Evaluation of metalloprotease inhibitors on hypertension and diabetic nephropathy

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The factors contributing to the development of hypertension and diabetic nephropathy remain poorly understood. Hyperglycemia leading to increases in the levels of glycosylated proteins, activation of the renin-angiotensin system, increased oxidative stress, and altered renal hemodynamics all appear to be involved (84). Regardless of the factors that initiate the renal injury, the progression to ESRD ultimately results in excess accumulation of extracellular matrix leading to glomerulosclerosis, renal interstitial fibrosis, tubular atrophy, and renal insufficiency. The control of the accumulation of extracellular matrix in the kidney is thought to be determined by the balance between the synthesis of matrix and its degradation by metalloproteases (MMPs). One of the factors that shift this balance is transforming growth factor-β (TGF-β).

Previous studies have shown that the expression of TGF-β in the kidney is elevated in both diabetes (3, 60, 61, 76) and hypertension (13, 22, 29, 43, 46, 66). Overproduction of TGF-β leads to apoptosis of podocytes (16, 61), mesangial cells (35), and tubular epithelial cells (4, 14), damage to the glomerular filtration barrier, and increased deposition of extracellular matrix. TGF-β also stimulates epithelial-to-mesenchymal transformation (EMT) (6, 9, 73, 79, 80), which promotes renal interstitial fibrosis.

Indeed, previous studies have reported that blockade of the TGF-β signal transduction cascade opposes the development of renal interstitial fibrosis in diabetic mice (8, 23, 83) and rats (2) as well as in salt-sensitive (13) and ANG II-dependent rat models of hypertension (43, 46).

Much less is known about the role of MMPs in the progression of diabetic and hypertension nephropathy. TGF-β increases the expression of tissue inhibitors of metalloproteases (TIMPS 1 and 2) that regulate the activity of MMPs that degrade extracellular matrix (1, 27, 46). It also increases the expression of tissue inhibitors of metalloproteases (TIMPS 1 and 2) that regulate the activity of MMPs that degrade extracellular matrix (1, 27, 46).

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polycystic kidney disease (51), unilateral ureteral obstruction (UUO) (28), and transplant nephropathy (54). Moreover, chronic administration of two MMP inhibitors, TISAM and Bay 12-9566, has been reported to slow the progression of renal injury in a UUO model of renal fibrosis (45) and transplant nephropathy (38).

There are several mechanisms by which an elevation in renal MMP activity could contribute to the development of progressive chronic kidney disease and renal fibrosis. First, Cheng and Lovett (9) reported that the ability of TGF-β to promote renal EMT is dependent on upregulation of MMP-2 activity, which normally degrades type IV collagen. The increase in renal MMP-2 activity is thought to disrupt tubular basement membranes, and this has been shown to be both necessary and sufficient to drive EMT in proximal tubular cultures (10, 82). MMPs may also contribute to the progression of chronic renal injury through the transactivation of the epidermal growth factor receptor (EGFR). Activation of certain metalloproteinas, including ADAM10 and ADAM17, increases the proteolytic release or “shedding” of transforming growth factor-α (TGF-α) and heparin-binding EGF (HB-EGF) from their membrane-bound “pro” forms in the kidney. Both TGF-α and HB-EGF are endogenous ligands of the EGFR (26, 34, 78) and stimulate the proliferation of fibroblasts, mesangial cells, and renal epithelial cells as well as the production of matrix proteins. Indeed, inhibitors of the EGFR have been reported to reduce the progression of renal fibrosis in animal models of ANG II hypertension (33) and polycystic kidney disease (40, 64, 69, 70). Finally, there are recent reports that MMP-2 and/or -3 promote the release of TGF-β from its large latent form bound to extracellular matrix (9, 41, 63, 72). This can activate a profibrotic feedback loop in which elevations in the renal synthesis of TGF-β stimulate the production of MMP-2, which in turn catalyzes the release of additional TGF-β from its bound latent forms.

The potential involvement of metalloproteases in EMT, renal cell proliferation, and TGF-β release makes this class of enzymes an attractive target for the development of new therapies for the treatment of progressive renal diseases. Thus the present study characterized the MMP-inhibitory profile of two novel compounds, 1-[(S)-2-carboxamide (XL081) and bis-4-[4-(chloro-phenoxy)-3,5-difluorobenzylsulfonyl]-1- hydroxy-4-(morpholin-4-ylcarbonyl)piperazine-2-carboxamide (XL081) and bis-4-[4-(chloro-phenoxy)-3,5-difluorobenzylsulfonyl]-3-hydroxycarbamoyl-piperazine-1-carboxylic acid 2-methoxy-ethyl ester, magnesium salt (XL784), and examined the effects of these inhibitors on the development of glomerulosclerosis and renal interstitial fibrosis in rat models of hypertension, Dahl salt-sensitive (Dahl S) and type 2 diabetic (T2DN) nephropathy.

METHODS

General. Experiments were performed using male Dahl S rats purchased from Charles River Laboratories and T2DN rats obtained from Physiogenex (Wauwatosa, WI). Dahl S rats are a well-established model of hypertension-induced focal glomerulosclerosis (12, 13, 18, 19, 55, 56, 75). The T2DN rat is a genetically modified version of the Goto-Kakizaki (GK) rat model of type II diabetes that has been shown to develop progressive proteinuria and focal glomerulosclerosis including thickening of the basement membrane, renal interstitial fibrosis, and the formation of glomerular nodules, which are common histological features of diabetic nephropathy (47). The rats in the present study had free access to food and water except when food was withdrawn overnight for measurement of fasting glucose levels. They were housed in the Animal Care Facility at the Woods Memorial Veterans Hospital (West Allis, WI), which is approved by the American Association for the Accreditation of Laboratory Animal Care, and all protocols were approved by the Animal Care Committee of the Medical College of Wisconsin.

Protocol 1: inhibitory profile of XL081 and XL784 on various MMPs. The inhibitory profiles of XL081 and XL784 on the activity of different MMPs were studied. Recombinant MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13 proteins at concentrations of 25, 40, 20, 20, and 37 nM, respectively, were preincubated with various concentrations of the MMP inhibitors ranging from 0.1 to 2,000 nM for 15 min in 40 ul of 50 mM Tris buffer (pH 7.5) containing 200 mM NaCl, 5 mM CaCl2, 20 μM ZnCl2, and 0.05% Brij. Then, the following isoform-selective MMP peptide substrates were added: MMP-1, 3.5 μM DNP-Pro-Leu-Ala-Leu-Trp-Ala-Arg-OH; MMP-2, 0.7 μM MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH2; MMP-3, 5 μM NBD-Arg-Pro-Lys-Pro-Leu-Ala-Nva-Trp-Lys-DME-NH2; MMP-8, 10 μM DNP-Pro-Leu-Ala-Tyr-Trp-Ala-Arg-OH; MMP-9, 5 μM MCA-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys-DNP-NH2; and MMP-13, 5 μM MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH2 and incubated for 4 h for MMP-1, 1 h for MMP-2, and 2 h for MMP-3, MMP-8, MMP-9, and MMP-13. After incubation, the plates were read in a fluorescence reader at wavelengths of excitation (Ex)280/emission (Em) 360 nm for MMP-1; Ex325/Em393 nm for MMP-2; Ex350/Em465 nm for MMP-3; Ex280/Em360 nm for MMP-8; Ex325/Em393 nm for MMP-9; and Ex325/Em393 nm for MMP-13.

The effects of the inhibitors on ADAM10 activity was measured by preincubating 5 nM of either the mouse or human recombinant enzyme with various concentrations of the test compounds ranging from 0.5 to 10,000 nM in 40 μl of a 50 mM HEPES buffer (pH 8.0) containing 100 mM NaCl, 1 mM CaCl2, and 0.01% NP-40. Then, 5 μM of a synthetic peptide substrate (DABCYL-Leu-Leu-Ala-Gln-Lys*-Leu-Arg-Ser-Ser-Arg-EDANS) was added. The reaction was incubated for 2 h at room temperature, and the increase in fluorescence was measured using wavelengths of Ex355/Em460 nm. All of these experiments were performed in triplicate, and the results were plotted as the percentage of control activity and IC50 concentrations determined.

Additional experiments were performed to access the pharmacological profile of the more potent MMP inhibitor, XL784. In these experiments, the effects of a high concentration of XL784 (10−5 M) was tested in vitro for its ability to compete for binding of agonists to 50 different receptors, ion channels, and enzymes using a commercial high-throughput screening assay (NovaScreen Biosciences, Hanover, MD). Each competitive binding assay was done in triplicate, and the entire panel was screened twice. We also determined the effects of XL784 on angiotensin-converting enzyme (ACE) activity in homogenates prepared from the kidney of normotensive Dahl S rats fed a AIN76 low-salt diet (0.1% NaCl, Dyets, Bethlehem, PA) using a fluorescent ACE assay based on the hydrolysis of a hip-his-leu substrate as previously described (53). Similar experiments were also performed to measure total renal tissue MMP activity using a FRET-peptide substrate enzyme assay (catalog no. 71158, AnaSpec, Fremont CA). Briefly, kidney samples (~0.25 mg) were homogenized in 1 ml of assay buffer that contained 50 mM Tris, 10 mM CaCl2, 150 mM NaCl, 0.1% (vol/vol), and Triton-X 100, pH 7.5. Tissue samples were centrifuged for 15 min at 10,000 g at 4°C, and the supernatant was collected. We incubated 50 μg of supernatant with 200 μl of the FRET-peptide substrate in 96-well plates at 4°C for 30 min, and the samples were protected from light. The fluorescent intensity of the samples were then read at Ex/Em = 490 nm/520 nm on a fluorescent plate reader.

In other experiments, we determined the exposure levels and half-lives of XL784 and XL081 following a 7-day repeat dosing. XL081 was administered once a day by oral gavage for 7 days at
Table 1. *In vitro* selectivity profiles of XL081 and XL784 on MMP activities

<table>
<thead>
<tr>
<th>Protease Type</th>
<th>XL081 IC50, nM</th>
<th>XL784 IC50, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human ADAM10</td>
<td>5.0 ± 1.6</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Murine ADAM10</td>
<td>3.8 ± 2</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>ADAM17 (TACE)</td>
<td>272 ± 1.1</td>
<td>73 ± 20</td>
</tr>
<tr>
<td>MMP-1</td>
<td>5.98 ± 2.6</td>
<td>1.900 ± 200</td>
</tr>
<tr>
<td>MMP-2</td>
<td>5.2 ± 1.5</td>
<td>0.81 ± 0.2</td>
</tr>
<tr>
<td>MMP-3</td>
<td>373 ± 2.6</td>
<td>120 ± 30</td>
</tr>
<tr>
<td>MMP-8</td>
<td>18.3 ± 1.5</td>
<td>10.8 ± 1.5</td>
</tr>
<tr>
<td>MMP-9</td>
<td>113 ± 1.5</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>MMP-13</td>
<td>4.0 ± 1.6</td>
<td>0.56 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SD from 2–5 separate experiments. MMP, matrix metalloproteinase.

doses of 300, 1,000, and 3,000 mg·kg⁻¹·day⁻¹ (6 rats/dose). XL784 was given at doses of 50, 100, 300, and 600 mg/kg (9 rats/dose). After 7 days of chronic dosing, the rats receive another oral dose on day 8, and blood samples were collected at 15 and 30 min and 1, 2, 3, and 24 h later for measurement of blood levels of the compounds by liquid chromatography/mass spectrometry (LC/MS).

The oral bioavailability of both compounds was also determined by comparing plasma levels measured in Sprague-Dawley rats at various time points following intravenous vs. oral administration of 300-mg/kg doses of each compound. Protein binding was determined by measuring plasma levels by tandem LC/MS (LC/MS/MS) in the plasma before and after ultrafiltration of the samples.

**Protocol 2: assessment of renal MMP and TGF-β1 levels during the development of hypertension in Dahl S rats**. Experiments were performed to determine whether MMPs and TGF-β1 levels increase during the development of hypertension in Dahl S rats. Experiments were performed using 9-wk-old Dahl S rats maintained on a low-salt diet (0.4% NaCl) and in a group of rats that were switched to a high-salt diet containing 8% NaCl for 3 wk. The animals were anesthetized with pentobarbital sodium (50 mg/kg ip), and the kidneys were collected for the measurement of MMP-2 and TGF-β1 levels. The kidneys were homogenized in RIPA buffer, centrifuged at 1,500 g, and MMP-2 levels were measured using a Quantikine ELISA kit (catalog no. DMP200, R&D Systems) that uses an antibody that recognizes the human, rat, and mouse isoforms. TGF-β1 levels were measured using a TGF-β1 ELISA (catalog no. G7591, Promega, Madison WI).

**Protocol 3: dose-ranging studies for the prevention of proteinuria in Dahl S rats**. These experiments were performed in eight groups of 9-wk-old Dahl S rats maintained on a low-salt diet from weaning to determine the doses of XL084 and XL784 that attenuate the development of proteinuria. Hypertension, proteinuria, and renal injury were induced by switching the rats to a high-salt diet containing 8% NaCl for 4 wk. The rats received daily oral doses of XL081 at doses of 50, 250, or 500 mg/kg or XL784 at doses of 50, 125, and 250 mg/kg. Control animals were treated with vehicle (corn oil) by gavage. The urinary excretion of protein was measured during the control period and weekly while the rats were treated with the high-salt diet. At the end of the experiment, the rats were anesthetized with pentobarbital sodium (50 mg/kg ip) 1 h after administration of the final dose, and a sample was collected for measurement of the plasma levels of these compounds. The kidney and heart were also collected and weighed and prepared for light microscopy using Masson trichrome stain to evaluate the degree of renal injury and fibrosis.

**Protocol 3: effects of XL081 on the development of hypertension and renal injury in Dahl S rats**. The effects of chronic administration of XL081 on the development of hypertension, proteinuria, and renal injury in Dahl S rats was compared with the renoprotective effects of the ACE inhibitor captopril. These experiments were performed in five groups of 9-wk-old Dahl S rats maintained from weaning on a low-salt diet (0.1% NaCl, AIN76 Dyets) to minimize renal injury. Group 1 was maintained on the low-salt diet for 4 additional wk. Groups 2–5 were challenged with a high-salt (8.0% NaCl) diet for 4 wk to induce hypertension and renal disease. Group 2 received daily oral doses of XL081 (100 mg/kg) by gavage. Group 3 received captopril (30 mg/kg) in the drinking water. Group 4 received XL081 (100 mg/kg) by gavage and captopril in the drinking water, while group 5 received daily oral gavage of vehicle (corn oil). The urinary excretion of protein was measured during the control period and weekly on the high-salt diet. After 24 days of treatment, the rats were anesthetized and a chronic catheter implanted in the femoral artery for measurement of arterial pressure. After a 3-day recovery period, blood pressure was measured on 3 consecutive days while the rats were freely moving in their home cages. At the end of the experiment, a plasma sample was collected for measurement of clinical chemistry, and the kidney and heart were collected for histological evaluation.

**Protocol 4: effect of XL784 on renal MMP activity in Dahl S rats in vivo**. These experiments were performed in three groups of 8-wk-old male Dahl S rats. Group 1 was maintained on a low-salt diet, and groups 2 and 3 were switched to a high-salt diet containing 8% NaCl. After 14 days on the various diets, groups 1 and 2 received a daily oral dose of vehicle (corn oil) by oral gavage, and group 3 received a 50 mg/kg dose of XL784 for 4 consecutive days. Two hours after administration of the final dose, the rats were euthanized with pentobarbital sodium (50 mg/kg ip), and the kidneys were collected. Whole kidney homogenates were prepared by centrifugation at 5,000 and 11,000 g, and MMP-2 protein levels were measured using a MMP-2 ELISA kit (catalog no. DMP200, R&D Systems). Total MMP activity in the renal homogenates was measured using a FRET-peptide substrate assay (catalog no. 71158, AnaSpec).

**Fig. 1.** Dose-related effects of a matrix metalloproteinase (MMP) inhibitor, XL784, on total MMP activity (*A*) and angiotensin-converting enzyme (ACE) activity (*B*) in homogenates prepared from the kidneys of Dahl S rats. Numbers in parentheses indicate the number of rats studied per group. Values are presented as means ± SE. *Significant difference from the corresponding value in samples treated with vehicle (P < 0.05).
levels are increased during the development of proteinuria and renal injury in T2DN rats. Three and 6-mo-old T2DN rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and the kidneys were collected for the measurement of MMP-2 and TGF-β1 levels. The kidneys were prepared as described above, and MMP-2 and TGF-β1 protein levels were measured using MMP-2 (catalog no. DMP200) and TGF-β1 ELISA kits (catalog no. MB100B, R&D Systems).

Protocol 6: renal MMP and TGF-β1 levels in T2DN rats. Experiments were performed to determine whether MMPs and TGF-β1

Protocol 7: effects of XL784 on the progression of diabetic nephropathy. These experiments were performed in 12-mo-old male T2DN rats (n = 53). The rats were uninephrectomized to accelerate the progression of renal injury. After a 2-wk recovery period, the animals were randomly assigned to five treatment groups (10 animals/group). Group 1 served as the control group and received a 5-ml/kg dose of vehicle (corn oil) by gavage daily. Group 2 received lisinopril in the drinking water at a concentration to deliver a dose of 20 mg·kg⁻¹·day⁻¹ and received a daily dose (5 ml/kg) of vehicle by gavage. Group 3 received a 50-mg/kg dose of XL784 daily by gavage. Group 4 received 20 mg·kg⁻¹·day⁻¹ of lisinopril in the drinking water and 50 mg/kg XL784 daily by gavage. Group 5 received a higher, 150-mg/kg dose of XL784 (150 mg/kg) daily by gavage. Urine was collected during a control period and biweekly for 4 mo to assess the degree of proteinuria. Blood pressure was recorded monthly using a tail-cuff device, and fasting glucose levels were determined during the control period and at the midpoint and end of the study. After 4 mo of drug treatment, the animals were anesthetized with isoflurane, a final blood sample was collected for measurement of clinical chemistry, and the heart and kidneys were collected for histological evaluation.

![Fig. 2. Comparison of renal MMP-2 (A) and transforming growth factor (TGF)-β1 (B) protein levels in Dahl salt-sensitive (S) rats fed either a low-salt (LS) or high-salt (HS) diet for 14 days. Numbers in parentheses indicate the number of rats studied per group. Values are presented as means ± SE. *Significant difference from the corresponding value in Dahl S rats fed a LS diet (P < 0.05).](image1)

![Fig. 3. Dose-related effects of the MMP inhibitors XL081 (A) and XL784 (B), on protein excretion in Dahl S rats fed a HS diet for 28 days. Numbers in parentheses indicate the number of rats studied per group. Values are presented as means ± SE. *Significant difference from the corresponding value in vehicle-treated rats (P < 0.05).](image2)
Statistical methods. Data are presented as means ± SE. The significance of differences in mean values was determined using an unpaired t-test (2 groups) or a repeated measures ANOVA followed by Dunn’s post hoc test. A P value <0.05 was considered to be statistically significant.

RESULTS

Inhibitory profile of XL081 and XL784 on various MMPs. The inhibitory profile of XL081 and XL784 on the activity of ADAM10, ADAM17, and six MMPs is presented in Table 1. XL784 is a highly potent, low-molecular-weight (1,122 g/mol) inhibitor of MMPs that has very limited aqueous solubility (20 μg/ml). XL784 potently inhibits MMP-2, MMP-13, and ADAM10 [TNF-α-converting enzyme (TACE)] activity in vitro, with IC₅₀ values in the range of 1–2 nM. XL784 also inhibited MMP-9 (IC₅₀ ~20 nM) activity and ADAM17 (IC₅₀ ~70 nM) also known as TACE. However, it exhibited low potency for inhibition of MMP-1 (IC₅₀ ~2,000 nM). XL081 is another low-molecular-weight (1,144 g/mol) inhibitor that is structurally related to XL784. It exhibits an inhibitory profile similar to XL784, but in general, is two to five times less potent at inhibiting MMP activity in vitro (Table 1). It is also less soluble than XL784, with an aqueous solubility of <5 μg/ml.

Additional experiments were performed to examine the ability of XL784 to inhibit MMP activity in homogenates prepared from the kidneys of Dahl S rats fed a low-salt diet (Fig. 1A). Over a range of concentrations from 0.1 to 10 μM, XL784 significantly inhibited total renal MMP activity in a concentration-dependent fashion. The free concentration of XL784 in these assays would be expected to be in the 2–200 nM range since this compound is extensively bound to both plasma and tissue proteins (>98%).

Since ACE is a Zn-MMP, we also determined the effects of XL784 on renal ACE activity. The results of these experiments are presented in Fig. 1B. Over a range of concentrations from 0.1 to 10 μM, XL784 had no significant effect on ACE activity in plasma from Dahl S rats maintained on a low-salt diet.

We also determined the pharmacological profile of XL784 to compete for binding of a wide variety of agonists to their receptors. These data are presented as supplemental data in Table S1 (all supplementary material for this article is available on the journal web site). XL784 at a concentration of 10⁻⁵ M, which is four orders of magnitude higher than its ED₅₀ for inhibition of MMP-2 activity, had very little effect on the binding of a wide variety of neurotransmitters, ion channel agonists, growth factors, and peptide hormones to their respec-
tive receptors. The notable exceptions are that this high concentration of XL784 reduced binding of glutamate to the NDMA receptor by 33%, histamine to the H2 receptor by 40%, oxytocin to its receptor by 31%, and nifedipine to the L-calcium channel by 37%.

**Exposure levels and half-lives of XL081 and XL784 in rats.** Percent oral bioavailability was 30% for XL784 and <10% for XL081. Thus the dose of XL081 used in the present study was higher than XL784. Peak blood levels measured 30 min after administration of XL081 averaged 5–10 μM following administration of 300, 1,000, and 3,000 mg/kg XL081. The half-life averaged 10 h following the 300-mg/kg dose and was prolonged to 26 h in rats receiving the 3,000-mg/kg dose. Minimal blood levels measured 24 h after administration of the 300-mg/kg dose were <1 μM. Similar results were seen following administration of XL784. Peak blood levels measured 30 min after administration of XL784 at doses ranging from 50 to 600 mg/kg averaged between 10 and 20 μM. Minimal blood levels measured 24 h after administration of XL784 at doses between 50 and 300 mg/kg averaged between 0.1 and 0.3 μM. Both compounds are extensively protein bound (>98%) so that the free levels attained following administration of the various doses of XL784 or 081 were in the range of 50–100 nM for the first 8 h and then fell to <10 nM 24 h after dosing.

**Effects of a high-salt diet on renal MMP-2 and TGF-β1 levels in Dahl S rats.** The results of these experiments are presented in Fig. 2. Renal expression of MMP-2 protein increased twofold in Dahl S rats fed a high-salt diet for 14 days. Renal TGF-β1 levels also increased by twofold in these animals.

**Dose-ranging studies in Dahl S rats.** The results of these experiments are presented in Fig. 3. Dahl S rats fed a high-salt diet and treated with vehicle rapidly developed hypertension, proteinuria, and renal injury. Protein excretion rose from <20 to >100 mg/day over a 4-wk period. Chronic administration of XL081 of doses of 50, 250, and 500 mg·kg⁻¹·day⁻¹ (Fig. 3A) or XL784 at doses of 50, 125, and 250 mg·kg⁻¹·day⁻¹ (Fig. 3B) markedly reduced proteinuria. The control rats treated with vehicle developed severe focal glomerular sclerosis with marked expansion of the mesangial matrix, renal interstitial fibrosis, and tubular necrosis (Fig. 4). The degree of glomerulosclerosis and renal fibrosis was reduced in animals treated with XL081 or XL784 (Fig. 4A). Glomerular injury scores were also significantly reduced in the rats treated with either MMP inhibitor (Fig. 4B).

**Effects of XL081 on the development of proteinuria in Dahl S rats.** A comparison of the effects of XL081 vs. the ACE inhibitor captopril on the development of hypertension and proteinuria in Dahl S rats is presented in Fig. 5. Blood pressure and proteinuria increased to 170 mmHg and 165 mg/day in Dahl S rats fed a high-salt diet for 4 wk (Fig. 5A). Chronic administration of captopril at a dose of 30 mg·kg⁻¹·day⁻¹ lowered MAP by ~20 mmHg, but it had no significant effect on the development of proteinuria (Fig. 5B). In contrast, XL081 (100 mg/kg) given either alone or in combination with captopril reduced the development of proteinuria by 50%. It also attenuated the development of hypertension. XL081 given alone or in combination with captopril markedly reduced the degree of mesangial matrix expansion, glomerular injury, and renal interstitial fibrosis (Fig. 6). In contrast, chronic administration of captopril had no significant effect on the degree of glomerulosclerosis or renal interstitial fibrosis in Dahl S rats fed a high-salt diet for 4 wk.

**Effects of XL784 on renal MMP activity in Dahl S rats in vivo.** We also determined the effectiveness of in vivo administration of XL784 (50 mg·kg⁻¹·day⁻¹) for 4 consecutive days to reduce renal MMP activity in Dahl S rats fed a high-salt diet for 14 days. The results of these experiments are presented in Fig. 7. Total MMP activity increased by 42% in Dahl S rats fed a high-salt diet for 14 days compared with the values observed in animals maintained on a low-salt diet. Treatment with XL784 normalized total MMP activity in the kidneys of Dahl S rats fed a high-salt diet to the same level as that seen in Dahl S rats maintained on a low-salt diet.

**Effects of XL784 on progression of renal disease in Dahl S rats with established hypertension and proteinuria.** Additional studies were performed to determine whether chronic administration of an MMP inhibitor could reverse the progression of proteinuria and renal disease in Dahl S rats with preexisting renal damage and established hypertension. These studies were done using the second-generation MMP inhibitor XL784, which is more soluble and has better oral bioavailability than XL081. In these experiments, Dahl S rats were fed a diet containing 4% NaCl for 5 wk to induce a stable hypertension (>160 mmHg) and preexisting proteinuria and renal injury. Proteinuria continued to progress from ~150 to 250 mg/day in control rats treated with vehicle. MAP rose to 190 ± 7 mmHg.
Chronic administration of the ACE inhibitor lisinopril (20 mg·kg⁻¹·day⁻¹) and the AT₁ blocker losartan (20 mg·kg⁻¹·day⁻¹) prevented the progression of proteinuria and reduced MAP by ~20 mmHg. Administration of XL784 (50 mg·kg⁻¹·day⁻¹) was as effective as blockade of the renin-angiotensin system at preventing the progression of proteinuria, but it did not lower MAP (Fig. 8B). Combination therapy with XL784, lisinopril, and losartan was very effective and reduced proteinuria from 150 to ~60 ± 9 mg/day. This was associated with a reduction in MAP to 135 ± 9 mmHg, which is not significantly different from the values seen in Dahl S rats maintained on a low-salt diet (Fig. 5A).

The effects of XL784 vs. lisinopril and losartan on the degree of renal and cardiac injury are presented in Figs. 9 and 10. Dahl S rats treated with vehicle exhibited severe glomerular sclerosis with expansion of the mesangial matrix, renal interstitial fibrosis, and tubular necrosis (Fig. 9A). Chronic treatment of the rats with lisinopril and losartan reduced but did not reverse the expansion of the mesangial matrix and the degree of glomerulosclerosis. Chronic administration of XL784 markedly reduced the degree of renal interstitial fibrosis and reduced the glomerular injury score a greater extent than did lisinopril and losartan (Fig. 9B). Combined therapy with XL784, lisinopril, and losartan markedly improved the degree of glomerulosclerosis and renal fibrosis, and the glomerular injury score fell to levels similar to levels typically seen in normotensive Dahl S rats fed a low-salt diet (compare Figs. 8B and 5B).

Dahl S rats fed a high-salt diet also exhibited cardiac hypertrophy and a marked degree of interstitial and perivascular cardiac fibrosis, as indicated by the blue staining of collagen in the hearts stained with Masson trichrome (Fig. 10A). Chronic treatment of the rats with lisinopril and losartan had no effect on the degree of cardiac fibrosis even though it reduced arterial pressure (Fig. 10B). Chronic treatment of the rats with XL784 treatment markedly attenuated the degree of cardiac fibrosis even though it had no effect on MAP. XL784 when used in combination with lisinopril and losartan completely abolished cardiac fibrosis.
kidneys of 6- vs. 3-mo-old T2DN rats (Fig. 11 A). MMP-2 protein levels were significantly elevated in the protein in the kidneys of T2DN rats are presented in Fig. 11. Similarly, renal TGF-β1 levels increased in 6- vs. 3-mo-old T2DN rats (Fig. 11 B).

The effects of the various treatments on fasting glucose levels in T2DN rats are presented in Fig. 12. All of the T2DN rats in the various treatment groups were diabetic at the beginning of the study, with fasting glucose levels averaging between 280 and 310 mg/dl. Chronic treatment of the rats with vehicle, lisinopril, or XL784 for 2 or 4 mo had no significant effect on fasting glucose levels (Fig. 12A) or glucose tolerance (Fig. 12B) in the various groups. Plasma cholesterol and triglyceride levels measured at the end of the study were elevated two- and fourfold in vehicle-treated T2DN rats (Table 2) relative to values typically reported in nondiabetic strains of rats (32). Chronic treatment of the rats with lisinopril, XL784, or combined therapy significantly reduced plasma cholesterol levels. Plasma triglyceride levels were not significantly altered by monotherapy with lisinopril or XL784, but it did decrease significantly in the group of rats receiving combined therapy. Plasma creatinine concentration and blood urea nitrogen (BUN) levels were elevated by two- to threefold in T2DN rats receiving vehicle (Table 2) vs. the normal range of 0.3 and 20 mg/dl we have typically reported in nondiabetic strains of rats (32). Plasma BUN levels were not significantly altered by any of the treatments, but plasma creatinine levels fell significantly in the group of rats treated with lisinopril, the low and high doses of XL784, and combined therapy.

The effects of lisinopril, a low (50 mg·kg⁻¹·day⁻¹) and high dose (150 mg·dl) of XL784, and combination therapy on blood pressure and the progression of proteinuria in T2DN rats is presented in Fig. 13. Baseline MAP estimated from systolic and diastolic pressures measured by a tail-cuff device averaged ~130 mmHg in all the groups (Fig. 13A). Blood pressure was not significantly altered in rats treated with vehicle or the low or high doses of XL784. Blood pressure decreased by 20–30 mmHg in the T2DN rats receiving lisinopril or combined therapy. Baseline proteinuria was elevated and averaged ~200 mg/day in all of the groups of T2DN rats compared with levels of proteinuria (<20 mg/day) typically seen in most nonhypertensive or diabetic strains of rats (Fig. 13B). Proteinuria increased over the 4-mo course of the study in the vehicle-treated animals. In contrast, proteinuria fell during the first month in rats treated with lisinopril, the low or high dose of XL784, or combination therapy with lisinopril and XL784. Similar results were seen by measuring changes in albumin excretion. Baseline albuminuria averaged >120 mg/day in all the groups. After 4 mo of treatment, albuminuria increased to 210 ± 23 mg/day in the vehicle-treated rats, but it fell significantly to 65 ± 14 mg/day in the rats treated with lisinopril, 102 ± 19 and 42 ± 12 mg/day in the rats treated with the low and high dose of lisinopril, and 24 ± 7 mg/day in the rats receiving lisinopril and XL784 combination therapy.

The effects of the various treatments on the progression of diabetic nephropathy are presented in Fig. 14. The kidneys obtained from the T2DN rats treated with vehicle exhibited severe renal injury, with tubular necrosis and dilation, formation of tubular casts, and renal interstitial fibrosis and inflam-
The glomeruli were markedly hypertrophied and exhibited thickening of the basement membrane and mesangial matrix expansion, leading to severe focal segmental glomerulosclerosis and fibrosis. Many of the glomeruli in the vehicle-treated rats exhibited infiltration of epithelial cells into Bowman's space. Treatment with lisinopril significantly reduced mesangial expansion, fibrosis of Bowman's space, tubular necrosis, and the degree of renal interstitial fibrosis and inflammation. Lisinopril also reduced the degree of glomerular injury (Fig. 14B). Monotherapy with the low dose of XL784 was as, or more effective, than lisinopril in reducing the degree of glomerulosclerosis, tubular necrosis, and renal interstitial fibrosis. It completely eliminated the epithelialization of Bowman's space. Finally, combination therapy with lisinopril and XL784 appeared to be the most effective at reducing the degree of glomerulosclerosis and renal interstitial fibrosis.

**DISCUSSION**

This present study examined the effects of two new MMP inhibitors on the development of proteinuria and renal injury in rat models of hypertension, Dahl S and T2DN. Both XL081 and XL784 are low-molecular-weight, orally active, selective inhibitors of MMP-2, MMP-13, and ADAM10, the latter being a type I integral membrane proteinase involved in diverse processes such as development, cell-cell interactions, and protein ectodomain shedding. XL784 potently inhibits these enzymes, with IC_{50} values of ~1–2 nM. XL784 also inhibits MMP-9 (IC_{50} ~20 nM) and ADAM17 (IC_{50} ~70 nM). However, XL784 displays low potency for inhibition of MMP-1 (IC_{50} ~2,000 nM). XL081 exhibits a similar inhibitory profile as XL784. The high selectivity of XL784 for MMP-2 over MMP-1 and its ability to target TACE was thought to mini-
mize the possibility of side effects, notably the skeletal muscle syndrome characterized by joint stiffness and inflammation that was previously observed in clinical trials of broad-spectrum MMP inhibitors as chemotherapeutic agents (52).

In the present study, both XL784 and XL081 were effective at reducing proteinuria at the lowest dose tested (50 mg·kg\(^{-1}\)·day\(^{-1}\)). The results of the pharmacokinetic analysis indicates that this dose would produce plateau-phase blood levels of 1–5 \(\mu\)M and free plasma levels between 20 and 100 nM given that these drugs are extensively protein bound (>98%). Thus the levels of XL784 attained in these studies should have been sufficient to produce sustained inhibition of MMP-2, -9, and -13 and ADAM10 and partial inhibition of MMP-3 and TACE activity for a better part of each day but not inhibit MMP-1 activity. Indeed, we were able to show that total renal MMP activity was reduced by 30% in Dahl S rats fed a high-salt diet. The free plasma levels attained in this study (<10\(^{-7}\) M) should have had little if any off target effect on the binding of neurotransmitters and peptides to their receptors and no significant effect on ACE activity.

Since we found that the levels of MMP-2 and TGF-\(\beta\)1 increased significantly in the kidneys of Dahl S rats during the development of hypertension-induced renal injury, we did dose-ranging studies to determine whether XL081 and XL784 at doses ranging from 50 to 500 mg/kg day would be effective in preventing the development of proteinuria and renal injury in Dahl S rats fed a high-salt diet. Both compounds attenuated the development of proteinuria and reduced renal fibrosis in a dose-dependent fashion. In subsequent studies, we found that administration of XL081 was more effective than captopril in reducing the degree of proteinuria, glomerulosclerosis, and renal injury in Dahl S rats fed a high-salt diet for 4 wk. Somewhat unexpectedly, we also found that XL081 attenuated the development of hypertension in Dahl S rats fed a high-salt diet, which raised the question of whether the renoprotective actions of XL081 were simply secondary to an antihypertensive effect or whether its renoprotective actions attenuate the severity of hypertension in Dahl S rats. Indeed previous studies

Fig. 10. Effects of the blockade of the renin-angiotensin system with lisinopril (20 mg·kg\(^{-1}\)·day\(^{-1}\)) and losartan (20 mg·kg\(^{-1}\)·day\(^{-1}\)), the MMP inhibitor XL784 (50 mg·kg\(^{-1}\)·day\(^{-1}\)), and combined therapy with lisinopril (20 mg·kg\(^{-1}\)·day\(^{-1}\)), losartan (20 mg·kg\(^{-1}\)·day\(^{-1}\)), and XL784 (50 mg·kg\(^{-1}\)·day\(^{-1}\)) on cardiac fibrosis in Dahl S rats fed a HS diet for 10 wk. Numbers in parenthesis indicate the number of rats studied per group. Values are presented as means ± SE. *Significant difference from the corresponding value in vehicle-treated rats (\(P < 0.05\)).
using diuretics (68, 74, 81) to prevent salt retention and genetic manipulation to prevent the development of hypertension clearly attenuate the development of renal injury in Dahl S rats. Similarly, servocontrol of renal perfusion pressure to prevent transmission of elevated systemic pressure markedly attenuates the development of glomerular injury and renal fibrosis in Dahl S rats fed a high-salt diet (44). On the other hand, several recent studies have indicated that administration of antioxidants (50) and immunosuppressants (42, 48) to reduce infiltration of immune cells and renal oxidative stress also reduce the degree of renal injury and that this attenuates the development of hypertension. We felt the most likely explanation for the lowering of blood pressure is that XL081 attenuated the development of glomerulosclerosis, which normally reduces the glomerular filtration rate, attenuates pressure natriuresis, and further promotes salt retention and the degree of hypertension in Dahl S rats fed a high-salt diet.

To further address the question of whether MMP inhibitors attenuate the development of proteinuria and renal injury by lowering arterial pressure or have more direct renoprotective effects, we studied the effects of XL784 in Dahl S rats that were fed a 4.0% NaCl diet for 5 wk to induce stable hypertension and preexisting renal injury. Administration of XL784 at a dose of 50 mg/kg was effective in vivo and sufficient to normalize the elevated renal MMP activity in Dahl S rats to a level similar to that seen in normotensive Dahl S rats fed the low-salt diet. Chronic treatment of the Dahl S rats with preexisting renal injury with XL784 prevented the progression of proteinuria without reducing arterial pressure and markedly reduced the degree of glomerulosclerosis and renal fibrosis. XL784 was equally effective as a combination of lisinopril and losartan at reducing proteinuria in these animals and was far more effective at reducing the degree of renal fibrosis. In other studies, we found that administration of XL784 given in combination with lisinopril and losartan had additive renoprotective effects and nearly restored proteinuria to the original control levels seen in the animals before the development of hypertension. The improvement in renal histology of these animals with long-standing hypertension and preexisting renal injury that were treated with XL784 or XL784 in combination with lisinopril and losartan was remarkable in that the degree of renal injury at the end of the study was similar to that seen in Dahl S rats that were maintained from birth on a low-salt diet to prevent the development of hypertension. XL784 also reduced the degree of interstitial and perivascular fibrosis in the Dahl S rats to a much greater degree than lisinopril and
losartan, even though blockade of the renin-angiotensin system lowered blood pressure while XL784 did not. Combined treatment with blockade of the renin-angiotensin system and XL784 eliminated the cardiac fibrosis. Since these animals had long-standing hypertension before the onset of the treatment, the only explanation for these remarkable findings is that XL784 promotes regression of cardiac fibrosis. Overall, these results indicate that chronic treatment of hypertensive Dahl S rats has the potential to reverse both preexisting glomerulosclerosis and renal interstitial fibrosis and cardiac fibrosis without lowering arterial pressure.

Next, we determined whether chronic blockade of MMP activity with XL784 would prevent the progression of renal injury in the T2DN rat model of diabetic nephropathy (47). We first confirmed that MMP-2 protein levels increased significantly along with the development of proteinuria in T2DN rats. In these studies, XL784 given alone or in combination with lisinopril reduced the degree of proteinuria and markedly reduced the degree of mesangial matrix expansion, glomerulosclerosis, tubular necrosis, and renal interstitial fibrosis. The improvement in renal histology in rats given XL784 was greater than that seen in rats treated with lisinopril alone, and combined treatment was more effective than either agent given alone. The renoprotective effects of XL784 in this diabetic model were not associated with changes in arterial pressure. In contrast, arterial pressure was reduced by 20 mmHg in the T2DN rats treated with lisinopril or lisinopril plus XL784. These data suggest that MMPs play a vital role in the development of hypertension- and diabetic-induced renal injury. The results further imply that inhibiting MMP activity is at least as effective as administering an ACE inhibitor and that chronic treatment with XL784 along with lowering arterial pressure by blocking the renin-angiotensin system has a great potential for the prevention and possibly reversal of hypertension- or diabetic-induced renal disease.

XL784 also lowered plasma cholesterol levels in T2DN rats. Since lisinopril had a similar effect, we suspect that this likely reflects the reduction in proteinuria seen in the treated animals. Indeed, plasma levels of cholesterol are usually mildly elevated in most models of chronic kidney disease associated with proteinuria due to the loss of lipid binding proteins in the urine.

The observation that chronic administration of an MMP inhibitor can prevent the progression of renal injury in Dahl S and T2DN rats with preexisting renal injury without altering arterial pressure was quite unexpected. Moreover, the mechanism by which this occurs remains to be studied in detail in future studies. There is evidence that the expression and activity of MMP-2 is diminished in the kidney of diabetic patients (15) and in animal models of both type 1 and 2 diabetes (24, 57, 63), and this has been assumed to promote collagen accumulation and renal fibrosis. However, the results of more recent studies suggest that MMP-2 activity is elevated in the serum or urine of type 1 diabetic patients (67) and in animal models of glomerulonephritis (36). Upregulation of renal MMP-2 activity has also been noted in several animal models of progressive renal injury including: the SHR (7), SPSHR (20) and the Ren-2 hypertensive rat (5), as well as anti-GBM and Thy-1 models of glomerulonephritis (25, 31), polycystic kidney disease (51), and following UUO (28) and SPSHR (20) and the Ren-2 hypertensive rat (5), as well as anti-GBM and Thy-1 models of glomerulonephritis (25, 31), polycystic kidney disease (51), and following UUO (28) and transplant nephropathy (54). Moreover, chronic blockade of

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**Table 2. Effects of XL784, lisinopril, or combined therapy on plasma lipids, creatinine, and blood urea nitrogen levels in type 2 diabetes nephropathy rats**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (11)</th>
<th>Lisinopril (8)</th>
<th>XL784 (10)</th>
<th>High-Dose XL784 (10)</th>
<th>XL784 + Lisinopril (11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol, mg/dl</td>
<td>213 ± 10</td>
<td>154 ± 8*</td>
<td>173 ± 13*</td>
<td>149 ± 6*</td>
<td>147 ± 8*</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>459 ± 51</td>
<td>525 ± 111</td>
<td>358 ± 87</td>
<td>370 ± 84</td>
<td>225 ± 25*</td>
</tr>
<tr>
<td>Blood urea nitrogen, mg/dl</td>
<td>39 ± 7</td>
<td>37 ± 5</td>
<td>25 ± 3</td>
<td>21 ± 2*</td>
<td>41 ± 5</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>1.2 ± 0.2</td>
<td>0.7 ± 0.1*</td>
<td>0.6 ± 0.1*</td>
<td>0.5 ± 0.1*</td>
<td>0.7 ± 0.1*</td>
</tr>
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</table>

Values are means ± SE. Numbers in parentheses indicate number of animals per group. *Significant difference for the corresponding value in vehicle-treated rats (P < 0.05).
MMP-2 has been reported to reduce the progression of renal injury in transplant nephropathy (38) and in the UUO model (45). There are several mechanisms that may explain how elevations in renal MMP activity could contribute to the development of renal fibrosis and progressive chronic kidney disease. One possibility is that MMP-2 and/or -3 promotes the release of TGF-β from the large latent forms bound to extracellular matrix (9, 41, 63, 72). TGF-β stimulates the synthesis of extracellular matrix protein such as fibronectin and collagen in fibroblasts, podocytes, and renal tubular and mesangial cells. TGF-β also stimulates the production of MMP-2, which would further activate a profibrotic positive-feedback loop in which elevations in MMP-2 activity catalyze the release of active TGF-β from its bound latent forms (10). TGF-β is known to play a critical role in the renal fibrosis associated with hypertension and diabetes (6, 12–13, 37, 46, 49, 59, 60, 83). Taken together, these data suggest that the inhibition of MMPs may reduce renal injury by inhibiting the release of TGF-β from latent stores in the tissue and reducing TGF-β stimulation of the synthesis of extracellular matrix.

Another mechanism by which MMP inhibitors may oppose the development of renal interstitial fibrosis associated with hypertension and diabetes is by inhibiting the development of EMT. Recent studies have indicated that the development of EMT in the kidney is dependent on upregulation of MMP-2, which normally degrades type IV collagen (10, 82). The increase in renal MMP-2 activity disrupts tubular basement membranes, and this has been shown to be both necessary and sufficient to drive EMT in proximal tubular cultures (10, 82).

Finally, MMPs may also contribute to the progression of chronic renal injury through the transactivation of the EGFR. Activation of certain metalloproteinases, including ADAM10 and ADAM17, increases the proteolytic release or “shedding” of TGF-α and heparin-binding EGF (HB-EGF) from their membrane-bound “pro” forms in the kidney. Both TGF-α and HB-EGF are endogenous ligands of the EGFR (26, 34, 78) and stimulate the proliferation of fibroblasts, myofibroblasts, mesangial cells, and renal epithelial cells as well as the production of matrix proteins. Uchiyama-Tanaka et al. (71) demonstrated that glomerular mesangial cells incubated with ANG II caused an elevation of fibronectin expression via HB-EGF and pretreatment with the MMP inhibitor batimastat attenuated the increase in fibronectin expression.
In summary, these results of the present study indicate that chronic administration of selective MMP inhibitors has the potential to delay the progression, and may even reverse, hypertension and diabetic nephropathy. They further suggest that activation of certain MMPs may play an important role in the development of glomerulosclerosis, tubular atrophy, and interstitial fibrosis in hypertension- and diabetic-induced nephropathy.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


