyme inhibition, mean±s.d. for at least three separate determinations.
[b]Additional results on rat A_1 and A_{2A} adenosine receptors are $n=1$ and included here for comparison.

Table 1b Comparison of selectivity of SLV320 and DPCPX as adenosine antagonists

Compound	*hA_1*	*hA_{2A}*	*hA_{2B}*	*hA_3*
SLV320	1	398	3981	200
DPCPX[a]	3.9	129	50	4000

Abbreviation: DPCPX, (8-cyclopentyl-1,3-dipropylxanthine).
Receptor binding experiments using cloned human receptors. The binding data represent K_i values given in nM.
[a]Fredholm *et al.* (2001).

adenosine receptor antagonist under development by Solvay Pharmaceuticals (Hannover, Germany), who supplied the compound for this study.

Results

In vitro *selectivity profile of SLV320*

In receptor binding experiments using cloned human receptors, SLV320 (for chemical structure see Figure 1) behaved as a potent and selective A_1 receptor ligand with selectivity factors of 200–4000 versus other adenosine receptor subtypes (see Table 1a). The selectivity factors are higher than those of the reference A_1 antagonist DPCPX (8-cyclopentyl-1,3-dipropylxanthine; see Table 1b).

In addition, the receptor-binding affinities as well as enzyme inhibitory properties of SLV320 were evaluated in a series of 94 receptors and 6 phosphodiesterases (PDE1–PDE6). The compound was thus tested in binding assays for affinity towards a broad range of receptors including adrenergic, muscarinic, nicotinic, dopaminergic, serotoninergic, histaminergic, glutamatergic, opioid, angiotensin, bradykinin and a number of neuropeptide receptors and uptake sites. A significant binding was measured only for the high-affinity rolipram-binding site on PDE4 (from mouse brain). SLV320 caused rolipram displacement from its binding site with a K_i of 79 nM, which was 79-fold less potent compared to its binding at the A_1 receptor site.

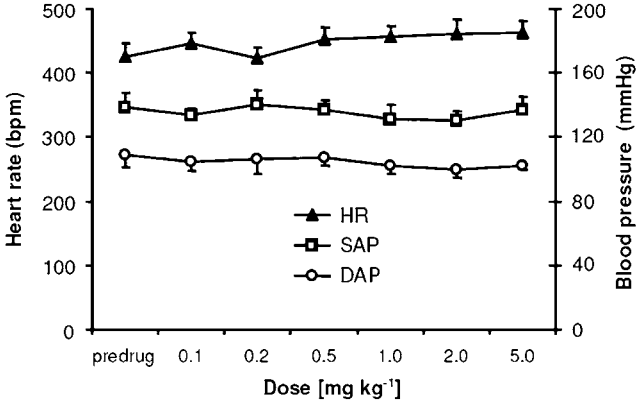

Figure 2 Effect of SLV320 i.v. on heart rate and blood pressure. Data shown are for HR (heart rate), SAP (systolic arterial pressure) and DAP (diastolic arterial pressure). The A_1 antagonist effects of SLV320 were investigated in male rats (anaesthetized with sodium thiopental) following cumulative i.v. dosing. In the investigated dose range of 0.1–5 mg kg^{-1} SLV320 caused no major haemodynamic effects of its own.

Haemodynamic effects of SLV320

The effect of SLV320 on systemic haemodynamics was assessed in anaesthetized male rats following i.v. administration over a range of 0.1–5 mg kg^{-1}. In this dose range, no haemodynamic effects of SLV320 on its own were observed with respect to heart rate and blood pressure (Figure 2).

However, in the setting of a transient A_1 adenosine receptor-mediated bradycardia (induced by i.v. bolus injection of adenosine, 100 μg kg^{-1}), oral pretreatment with SLV320 (administered 10 min before the adenosine challenge) dose-dependently reduced the effect of exogenous adenosine (Figure 3). The ED_{50} of this A_1 antagonistic response was calculated to be 0.49 mg kg^{-1}. By comparison, the ED_{50} determined following i.v. administration of SLV320 was 0.25 mg kg^{-1} (data not shown). In addition to its bradycardic effect, adenosine caused a concomitant transient drop in blood pressure, an effect known to be mediated by vascular A_2 receptors; this hypotension was more weakly affected and only by higher i.v. doses of SLV320

($>2\,mg\,kg^{-1}$); thus at an i.v. dose of $5\,mg\,kg^{-1}$, SLV320 diminished the adenosine-dependent hypotension by $44.6\pm5.5\%$. This confirms the selectivity of the compound towards A1 receptors *in vivo*.

5/6 nephrectomy and uraemic cardiomyopathy

Mortality and body weight gain. After 5/6 nephrectomy was performed, group size was 12 rats per group. Only one rat in the 5/6 NX + SLV320 group died shortly before study end. Mean body weight gain during the study period was $70\pm1.5\%$, and there was no difference between the groups (Kruskal–Wallis, $P=0.14$).

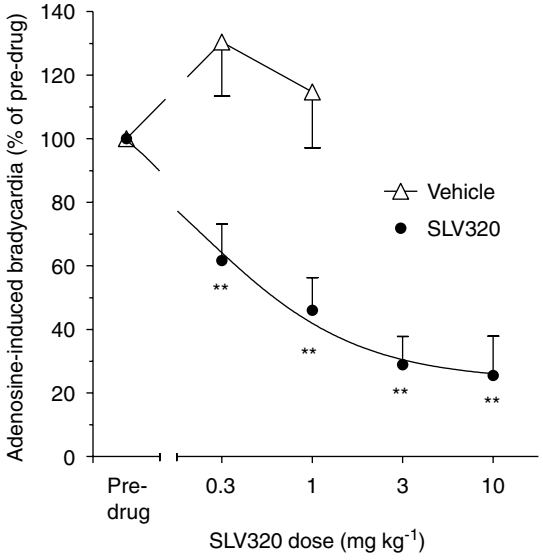

Figure 3 Dose-dependent suppression of adenosine-induced bradycardia (adenosine dose: $100\,\mu g\,kg^{-1}$ i.v.) by cumulative oral SLV320 treatment. Data shown are percentage values, as compared to the full extent (100%) of heart rate reduction measured at the beginning of each experiment (predrug value). Data are means ± s.e.m. ($n=2$–8). $^{**}P<0.01$ versus predrug value. Statistical analysis was achieved by one-way ANOVA followed by Dunnett's multiple comparison post-test to detect significant differences between the groups.

Blood pressure. Before 5/6 nephrectomy and treatment, there were no differences in blood pressure between the study groups. In week 1, the 5/6 NX + SLV320 group exhibited a significantly higher blood pressure compared to the untreated 5/6 NX group, whereas for the remainder of the study no differences between both nephrectomized groups were detected (see Table 2).

Plasma and urinary parameters and GFR. At the end of the study (week 12), animals were placed in metabolic cages to obtain urine samples, and during the final anaesthesia, blood samples were taken for analysis. The results of the urine and plasma analyses are illustrated in Table 3.

GFR significantly decreased in both nephrectomized groups versus sham controls; no effects of treatment with SLV320 on healthy or nephrectomized animals were detected. The same pattern was present when plasma urea and creatinine levels were assessed. When urinary albumin excretion was assessed, no difference was found between the nontreated and SLV320-treated sham-operated groups. Both nephrectomized groups exhibited a significantly increased urinary albumin excretion compared to sham animals. However, this albuminuria was significantly attenuated by about 50% by treatment with SLV320 (see Table 3).

The plasma levels of CK, ALT and AST did not differ between the sham-operated groups. However, there was a trend to higher CK levels and a significant increase in ALT and AST plasma levels in untreated nephrectomized animals when compared to sham animals. Treatment with SLV320

Table 2 Systolic blood pressure during the time course of the study (mm Hg)

Time	*Sham*	*Sham + SLV320*	*5/6 NX*	*5/6 NX + SLV320*
Pre-OP	105 ± 5	113 ± 5	110 ± 3	117 ± 7
Week 1	127 ± 2	137 ± 3*	119 ± 3	131 ± 3†
Week 4	122 ± 3	124 ± 4	134 ± 4	124 ± 4
Week 9	116 ± 5	127 ± 4	127 ± 4	123 ± 4
Week 12	118 ± 4	133 ± 4*	130 ± 4*	120 ± 4

Values are given as mean ± s.e.m.
$^{*}P<0.05$ versus Sham; $^{\dagger}P<0.05$ versus 5/6NX.

Table 3 Body weight, plasma analytes, GFR and albumin excretion at study end

Parameter	*Sham*	*Sham + SLV320*	*5/6 NX*	*5/6 NX + SLV320*
Body weight (g)	472 ± 14	434 ± 9*	428 ± 11*	438 ± 22
CK (U/l)	481 ± 159	255 ± 31	1267 ± 324	196 ± 64‡
AST (U/l)	41 ± 2	41 ± 1	60 ± 5*	35 ± 1‡
ALT (U/l)	36 ± 1	31 ± 1	43 ± 1*	32 ± 1‡
Potassium (mM)	4.1 ± 0.2	3.8 ± 0.2	4.8 ± 0.4	4.0 ± 0.2
CRP (mg/l)	4.1 ± 0.3	3.9 ± 0.2	3.7 ± 0.2	3.9 ± 0.2
Glucose (mM)	12.2 ± 0.9	10.7 ± 0.7	9.7 ± 0.6	10.5 ± 0.4
Creatinine (mg/l)	4.7 ± 0.3	4.3 ± 0.2	7.3 ± 0.3**	7.6 ± 0.4**
Urea (mg/l)	362 ± 13	360 ± 9	665 ± 42**	643 ± 36**
Albumin (g/l)	36.8 ± 0.8	37.8 ± 0.5	33.6 ± 0.8*	35.3 ± 0.5
GFR (ml/min/100 g)	0.45 ± 0.05	0.52 ± 0.02	0.32 ± 0.02*	0.31 ± 0.01*
Urinary albumin excretion (mg/24 h)	0.06 ± 0.02	0.07 ± 0.02	2.31 ± 0.39**	1.08 ± 0.19**,†

Abbreviations: CRP, C-reactive protein; GFR, glomerular filtration rate.
Values are given as mean ± s.e.m.
$^{*}P<0.05$; $^{**}P<0.001$ versus sham; $^{\dagger}P<0.05$; $^{\ddagger}P<0.001$ versus 5/6NX.

Table 4 Cardiac histology and immunohistochemistry

Parameter	*Sham*	*Sham + SLV320*	*5/6 NX*	*5/6 NX + SLV320*
Heart weight (% body weight)	0.29 ± 0.01	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.02
Laminin (% of section)	5.3 ± 1.0	5.3 ± 0.4	6.8 ± 1.1	5.2 ± 1.5
Fibronectin (% of section)	7.2 ± 1.2	7.3 ± 1.1	8.1 ± 1.7	7.5 ± 1.3
Perivascular fibrosis (score)	2.6 ± 0.1	2.5 ± 0.1	2.7 ± 0.1	2.6 ± 0.1
Media/lumen ratio	1.1 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1

Values are given as mean ± s.e.m.
Unpaired *t*-test was applied to detect significant differences between the study groups.

completely (and significantly) suppressed this increase in nephrectomized animals.

Plasma levels of albumin were significantly lower in untreated nephrectomized animals versus sham-operated animals, while nephrectomized animals treated with SLV320 did not differ from sham controls. Plasma glucose and CRP levels were not different between the study groups. The plasma potassium concentration tended to be elevated in the nontreated, but not in the SLV320-treated, nephrectomized group.

Cardiac and renal histology. The histology results are illustrated in Table 4. Regarding heart weight, media/lumen ratio of cardiac arteries and perivascular fibrosis, no differences were detected between the study groups. Immunohistochemistry revealed a significant increase of collagen I and III in untreated nephrectomized rats when compared to sham-operated animals. However, this effect was totally suppressed in nephrectomized animals treated with SLV320 (Figure 4; for illustration of immunostaining, see Figure 5).

In the kidney, no effect of treatment with SLV320 on fibrosis was detected (data not shown).

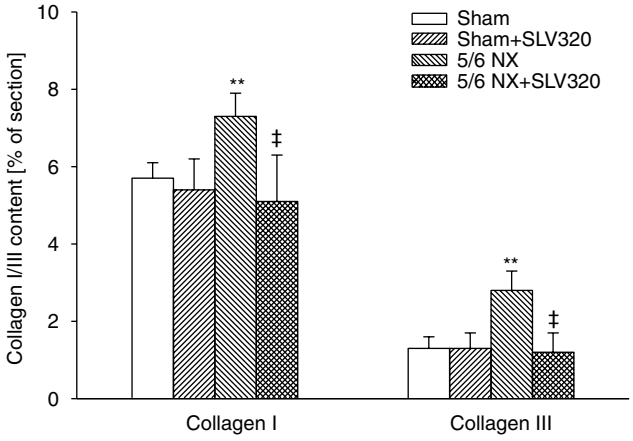

Figure 4 Collagen I and III in rat heart. Values are given as mean ± s.e.m. Unpaired *t*-test was applied to detect significant differences between the study groups. $^{**}P<0.001$ versus sham, $^{‡}P<0.001$ versus 5/6NX.

Discussion

This study describes the pharmacological properties of a novel A_1 adenosine receptor antagonist SLV320 and its protective effects on target organ damage in a rat model of CRF. We demonstrated that SLV320 is a selective A_1 receptor antagonist *in vitro* and *in vivo*. Importantly, these investigations have revealed for the first time that blockade of an adenosine A_1 receptor had protective effects in the heart and kidney, by attenuating cardiac fibrosis and albuminuria in rats with 5/6 nephrectomy and that these protective effects occurred without changes in blood pressure.

Our receptor binding experiments demonstrated that SLV320 is a selective and potent adenosine A_1 antagonist ($K_i = 1.0$ nM) with a selectivity factor of at least 200 versus other adenosine receptor subtypes and an even higher selectivity factor versus most other receptors. The selectivity factors are higher than those of the reference A_1 antagonist DPCPX (8-cyclopentyl-1,3-dipropylxanthine). Functional A_1 antagonism could be confirmed in the rat: adenosine-induced bradycardia was prevented with ED_{50} values of 0.25 and 0.49 mg kg^{-1} after i.v. and oral application respectively.

We assessed the clinical potential of SLV320 in preventing cardiac target organ damage in CRF using rats with 5/6 nephrectomy. Treatment was tolerated well without any observable impact of SLV320 on mortality, or on any of the screening parameters assessed in the plasma.

Regarding cardiac outcome, there was no difference between all study groups in cardiac weights, indicating that no significant cardiac hypertrophy was present in our mild uraemic model. However, there was a trend towards higher CK plasma levels and significantly higher ALT and AST plasma levels in the untreated nephrectomized group compared to sham controls. This effect was completely abolished by treatment with SLV320. As CRP levels were not different between the groups in our study, we conclude that the ALT, AST and CK elevations in untreated nephrectomized animals were of cardiac origin, which was responsive to treatment with SLV320.

This is the first study to show that an A_1 adenosine receptor antagonist inhibits myocardial fibrosis, without affecting blood pressure: our immunohistochemistry results demonstrate that uraemia indeed enhanced cardiac collagen I and III contents in our model and that treatment with SLV320 in nephrectomized animals lowered the amount of collagen I and III to baseline levels. The occurrence of cardiac fibrosis in our model is in line with data from animal experiments as well as human studies revealing that uraemia promotes cardiac fibrosis, independently of hypertension. (Amann *et al.*, 1998a, b). Our finding of a crucial role of the

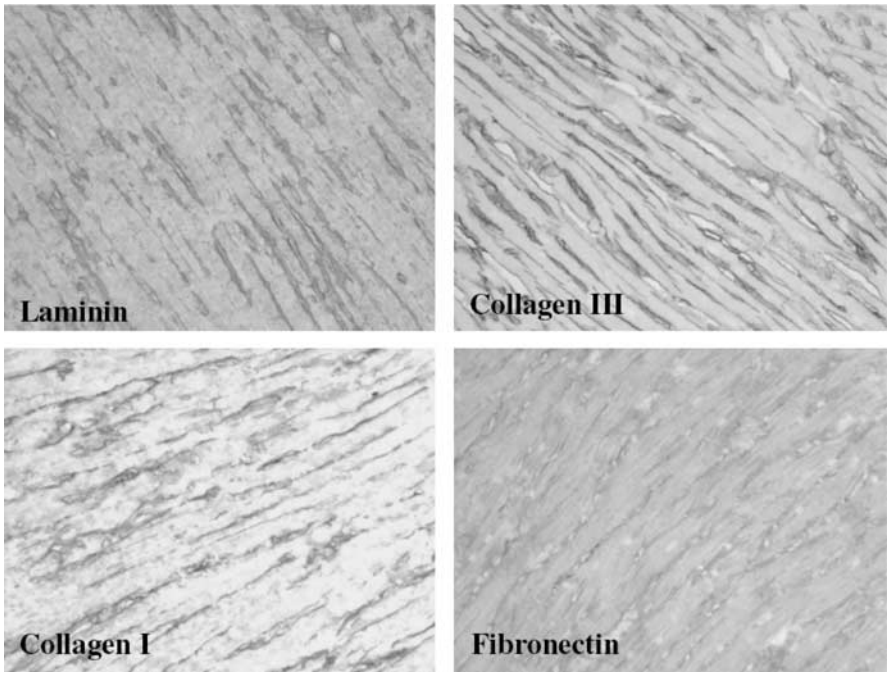

Figure 5 Photomicrographs of rat heart. Typical sections of the heart of 5/6 NX rats with immunostaining for the matrix proteins laminin, collagen I, III and fibronectin. Magnification $\times 200$. For comparison between the groups, see Figure 4.

A_1 receptor in the development of cardiac fibrosis is supported by a very recent study dealing with a transgenic mouse model characterized by an inducible, cardiac overexpression of A_1 receptors (Funakoshi *et al*., 2006). They demonstrated that cardiac overexpression of the A_1 receptor in mice leads to remarkable cardiac fibrosis, which could be reversed by switching off the transgenic overexpression. The pathophysiological pathways involved in this newly discovered link between the A_1 receptor and cardiac fibrosis are not yet elucidated. As a possible explanation, we suggest that A_1 receptors may mediate the chemoattractant response of neutrophils, and blockade of A_1 receptors was shown to be beneficial in the setting of ischaemia-reperfusion injury (Forman *et al*., 2000). As an alternative explanation, we suggest that blockade of the cardiac A_1 adenosine receptors may increase the action of adenosine at A_2 receptors. It is well known that adenosine exerts inhibitory effects on cardiac fibroblast proliferation and matrix synthesis via A_2 receptors (Dubey *et al*., 1998, 2001; Chen *et al*., 2004). Thus the observed effects may possibly be attributed to enhanced agonist activity at cardiac A_2 receptors by endogenous adenosine, as a consequence of blocking the A_1 receptors by SLV320.

Adenosine is released under conditions of ischaemic stress and this is believed to serve as protection against tissue injury (Ely and Berne, 1992). This raises concern about the application of adenosine antagonists to patients with CRF who are a high-risk population with regard to ischaemic heart disease. Nevertheless, there is a growing body of evidence from different animal models dealing with A_1 receptor antagonists in myocardial infarction which demonstrates that administration of these compounds can actually reduce infarct size or at least has no effect on infarct size at all (Gross *et al*., 1997; Forman *et al*., 2000; Auchampach *et al*., 2004). Moreover, in a rodent model of hypertensive dilatative cardiomyopathy, an A_1 receptor antagonist exhibited a favourable action on cardiac function (Jackson *et al*., 2001). These data are supported by experiments using mixed A_1/A_2 adenosine receptor agonists: they demonstrated that cardioprotection offered by these drugs is not attenuated by A_1 antagonists, but by A_2 antagonists thus linking cardioprotection to the A_2 receptor (Xu *et al*., 2001; Kis *et al*., 2003).

Kidney function – as assessed by plasma urea, creatinine and GFR – was significantly impaired in animals subjected to 5/6 nephrectomy when compared to sham controls, as expected. Treatment with SLV320 did not affect these parameters.

Albuminuria was enhanced in nephrectomized animals compared to sham controls, and treatment with SLV320 attenuated urinary albumin excretion by about 50%. As albuminuria is an early and sensitive marker of renal and cardiovascular risk (Basi and Lewis, 2006), we conclude that treatment with SLV320 indeed exerts beneficial effects on renal disease progression, but long-term studies are needed to establish if this effect is translated into GFR preservation when the observation period is prolonged.

During the experiment, we did not observe any sustained differences in blood pressure between all study groups. This finding can be explained by the fact that in our specific model of renal mass ablation, the blood pressure normally rises slowly over time (Gretz, 1995). Given the fact that in our present study, we produced a comparatively mild uraemia as indicated by lower plasma creatinine, higher GFR and lower mortality when compared with previous settings (Kalk *et al*., 2006), it is evident

that hypertension was likely to be absent within the time course of our study.

In conclusion, the present study is the first to describe the *in vitro* and *in vivo* properties of a novel A_1 receptor antagonist, SLV320. We demonstrated that SLV320 is a selective A_1 receptor antagonist, which attenuates cardiac fibrosis and albuminuria without affecting blood pressure, in a rat model of CRF.

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Conflict of interest

YF, DZ, G-WB and BH are research employees of Solvay Pharmaceuticals, Hannover.

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