Aus dem Center for Cardiovascular Research
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Renal effects of the dipeptidyl peptidase inhibitor linagliptin in non-diabetic rats with 5/6 nephrectomy

zur Erlangung des akademischen Grades
Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät
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Table of contents

Abstrakt (Deutsch)

Dipeptidylpeptidase 4 (DPP4)-Inhibitoren verzögern glukoseunabhängig die Progression des chronischen Nierenversagens (CNV) in experimentellen Modellen der diabetischen Nephropathie. In dieser Arbeit wurden renoprotektive Effekte des DPP4-Inhibitors Linagliptin in einem nicht-diabetischen 5/6-Nephrektomie Rattenmodell untersucht und mit dem Angiotensin-II-Rezeptorblocker (ARB) Telmisartan verglichen. Die Tiere wurden in 4 Gruppen aufgeteilt: Sham-Operation plus Placebo; 5/6-Nephrektomie plus Placebo; 5/6-Nephrektomie plus Linagliptin; und 5/6-Nephrektomie plus Telmisartan. Die Behandlung mit Linagliptin führte zu einer signifikanten Reduktion (48%) der interstitiellen Fibrose in der Niere im Vergleich zu Placebo behandelten Tieren. Telmisartan bewirkte eine numerische Reduktion (24%) der renalen interstitiellen Fibrose, die jedoch nicht statistisch signifikant war. Die Albumin-Kreatinin-Ratio im Urin wurde sowohl durch Linagliptin (66%) als auch durch Telmisartan (92%) signifikant gesenkt. Die Behandlung mit Telmisartan war mit einer signifikanten Reduktion des Blutdrucks verbunden, bei mit Linagliptin behandelten Tieren war diesbezüglich kein Effekt zu beobachten. Eine massenspektrometrische Analyse von Peptiden zeigte dass Linagliptin im Vergleich zu Placebo eine unterschiedliche Regulation von 552 plasmatischen und 320 renalen Peptiden bewirkte. Im Vergleich zu Placebo, fanden sich bei Telmisartan 108 plasmatische und 363 renale unterschiedlich regulierte Peptide. Linagliptin führte zu einer Hochregulation von Peptiden des Typ 1 Kollagens, des Apolipoproteins C1 und des Heterogeneous Nuclear Ribonucleoproteins A2/B1, einem in der Signalkaskade des atrialen natriuretischen Peptids involviertem Faktor. Telmisartan war mit einer Hochregulation von Angiotensin-II verbunden. Zur Bestätigung der Ergebnisse wurde in einer weiteren Studie die Wirkung von Linagliptinin in 5/6-nephrektomierten Wildtyp- und DPP4-defizienten Ratten untersucht. Linagliptin zeigte sich in Wildtyp-Ratten genauso wirksam wie in den DPP4 defizienten Tieren. Zusammenfassend entfaltete Linagliptin im Vergleich zu Telmisartan vergleichbare positive Effekte auf die Progression des CNVs in nicht-diabetischen Ratten mit 5/6-Nephrektomie. Daten dieser Studie deuten ferner darauf hin, dass die der Renoprotektion zugrunde liegenden Mechanismen der beiden untersuchten Pharmaka über unterschiedliche Signalwege mediiert werden.

Abstract (English)

Dipeptidyl peptidase (DPP)-4 inhibitors delay chronic kidney disease (CKD) progression in experimental diabetic nephropathy in a glucose-independent manner. Here we compared the effects of the DPP-4 inhibitor linagliptin versus telmisartan in preventing CKD progression in non-diabetic rats with 5/6 nephrectomy. Animals were allocated to 1 of 4 groups: sham operated plus placebo; 5/6 nephrectomy plus placebo; 5/6 nephrectomy plus linagliptin; and 5/6 nephrectomy plus telmisartan. Interstitial fibrosis was significantly decreased by 48% with linagliptin but a non-significant 24% with telmisartan versus placebo. The urine albumin-to-creatinine ratio was significantly decreased by 66% with linagliptin and 92% with telmisartan versus placebo. Blood pressure was significantly lowered by telmisartan, but it was not affected by linagliptin. As shown by mass spectrometry, the number of altered peptide signals for linagliptin in plasma was 552 and 320 in the kidney. For telmisartan, there were 108 peptide changes in plasma and 363 in the kidney versus placebo. Linagliptin up-regulated peptides derived from collagen type I, apolipoprotein C1, and heterogeneous nuclear ribonucleoproteins A2/B1, a potential downstream target of atrial natriuretic peptide, whereas telmisartan up-regulated angiotensin II. A second study was conducted to confirm these findings in 5/6 nephrectomy wild-type and genetically deficient DPP-4 rats treated with linagliptin or placebo. Linagliptin therapy in wild-type rats was as effective as DPP-4 genetic deficiency in terms of albuminuria reduction. Thus, linagliptin showed comparable efficacy to telmisartan in preventing CKD progression in non-diabetic rats with 5/6 nephrectomy. However, the underlying pathways seem to be different.

Detailed declaration of own work / Affidavit

I, Oleg Tsuprykov, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "*Renal effects of the dipeptidyl peptidase inhibitor linagliptin in non-diabetic rats with 5/6 nephrectomy*" I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

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Detailed Declaration of Contribution

Oleg Tsuprykov had the following share in the following publication:

Tsuprykov O, Ando R, Reichetzeder C, von Websky K, Antonenko V, Sharkovska Y, Chaykovska L, Rahnenführer J, Hasan AA, Tammen H, Alter M, Klein T, Ueda S, Yamagishi S, Okuda S, Hocher B. The dipeptidyl peptidase inhibitor linagliptin and the angiotensin II receptor blocker telmisartan show renal benefit by different pathways in rats with 5/6 nephrectomy. Kidney Int. 2016;89(5):1049-61.

Contribution in detail:

The overall contribution of Oleg Tsuprykov in this study was at least 50%. Oleg Tsuprykov's detailed contribution in this study included the following activities: systolic blood pressure measurement (about 50%), organ harvesting (about 30%), histological analysis of kidney samples (about 80%), protein expression analysis (100%), statistical analysis of the acquired data (100%), writing the manuscript (about 80%), preparation of the figures for the manuscript (100%).

Signature, date and stamp of the supervising University teacher

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Journals 1 - 20 (of 77)

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Page 1 of 4

[1 | 2 | 3 | 4]

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☐	3	J AM SOC NEPHROL	1046-6673	32376	8.491	9.086	2.460	274	8.7	0.05665	3.404
☑	4	KIDNEY INT	0085-2538	38159	7.683	7.839	2.658	243	>10.0	0.05176	2.759
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☐	6	AM J KIDNEY DIS	0272-6386	19966	6.269	5.684	1.766	197	9.3	0.03364	2.091
☐	7	NAT REV UROL	1759-4812	1844	5.957	5.629	1.319	47	3.5	0.00834	1.892
☐	8	J UROLOGY	0022-5347	46087	4.700	4.169	1.345	470	>10.0	0.06075	1.347
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☐	10	BJU INT	1464-4096	19306	4.387	3.400	1.456	270	6.5	0.04017	1.045
☐	11	NEPHROL DIAL TRANSPL	0931-0509	22676	4.085	3.459	1.015	326	7.1	0.04401	1.114
☐	12	PROSTATE CANCER P D	1365-7852	1655	3.803	3.196	0.702	57	5.2	0.00431	0.995
☐	13	PROSTATE	0270-4137	7079	3.778	3.277	0.889	189	6.9	0.01175	0.868
☐	14	SEMIN NEPHROL	0270-9295	2332	3.773	3.459	0.322	59	6.9	0.00648	1.465
☐	15	AM J PHYSIOL-RENAL	1931-857X	16715	3.390	3.395	0.605	258	8.4	0.02478	1.017
☐	16	EUR UROL SUPPL	1569-9056	685	3.364	1.833	3.500	4	7.9	0.00104	0.601
☐	17	CURR OPIN NEPHROL HY	1062-4821	3078	3.232	3.278	0.747	83	6.3	0.00754	1.155
☐	18	NEUROUROL URODYNAM	0733-2467	4138	3.128	2.743	0.661	124	6.1	0.00859	0.791
☐	19	UROL ONCOL-SEMIN OBJ	1078-1439	3621	2.921	2.739	1.431	144	3.1	0.01089	0.802
☐	20	KIDNEY BLOOD PRESS R	1420-4096	1336	2.908	2.235	0.175	63	3.8	0.00290	0.488

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Journals 1 - 20 (of 77)

[1 | 2 | 3 | 4]

Page 1 of 4

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The dipeptidyl peptidase inhibitor linagliptin and the angiotensin II receptor blocker telmisartan show renal benefit by different pathways in rats with 5/6 nephrectomy

OPEN

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Dipeptidyl peptidase (DPP)-4 inhibitors delay chronic kidney disease (CKD) progression in experimental diabetic nephropathy in a glucose-independent manner. Here we compared the effects of the DPP-4 inhibitor linagliptin versus telmisartan in preventing CKD progression in non-diabetic rats with 5/6 nephrectomy. Animals were allocated to 1 of 4 groups: sham operated plus placebo; 5/6 nephrectomy plus placebo; 5/6 nephrectomy plus linagliptin; and 5/6 nephrectomy plus telmisartan. Interstitial fibrosis was significantly decreased by 48% with linagliptin but a non-significant 24% with telmisartan versus placebo. The urine albumin-to-creatinine ratio was significantly decreased by 66% with linagliptin and 92% with telmisartan versus placebo. Blood pressure was significantly lowered by telmisartan, but it was not affected by linagliptin. As shown by mass spectrometry, the number of altered peptide signals for linagliptin in plasma was 552 and 320 in the kidney. For telmisartan, there were 108 peptide changes in plasma and 363 in the kidney versus placebo. Linagliptin up-regulated peptides derived from collagen type I, apolipoprotein C1, and heterogeneous nuclear ribonucleoproteins A2/B1, a potential downstream target of atrial natriuretic peptide, whereas telmisartan up-regulated angiotensin II. A second study was conducted to confirm these findings in 5/6 nephrectomy wild-type and genetically deficient DPP-4 rats treated with linagliptin or placebo. Linagliptin therapy in wild-type rats was as effective as DPP-4 genetic deficiency in terms of albuminuria reduction. Thus, linagliptin showed comparable efficacy to telmisartan in preventing CKD progression in non-diabetic rats with 5/6 nephrectomy. However, the underlying pathways seem to be different.

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Chronic kidney disease (CKD) is a major global health problem associated with significant morbidity and mortality. The prevalence of CKD is considered to be 8% to 16% worldwide.[1] Although hypertension and diabetes mellitus are known to be the leading causes of CKD, a variety of other risk factors including dyslipidemia, ischemia, infection, toxins, and autoimmune and inflammatory diseases contribute to the development and progression of CKD.[2]

Reducing blood pressure (BP) using angiotensin II receptor blockers or angiotensin-converting enzyme inhibitors is the first-line therapy for delaying CKD progression.[3] However, in patients who do not sufficiently respond to renin-angiotensin system inhibitors, or in whom this drug class causes major side effects, the current CKD treatment standards need to be improved.

Dipeptidyl peptidase (DPP)-4 inhibitors ("gliptins") have been approved for the treatment of type 2 diabetes mellitus since 2006. Several studies have reported that DPP-4 inhibitors exert beneficial effects on renal morphology and function in rodent diabetes models.[4–8] These renal effects have been demonstrated mostly in studies investigating hyperglycemic conditions.

It is well known that the antidiabetic effects of DPP-4 inhibitors are mediated via increases in levels of the incretin hormones glucagon-like peptide (GLP)-1 and glucose-dependent insulinotropic polypeptide (GIP). However, there is a broad range of other substrates for the DPP-4 enzyme including brain natriuretic peptide, substance P, peptide YY, neuropeptide Y, and stromal cell-derived factor-1 alpha (SDF-1α), which are thought to contribute to beneficial renal and cardiovascular effects;[9,10] however, the underlying mechanisms have not yet been fully elucidated.

In this study, we first evaluated the glucose-independent renal effects of the DPP-4 inhibitor linagliptin in a rat 5/6 nephrectomy (5/6 Nx) model, one of the most valuable and extensively investigated experimental CKD animal models,[11] and investigated the underlying molecular mechanisms, in comparison with those of telmisartan, one of the most commonly used angiotensin II receptor blockers. We furthermore examined the effect of linagliptin on systolic BP (SBP) and urinary protein excretion in DPP-4-deficient mutant Fisher rats (DPP-4$^{-/-}$).

RESULTS

SBP and kidney function

Baseline SBP levels in the first study (Core study) and in the second study (Confirmation study) are presented in Table 1. SBP was not significantly affected by 5/6 Nx in any of the studies (Table 1). At the end of study 1, SBP was pronouncedly lowered by telmisartan (–47.1 mm Hg, $P < 0.001$ vs. placebo), whereas the BP-lowering effect by linagliptin was not significant (–10.8 mm Hg, NS, vs. placebo) (Table 1, Core study). At the end of study 2, neither DPP-4 genetic ablation alone nor the one with linagliptin administration on its top showed any BP-lowering effect (Table 1, Confirmation study).

Baseline parameters of protein excretion in both studies did not differ among the treatment groups (Figure 1a and b). At the end of study 1, urinary albumin-to-creatinine ratio was approximately 14-fold higher ($P < 0.001$) in the placebo group versus the sham control (Figure 1a), whereas it was significantly reduced following treatment with telmisartan (–92%; $P < 0.001$) and linagliptin (–66%; $P < 0.001$) versus placebo. Neither linagliptin nor telmisartan lowered plasma cystatin C (Table 1, Core study). At the end of study 2, urinary total protein-to-creatinine ratio was approximately 22-fold ($P < 0.001$) higher in placebo-treated 5/6 Nx wild-type rats versus sham control rats (Figure 1b). In 5/6 Nx rats, DPP-4 knockout decreased urinary total protein-to-creatinine ratio by 66% ($P < 0.001$ vs. 5/6 Nx + wild type) at 4 weeks of the study. In 5/6 Nx DPP-4$^{-/-}$ rats, linagliptin decreased urinary total protein-to-creatinine ratio by 50% ($P < 0.01$ vs. 5/6 Nx + wild type) at 4 weeks of the study.

Kidney weight and morphology

At the end of study 1, relative kidney weight was significantly higher in placebo-treated 5/6 Nx rats versus sham control rats (Table 1, Core study). Telmisartan significantly decreased kidney weight versus placebo ($P < 0.05$), whereas linagliptin had no effect. Compared with the sham group, 5/6 Nx numerically increased renal interstitial fibrosis by 69% (P = NS) and glomerular size by 28% ($P < 0.01$) (Figure 2). These findings were significantly attenuated by linagliptin treatment: renal interstitial fibrosis and glomerular hypertrophy (determined by glomerular size) decreased by 48% ($P < 0.05$) and 18% ($P < 0.05$), respectively, versus placebo (Figure 2). Telmisartan did not show any beneficial effects on kidney morphology (Figure 2). The glomerulosclerosis index was elevated in 5/6 Nx + placebo rats ($P < 0.05$ vs. sham) and was not restored by either telmisartan or linagliptin (Table 1, Core study). Media-to-lumen ratio of intrarenal arteries and renal perivascular fibrosis showed no differences between the treatment groups (Table 1, Core study).

Markers of renal fibrosis and inflammation

In this study, 5/6 Nx resulted in an increase of fibrotic marker collagen type I and inflammatory pan-macrophage marker CD68 renal protein expression as determined by western blot (Figure 3a and b). Linagliptin, but not telmisartan, restored collagen type I renal protein expression ($P < 0.05$ vs. placebo) (Figure 3a). In contrast, telmisartan normalized CD68 expression level ($P < 0.05$ vs. placebo) (Figure 3b), whereas linagliptin did not. Renal protein expression of the other profibrotic markers, such as collagen type III, transforming growth factor beta 1 (TGF-β1), phospho-SMAD2–to–total SMAD2 ratio, and phospho-SMAD3–to–total SMAD2/3 ratio did not show significant differences between the study groups (Table 1, Core study). Additional data on renal fibrosis-associated gene expression levels are summarized in Table 1, Core study.

Urinary DPP-4 activity and malondialdehyde excretion

Urinary DPP-4 activity, as determined by urinary DPP-4 activity-to-creatinine ratio, was decreased by linagliptin at week 18 ($P < 0.01$ vs. placebo) (Figure 4a). Oxidative stress in the kidney, determined by urinary malondialdehyde-to-creatinine ratio, was reduced by telmisartan at week 18 ($P < 0.01$ vs. placebo), but not by linagliptin (Figure 4b).

Plasma DPP-4 activity, GLP-1, GIP, SDF-1α, and glucose

At the end of study 1, DPP-4 activity in plasma was reduced by 72% with linagliptin ($P < 0.001$ vs. placebo) and was not affected by telmisartan (Table 1, Core study). The plasma concentration of total incretins (sum of active and inactive levels) was not significantly different between 5/6 Nx + placebo rats and sham (P = NS for GLP-1 and P = NS for GIP) (Table 1, Core study). Telmisartan did not affect total incretin levels, whereas linagliptin decreased total GIP levels ($P < 0.01$ vs. placebo), but not GLP-1 levels (Table 1, Core study). Linagliptin resulted in a 40.5-fold increase in active GLP-1 and a 2.2-fold increase in active GIP ($P < 0.001$ vs. placebo for both), whereas telmisartan had no effect on active incretin plasma levels (Figure 5a and b). In addition,

Table 1 | SBP, renal morphology, and function, and expression levels of fibrosis-associated genes

Core study (study 1)				
Parameter	**Sham + placebo**	**5/6 Nx + placebo**	**5/6 Nx + telmisartan**	**5/6 Nx + linagliptin**
Systolic blood pressure (mm Hg)				
Baseline SBP	128.50 ± 2.82	114.60 ± 2.41	115.80 ± 2.05	118.80 ± 2.22
SBP at week 7	121.88 ± 3.42	125.62 ± 3.14	91.90 ± 2.96[a]	114.00 ± 2.87[b]
SBP at week 12	128.48 ± 4.18	132.42 ± 4.65	88.89 ± 2.34[a]	125.29 ± 4.14
SBP at week 17	121.00 ± 4.51	134.30 ± 3.98	87.22 ± 3.85[a]	123.50 ± 3.33
Kidney morphology				
Final body weight (g)	591.0 ± 34.1	588.3 ± 10.4	542.1 ± 19.7	560.3 ± 18.1
Relative weight of left kidney (mg/g)	2.85 ± 0.14	4.39 ± 0.29[c]	3.47 ± 0.11[b]	4.32 ± 0.24[c]
GS index (score)	1.72 ± 0.05	2.02 ± 0.07[d]	1.87 ± 0.06	1.90 ± 0.04
Media-to-lumen ratio	2.66 ± 0.18	2.62 ± 0.12	2.69 ± 0.18	2.62 ± 0.22
Renal perivascular fibrosis (score)	1.92 ± 0.26	1.88 ± 0.13	1.73 ± 0.17	1.77 ± 0.10
Plasma parameters				
Final plasma glucose (mmol/l)	8.08 ± 0.29	7.65 ± 0.26	8.13 ± 0.26	7.67 ± 0.23
Cystatin C (ng/ml)	695 ± 37.83	1528 ± 97.65[c]	1356 ± 61.38[d]	1443 ± 72.66[e]
MCP-1 in plasma	423.3 ± 72.44	665.9 ± 42.74[e]	533.2 ± 35.78	664.1 ±39.17[e]
DPP-4 activity in plasma, arbitrary units	173,721 ± 6733	165,082 ± 6957	151,075 ± 6102	46,198 ± 1792[a,c]
Total GLP-1 (pg/ml)	14.26 ± 5.73	23.13 ± 3.33	27.48 ± 4.08	24.54 ± 3.48
Total GIP (pg/ml)	227.3 ± 21.10	368.0 ± 39.01	312.7 ± 31.46	210.1 ± 39.63[f]
Total SDF-1α (ng/ml)	221.5 ± 107.2	802.1 ± 388.7	910.3 ± 444.7	1301.0 ± 194.6[b,e]
Relative protein expression in the kidney				
Collagen type III (relative protein expression)	1.00 ± 0.08	1.33 ± 0.09	1.20 ± 0.09	1.29 ± 0.09
TGF-β1 (relative protein expression)	1.00 ± 0.10	1.25 ± 0.10	0.93 ± 0.10	1.04 ± 0.08
Phospho-SMAD2 (relative protein expression)	1.00 ± 0.21	1.21 ± 0.12	0.91 ± 0.11	0.90 ± 0.16
Total SMAD2 (relative protein expression)	1.00 ± 0.10	1.12 ± 0.09	0.92 ± 0.08	0.88 ± 0.06
Phospho-SMAD2/total SMAD2 ratio	1.00 ± 0.23	1.08 ± 0.15	0.89 ± 0.13	1.04 ± 0.16
Phospho-SMAD3 (relative protein expression)	1.00 ± 0.12	1.09 ± 0.21	1.34 ± 0.19	1.19 ± 0.16
Total SMAD2/3 (relative protein expression)	1.00 ± 0.11	1.06 ± 0.08	0.82 ± 0.05	0.95 ± 0.08
Phospho-SMAD3/total SMAD2/3 ratio	1.00 ± 0.13	1.17 ± 0.34	1.62 ± 0.25	1.39 ± 0.25
Relative gene expression in the kidney				
Collagen type Iα1 (relative gene expression)	1.00 ± 0.25	5.23 ± 1.54[e]	1.94 ± 0.28	3.27 ± 0.97[d]
Collagen type IIIα1 (relative gene expression)	1.00 ± 0.35	4.06 ± 1.20[d]	1.40 ± 0.22	2.77 ± 0.88
TGF-β1 (relative gene expression)	1.00 ± 0.37	2.01 ± 0.37[d]	0.94 ± 0.10[b]	1.50 ± 0.27
TIMP-1 (relative gene expression)	1.00 ± 0.52	2.38 ± 0.46[d]	0.81 ± 0.11[b]	1.80 ± 0.51
GLP-1 receptor (relative gene expression)	1.00 ± 0.30	1.27 ± 0.29	2.02 ± 0.80	1.93 ± 0.48

Confirmation study (study 2)				
Parameter	**Sham + placebo**	**5/6 Nx + placebo**	**5/6 Nx + DPP-4$^{-/-}$ + placebo**	**5/6 Nx + DPP-4$^{-/-}$ + linagliptin**
Baseline SBP (mm Hg)	115.8 ± 7.7	118.5 ± 6.6	121.2 ± 8.7	116.8 ± 6.0
SBP at week 4 (mm Hg)	108.4 ± 10.0	159.4 ± 30.5	120.6 ± 12.3	133.9 ± 24.3

DPP-4, dipeptidyl peptidase-4; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide 1; GS, glomerulosclerosis; MCP-1, monocyte chemoattractant protein 1; Nx, nephrectomy; SBP, systolic blood pressure; SDF-1α, stromal cell-derived factor-1 alpha; TGF-β1, transforming growth factor beta 1; TIMP-1, tissue inhibitor of metalloproteinase 1.
Values are given as mean ±SEM.
[a]$P < 0.001$ versus 5/6 Nx + placebo.
[b]$P < 0.05$ versus 5/6 Nx + placebo.
[c]$P < 0.001$ versus sham + placebo.
[d]$P < 0.05$ versus sham + placebo.
[e]$P < 0.01$ versus sham + placebo.
[f]$P < 0.01$ versus 5/6 Nx + placebo.

linagliptin increased total SDF-1α plasma levels ($P < 0.05$ vs. placebo) (Table 1, Core study). Final plasma glucose concentrations were not significantly different among the groups (Table 1, Core study).

Plasma multianalyte profiling

Levels of plasma osteopontin, beta-2-microglobulin, macrophage colony-stimulating factor 1, and monocyte chemoattractant protein 1 were increased in placebo-treated 5/6 Nx rats (Figure 6 and Table 1, Core study). Linagliptin lowered plasma levels of calbindin ($P < 0.05$). Telmisartan decreased osteopontin ($P < 0.01$) and macrophage colony-stimulating factor 1 levels ($P < 0.01$). Neither drug treatment significantly reduced beta-2-microglobulin and monocyte chemoattractant protein 1 levels compared with the placebo-treated 5/6 Nx group (Figure 6c and Table 1, Core study).

Liquid chromatography and mass spectrometry of plasma and kidney samples

At the end of study 1, 20 plasma ($n = 6$ for 5/6 Nx + placebo; $n = 7$ for 5/6 Nx + telmisartan; $n = 7$ for 5/6 Nx + linagliptin)

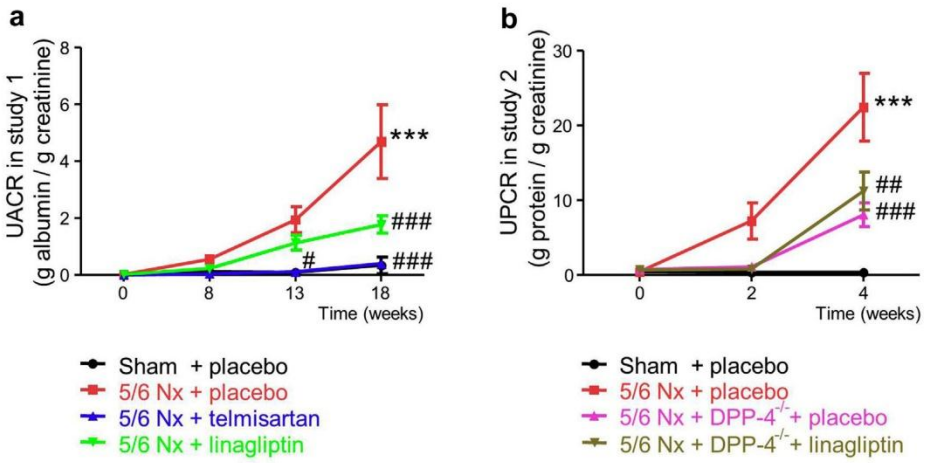

Figure 1 | UACR in study 1 and UPCR in study 2. (**a**) Urine albumin-to-creatinine ratio (UACR) in the core study (study 1) and (**b**) urine total protein-to-creatinine ratio (UPCR) in the confirmation study (study 2). Values are given as mean ± SEM. $^{***}P < 0.001$ versus sham + placebo; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ versus 5/6 Nx + placebo. DPP-4, dipeptidyl peptidase-4; Nx, nephrectomy.

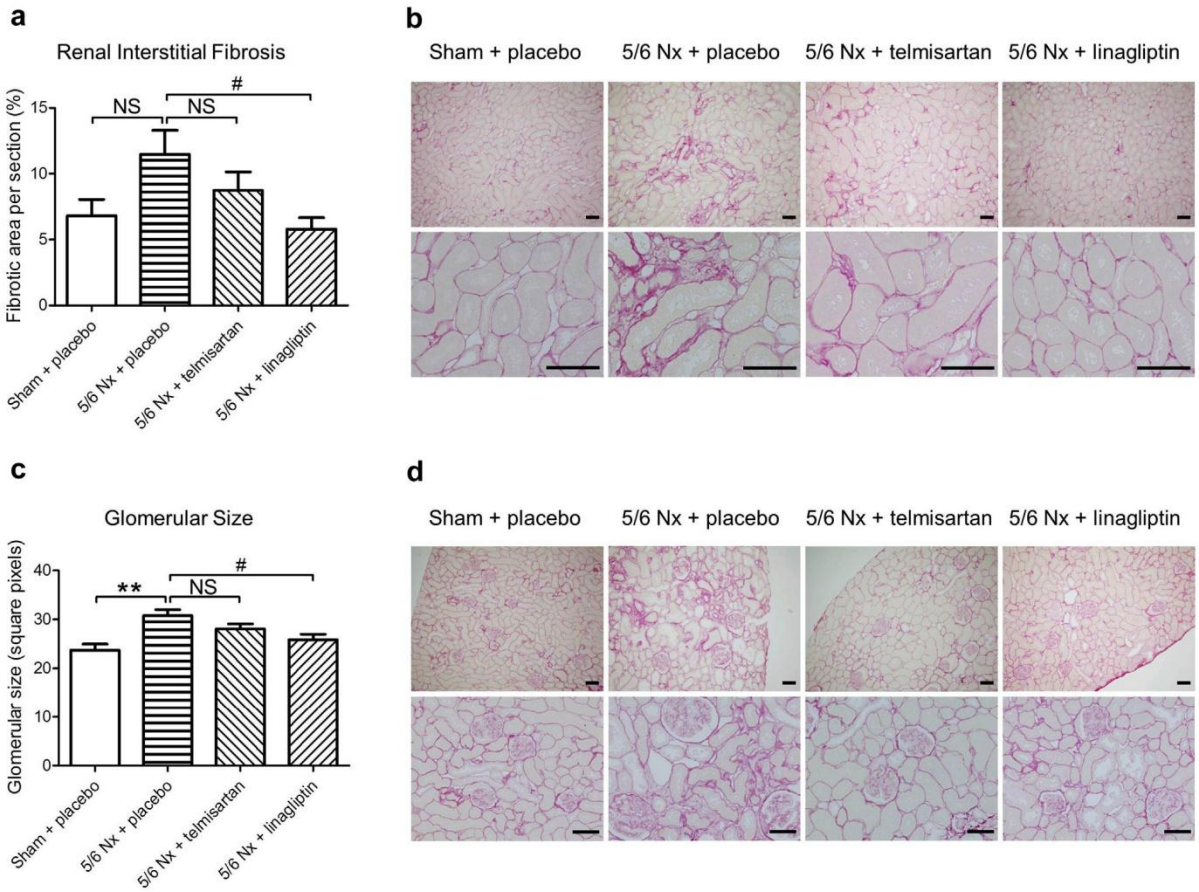

Figure 2 | Kidney morphology. (**a**) Renal interstitial fibrosis, (**b**) typical photomicrographs of the kidneys stained with sirius red for interstitial fibrosis, (**c**) glomerular size, and (**d**) typical photomicrographs of the kidneys stained with sirius red for glomerular hypertrophy. Values are given as mean ± SEM. Bar = 100 µm. $^{**}P < 0.01$ versus sham + placebo; $^{\#}P < 0.05$ versus 5/6 Nx + placebo. NS, not significant; Nx, nephrectomy.

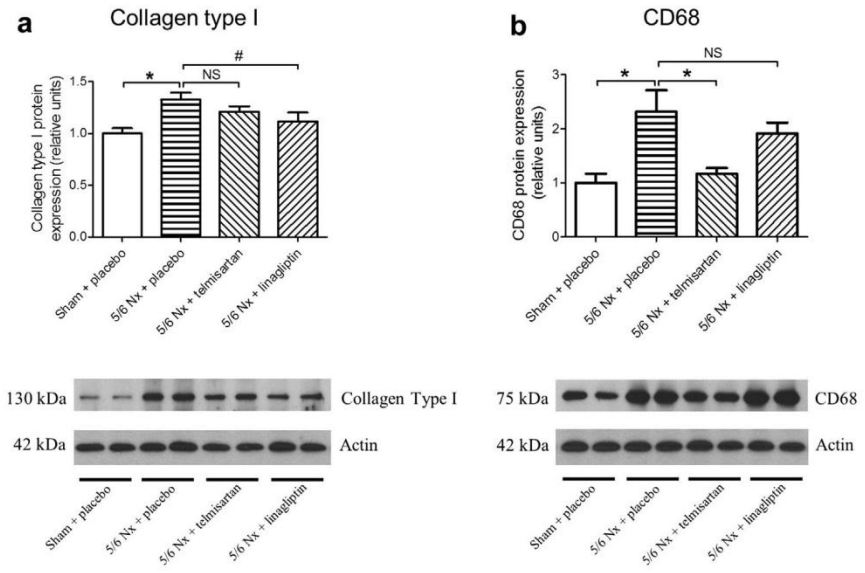

Figure 3 | Collagen type I and CD68 renal protein expression. Renal relative protein expression of (**a**) collagen type I and (**b**) cluster of differentiation 68 (CD68). Values are given as mean ± SEM. $^{*}P < 0.05$ versus sham + placebo; $^{\#}P < 0.05$ versus 5/6 Nx + placebo. NS, not significant; Nx, nephrectomy.

and 15 kidney samples ($n = 5$ per group) were analyzed by liquid chromatography and mass spectrometry to reveal effects related to telmisartan or linagliptin treatment. The analysis showed qualitative and quantitative differences between study groups. Compared to placebo, the number of statistically significant different signals for linagliptin in plasma was 552 (309 up- and 243 down-regulated) and 320 in kidney (180 up- and 140 down-regulated), and for telmisartan, 108 in plasma (66 up- and 42 down-regulated) and 363 in kidney (162 up- and 201 down-regulated). Among each group, the signals showed overlapping (26 in plasma and 65 in kidney) (Figure 7a and b).

Subsequently, the amino acid sequences of peptides with the highest signal-to-noise ratios and absence of cysteine bridges were directly identified in plasma (Figure 8a) and kidney (Figure 8b) by means of tandem mass spectrometry. Linagliptin treatment resulted in up-regulation of 4 peptides derived from collagen type I alpha 1 (3 of them in the kidney

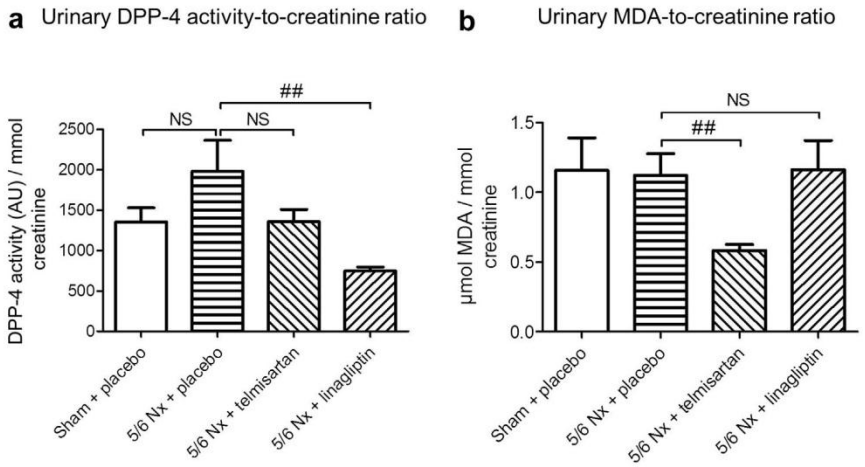

Figure 4 | Urinary DPP-4 activity and MDA urinary excretion. (**a**) Urinary dipeptidyl peptidase-4 (DPP-4) activity-to-creatinine ratio and (**b**) urinary malondialdehyde (MDA)-to-creatinine ratio. To control for variations in urinary flow rate, DPP-4 activity and MDA urinary excretion were normalized to creatinine. Values are given as mean ± SEM. $^{\#\#}P < 0.01$ versus 5/6 Nx + placebo. NS, not significant; Nx, nephrectomy.

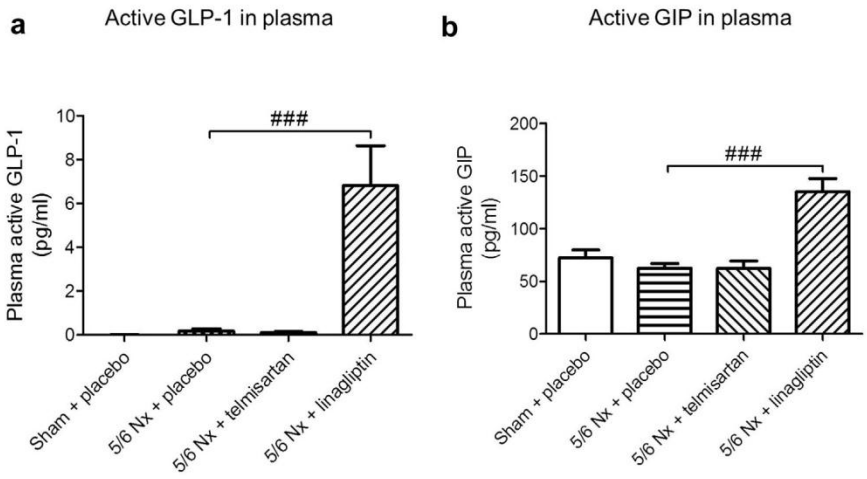

Figure 5 | Plasma concentration of active incretins. (**a**) Plasma concentration of active glucagon-like peptide 1 (GLP-1) and (**b**) active glucose-dependent insulinotropic polypeptide (GIP). Values are given as mean ± SEM. $^{###}P < 0.001$ versus 5/6 Nx + placebo. Nx, nephrectomy.

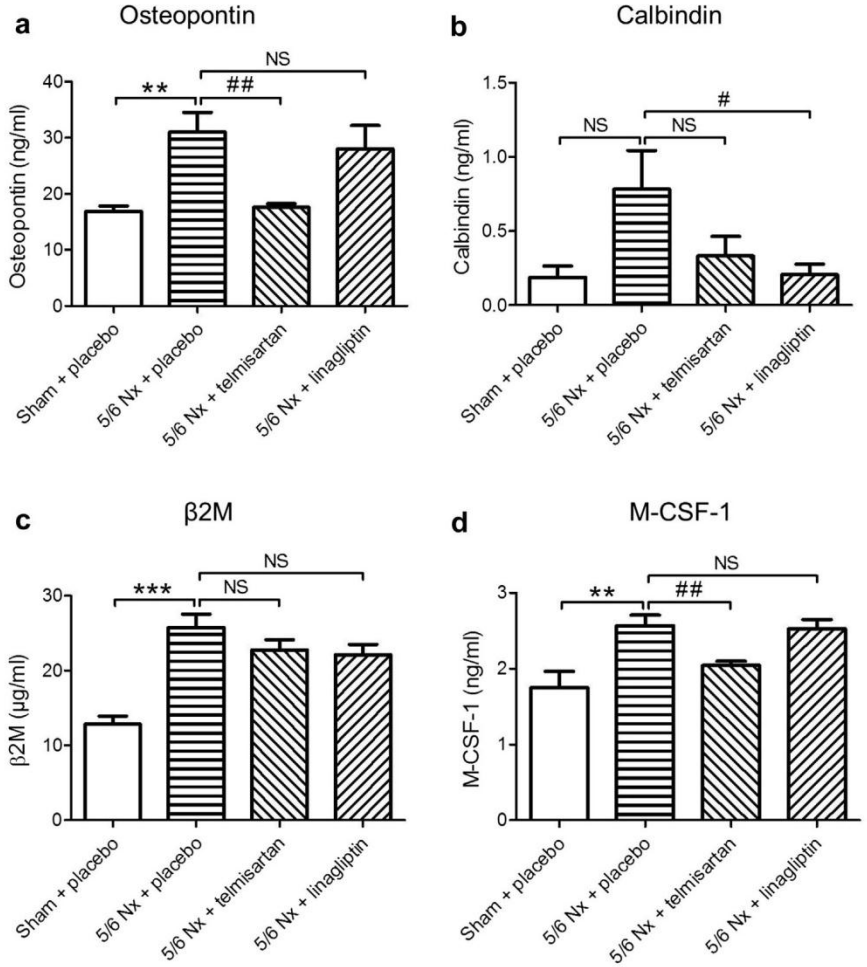

Figure 6 | Plasma parameters. Plasma concentration of (**a**) osteopontin, (**b**) calbindin, (**c**) beta-2 microglobulin (β2M), and (**d**) macrophage colony-stimulating factor 1 (M-CSF-1). Values are given as mean ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$ versus sham + placebo; $^{#}P < 0.05$; $^{##}P < 0.01$ versus 5/6 Nx + placebo. NS, not significant; Nx, nephrectomy.

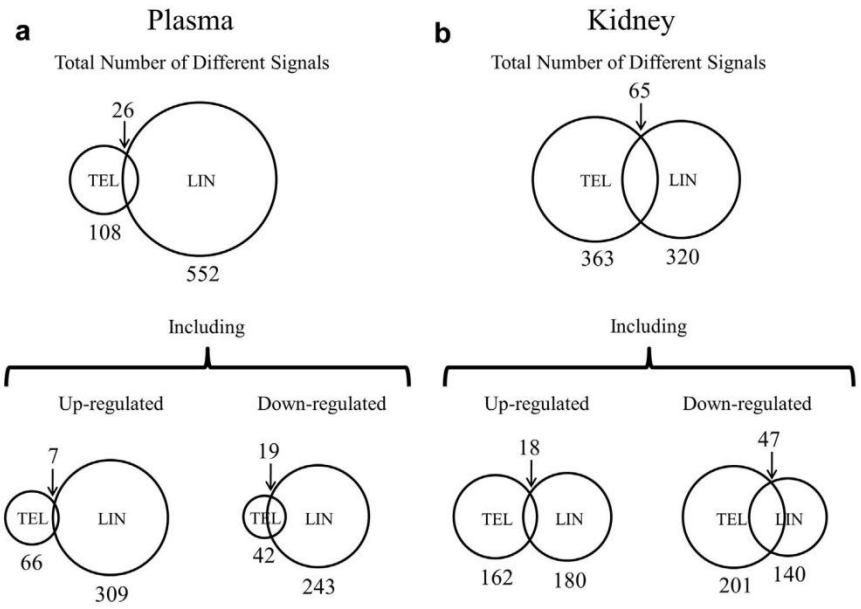

Figure 7 | Results of univariate analyses of LC-MS data. The circles represent the total number of statistically significant different signals as well as up- and down-regulated signals in 5/6 Nx + telmisartan (TEL) and 5/6 Nx + linagliptin (LIN) groups in comparison to 5/6 Nx + placebo in (**a**) plasma and (**b**) kidney according to the results of univariate analysis of liquid chromatography and mass spectrometry (LC-MS) data. Arrows indicate the number of overlapping signals. Statistical thresholds: $P < 0.01$, area under the receiver-operating characteristics curve > 0.9, $r > 0.6$ (for plasma); $P < 0.01$, area under the receiver-operating characteristics curve > 0.95, $r > 0.7$ (for kidney). Nx, nephrectomy.

and all 4 in plasma), 3 peptides derived from apolipoprotein C1 (Apo-C1) (in plasma only), and 2 peptides derived from heterogeneous nuclear ribonucleoproteins A2/B1 (HNRNPA2B1) (in kidney only). All aforementioned peptides contain an N-terminal proline at position 2, representing a part of the DPP-4 consensus cleavage motif, thus confirming validity of the DPP-4 inhibition. Telmisartan treatment led to an up-regulation of angiotensin II in plasma, indicating the reliability of the results, because an elevation of angiotensin II is a known effect of angiotensin II receptor blockers therapy.[12]

DISCUSSION

In the 5/6 Nx model of CKD, telmisartan profoundly lowered SBP, whereas linagliptin showed no BP-lowering effect. Both drugs reduced albumin excretion. Linagliptin's action was pronounced with respect to reduction of renal interstitial fibrosis and glomerular hypertrophy. In contrast, telmisartan's

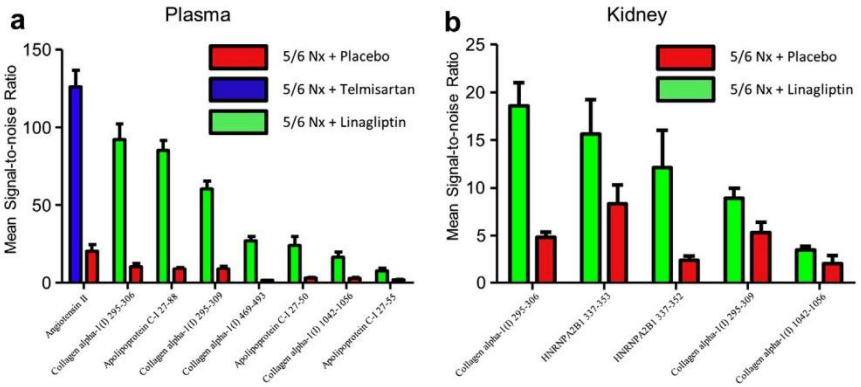

Figure 8 | Identified peptides sorted by their mean signal-to-noise ratios. The bar plots depict the signal-to-noise ratios (SNRs) for identified peptides in (**a**) plasma and (**b**) kidney. Values are given as mean ± SEM. HNRNPA2B1, heterogeneous nuclear ribonucleoproteins A2/B1; Nx, nephrectomy.

beneficial action was mostly due to its BP-lowering effect. The extent of the beneficial effects exerted by each drug was different. Antiproteinuric action of linagliptin treatment was as effective as a complete DPP-4 genetic deficiency and add-on linagliptin treatment in DPP-4-deficient mutant Fisher rats did not further improve the outcome. DPP-4 inhibition by linagliptin was associated with significant increases in plasma active GLP-1, GIP, and total SDF-1α concentrations, as well as decreases in plasma and urinary DPP-4 activity.

Translation to clinical science

The more pronounced the proteinuria levels are, the faster CKD progresses and the higher the risk of cardiovascular complications. Therefore, reducing proteinuria is one of the key treatment goals in CKD. In this study, linagliptin showed an antialbuminuric effect. Other reports,[4,13,14] including our own,[6] have also demonstrated the antialbuminuric action of an incretin-based therapy. In endothelial nitric oxide synthase knockout mice with streptozotocin-induced diabetic nephropathy, linagliptin significantly reduced albumin excretion to a greater extent than telmisartan.[6] This is consistent with findings from a pooled *post hoc* analysis of 4 studies in 217 patients with type 2 diabetes mellitus and renal dysfunction treated with linagliptin in addition to stable doses of renin-angiotensin system inhibitors.[14] In another study of 36 patients with type 2 diabetes mellitus, sitagliptin significantly lowered urinary albumin-to-creatinine ratio.[13] A reduction in albumin excretion alone may not only be a sign of improvement, but it may also be causally linked to a reduction in disease progression.[15] Min *et al.*[16] reported that in a mouse model of renal fibrosis, induced by unilateral ureteral obstruction, the DPP-4 inhibitor LC15-0444 lowered albuminuria and renal fibrosis.

Renal fibrosis and proteinuria are strong predictors of the clinical progression of CKD.[17–23] If drugs can influence these surrogate outcomes, the likelihood that hard clinical outcomes such as a reduction in all-cause and cardiovascular mortality will be affected is high. Therefore, the finding that linagliptin reduces kidney fibrosis and proteinuria is important from a translational perspective. Plasma glucose levels were not affected by linagliptin (Table 1, Core study), indicating that the beneficial effects observed with linagliptin are glucose-independent. In addition, the beneficial effects of linagliptin were similar regardless of the rats' genetic background (different strains, both wild type and DPP-4$^{-/-}$), suggesting that our findings are generally applicable and therefore may be translated into clinical science.

Effects of telmisartan on CKD progression

As expected, treatment with telmisartan resulted in potent reductions in SBP and albuminuria. In addition, telmisartan restored plasma levels of kidney injury marker osteopontin (Figure 6a). Telmisartan exerted potent anti-inflammatory/immunomodulatory effects as shown by decreases in renal pan-macrophage marker CD68 protein expression (Figure 3b) and lowering plasma levels of macrophage colony-stimulating factor 1 (Figure 6d). In contrast, linagliptin showed no anti-inflammatory/immunomodulatory effects. Telmisartan significantly lowered urinary malondialdehyde excretion most likely due to its antihypertensive effect.

Potential mechanism of linagliptin to reduce CKD progression

In the current study, we adopted a 2-pronged approach to assess potential molecular mechanisms explaining effects of DPP-4 inhibition on kidney morphology and function in 5/6 Nx rats. We used a candidate-pathway approach and a non-hypothesis-driven peptidomics approach to explore in depth the underlying mechanisms of DPP-4 inhibition. The candidate approach revealed that plasma levels of DPP-4 substrates such as active GLP-1, active GIP, and total SDF-1 were elevated by linagliptin in our CKD model.

We likewise tested candidate pathways such as TGF-β/SMAD2/3 signaling. Other groups demonstrated that DPP-4 inhibition causes a suppression of TGF-β/SMAD2/3 signaling mainly in animal models of diabetic CKD[4,24,25] and kidney cell lines exposed to high glucose concentrations.[26,27] However, in our nondiabetic CKD model, we found no evidence supporting a potential modulation of the TGF-β/SMAD2/3 pathway by linagliptin (Table 1, Core study). Concerning the finding of increased plasma GLP-1, we have the opinion that the GLP-1 pathway does not contribute to a major extent to the renoprotective effects of linagliptin, because preliminary data from an ongoing study are showing that 5/6 Nx GLP-1 receptor knockout (GLP-1r$^{-/-}$) mice develop interstitial fibrosis and decreased glomerular filtration rate; however, linagliptin treatment still improved kidney morphology and function (unpublished data by B. Hocher *et al.*). These data strongly suggest that the GLP-1r pathway seems not to be involved in the renoprotective effects of linagliptin in our model. In a nondiabetic rat Thy-1 glomerulonephritis model Higashijima *et al.*[28] showed that both alogliptin and anagliptin reduced the number of CD68-positive inflammatory macrophages in the kidney directly via GLP-1–dependent signaling. Because the GLP-1r pathway seems to be less important in the pathogenesis of CKD after 5/6 Nx, we did not see effects on GLP-1r–mediated kidney inflammation as it was reported by Higashijima *et al.*[28] in the Thy-1 glomerulonephritis model. Taken together, anti-inflammatory effects of DPP-4 inhibition seem to be more important in CKD models with a pronounced kidney inflammation. The impact of the increased plasma concentrations of the DPP-4 substrates GIP and SDF-1α on the progression of kidney disease after linagliptin treatment is unknown and needs to be addressed in further studies.

The mass spectrometric analysis revealed an increased abundance of collagen type I alpha 1 fragments with N-terminal proline in the position 2—a preferable cleavage motif for DPP-4.[29] Together with our findings of a decreased collagen I expression in the kidney, this might indicate an influence of linagliptin on collagen I homeostasis. However, an alternative and more likely explanation of increased collagen fragments in plasma and kidney samples after linagliptin treatment is related to the fact that matrix-metalloproteases–digested collagen

fragments are substrates of DPP-4 and consequently increase after DPP-4 inhibition.[30]

Apo-C1 is an important biomolecule, participating in lipid metabolism, acting via inhibition of plasma cholesteryl ester transfer protein, an enzyme promoting the transfer of cholesterol esters and triglycerides between plasma and lipoproteins.[31] DPP-4 is able to cleave N-terminal dipeptide containing proline in position 2 from a full-length Apo-C1 molecule, turning it into a truncated form.[32] Our data of an increased concentration of a nontruncated Apo-C1 in the plasma after linagliptin treatment are in agreement with a recent study by Skinner *et al.*[33] using sitagliptin—another DPP-4 inhibitor. In contrast to their study, in plasma we found a >95% conversion rate in placebo and an approximately 50% conversion rate after linagliptin treatment (data not shown). Although the exact biological role of Apo-C1 truncation is not clear yet, it is hypothesized that Apo-C1 regulates protein-protein interaction with receptors involved in lipid metabolism.[32] The levels of proatherogenic low-density lipoproteins were reported to be elevated as a result of any disturbances of Apo-C1 plasma concentrations (both increase and decrease).[34] However, in our study, we mainly saw effects on kidney fibrosis. The link between Apo-C1 and kidney fibrosis needs to be established in future studies. Moreover, it needs to be demonstrated that a prolongation of the half-life of full-length plasma Apo-C1 might be an additional beneficial effect of DPP-4 inhibition.

We likewise found an up-regulation of HNRNPA2B1 fragment in the kidney of linagliptin-treated 5/6 Nx rats. The representatives of type A and B HNRNPs have a high degree of amino acid sequence similarity.[35] HNRNPA1 phosphorylation plays a major role in the downstream nuclear signaling of atrial natriuretic peptide through cyclic guanosine monophosphate and cyclic guanosine monophosphate–dependent protein kinase.[36] In the kidney, a disturbance of atrial natriuretic peptide–mediated cyclic guanosine monophosphate synthesis is known to be a trigger of fibrosis.[36] Thus, an activation of the atrial natriuretic peptide–dependent guanylate cyclase pathway may contribute to the antifibrotic properties of linagliptin.[37,38]

Based on our current data, which is derived from a candidate-pathway approach in combination with an open approach, and our current understanding of the mode of action of DPP-4 inhibitors,[10] we conclude that the pharmacological effects of linagliptin cannot be explained by interacting with a single pathway. In contrast, our hypothesis is that the beneficial effects of linagliptin are attributed to the simultaneous interference with multiple pathways. This hypothesis is supported by our findings of the peptidomics analysis in plasma (Figure 7a).

In the 5/6 Nx model, linagliptin reduced albuminuria as effectively as genetic DPP-4 deficiency and a combination of both did not further reduce albuminuria. This suggests that the antiproteinuric effects of linagliptin are only due to the inhibition of DPP-4 activity and not due to potential pleiotropic effects of linagliptin.

Limitations

Although we confirmed that linagliptin treatment increased plasma active GLP-1 and GIP, as well as total SDF-1α levels, we did not evaluate other substrates of DPP-4 such as brain natriuretic peptide, substance P, peptide YY, neuropeptide Y, meprin A subunit β, and high-mobility group protein B,[10,39] any of which may be responsible for the observed beneficial actions. Additional studies using receptor knockout models for these potential DPP-4 substrates will be needed to elucidate further the mechanisms responsible for the renal effects of linagliptin. A further limitation is the prevention style of the study, which does not mimic clinical conditions. Furthermore, only peptides with the highest signal-to-noise ratios and absence of cysteine bridges were sequenced after being detected by liquid chromatography and mass spectrometry, thus we cannot exclude that peptides with a lower signal-to-noise ratio might have played an important role as well. In addition, we found 26 peptides in plasma and 65 peptides in kidney (Figure 7a and b), which were similarly regulated by telmisartan and linagliptin, indicating potentially overlapping renoprotective pathways of both drugs. However, based on our strategy to start first sequencing peptides with the highest signal-to-noise ratio and the absence of cysteine bridges, these peptides were not identified yet.

Conclusion

In conclusion, this study provides evidence that linagliptin delays renal disease progression in a nondiabetic, non-glucose-dependent rodent CKD model. DPP-4 inhibition with linagliptin—and potentially other DPP-4 inhibitors also —may therefore be a novel approach for the treatment of CKD in general. Clinical proof-of-concept studies are needed to evaluate the safety and efficacy of linagliptin in patients with nondiabetic CKD.

MATERIALS AND METHODS

Animals

Two independent studies were performed. The Core study (study 1) was carried out in 50 male Wistar rats purchased from Charles River Laboratories International, Inc. (Wilmington, MA) at the age of 8 weeks. The experiment was approved by the Committee on the Ethics of Animal Experiments (Landesamt fuer Gesundheit und Soziales), Berlin, Germany. The second "confirmation" study (study 2) was conducted in 45 male Sprague-Dawley and F344/DuCrlCrlj (F344) genetically DPP-4–deficient rats purchased from Charles River Laboratories (Yokohama, Japan) at the age of 8 weeks. All experimental procedures were carried out in accordance with the National Research Council of the National Academies Guide for Care and Use of Laboratory Animals and approved by the ethics committee of Kurume University, Japan.

Study design

One week after purchase, animals in study 1 were randomly assigned to 1 of 4 groups: sham operation + placebo ($n = 6$); 5/6 Nx + placebo ($n = 15$); 5/6 Nx + linagliptin ($n = 14$); 5/6 Nx + telmisartan ($n = 15$). The 5/6 Nx operation was performed as follows: uninephrectomy at week 1, followed at week 3 by amputation of the poles of the remaining kidney (Figure 9). Sham operations were

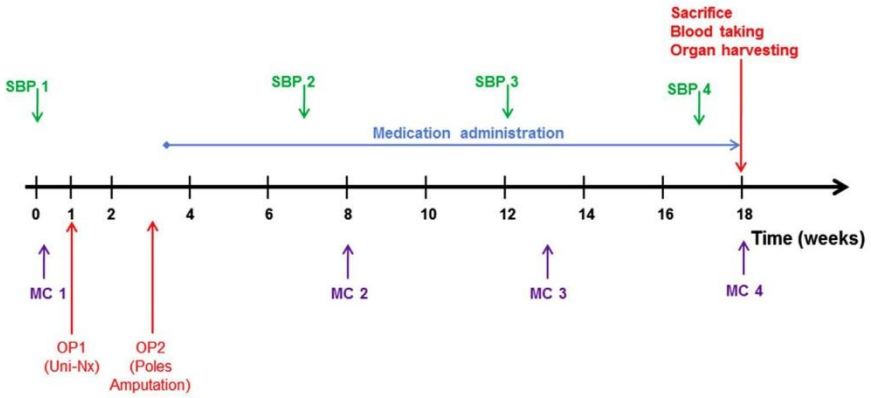

Figure 9 | Time course of the Core study (study 1). The experiment was started when the rats were 8 weeks old. MC, metabolic cages (to obtain 24-hour urine samples); OP1, uninephrectomy operation on the left side; OP2, amputation of the poles of right kidney; SBP, systolic blood pressure measurements; Uni-Nx, uninephrectomy.

performed at the same time points. The duration of study 1 was 18 weeks. During the study, SBP was measured by the tail-cuff method at weeks 0 (baseline), 7, 12, and 17. The animals were placed in metabolic cages to obtain 24-hour urine samples at weeks 0 (baseline), 8, 13, and 18. The rats were sacrificed at week 18 and blood samples were taken and organs harvested.

In study 2, wild-type and DPP-4$^{-/-}$ rats were subjected to either the sham (wild-type rats only) or 5/6 Nx operation. The 5/6 Nx operation was performed as follows: at 8 weeks of age, the poles of right kidney were amputated (week −1), followed 1 week later by uninephrectomy (week 0, baseline) (Figure 10). Sham operations were performed at the same time points. DPP-4$^{-/-}$ + 5/6 Nx rats were subdivided into placebo and linagliptin-treatment groups. The rats were given oral linagliptin using a stainless steel tube for 4 weeks (9 to 13 weeks of age). Thus, the study groups were as follows: wild-type rats + sham + placebo ($n = 10$); wild-type rats + 5/6 Nx + placebo ($n = 19$); DPP-4$^{-/-}$ + 5/6 Nx + placebo ($n = 8$); DPP-4$^{-/-}$ + 5/6 Nx + linagliptin ($n = 8$). The duration of study 2 was 4 weeks. Final body weight, SBP, and urinary total protein were analyzed. SBP was measured using the tail-cuff method at weeks 0 (baseline) and 4 (Figure 10). The animals were placed in metabolic cages to obtain 24-hour urine samples at weeks –1, 2, and 4. Animals were sacrificed after 4 weeks of treatment.

Drug treatments

Telmisartan and linagliptin were provided by Boehringer Ingelheim Pharma (Biberach an der Riss, Germany). In study 1, linagliptin (83 mg/kg in chow) and telmisartan (5 mg/kg/day in drinking water) were administered from day 4 after the first surgery until sacrifice. The dose of linagliptin corresponds to a dose of approximately 3 mg/kg/day; this dose has been used in previous studies.[40,41] In study 2, the dose of linagliptin (administered via oral gavage daily) corresponds to a dose of approximately 3 mg/kg/day. Control animals received vehicle only.

Renal morphometry

Renal morphology parameters were measured as described previously.[42,43] Briefly, interstitial fibrosis was evaluated after sirius red staining using computer-aided histomorphometry devices. Glomerular size was assessed by measuring ≥50 glomeruli in each longitudinal sirius red–stained kidney section using ImageJ software (National Institutes of Health, Bethesda, MD).[44] Glomerulosclerosis was

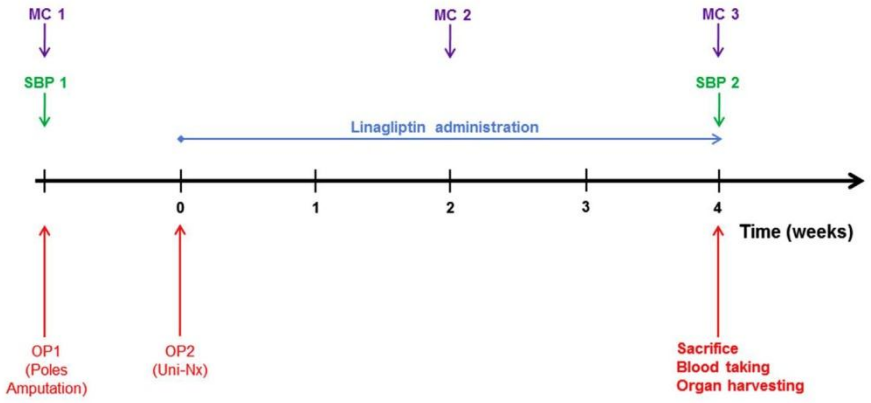

Figure 10 | Time course of the confirmation study (study 2). The study started when the rats were 8 weeks old. MC, metabolic cages (to obtain 24-hour urine samples); OP1, amputation of the poles of right kidney; OP2, uninephrectomy operation on the left side; SBP, systolic blood pressure measurements; Uni-Nx, uninephrectomy.

Table 2| List of primary antibodies used

Product name	Manufacturer	Catalog no.	Host species	Dilution
Actin	Sigma-Aldrich (St. Louis, MO)	A5060	Rabbit	1:50,000
Collagen type I	Acris Antibodies GmbH (Herford, Germany)	R1038	Rabbit	1:1000
Collagen type III	Acris Antibodies	13548-1-AP	Rabbit	1:1000
CD68 (ED-1)	Santa Cruz Biotechnologies (Santa Cruz, CA)	sc-59103	Mouse	1:1000
Phospho-SMAD2	Merck Millipore (Billerica, MA)	04-953	Rabbit	1:1000
Total SMAD2	Santa Cruz Biotechnologies	sc-6200	Goat	1:500
Phospho-SMAD3	Cell Signaling (Beverly, MA)	C25A9	Rabbit	1:2000
Total SMAD2/3	Santa Cruz Biotechnologies	sc-6202	Goat	1:500
TGF-β1	Santa Cruz Biotechnologies	sc-146	Rabbit	1:500

CD68, cluster of differentiation 68; TGF-β1, transforming growth factor beta 1.

quantified as percentage of periodic acid–Schiff–positive area within the glomerulus using a subjective semiquantitative score system (grades I–IV) by 2 investigators who were blinded to the study groups. Media/lumen ratio was measured using ImageJ based on analysis of photomicrographs of intrarenal arteries after Elastica van Gieson staining. Perivascular fibrosis was judged after sirius red staining using a semiquantitative score by 2 blinded independent investigators.

Protein expression analysis

Kidney tissue was lysed in urea/thiourea buffer (2 M thiourea, 7 M urea, 2% sodium dodecyl sulfate, 1% dithiothreitol). All subsequent steps were performed as previously described.[45] Details of the primary antibodies used are presented in Table 2. Final results were calculated as a ratio between the protein of interest and actin expression.

Real-time polymerase chain reaction—quantitation of gene expression

Total RNA was extracted from 50 mg of snap-frozen tissue by homogenization in peqGOLD TriFast reagent (Peqlab, Erlangen, Germany). Residual genomic DNA was removed with TURBO DNase (Life Technologies Co., Carlsbad, CA). RNA concentration and purity were assayed by spectrophotometry. First-strand cDNAsynthesis was performed with random hexamer primer and 1 μg of RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies).

Sequences from the Ensembl database and Primer3 software (available online) were used to create specific intron-spanning primers for the target genes.[46,47] Primer sequences are displayed in Table 3. Synthesized primers were obtained from Sigma-Aldrich (St Louis, MO) or Biolegio (Nijmegen, the Netherlands).

The SensiFAST SYBR Lo-ROX kit (Bioline, London, UK) was used for real-time polymerase chain reaction amplification with 10 ng of cDNA as template and 5– to 10 pmol of each primer. Polymerase chain reaction was performed on an Mx3000P thermal cycler (Stratagene, La Jolla, CA); all samples were analyzed in triplicate. The amplification efficiency of every reaction was checked by linear regression method.[48] Expression of the gene of interest was divided by the housekeeping gene (*GAPDH*) and expressed as fold change compared with that of the sham group.

Plasma and urine analyses

Urinary creatinine and albumin levels were quantitatively determined by commercially available detection kits (Immundiagnostik, Bensheim, Germany). Plasma samples were taken at study end and analyzed for biomarker patterns by Rat KidneyMAP (version 1.0) and Rodent MAP (version 3.0) multianalyte profiling platform (Myriad RBM, Austin, TX). DPP-4 activity was measured as reported previously.[49] At study end, total GLP-1 and active GLP-1 (detecting 7-36 amide and 7-37) concentrations were determined by an enzyme-linked immunosorbent assay (total GLP-1: cat. no. K150JVC-1; active GLP-1: cat. no. K150JWC-1; Meso Scale Discovery, Gaithersburg, MD). For active GIP analysis, the rat enzyme-linked immunosorbent assay (cat. no. 27202; Immuno-Biological Laboratories IBL, Minneapolis, MN) was used; for total GIP, the rat/mouse enzyme-linked immunosorbent assay (cat. no. EZRMGIP-55K; Millipore, Darmstadt, Germany) was used. Malondialdehyde was detected in urine using the MDA HPLC kit (cat. no. KC1900; Immundiagnostik, Bensheim, Germany). Final plasma glucose levels were determined using a clinical glucose assay reagent (Infinity Glucose Reagent; cat. no. TR15421; Thermo Fisher Scientific, Waltham, MA). In study 2, urinary total protein levels were measured with a pyrogallol red-molybdate complex using Micro TP-AR2 kit (Wako Pure Chemical Industries, Osaka, Japan) and urinary creatinine concentration was measured by a colorimetric method using a Determiner-L Cre kit (Kyowa Medex Co., Ltd, Tokyo, Japan).

Table 3| Real-time polymerase chain reaction primers

Gene	Primer sequence	Length	Exons
TGF-β1	+ CCAAGGAGACGGAATACAGG	101	2–4
ENSRNOG00000020652	– GTTTGGGACTGATCCCATTG		
TIMP-1	+ CGGACCTGGTTATAAGGGCTA	104	2–4
ENSRNOG00000010208	– GAATCCTTTGAGCATCTTAGTCATC		
Collagen type Iα1	+ TGGATTCCAGTTCGAGTATGG	129	49–50
ENSRNOG00000003897	– GCTACGCTGTTCTTGCAGTG		
Collagen type IIIα1	+ CAATGTAGATGAATTGGGATGC	119	1–2
ENSRNOG00000003357	– TGTCATCACAGAGGACAGATCC		
GLP-1 receptor	+ CAATCGGGGTCAACTTCCT	109	10–12
ENSRNOG00000001152	– GACTTCGCGAGTCTGCATTT		
GAPDH	+ CCATCAACGACCCCTTCAT	150	3–4
ENSRNOG00000018630	– GATCTCGCTCCTGGAAGATG		

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLP-1, glucagon-like peptide 1; TGF-β1, transforming growth factor beta 1; TIMP-1, tissue inhibitor of metalloproteinase.

Liquid chromatography and mass spectrometry of plasma and kidney samples

Proteins were removed by centrifugal ultrafiltration and mass spectrometric data were acquired as described previously.[30,50] Briefly, after protein depletion a 0.300 ml equivalent of plasma or 7.5 mg equivalent of kidney tissue by reversed phase liquid chromatography. After separation, each fraction was subjected to matrix-assisted laser desorption ionization time of flight mass spectrometry in linear mode. After mass spectrometry–data acquisition, spectra were analyzed, including peak recognition and visualization using the software package Spectromania developed in-house and R[51] including the MALDIquant[52] package. Peptide identification was achieved by matrix-assisted laser desorption ionization time of flight mass spectrometry/mass spectrometry. Tandem mass spectrometry spectra were subsequently noise filtered and peak deisotoped and saved in Mascot (Matrix Science, London, UK) generic file format and submitted to the Mascot search engine. Cascading searches including several post-translational modifications in UNIPROT (version 2015_09, www.uniprot.org) were performed.

Statistical analysis

All data are expressed as means ± SEM. Statistical analyses were performed using GraphPad Prism (version 5; GraphPad Software, San Diego, CA). For the statistical analysis of body weight, SBP, urinary albumin-to-creatinine ratio, urinary total protein-to-creatinine ratio, a 2-way analysis of variance with Bonferroni *post hoc* test was used. In all other cases, 1-way analysis of variance was used followed either by Dunn test when the data were not normally distributed or by Dunnett test when the data were normally distributed. In all cases, differences were considered statistically significant if $P < 0.05$. To find relevant signals within the mass spectrometric data, statistical analysis including 2-sample *t*-test (Welch), Pearson product moment correlation coefficient, receiver-operating characteristics,[53] and determination of the signal-to-noise ratio for each signal were carried out. The thresholds for significant differences were set for plasma at $P < 0.01$, $r > 0.6$, the area under the receiver-operating characteristics curve > 0.9 and $P < 0.01$, $r > 0.7$, area under the receiver-operating characteristics curve > 0.95 for kidney tissue.

DISCLOSURE

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Curriculum vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

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