



Pharmacological effects of a vitamin K₁ 2,3-epoxide reductase (VKOR) inhibitor, 3-acetyl-5-methyltetronic acid, on cisplatin-induced fibrosis in rats

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ABSTRACT. Cisplatin (CDDP) is a chemotherapeutic agent that is widely used in the treatment of lymphomas and solid malignancies. However, its clinical usage is limited by its severe side effects in the kidneys. Glomerular and tubular injuries in the kidneys commonly progress to interstitial fibrosis and, ultimately, the end stage of renal failure. We previously reported that 3-acetyl-5-methyltetronic acid (AMT) had inhibitory effects on rat renal vitamin K₁ 2,3-epoxide reductase (VKOR) *in vitro* and also suppressed mesangial cell proliferation and, consequently, the formation of fibrosis via the vitamin K-dependent activation of the growth arrest-specific 6 (Gas6)/Axl pathway in anti-Thy-1 glomerulonephritis (Thy-1 GN) in rats. In the present study, we demonstrated that AMT alleviated the progression of renal fibrosis in CDDP-treated rats. The repeated intravenous administration of AMT for 28 days dose-dependently suppressed increases in plasma urea nitrogen and plasma creatinine levels as well as creatinine clearance in CDDP-treated rats. Furthermore, the treatment suppressed the expression of α -smooth muscle actin (SMA)-positive cells and ameliorated the extracellular matrix accumulation of collagen III, indicating an antifibrotic effect. In conclusion, our toxicological and histopathological results demonstrated quantitatively the pharmacological inhibitory effects of AMT on the progression of renal fibrosis in CDDP-treated rats.

KEY WORDS: 3-acetyl-5-methyltetronic acid (AMT), cisplatin (CDDP), growth arrest-specific 6 (Gas6)/Axl signaling pathway, renal fibrosis, vitamin K₁ 2,3-epoxide reductase (VKOR) inhibition

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Cisplatin (CDDP) is a chemotherapeutic agent that is widely used in the treatment of lymphomas and solid malignancies. However, its clinical usage is limited by its severe side effects in normal tissues including the kidneys, with many patients discontinuing the treatment [2, 3]. CDDP-induced nephrotoxicity is characterized by an acute inflammatory response that is related to apoptosis and necrosis through the involvement of leukocytes, adhesion molecules, cytokines and chemokines released from inflammatory cells, and the affected cells themselves, and eventually leads to interstitial fibrosis [4, 15]. Glomerular and tubular injuries in the kidneys commonly progress to interstitial fibrosis and, ultimately, the end stage of renal failure [10]. In animal experiments using rats, an intravenous CDDP-treatment induced renal acute focal damage in tubules with the consequent dilatation of affected tubules and formation of luminal casts, and simultaneous increases were observed in plasma urea nitrogen (UN) and plasma creatinine levels due to renal injury [9, 25]. Therefore, extensive interstitial fibrosis has been reported in the kidneys in the chronic stage 14 days after the administration of CDDP to rats [9]. Antioxidants have been shown to prevent acute renal injury in a rat model due to their radical-scavenging effects [1, 7]. CDDP, which is taken up by renal tubular cells, causes cytotoxicity through a combination of insults: the production of reactive oxygen species (ROS), mitochondrial dysfunction, inhibition of protein synthesis and DNA injury [12]. Antioxidants reduce cytotoxicity by scavenging ROS, and suppress apoptosis, necrosis and inflammatory progression in the kidneys. Yamamoto *et al.* recently reported that an anti-inflammatory agent, a cyclooxygenase (COX)-2-selective inhibitor, ameliorated CDDP-induced renal lesions [21]. In their study, the COX-2 inhibitor was suggested to reduce the infiltration of macrophages in affected renal lesions and decreased the production of TGF- β , thereby reducing the number of anti- α -smooth muscle actin (SMA)-positive myofibroblasts, which are responsible for the development of fibrosis.

Vitamin K₁ 2,3-epoxide reductase (VKOR) is a protein that converts the epoxide of vitamin K back to vitamin K, a co-factor

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that is essential for the post-translational γ -carboxylation of several blood coagulation factors [23]. It is also involved in many physiological processes, such as cell proliferation, migration and differentiation as well as cell survival during development and tissue repair via the vitamin K-dependent activation of the Growth arrest-specific 6 (Gas6)/Axl signaling pathway [22]. We previously demonstrated that the low molecular weight compound, 3-acetyl-5-methyltetronic acid (AMT), had inhibitory effects on rat kidney VKOR *in vitro* and also suppressed mesangial cell proliferation and, consequently, the formation of fibrosis formation via the Gas6/Axl pathway in anti-Thy-1 glomerulonephritis (Thy-1 GN) in rats [19]. Previous studies have reported the importance of Gas6/Axl in these physiological processes. However, to best of our knowledge, the influence of the inhibitory effects of VKOR on the Gas6/Axl pathway in CDDP-induced rat renal failure has not yet been examined. Therefore, we have investigated the pharmacological effects of the VKOR inhibitor, AMT, on CDDP-induced fibrosis in rats.

MATERIALS AND METHODS

Materials

AMT was synthesized at Pharmaceutical Research Laboratories, Toray Industries, Inc. (Tokyo, Japan). CDDP was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The monoclonal mouse anti-human SMA antibody, Clone 1A4, was purchased from Dako A/S (Glostrup, Denmark). A polyclonal rabbit anti-rat collagen III antibody was purchased from AbD Serotec (Oxford, U.K.).

Animal experiments

Male Crl:SD rats (6 weeks old) from Charles River Japan (Yokohama, Japan) were maintained at 19–25°C under a 12-hr dark/light cycle. Rats were allowed unlimited access to chow (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Animal experiments were conducted according to the Guidelines for Animal Experiments, Research and Development Division, Toray Industries, Inc. Rats were divided into the following four groups: (1) saline-treated control group; (2) AMT 0 mg/kg/day (saline)-treated group after the administration of CDDP (6 mg/kg); (3) AMT 10 mg/kg/day-treated group after the administration of CDDP (6 mg/kg); and (4) AMT 30 mg/kg/day-treated group after the administration of CDDP (6 mg/kg), and each group had six animals. Saline or CDDP was intravenously bolus-injected via the tail vein on Day 0. Saline or AMT (saline used for a vehicle) was intravenously bolus-injected once a day for 28 days (Day 0 to 27). Body weights were measured before the administration of CDDP on Day 0 and before the administration of saline or AMT on Days 5, 10, 15 and 20 and after a 24-hr urine collection on Day 28. Urine was collected for 24 hr after the administration of AMT on Days 4 and 27. On Days 5 and 28 after urine collection, rats were placed under inhaled anesthesia with isoflurane, and blood was withdrawn from the jugular vein (Day 5) and abdominal aorta (Day 28), respectively.

Urinalysis and blood chemistry

A urinalysis was conducted on 24-hr pooled urine. Total protein, creatinine and albumin were measured with an Auto Analyzer 7070 (Hitachi, Tokyo, Japan). Heparinized blood was centrifuged (approx. 1,600 \times g, 15 min, 4°C) to obtain plasma, and a blood chemistry examination was conducted. Total protein, albumin, UN and creatinine were measured with the Auto Analyzer 7070 (Hitachi). Creatinine clearance (Ccr) was calculated from the concentration of creatinine in the urine, urine flow rate and creatinine concentration in plasma.

Renal pathology and histopathology

After blood collection on Day 28, the kidneys were removed and weighed, and gross pathology was conducted. Relative organ weights were calculated based on body weights. The kidneys were fixed in 10 vol% neutral buffered formalin at 4°C for 24 hr. They were embedded in paraffin, and sections (5- μ m thick) were stained with hematoxylin and eosin (HE) for light microscopy. A histopathological assessment was performed in an open-label study in addition to the following grades along with other common toxicological studies: –, no change; -/+, very slight change; +, slight change; ++, moderate change; +++, marked change.

Immunochemical staining and quantification

Immunohistochemical staining for α -SMA and collagen III was performed on formalin-fixed, paraffin-embedded sections. Paraffin sections were dewaxed and washed in phosphate buffered saline (PBS). In collagen III-staining, heat-induced antigen retrieval was achieved by incubating sections in 0.1 mol/l Tris-HCl buffer (pH 9.0) overnight at 80°C. In α -SMA-staining, a wet autoclave pretreatment for antigen retrieval was performed in 0.01 mol/l citrate buffer (pH 6.0) for 5 min at 121°C. Sections were washed in PBS, and endogenous peroxidase was blocked by incubation with 0.3% H₂O₂ in PBS for 30 min. Sections were then incubated with primary antibodies against α -SMA and collagen III, respectively. All primary antibodies were diluted in PBS containing 20% goat serum overnight at 4°C. Binding was detected by EnVision Dual Link System-HRP (Dako, Carpinteria, CA, U.S.A.) for collagen III and LSAB 2 System-HRP (Dako) for α -SMA. Peroxidase activity was visualized using 3, 3'-diaminobenzidine tetrahydrochloride (Dako) for 1 min.

In order to quantify immunohistochemical data, TIFF images of kidney sections were acquired by a digital slide scanner (NanoZoomer C9600; Hamamatsu Photonics K.K., Hamamatsu, Japan) with an Olympus UplanApo 20-fold magnification/ NA 0.7 objective lens. Five random areas of images of the cortical and outer medullary fields (1,600 \times 1,200 pixels) in each section were captured using the viewer software (NDP view; Hamamatsu Photonics K.K.). Collagen III- and α -SMA-positive areas in each field

Table 1. Urinalysis following repeated intravenous administrations of AMT on Days 5 (A) and 28 (B) in CDDP-treated rats

Group	AMT	Total Protein (mg/day)	Albumin (mg/day)	Creatinine (mg/day)	Albumin/Creatinine (mg/mg)	Creatinine Clearance (ml/min)
(A) Day 5						
Control	-	3.26 ± 0.46	0.85 ± 0.27	4.6 ± 0.3	0.19 ± 0.06	1.68 ± 0.08
CDDP-treatment	0 mg/kg/day	8.86 ± 1.33 ^{##}	39.22 ± 7.08 ^{###}	6.6 ± 0.5 ^{##}	6.02 ± 1.20 ^{###}	0.55 ± 0.14 ^{###}
	10 mg/kg/day	7.72 ± 1.69	31.92 ± 11.45	4.7 ± 0.5	6.54 ± 1.64	0.87 ± 0.23
	30 mg/kg/day	2.84 ± 0.66 ^{**}	4.73 ± 2.42 ^{***}	4.0 ± 0.3	1.15 ± 0.54 ^{**}	1.22 ± 0.10 ^{**}
(B) Day 28						
Control	-	4.31 ± 0.25	0.83 ± 0.22	7.53 ± 0.38	0.11 ± 0.03	2.34 ± 0.13
CDDP-treatment	0 mg/kg/day	1.77 ± 0.29 ^{##}	1.08 ± 0.40	4.46 ± 0.54 ^{###}	0.25 ± 0.09	0.33 ± 0.09 ^{###}
	10 mg/kg/day	3.36 ± 0.43 ^{**}	1.06 ± 0.39	6.14 ± 0.59 [*]	0.19 ± 0.06	1.22 ± 0.22 ^{***}
	30 mg/kg/day	3.52 ± 0.33 ^{**}	0.94 ± 0.28	7.68 ± 0.18 ^{***}	0.12 ± 0.04	2.13 ± 0.09 ^{***}

These data were represented as means ± SEM (n=6). Comparison to control group, ^{###}P<0.001; ^{##}P<0.01. Comparison to AMT 0 mg/kg/day group, ^{***}P<0.001; ^{**}P<0.01; ^{*}P<0.05.

were assessed quantitatively using the image analysis software WinROOF ver. 6.3 (Mitsuya Shoji Corp., Fukui, Japan).

Statistical analysis

Values are expressed as the means ± SEM. Data on body weight, urinalysis and blood chemistry were statistically analyzed using SAS Ver. 9.3 (SAS institute Japan, Tokyo, Japan). The statistical significance of differences in mean values between control rats (no CDDP treatment) and CDDP-treated rats (no AMT treatment, 0 mg/kg/day), and between CDDP-treated rats (no AMT treatment, 0 mg/kg/day) and each AMT treatment group (10 and 30 mg/kg/day after the CDDP treatment) were assessed with the one-way analysis of variance (ANOVA) and the LSD comparison test. Differences among means were considered significant at *P* values of <0.05, 0.01 and 0.001.

RESULTS

Urinalysis and blood chemistry

Five days after the administration of CDDP, urinary albumin levels and the albumin-to-creatinine ratio (ACR) were markedly increased in the AMT 0 mg/kg/day group (Table 1). However, the AMT treatment (30 mg/kg/day) significantly suppressed these increases in the urinalysis, whereas a slight or almost no change in urinary albumin levels and ACR was observed in the AMT 10 mg/kg/day group compared to the AMT 0 mg/kg/day group. In blood chemistry, plasma UN and creatinine levels were markedly increased in the AMT 0 mg/kg/day group; however, these increases were significantly inhibited by AMT in a dose-dependent manner in CDDP-treated rats (Table 2). Five days after the CDDP treatment, Ccr was slightly decreased, whereas the AMT (30 mg/kg/day) treatment significantly ameliorated deteriorations in renal function (Table 1).

Twenty-eight days after the CDDP treatment, no significant difference was observed in the level of urinary Alb between control and AMT 0 mg/kg/day groups (Table 1). In blood chemistry, plasma UN and creatinine levels increased on Day 28, and the AMT treatments dose-dependently suppressed these increases in both levels (Table 2). A marked decrease in Ccr was still present on Day 28 in AMT 0 mg/kg/day group; however, the AMT treatments (10 and 30 mg/kg/day) significantly suppressed deteriorations in renal function in a dose-dependent manner.

Gross pathology and histopathology

A marked increase was observed in relative kidney weights (Table 3), and pathologically, enlargement, rough-surface and discoloration of the kidneys were macroscopically observed in the AMT 0 mg/kg/day group. However, dose-dependent recovery occurred significantly in relative kidney weights and were also observed in these pathological findings with the AMT treatments (10 and 30 mg/kg/day).

Regarding histopathological findings, marked hyaline casts and tubular dilatation were observed in the cortico-medullary junctions in the AMT 0 mg/kg/day group, and the infiltration of mononuclear cells around these tubules and within their lumina was also detected (Table 4). Renal tubules with lumina of various sizes were observed in the cortical and outer medulla fields of the kidneys, while spindle-shaped fibroblastic cells and mononuclear cells were prominent in the interstitium around the renal tubules, indicating progressive fibrosis (Fig. 1). Therefore, slight fibrosis in the cortex and moderate fibrosis in the outer medulla were histopathologically caused by the CDDP treatment on Day 28. However, pathological findings after AMT treatments revealed a dose-dependent amelioration in renal injuries induced by CDDP; of the six rats examined, two had very slight and four had no fibrosis in the cortex, while one had slight and five had very slight fibrosis in the outer medulla with the AMT treatment (30 mg/kg/day) for 28 days (Table 4).

Table 2. Blood chemistry following repeated intravenous administrations of AMT on Days 5 (A) and 28 (B) in CDDP-treated rats

Group	AMT	Total Protein (g/dl)	Albumin (g/dl)	BUN (mg/ml)	Creatinine (mg/ml)
(A) Day 5					
Control	-	5.84 ± 0.07	4.48 ± 0.05	12.3 ± 0.7	0.19 ± 0.00
CDDP-treatment	0 mg/kg/day	6.02 ± 0.12	4.39 ± 0.09	77.7 ± 12.4 ^{###}	1.11 ± 0.26 ^{###}
	10 mg/kg/day	5.76 ± 0.12	4.12 ± 0.07	28.8 ± 5.6 ^{***}	0.47 ± 0.09 ^{**}
	30 mg/kg/day	5.73 ± 0.11	3.85 ± 0.16 [*]	18.1 ± 0.8 ^{***}	0.23 ± 0.01 ^{***}
(B) Day 28					
Control	-	5.69 ± 0.04	4.19 ± 0.04	17.6 ± 0.4	0.23 ± 0.01
CDDP-treatment	0 mg/kg/day	5.40 ± 0.21	3.94 ± 0.13	107.8 ± 25.8 ^{###}	1.22 ± 0.22 ^{###}
	10 mg/kg/day	5.61 ± 0.08	3.97 ± 0.07	22.2 ± 3.7 ^{***}	0.39 ± 0.05 ^{***}
	30 mg/kg/day	5.60 ± 0.03	3.91 ± 0.10	19.0 ± 0.4 ^{***}	0.25 ± 0.01 ^{***}

These data were represented as means ± SEM (n=6). Comparison to control group, ^{###}P<0.001. Comparison to AMT 0 mg/kg/day group, ^{***}P<0.001; ^{**}P<0.01; ^{*}P<0.05.

Table 3. Relative kidney weight following repeated intravenous administrations of AMT on Day 28 in CDDP-treated rats

Group	AMT	Relative kidney weight (g/100 g B.W.)		
		Left	Right	Total
Control	-	0.349 ± 0.005	0.344 ± 0.006	0.692 ± 0.008
CDDP-treatment	0 mg/kg/day	0.928 ± 0.177	0.913 ± 0.166	1.841 ± 0.342 ^{###}
	10 mg/kg/day	0.492 ± 0.052	0.496 ± 0.057	0.988 ± 0.109 ^{**}
	30 mg/kg/day	0.345 ± 0.004	0.345 ± 0.004	0.69 ± 0.008 ^{***}

These data were represented as means ± SEM (n=6). Comparison to control group, ^{###}P<0.001. Comparison to AMT 0 mg/kg/day group, ^{***}P<0.001; ^{**}P<0.01.

Table 4. Histopathological findings of kidneys following repeated intravenous administrations of AMT on Day 28 in CDDP-treated rats

Findings	Test article: Dose (mg/kg/day): No. of animals:	Saline				AMT			
		-	0	10	30	-	0	10	30
Not remarkable		6	6	6	6	0	0	0	0
Hyaline cast	+	0	0	6	5	0	0	0	0
	-/+	0	0	0	0	0	0	0	3
	None	6	6	0	1	0	0	0	3
Tubular dilatation, cortico-medullary junction	+++	0	0	3	0	0	0	0	0
	++	0	0	3	4	0	0	0	0
	+	0	0	0	0	0	0	0	1
	-/+	0	0	0	0	0	0	2	0
	None	6	6	0	0	0	0	0	5
Interstitial fibrosis, outer medulla	++	0	0	6	2	0	0	0	0
	+	0	0	0	3	0	0	0	1
	-/+	0	0	0	0	0	0	1	5
	None	6	6	0	0	0	0	0	0
Interstitial fibrosis, cortex	+	0	0	6	3	0	0	0	0
	-/+	0	0	0	0	0	0	3	2
	None	6	6	0	0	0	0	0	4

Grade: -/+, very slight; +, slight; ++, moderate; +++, marked.

Immunohistochemical staining and quantification

An immunohistochemical examination of α -SMA and collagen III was conducted to quantify the development and formation of fibrosis in the kidneys after the CDDP treatment. Alpha-SMA-positive cells were observed, especially around dilated tubules in the cortico-medullary junctions, and the number of which was markedly higher in the AMT 0 mg/kg/day group than in the

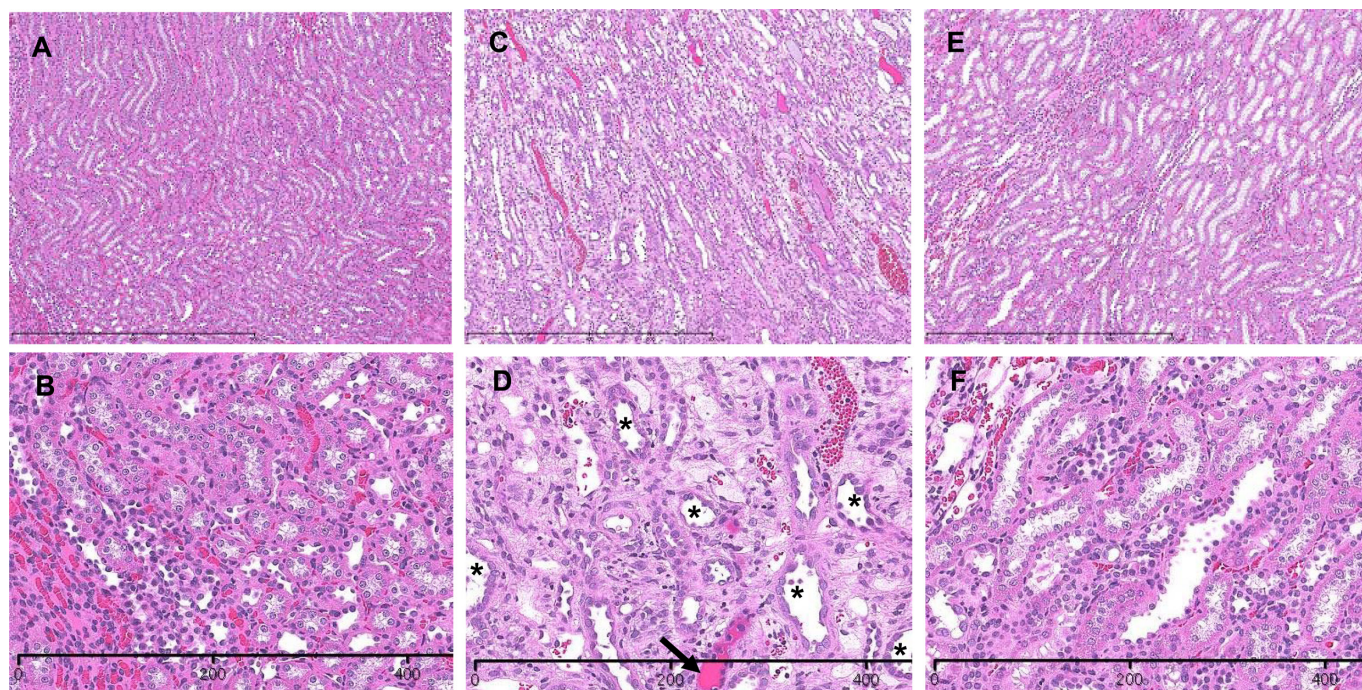


Fig. 1. Histopathology of kidneys at 28 days after the administration of saline or CDDP in saline-treated (AMT 0 mg/kg/day) rats (A and B), CDDP-treated rats without the AMT treatment (C and D) and CDDP-treated rats with the AMT treatment (30 mg/kg/day) (E and F). In the cortex and outer medulla of the kidneys in CDDP-treated rats, tubular dilation (*) and hyaline casts (arrow) in tubules were observed, and spindle-shaped fibroblastic cells and mononuclear cells were prominent in the interstitium around the renal tubules, indicating more progressive fibrosis from that in control rats. These histopathological changes were alleviated in AMT-treated rats. HE staining. Each bar shows 800 μ m. AMT, 3-acetyl-5-methyltetronic acid; CDDP, cisplatin.

control group (Fig. 2). These fibrotic areas in the cortex and outer medulla significantly decreased in a dose-dependent manner with the AMT treatments. In control kidneys, the expression of collagen III was highly localized in vascular smooth muscle cells and perivascular fibrous tissues; however, similar to α -SMA, collagen III also markedly increased in the interstitial area around the affected tubules in the AMT 0 mg/kg/day group (Fig. 3). The AMT treatments caused the dose-dependent ameliorations in extracellular matrix accumulation, and the intravenous administration of AMT (30 mg/kg/day) for 28 days led to the same levels of collagen III being expressed, especially in the outer medulla of the kidneys, as these in the control group.

DISCUSSION

In the present study, we demonstrated that the VKOR inhibitor, AMT, prevented renal tubulointerstitial fibrosis induced by CDDP. We previously reported that AMT exhibited inhibitory effect on rat kidney VKOR *in vitro* and also suppressed mesangial cell proliferation and, consequently, the formation of fibrosis via the vitamin K-dependent activation of the Gas6/Axl pathway in anti-Thy-1 glomerulonephritis (Thy-1 GN) in rats [19]. Gas6 structurally belongs to the family of plasma vitamin K-dependent proteins and is secreted by a wide variety of cell types including mesangial cells and epithelial cell [23]. Furthermore, its receptor tyrosine kinase Axl transduces signals from the extracellular matrix into the cytoplasm by binding the growth factor Gas6. The Gas6/Axl signaling pathway plays a key role in many physiological processes including cell proliferation, migration and differentiation as well as cell survival during development and tissue repair [8, 20, 24]. In Thy-1 GN, the expression of Gas6 and Axl was shown to be markedly increased in the kidneys, with the progression of mesangial cell proliferation and extracellular matrix production consequently being observed [22]. In clinical studies, Gas6 and Axl levels were found to increase in various kidney diseases. Fiebeler *et al.* assessed the expression of Gas6 and Axl in 26 human renal specimens from patients with various types of kidney diseases and normal renal tissues and showed that Gas6 and Axl were upregulated in the renal tubules, brush border, and glomeruli of these patients' kidneys [5]. Lee *et al.* showed that plasma Gas 6 levels were significantly higher in patients with chronic kidney disease than in normal controls, which suggested that the abnormal upregulation of circulating Gas6 was associated with renal disease and inversely proportional to renal function [11]. Therefore, Gas6/Axl is a crucial modulator of many types of physiological processes and is closely related to renal injury.

In the rat model of CDDP-induced fibrosis, renal function was shown to deteriorate from 3 to 7 days after the administration of CDDP, and a marked increase in the plasma levels of UN and creatinine, which are parameters of renal injury, has been observed in the acute stage [9]. In the present study, plasma UN and creatinine levels both increased 5 days after the CDDP

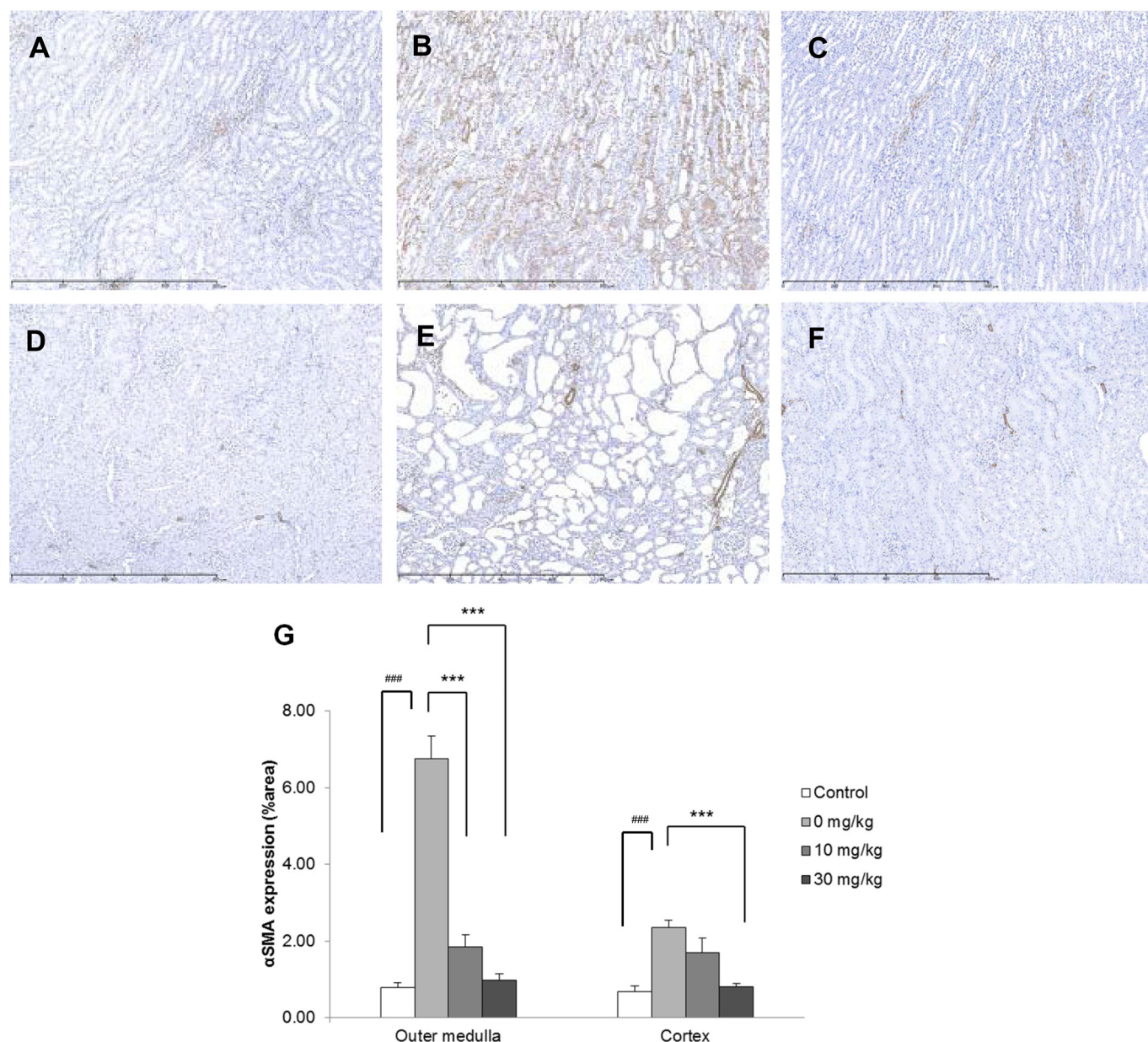


Fig. 2. Immunohistochemistry of α -SMA in the outer medulla (A–C) and cortex (D–F) of kidneys 28 days after the administration of CDDP in saline-treated control rats (A and D), CDDP-treated rats with the saline treatment (AMT 0 mg/kg/day) (B and E) and CDDP-treated rats with the AMT treatment (30 mg/kg/day) (C and F). Alpha-SMA-positive cells were observed around dilated tubules in the cortico-medullary junctions. The expression areas of α -SMA-positive cells in the outer medulla and cortex were quantified by an imaging analysis (G). Alpha-SMA-positive myofibroblastoma in the cortex and outer medulla significantly decreased in a dose-dependent manner with the AMT treatments. Each bar shows 800 μ m. These data were represented as means \pm SEM (n=6). Comparison to control group, ### P <0.001. Comparison to AMT 0 mg/kg/day group, *** P <0.001. AMT, 3-acetyl-5-methyltetronic acid; CDDP, cisplatin; SMA, smooth muscle actin.

treatment. Moreover, urinary albumin levels and ACR also increased, while Ccr was markedly decreased on Day 5. CDDP, when administered, is taken up by renal tubular cells, especially the proximal tubular cells of the inner cortex and outer medulla [2]. The mechanisms underlying CDDP-induced nephrotoxicity are complex and involve multiple pathways; peroxidation of the cell membrane, mitochondrial dysfunction, the inhibition of protein synthesis and DNA injury [12, 16]. As a consequence of these processes, the administered CDDP produces increases in necrosis and apoptosis, and consequently, regeneration in the kidney [12]. Cytokines and chemokines released from renal epithelial and inflammatory cells simultaneously induce the migration of inflammatory cells [4, 15]. In CDDP-treated rats, inflammatory cell infiltration has been observed in renal tubules from the acute stage, a few days after the administration of CDDP [13]. Gas6 is known to promote inflammation by enhancing interactions between endothelial cells, platelets, and leukocytes and also enhances inflammatory cell infiltration into inflamed tissues [18]. In the acute stage, 5 days after the CDDP treatment, blood chemistry and urinalysis tests revealed that the AMT treatments (10 and

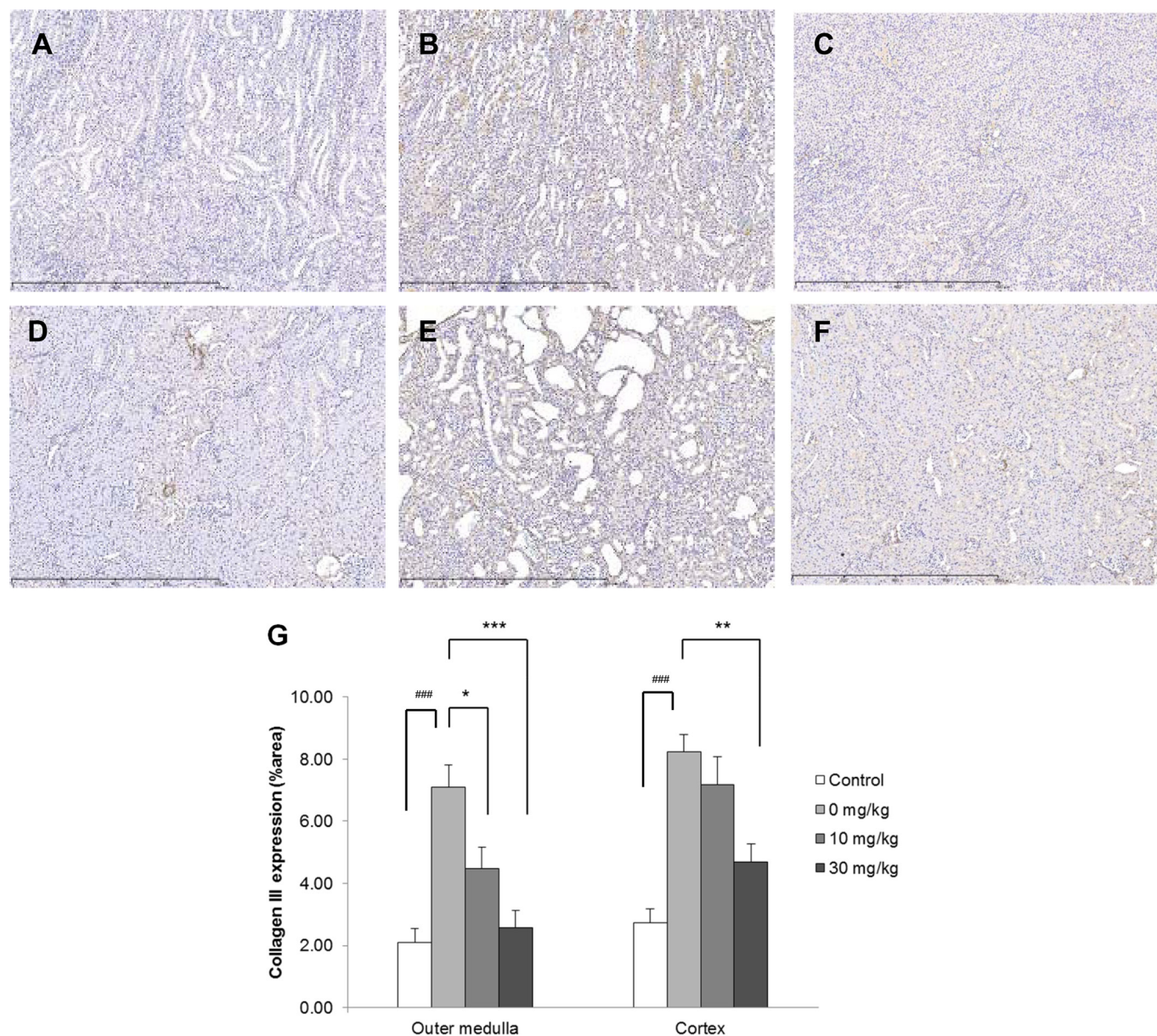


Fig. 3. Immunohistochemistry of collagen III in the outer medulla (A–C) and cortex (D–F) of kidneys 28 days after the administration of CDDP in saline-treated control rats (A and D), CDDP-treated rats with the saline treatment (AMT 0 mg/kg/day) (B and E) and CDDP-treated rats with the AMT treatment (30 mg/kg/day) (C and F). The expression areas of the extracellular matrix in the outer medulla and cortex were quantified by an imaging analysis (G). The AMT treatments caused the dose-dependent ameliorations in extracellular matrix accumulation. The AMT (30 mg/kg/day) treatment for 28 days led to the same levels of collagen III being expressed, especially in the outer medulla of the kidneys, as those in the control group. These data were represented as means \pm SEM (n=6). Comparison to control group, ### P <0.001. Comparison to AMT 0 mg/kg/day group, * P <0.05; ** P <0.01; *** P <0.001. AMT, 3-acetyl-5-methyltetronic acid; CDDP, cisplatin.

30 mg/kg/day) significantly suppressed renal injury in a dose-dependent manner. In our previous study, in which AMT was shown to inhibit mesangial cell proliferation and prevent the progression of renal injury in Thy-1 GN rats, we reported that AMT had inhibitory effects on VKOR and reached a sufficient concentration in the kidneys to inhibit the Gas6/Axl pathway via VKOR [19]. Unfortunately, except for the inhibitory effects of VKOR and tissue distribution of AMT, we do not have any other pharmacological information on this compound; no data are available on the other physiological effects of this compound, such as direct anti-inflammatory and renal antihypertensive effects observed with COX and ARB/ACE inhibitors, respectively. However, AMT did not exhibit any radical scavenging effects (data not shown). Due to its VKOR inhibitory activity and tissue distribution, we speculated that the amelioration of renal injury and renal function at the acute stage in CDDP-treated rats was based on the inhibition of the Gas6/Axl pathway via the VKOR inhibitory effects of AMT. In addition, we have reported anti-coagulation effect caused by VKOR inhibition of AMT in rats [19], and we observed no anti-coagulation effects of clinical signs and gross pathological findings in the

rat treated with the same dose levels used in the present study.

Tubulointerstitial fibrosis induced by CDDP is progressive in the chronic stage, with the fibrotic area increasing, especially 14 days after the administration of CDDP when acute renal disease has recovered [9]. In the present study, we quantified tubulointerstitial fibrosis by an immunohistochemical examination with α -SMA and collagen III, and a large increase in the fibrotic area was observed 28 days after the CDDP treatment. Moreover, in CDDP-treated rats, fibrotic areas were significantly smaller in the AMT-treatment groups than in the vehicle-treated group in a dose-dependent manner. Alpha-SMA is a marker of myofibroblasts, which differentiate from interstitial cells and mesenchymal cells in the kidneys, and myofibroblasts produce several components of the extracellular matrix, such as collagen [14]. Gas6 and its receptor Axl are expressed in macrophages, progenitor cells and myofibroblasts, and a Gas6 deficiency has been shown to reduce inflammation and the activation of myofibroblasts. Fourcot *et al.* reported that liver fibrosis in Gas6 ($-/-$) mice was linked to the inhibition of the inflammatory response that controls lipid metabolism and myofibroblast activation [6]. We speculated that the inhibition of the Gas6/Axl signaling pathway by the AMT treatment regulated inflammatory cells, and suppressed their infiltration and consequent fibrosis in affected kidneys in CDDP-treated rats.

In the present study, we showed that AMT had pharmacological effects on CDDP-induced nephrotoxicity in the acute and chronic stages. In acute stage, 5 days after the administration of CDDP, renal function parameters, such as creatinine clearance and BUN were lower in the AMT 10 mg/kg/day group than in the AMT 0 mg/kg/day group; however, urinary albumin levels were higher and similar between the AMT 10 mg/kg/day and AMT 0 mg/kg/day groups in CDDP-treated rats. A previous study reported that urinary albumin was increased not only due to renal dysfunction caused by CDDP, but also the direct inhibition of vacuolar H⁺-ATPase caused by CDDP and a decrease in receptor-mediated endocytosis in the renal proximal tubules [17]. Therefore, after the administration of CDDP, the AMT 10 mg/kg/day treatment inhibited acute renal injury and ameliorated some renal function parameters based on the inhibition of the Gas6/Axl pathway via the inhibitory effects of VKOR, which may reduce the infiltration of inflammatory cells and cell proliferation, but did not inhibit the direct effects of CDDP on vacuolar H⁺-ATPase in the kidneys. In the chronic stage, AMT was also shown to reduce the activation of myofibroblasts and renal fibrosis in the kidneys of CDDP-treated rats. On day 28 after the administration of CDDP, creatinine clearance in the AMT 30 mg/kg/day group was significantly lower than that in the AMT 0 mg/kg/day group in CDDP-treated rats, even though it was not significantly lower on day 5. Therefore, the activation of the Gas6/Axl pathway was also involved in the chronic stage in CDDP-treated rats, and the inhibition of this pathway by AMT had antifibrotic effects. These pharmacological effects in CDDP-treated rats were attributed to inhibitory effects on the Gas6/Axl signaling pathway due to the inhibitory activity of VKOR and tissue distribution of AMT. However, the significance of the Gas6/Axl pathway in CDDP-induced renal injury remains unclear, and the mechanisms underlying the effects of AMT have not yet been elucidated. The pharmacological amelioration caused by AMT in renal toxicity and fibrosis currently remains unclear; however, it still warrants investigation due to its strong therapeutic effects in the experimental animal model in the present study. Further studies are needed in order to elucidate the therapeutic mechanisms responsible.

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