Cis-dichlorodiammineplatinum Upregulates Angiotensin II Type 1 Receptors through Reactive Oxygen Species Generation and Enhances VEGF Production in Bladder Cancer

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Abstract

We previously reported that angiotensin II type 1 receptor (AT1R) antagonists enhanced the cytotoxicity of cis-dichlorodiammineplatinum (CDDP) in a bladder cancer xenograft model. To elucidate the synergistic mechanism, we investigated whether reactive oxygen species (ROS) generation induced by CDDP may affect the regulation of AT1R expression. Five invasive human bladder cancer cell lines, T24, UMUC-3, 5637, KU-1, and KU-19-19, were used in the in vitro study. For the in vivo study, T24 cells were used. We also examined AT1R and vascular endothelial growth factor (VEGF) expression in human bladder cancer specimens that had been treated with CDDP-based chemotherapy. The in vitro study showed that AT1R expression was significantly upregulated by CDDP in T24, KU-1, and KU-19-19 cells. On the other hand, AT1R expression was not changed in UMUC-3 and 5637 cells. ROS generation was also significantly upregulated by CDDP in T24, KU-1, and KU-19-19 cells. The upregulation of AT1R expression induced by CDDP was significantly suppressed by scavenging free radicals. Angiotensin II induced VEGF production in CDDP-treated cells; however, the AT1R antagonist significantly inhibited the increase in VEGF. The in vivo study results also showed that CDDP treatment upregulated AT1R expression, resulting in increased VEGF. Clinical specimens from patients who underwent cystectomy after neoadjuvant CDDP-based chemotherapy showed significantly higher AT1R and VEGF expression than corresponding transurethral resection specimens. Our findings indicate that CDDP upregulates AT1R expression through ROS generation and enhances VEGF production. Therefore, AT1R blockade may be an effective strategy for bladder cancer in combination with CDDP-based chemotherapy.

Introduction

Angiotensin II (Ang II) is a key biological peptide in the renin-angiotensin system that regulates blood pressure and renal hemodynamics. There are two major subtypes of Ang II receptor: Ang II type 1 receptor (AT1R) and type 2 receptor (AT2R). AT1R antagonists (ARB) are widely used as antihypertensive drugs and do not have severe side effects (1). Concern regarding the potential role of Ang II in angiogenesis and promotion of tumor growth has been growing in the past few decades (2–6), and Lever et al. (7) reported the first clinical evidence that long-term Ang II blockade might have a protective effect against carcinogenesis.

We previously showed that Ang II-AT1R signaling led to potent induction of vascular endothelial growth factor (VEGF) in murine renal cancer cells, bladder cancer cells, and prostate cancer cells (8–10). However, previous studies have indicated that ARB alone did not completely suppress tumor growth at doses greater than achievable doses. To determine the clinical potency of ARB, we examined the efficacy of AT1R antagonists in combination therapy with chemotherapeutic agents (11, 12) and found that combination therapy with cis-dichlorodiammineplatinum (CDDP) and ARB at clinically achievable doses enhanced CDDP-induced cytotoxicity in a mouse xenograft model (12). However, the detailed molecular mechanisms of the synergistic actions in this combination therapy have not yet been fully elucidated.

CDDP is commonly considered to be the most effective DNA-damaging agent for the treatment of advanced bladder cancer. CDDP binds to DNA to form covalent platinum DNA adducts (13), acts as a DNA alkylation, and is a potent generator of reactive oxygen species (ROS). Through these mechanisms, CDDP induces multiple cellular responses, including inhibition of DNA repair and transcription, cell cycle arrest, and apoptosis (14).
For bladder cancer, in previous studies, we found that ROS generation induced by CDDP particularly affected cell viability (15, 16). In the present study, to elucidate the mechanism of the synergistic actions of combination therapy consisting of CDDP and ARB, we focused on the association between the ability of CDDP to act as a strong inducer of ROS generation and AT1R expression in cancer cells.

In vascular smooth muscle cells, the regulation of AT1R expression has been investigated, and various kinds of factors such as cytokines, growth factors, nitric oxide, oxide low density lipoprotein (17, 18), and ROS are recognized as significant inducers of AT1R expression (19, 20). Although upregulation of AT1R expression leads to an elevated functional response of Ang II stimulation, regulation of AT1R expression in cancer cells had not yet been fully elucidated. To the best of our knowledge, there has been no finding suggesting an association between AT1R expression and anticancer agents, although anticancer agents can induce a significant amount of ROS generation (21).

In the present study, we examined the regulation of AT1R expression and regulation of VEGF production during treatment with CDDP-based chemotherapy in advanced bladder cancer. Next, focusing on ROS generation, we investigated the association between ROS generation and AT1R expression in cancer cells, and whether CDDP, as a strong inducer of ROS generation, could modulate AT1R expression through the increased level of ROS generation. Therefore, the present study was undertaken to investigate the detailed mechanisms of the synergistic actions of combined CDDP and ARB therapy in advanced bladder cancer.

Materials and Methods

Cell lines and agents
Five invasive human bladder cancer cell lines, T24, UMUC-3, S637, KU-1, and KU-19-19, were used. KU-1 and KU-19-19 cells, which are aggressive human bladder cancer cell lines, were established in our laboratory (22, 23), whereas T24, UMUC-3, and S637 were obtained from the American Type Culture Collection. All cells were routinely maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Dainippon Pharmaceutical) at 37°C in a humidified 5% CO2 atmosphere. CDDP was kindly supplied by Nippon Kayaku Co., and the ARB olmesartan and olmesartan medoxiomil was provided by Daiichi Sankyo. Edaravone (a free radical scavenger) was obtained from Wako Pure Chemical Industries. Mouse monoclonal antibody for β-actin was purchased from Sigma. Rabbit polyclonal antibody for VEGF and AT1R were purchased from Santa Cruz Biotechnology, Inc.

Clinical samples and clinicopathologic features
Archival paraffin-embedded sections were obtained from 20 patients who underwent transurethral resection of a bladder tumor (TUR-BT) and then total cystectomy for advanced bladder cancer at our institution between 2003 and 2009. A total of eight patients received CDDP-based chemotherapy as neoadjuvant chemotherapy before cystectomy. The clinicopathologic features of the patients are listed in Table 1.

Murine xenograft bladder cancer model
Six-week-old athymic nude BALB/C mice with an average body weight of 20 g were obtained from Sankyo Lab Service. T24 cells (2 × 10⁶ cells), suspended in 100 μL of Matrigel (Becton Dickinson Labware), were implanted s.c. into the flank of each mouse. The animals were carefully monitored, and the tumor was measured twice a week. Tumor volume was calculated using the following formula: tumor volume (mm³) = length × width × height × 0.52. To investigate the regulation of AT1R and VEGF expression in the tumor tissue, the mice were assigned to one of two groups: control or CDDP only, each consisting of five animals. After the tumors had reached a volume of ~100 mm³, CDDP (10 mg/kg) was administered i.p. The animals were killed 24 hours later, and the subcutaneous tumors were harvested to investigate the regulation of AT1R and VEGF expression in tumor tissue.

To examine the efficacy of ARB only in vivo, the animals were assigned to one of three groups: control and ARB olmesartan medoxiomil (1 or 10 mg/kg/d) only, each consisting of 10 mice. On day 14 after cancer cell implantation, the mice were administered olmesartan medoxiomil (1 or 10 mg/kg/d) by gavage. On the 28th day, the animals were killed and the subcutaneous tumors were harvested. Next, to evaluate the efficacy of the combination therapy, the animals were assigned to one of four groups: control, CDDP only, or combined CDDP and olmesartan medoxiomil (1 or 10 mg/kg/d), each consisting of 10 mice. On day 14 after cancer cell implantation, the mice were administered olmesartan medoxiomil (1 or 10 mg/kg/d) by gavage. CDDP was administered i.p. on the 21st day. On the 28th day, the animals were killed and the subcutaneous tumors were harvested. These experiments were carried out according to Japanese government guidelines, and the protocol was approved by the Animal Care Committee of Keio University.

Cell growth assay
All cell lines were seeded at a density of 1 × 10⁴ per well into 96-well culture plates. Following 24- or 48-hour incubation in RPMI 1640 with 10% fetal bovine serum, the cells were incubated for the appropriate time with various concentrations of CDDP. To examine the Ang II-induced effects on cell growth, the cell lines were incubated with various concentrations of Ang II and olmesartan. Cell viability was determined using a Premix WST-1 Cell Proliferation Assay System (Takara Bio, Inc.) to examine the cytotoxic effect of each agent. The absorbance value of each well was determined in a microplate spectrophotometer (Bio-Rad Laboratories, Inc.).

Measurement of intracellular level of ROS
Dichlorodihydrofluorescein diacetate, which permeates into cells and interacts with intracellular ROS to generate
fluorescent dichlorofluorescein (DCF), was used to measure the intracellular levels of ROS generated by CDDP. The amount of ROS was estimated from DCF production (Cell Biolabs, Inc.). Fluorescence intensity was determined in the cells at 480-nm excitation/530-nm emission by using a fluorometric plate reader. Cells were incubated at a density of 1 × 10⁴ per well in 96-well culture plates for 24 hours. After this preincubation period, the medium was discarded and the attached cells were washed with PBS, and then exposed to DCFH-DA solution at 37°C. Next, the cells were exposed to 10 μmol/L CDDP for 120 minutes and then DCF production was measured.

**Cell extracts and Western blot analysis**

Whole-cell extracts were obtained using radioimmunoprecipitation assay buffer (Cell Signaling Technology Japan) containing protease inhibitors, according to the manufacturer’s protocol. The extracted whole protein (50 μg) with sample buffer containing 2-mercaptoethanol was separated on 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories) and then incubated with 5% skim milk overnight. The primary antibodies for VEGF and AT1R at a dilution of 1:200 were reacted. Membranes were then incubated with appropriate secondary antibodies. The signals were detected by enhanced chemiluminescence reagents (ECL plus Western Blotting Detection System; Amersham Pharmacia Biotech) and analyzed. The intensity was quantified using an LAS 3000 system (Fuji Film).

**Real-time quantitative PCR**

The cells were lysed with RNAiso reagent (Takara Bio, Inc.), and then the RNA was quantified by the ratio of absorbance at 260/280 nm. First-strand cDNA was synthesized from RNA (1 μg) by using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a 20 μL reaction. The reaction mixture (1 μL) was then used as a template in a TaqMan Fast real-time quantitative PCR assay using a StepOne real-time PCR System (Applied Biosystems). The primers and TaqMan probe sets (TaqMan Gene Expression Assays, Inventoried) for AT1R were as follows: forward, 5′-CTTCGACGCA-CAATG-CTTGT-3′; reverse, 5′-CTGCCAGCAGCCA-AATGA-3′. The human β-actin endogenous control (Hs99999903_m1) was purchased from Applied Biosystems (sequences not disclosed). The AT1R to β-actin mRNA ratios were calculated for each sample to evaluate the relative mRNA expression.

**Immunostaining for AT1R and VEGF**

Formalin-fixed paraffin-embedded sections (4 μm) were deparaffinized, rehydrated, and washed in PBS.

| Table 1. Changes in expression of AT1R and VEGF induced by CDDP-based chemotherapy in human bladder cancer specimens |
|---|---|---|---|---|---|---|---|
| **Case no.** | **Sex** | **Age** | **Histology of cystectomy** | **AT1R of TUR-BT** | **AT1R of cystectomy** | **VEGF of TUR-BT** | **VEGF of cystectomy** |
| 1 | Male | 56 | UC G3 pT2a | − | − | + | + |
| 2 | Male | 59 | UC G3 pT3a | + | ++ | + | + |
| 3 | Male | 53 | UC G3 pT3a | − | +++ | − | + |
| 4 | Male | 64 | UC>AC G3 pT3a | + | + | + | + |
| 5 | Male | 61 | UC>AC G3 pT3a | ++ | ++ | + | + |
| 6 | Male | 56 | UC G3 pT3a | − | − | ++ | +++ |
| 7 | Female | 76 | UC G3 pT3a | + | +++ | + | +++ |
| 8 | Male | 78 | UC G3 pT2b | + | ++ | + | ++ |
| Patients without CDDP-based neoadjuvant chemotherapy |
| 1 | Male | 61 | UC G3 pT2a | + | + | ++ | ++ |
| 2 | Male | 69 | UC G3 pT4 | +++ | +++ | + | + |
| 3 | Male | 73 | UC G3 pT2b | ++ | ++ | + | + |
| 4 | Male | 58 | UC G2 pT2b | − | − | + | + |
| 5 | Male | 73 | UC>SCC G2 pT1 | − | − | ++ | ++ |
| 6 | Male | 70 | UC>SCC G3 pT4 | + | + | − | + |
| 7 | Female | 75 | UC G3 pT3a | ++ | + | − | − |
| 8 | Female | 74 | UC G2 pT2a | ++ | + | + | + |
| 9 | Female | 72 | UC>AC G3 pT2b | − | − | ++ | ++ |
| 10 | Male | 77 | UC>SCC G3 pT3a | − | − | ++ | ++ |
| 11 | Male | 69 | UC G3 pT3a | ++ | + | + | + |
| 12 | Male | 56 | UC G3=2 pT3a | ++ | + | + | + |
| Abbreviations: UC, urothelial carcinoma; AC, adenocarcinoma; SCC, squamous cell carcinoma; G, grade. |
Endogenous peroxidase was quenched, and sections were blocked with skim milk. Primary antibodies of VEGF and AT1R were then applied at room temperature for 1 hour. After washing with PBS, they were incubated with secondary antibodies against rabbit IgG conjugated to a peroxidase-labeled dextran polymer for 1 hour. After washing the slides, they were counterstained with 10% hematoxylin for 1 to 2 minutes.

The intensity of AT1R and VEGF staining tumor lesions was stratified from 0 to 3 (0, no staining; 1, slight staining; 2, medium staining; 3, strong staining), and the immunopositivity of staining cancer cells was

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**Figure 1.** AT1R expression in various cell lines, and regulation of AT1R expression induced by CDDP. *, P < 0.05, compared with control. Each value represents the mean ± SEM of at least three individual experiments. A, AT1R expression in five bladder cancer cell lines. B, time response to CDDP of UMUC-3, 5637, KU-1, and KU-19-19 cells with respect to AT1R expression. C, dose and time response to CDDP of T24 cells with respect to AT1R expression. Ctrl, control.
assessed in at least 10 representative areas by two independent investigators operating in a blinded fashion with ×400 magnification. Staining was scored according to the method of Kosugi et al. (9).

Enzyme-linked immunosorbent assay for VEGF in conditioned medium

Cells were seeded in 60-mm² dishes and allowed to attach at 37°C in a humidified 5% CO₂ atmosphere. After 24 hours, the medium was replaced with 10% fetal bovine serum medium with or without 10 μmol/L CDDP for 12 hours and then with serum-free medium for 4 hours. Next, the cells were incubated with conditioned medium (10⁻⁸ mol/L Ang II, 10⁻⁸ mol/L Ang II and 10⁻⁷ mol/L olmesartan, or 10⁻⁷ mol/L olmesartan). After 12 hours, the supernatant was collected and VEGF was measured using a commercially available enzyme-linked immunosorbent assay kit (Quantikine, R&D Systems). The protein level for VEGF was quantified by comparing its optical density to the standard curve for VEGF and normalizing it for the cell number.

Statistical analysis

All data are presented as the mean ± SEM. Statistical analyses were done using the Mann-Whitney U test.
P values <0.05 were accepted as statistically significant. All statistical analyses were done using commercially available statistical software.

**Results**

**AT1R expression in five bladder cancer cell lines and upregulation of AT1R through CDDP exposure**

AT1R expression in the five bladder cancer cell lines, UMUC-3, 5637, T24, KU-1, and KU-19-19, was evaluated using Western blot analysis (Fig. 1A). Each cell line expressed AT1R, and AT1R expression by T24 and KU-1 cells was particularly strong compared with the other three cell lines. Next, the effects of CDDP on the expression of AT1R were evaluated using Western blot analysis. CDDP (10 μmol/L) upregulated AT1R expression in KU-1 and KU-19-19 cells (Fig. 1B). AT1R expression in T24 cells was upregulated most significantly and in a time- and dose-dependent manner (Fig. 1C). CDDP did not show any significant regulation of AT1R expression in UMUC-3 and 5637 cells.

**Antiproliferative effects of CDDP and upregulation of ROS generation in five bladder cancer cells treated with CDDP**

The antiproliferative effect of CDDP in the five bladder cancer cell lines was evaluated. WST-1 assay at 24 hours after CDDP exposure showed that the cell viability of T24, KU-1, and KU-19-19 cells was strongly affected by CDDP compared with that of UMUC-3 and 5637 cells (Fig. 2A). To investigate the association between the sensitivity of CDDP and ROS generation induced by CDDP, we measured DCF production after CDDP exposure in each cell line. CDDP (10 μmol/L) significantly upregulated ROS generation in T24, KU-1, and KU-19-19 cells; however, in UMUC-3 and 5637 cells, it did not show any significant effect on ROS generation at 120 minutes (Fig. 2B). These results were correlated with the regulation of AT1R expression induced by CDDP in each cell line.
Edaravone, a free radical scavenger, inhibited upregulation of AT1R induced by CDDP exposure

To examine whether ROS generation induced by CDDP could affect AT1R expression in cancer cells, we used edaravone, a free radical scavenger, to exclude the effect of ROS generation induced by CDDP on AT1R expression. First, to evaluate the edaravone-induced effects on cell growth, T24 cells were incubated with various concentrations of edaravone. Edaravone showed no effects on cell proliferation for 12 hours. Similar results were also observed for 24 hours.

Next, T24 cells were treated with or without 10 μmol/L CDDP and/or 10^{-3} mol/L edaravone for 6 and 12 hours. AT1R expression was evaluated at each time point. Real-time quantitative PCR showed that CDDP significantly induced an increase in AT1R mRNA compared
with CDDP-untreated cells at 12 hours, whereas edaravone significantly reduced CDDP-induced AT1R upregulation (Fig. 3A). Similarly, Western blotting showed that edaravone reduced CDDP-induced AT1R upregulation at 12 hours, although edaravone did not affect any AT1R expression without CDDP exposure (Fig. 3B).

Effects of AT1R blockade by olmesartan on CDDP-treated cells in vitro

To examine the Ang II–inducing effects on cell growth, T24 cells were incubated with various concentrations of CDDP with or without Ang II and olmesartan for 24 hours (Fig. 3C). In vitro proliferation assay at clinically achievable concentrations of Ang II (10⁻⁸ to 10⁻⁷ mol/L) and olmesartan (10⁻⁷ to 10⁻⁶ mol/L) showed no effects on cell proliferation, although neither Ang II nor olmesartan affected the viability of CDDP-treated cells at any of the concentrations tested. Similar results were observed for 48 hours.

CDDP enhanced VEGF production through upregulation of AT1R expression

We assessed VEGF production in T24 cells treated with CDDP to examine whether AT1R signaling is related to VEGF increases during CDDP exposure. To determine the effect of Ang II on VEGF production, T24 cells treated with CDDP were exposed to Ang II (10⁻⁸ mol/L) for 12 hours under normal levels of oxygen with or without olmesartan (10⁻⁷ mol/L). VEGF in the supernatant was assessed by enzyme-linked immunosorbent assay at each time point. Ang II strongly induced VEGF production (8.7 ± 0.7 pg/10⁵ cells) compared with Ang II–untreated cells (6.3 ± 0.5 pg/10⁵ cells), whereas olmesartan significantly inhibited Ang II–induced VEGF production (6.2 ± 0.8 pg/10⁵ cells; Fig. 3D).

CDDP upregulated AT1R expression and enhanced VEGF production in a murine xenograft bladder cancer model

We examined whether Ang II induced AT1R and VEGF expression in a mouse T24 xenograft model (Fig. 4A–D). Although both the control group and CDDP-treated group expressed AT1R, the latter exhibited significantly higher AT1R and VEGF expression than the control group. When AT1R expression and VEGF expression in tumor tissue were quantified by Western blotting, similar results were obtained (Fig. 4E).

Effects of combination therapy with CDDP and AT1R antagonist on tumor growth in a murine xenograft bladder cancer model

First, we examined the efficacy of ARB monotherapy in the T24 xenograft model. As shown in Fig. 4F, the ARB olmesartan medoxiomil (1 or 10 mg/kg) was administered daily by gavage from day 14 after cancer cell implantation, and the tumor growth was more strongly suppressed at the higher dose of olmesartan medoxiomil.

Next, we investigated the efficacy of combination therapy with CDDP and ARB in vivo. As shown in Fig. 4G, the combination with CDDP and olmesartan medoxiomil (1 mg/kg) administered daily by gavage significantly suppressed tumor growth in the murine xenograft bladder cancer model. The tumor volume was 64.5% in the CDDP and olmesartan medoxiomil (1 mg/kg) combination group compared with the CDDP-only group on day 28 after cancer cell implantation, whereas tumor growth was more strongly suppressed at the higher dose of olmesartan medoxiomil (10 mg/kg).

Changes in expression of AT1R and VEGF after CDDP-based chemotherapy in human bladder cancer specimens

To determine the effects of CDDP on AT1R and VEGF expression in bladder cancer, we examined TUR specimens and surgical specimens from patients with and without neoadjuvant CDDP-based regimens before cystectomy. The results in 20 patients are presented in Table 1. Higher AT1R expression in residual cancer cells after CDDP-based chemotherapy compared with before chemotherapy in corresponding TUR specimens was observed in four of eight patients. Of the four patients who exhibited higher AT1R expression, three showed higher VEGF expression in residual cancer cells (Fig. 5A–D). On the other hand, although AT1R expression in TUR specimens was observed in 8 of 12 patients, no significant change in AT1R expression was observed in the

![Figure 5](https://example.com/figure5.png)

Figure 5. Serial photo panels showing a typical human bladder cancer specimen before (A, C) and after treatment with CDDP-based chemotherapy (B, D). Immunohistochemical staining for AT1R expression (A, B) and VEGF production (C, D). Magnification, ×400. Scale bars, 50 μm.
corresponding cystectomy specimens without CDDP-based chemotherapy. Similar results were observed for VEGF expression.

Discussion

In the present study, focusing on ROS generation in bladder cancer cells, we analyzed two different molecular events that followed CDDP treatment. First, VEGF production was enhanced through the upregulation of AT1R expression induced by CDDP exposure. Second, using a free radical scavenger, its upregulation of AT1R expression was significantly suppressed. These results indicated that ROS generation in cancer cells affects AT1R expression, and CDDP, being a strong inducer of ROS generation, enhances Ang II-dependent VEGF production by increasing AT1R expression. Based on these molecular mechanisms, we also showed the efficacy of combination therapy with CDDP and ARB at clinically achievable doses in a murine xenograft bladder cancer model. To the best of our knowledge, this is the first report that shows the detailed mechanism of the regulation of AT1R expression after CDDP-based chemotherapy, resulting in increased VEGF.

Bladder cancer is one of the most aggressive epithelial tumors and is characterized by a high rate of early systemic dissemination. Patients with metastatic bladder cancer are routinely treated with CDDP-based systemic chemotherapy, such as the M-VAC (methotrexate, vinblastine, doxorubicin, cisplatin) and/or GC (gemcitabine, cisplatin) regimens, particularly in the setting of unresectable, diffusely metastatic, measurable disease. Recently, although CDDP-based neoadjuvant chemotherapy has been recognized to improve the prognosis of patients who undergo cystectomy to treat locally advanced bladder cancer (24, 25), the indication for CDDP-based chemotherapy has been increasing compared with in the past. Although the response rates remain inadequate, and despite continuous efforts to identify and develop more effective substitutions, the results thus far have not been satisfactory. CDDP-based regimens still constitute the gold standard for the treatment of advanced bladder cancer. Therefore, potential synergistic combination therapies with CDDP should be important lines of further research.

Ang II is associated with angiogenesis. We previously reported a potential role of the antiangiogenic effect of ARB against murine renal cancer, human bladder cancer, and prostate cancer through their suppression of VEGF production (8–10). To explain the mechanism of Ang II–AT1R signaling, we also reported that Ang II significantly activated HIF-1α and Ets-1, resulting in the upregulation of angiogenesis in castrated resistant prostate cancer under normal oxygen conditions (26). To further clarify the clinical potency of ARB, we have shown that ARB at clinically achievable doses enhanced CDDP-induced cytotoxicity in a mouse xenograft model of bladder cancer (12).

CDDP is commonly considered to be the most effective agent for the treatment of advanced bladder cancer. We previously reported that ROS generation in cancer cells induced by CDDP was significantly associated with cell viability (15, 16). The efficacy of combination therapy with CDDP and ARB in clinical experiments was evaluated retrospectively in non–small-cell lung cancer. Wilop et al. (27) reported that the addition of ACEI and ARB to platinum-based first-line chemotherapy contributed to prolonged survival. However, at present, no synergistic mechanism for this combination therapy has been fully evaluated, although this modality may be easily achievable without severe clinical side effects.

We investigated the association between the upregulation of AT1R expression and ROS generation induced by CDDP by using five bladder cancer cell lines. AT1R expression was significantly upregulated in T24, KU-1, and KU-19-19 cells, whereas ROS generation in these cells was also significantly upregulated in the early phase after CDDP exposure. However, UMUC-3 and 5637 cells, in which ROS generation was not significantly upregulated in the same phase, did not show any significant regulation of AT1R expression.

Edaravone is a potent free radical scavenger and is widely used to inhibit oxidative damage to cultured endothelial cells (28) and to protect the brain from postschismic reperfusion injury (29, 30). In the present study, using this agent, we showed that suppressing ROS generation induced by CDDP exposure could significantly reduce the upregulation of AT1R expression.

We examined whether AT1R upregulation induced by CDDP exposure affected VEGF production. The in vitro study showed that Ang II significantly enhanced VEGF production in CDDP-treated cells, and ARB at clinically achievable doses significantly inhibited the increase in VEGF. We also examined this AT1R signaling by using a murine xenograft model and observed the upregulation of expression of AT1R and VEGF in CDDP-treated tumors. These results suggest that a possible molecular mechanism of tumor aggressiveness induced by the upregulation of AT1R expression is through increased ROS generation after CDDP exposure. Whereas VEGF is regulated by many mechanisms in cancer cells, it may be controversial whether the AT1R pathway is a major regulator of VEGF production or whether other mechanisms exist at this point. However, our data suggested that ARB might block at least one of the mechanisms in VEGF production.

Using human bladder cancer specimens, we next investigated whether there was any regulation of AT1R and VEGF expression after CDDP-based chemotherapy. Immunostaining revealed some clinical specimens from patients who underwent cystectomy after neoadjuvant CDDP-based chemotherapy showed significantly higher AT1R and VEGF expression compared with the corresponding TUR specimens (Table 1; Fig. 5). These clinical results were quite consistent with our experimental results. However, only one patient who underwent neoadjuvant chemotherapy received the minimum dose
of ARB for hypertension. Due to the small number of patients and low dose of ARB, we could not evaluate the precise therapeutic effect of ARB in combination with CDDP-based chemotherapy.

Several reports showed the potent ability of tumor cells in promoting more increased cytokines after chemotherapeutic agents (31–33). In particular, in CDDP-based chemotherapy, Inoue et al. (34) reported that VEGF was overexpressed within post-M-VAC residual tumors compared with pretherapy tumor specimens by using human bladder cancer specimens. We previously reported the efficacy of a combination therapy consisting of paclitaxel and an ARB by using a bladder cancer xenograft model (11). Although no additional effect on tumor cytotoxicity was observed in vitro, the enhancement of tumor suppression was induced in vivo. However, the dose of ARB in the combination therapy with paclitaxel was still approximately two times higher than that of CDDP. Moreover, we examined the regulation of AT1R expression in T24 cells after introducing paclitaxel at the same cytotoxic dose as that of CDDP in vitro. There was no significant regulation of AT1R expression or increase in ROS generation (data not shown). Therefore, we propose that the combination of an ARB with other nonplatinum chemotherapeutic agents such as paclitaxel might have an additive effect, which is not as synergistic as that of CDDP, on tumor growth through angiogenic inhibition.

CDDP-based chemotherapy still remains the gold standard treatment for advanced bladder cancer. However, our findings suggest that CDDP exposure may induce angiogenic potentials in bladder cancer cells, resulting in CDDP resistance. Taken together, CDDP treatment may work as a double-edged sword, and this molecular mechanism is particularly interesting in terms of effective regimens for advanced bladder cancer. In the present study, using a murine xenograft model in bladder cancer, we clearly showed the efficacy of combination therapy consisting of CDDP and ARB at clinically achievable doses.

In efforts to improve the treatment of advanced bladder cancer, standard chemotherapeutic agents have recently been changed from a M-VAC regimen to a GC regimen due to the reduction in severe side effects. However, the GC regimen still has some side effects. Therefore, it is important to identify more appropriate regimens, taking into consideration the therapeutic effects as well as the side effects. The present findings suggest that CDDP-based chemotherapy induces angiogenic potential in bladder cancer cells and that antiangiogenic agents may be combined with CDDP-based chemotherapy. Because the antihypertensive agents ARBs are already in clinical use without severe side effects, and if these drugs can suppress tumor progression by inhibiting their activated angiogenic potentials at clinically achievable doses, then we propose that they may be an effective modality in the treatment of cancer.

In summary, CDDP upregulated AT1R expression through ROS generation and enhanced VEGF production induced by Ang II in bladder cancer. These findings suggest a new molecular mechanism for Ang II–AT1R signaling when anticancer agents are administered in combination with an ARB. We propose that patients who require CDDP-based chemotherapy due to invasive bladder cancer are potential candidates for this combined modality, although further investigation is warranted with respect to appropriate patient selection.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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