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Anti-cancer effects of newly developed chemotherapeutic agent, glycoconjugated palladium (II) complex, against cisplatin-resistant gastric cancer cells

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Abstract

Background: Cisplatin (CDDP) is the most frequently used chemotherapeutic agent for various types of advanced cancer, including gastric cancer. However, almost all cancer cells acquire resistance against CDDP, and this phenomenon adversely affects prognosis. Thus, new chemotherapeutic agents that can overcome the CDDP-resistant cancer cells will improve the survival of advanced cancer patients.

Methods: We synthesized new glycoconjugated platinum (II) and palladium (II) complexes, $[PtCl_2 (L)]$ and $[PdCl_2 (L)]$. CDDP-resistant gastric cancer cell lines were established by continuous exposure to CDDP, and gene expression in the CDDP-resistant gastric cancer cells was analyzed. The cytotoxicity and apoptosis induced by $[PtCl_2 (L)]$ and $[PdCl_2 (L)]$ in CDDP-sensitive and CDDP-resistant gastric cancer cells were evaluated. DNA double-strand breaks by drugs were assessed by evaluating phosphorylated histone H2AX. Xenograft tumor mouse models were established and antitumor effects were also examined *in vivo*.

Results: CDDP-resistant gastric cancer cells exhibit ABCB1 and CDKN2A gene up-regulation, as compared with CDDP-sensitive gastric cancer cells. In the analyses of CDDP-resistant gastric cancer cells, $[PdCl_2 (L)]$ overcame cross-resistance to CDDP *in vitro* and *in vivo*. $[PdCl_2 (L)]$ induced DNA double-strand breaks.

Conclusion: These results indicate that $[PdCl_2 (L)]$ is a potent chemotherapeutic agent for CDDP-resistant gastric cancer and may have clinical applications.

Keywords: Glycoconjugated platinum (II) complex, Glycoconjugated palladium (II) complex, Cisplatin, Drug resistance, Gastric cancer

Background

Cancer is a leading cause of death worldwide, and according to the WHO mortality database (as at November 2006), gastric cancer is the second leading cause of cancer death after lung cancer.

Cisplatin (CDDP) is the most frequently used chemotherapeutic agent for various types of advanced cancer and is used in combination regimens. Some CDDP-based combination chemotherapy regimens have also shown high response rates [1]. Based on recent Japanese phase III trials for metastatic gastric cancer, S1 plus cisplatin combination chemotherapy was established as the standard first-line chemotherapy [2].

However, CDDP-based combination chemotherapy regimens have several disadvantages, including side effects such as nephrotoxicity, neurotoxicity, ototoxicity and vomiting. In addition, some tumors acquire resistance to CDDP, reducing its efficacy [3,4]. Several mechanisms are involved in CDDP resistance [5]. Such mechanisms include decreased intracellular drug accumulation and/or increased drug efflux [6-9], drug inactivation

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by increased levels of cellular thiols [6,10], increased nucleotide excision-repair activity [9,11] and evasion of apoptosis [6,12]. Thus, for continued progress in cancer therapy, more effective drugs must be found.

Cancer cells take in higher levels of glucose than normal cells, a phenomenon known as the Warburg effect [13]. To achieve lower undesired toxicity, enhanced solubility and tumor selectivity, we have developed and have reported several glycoconjugated drugs [14,15]. Another strategy to design new antitumor agents related to CDDP is to change the nature of the central metal ion [16,17]. As palladium (Pd) chemistry is similar to that of platinum (Pt), Pd complexes (II) are expected to exhibit antitumor activities similar to those of Pt. Attempts have been made to synthesize Pd (II) complexes with such activities, as Pd complexes are expected to have less kidney toxicity than Pt complexes [18].

In this study, we synthesized a new glycoconjugated Pt (II) complex and a new glycoconjugated Pd (II) complex, and analyzed its cytotoxicity, ability to induce apoptosis, and ability to induce DNA double-strand breaks in CDDP-sensitive and CDDP-resistant gastric cancer cell lines *in vitro* and *in vivo*.

Methods
Drugs
Reagents and solvents used in this study were commercial products of the highest available purity. The Pt (II) and Pd (II) complexes were easily prepared using the one-pot reaction of Pt (II) or Pd (II) salt, amino sugar and pyridine aldehyde derivative without isolation of a Schiff base ligand (L) as follows.

$[PtCl_2$ (L)] (L = 2-deoxy-2-[(2-pyridinylmethylene) amino]-α-D-glucopyranose):Dichloro (2-deoxy-2-[(2-pyridinylmethylene)amino]-α-D-glucopyranose) Pt. An aqueous (50 mL) solution of D (+)-glucosamine • hydrochloride (0.65 g, 3.0 mmol) was neutralized with $NaHCO_3$ (0.26 g, 3.1 mmol). To this solution, a MeOH (50 mL) solution of 2-pyridinecarbaldehyde (0.32 g, 3.1 mmol) was added, followed by stirring for 2 h and addition of K_2 $[PtCl_4]$ (1.25 g, 3.0 mmol) in 30 mL of H_2O. The reaction was continued for another 41 h at room temperature. The mixture was concentrated by evaporation and the resulting residue was purified by silica gel column chromatography (eluent: acetone) to give a pale yellow powder (1.07 g, 67%). Single crystals were obtained by recrystallization from MeOH/Et_2O. Anal. calcd for $[PtCl_2$ (L)], $C_{12}H_{18}Cl_2N_2O_5Pt$, C; 26.98, H; 3.02, N; 5.24. found for C; 27.13, H; 2.97, N; 5.07. MS (FAB, pos): m/z = 498 $[M-Cl]^+$.

$[PdCl_2$ (L)] (L = 2-deoxy-2-[(2-pyridinylmethylene) amino]-α-D-glucopyranose):Dichloro (2-deoxy-2-[(2-pyridinylmethylene)amino]-α-D-glucopyranose) palladium. This complex was prepared by following a

similar procedure as described above for $[PtCl_2$ (L)] using Na_2 $[PdCl_4]$ instead of K_2 $[PtCl_4]$. The complex was dissolved in MeOH and insoluble materials were removed by filtration. The filtrate was concentrated by evaporation to give a pale yellow powder (1.1 g, 83%). This complex was purified by recrystallization from MeOH/Et_2O. Anal. calcd for $[PtCl_2$ (L)], $C_{12}H_{18}Cl_2N_2O_5Pd$, C; 32.35, H; 3.62, N; 6.29. found for C; 32.02, H; 3.51, N; 6.01. MS (FAB, pos): m/z = 431 $[M-HCl + Na]^+$.

CDDP and CBDCA were purchased from Bristol-Myers Co. (Tokyo, Japan). L-OHP was purchased from Yakult (Tokyo, Japan).

Measurements
Elemental analysis was carried out on a Perkin-Elmer 240C or a Fisons Instruments EA1108 Elemental Analyzer. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-GSX400 in *N,N*-dimethylformamide-d_7 (DMF-d_7)/D_2O. Mass spectra were obtained on a JEOL JMS-700 T Tandem MS-station mass spectrometer.

Crystallography
Suitable crystals for X-ray crystallography were obtained by slow recrystallization of $[PtCl_2$ (L)] and $[PdCl_2$ (L)] from a minimal amount of methanol and ether mixtures. Crystallographic data (excluding structure factors) for the structure reported in this paper were deposited with the Cambridge Crystallographic Data Center as supplementary publication no. CCDC-835397. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB21EZ, UK (Fax: (+44) 1223-336-033; E-mail: deposit@ccdc.cam.ac.uk).

Cell culture
The human gastric cancer cell lines MKN28 (Japanese Cancer Research Resources Bank, No. 0253) and MKN45 (Japanese Cancer Research Bank, No. 0254) were cultured in RPMI1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 1% ampicillin and streptomycin. Cells were cultured under an atmosphere of 5% CO_2 at 37°C.

Establishment of CDDP-resistant sublines from MKN28 and MKN45
CDDP-resistant MKN28 (MKN28 (CDDP)) and CDDP-resistant MKN45 (MKN45 (CDDP)) were established by continuous exposure to CDDP starting at 0.5 μmol/L and increasing in a stepwise manner to 10 μmol/L for more than 5 months. Experiments with these sublines were performed after maintenance in CDDP-free medium for 2–3 weeks.

RT2 Profiler PCR arrays for human cancer drug resistance & metabolism

Total RNA (1 μg) from MKN45 (0) or MKN45 (CDDP) was converted to cDNA and used to screen inflammatory cytokines and receptors using quantitative real-time PCR arrays according to the manufacturer's instructions (SuperArray Bioscience). Reactions were cycled in an ABI Prism 7500 FAST sequence detector (Applied Biosystems) and acquired data were analyzed using the DDCt method to determine the expression levels of each transcript normalized against the expression level of housekeeping gene controls. A gene-wise, two-sample t-test was performed for each transcript to identify statistical differences in expression between MKN45 (0) or MKN45 (CDDP).

In vitro treatment

Cell viability was determined by WST-8 cell proliferation assay. Gastric cancer cells were seeded into 96-well culture plates at 5×10^3 cells/100 μL/well and incubated overnight. Cells were treated for 48 h with graded concentrations of CDDP (0–200 μmol/L), [PtCl$_2$(L)] (0–200 μmol/L), [PdCl$_2$(L)] (0–200 μmol/L), L-OHP (0–100 μmol/L) or CABDA (0–400 μmol/L). After treatment, cells were incubated with cell a counting kit-8 (Dojindo, Kumamoto, Japan) for 4 h and absorption at 450 nm was measured with a microscope reader (SPECTRA MAX340; Molecular Devices, Silicon Valley, CA). Cell viability was expressed as a percentage vs. untreated control cells and half maximal (50%) inhibitory concentration (IC$_{50}$) was calculated. Resistance factor (RF) is defined as the relative ratio of IC$_{50}$ values in both cell lines (MKN28 (CDDP)/MKN28 (0) or MKN45 (CDDP)/MKN45 (0)).

Assessment of apoptosis

Apoptosis was assessed by analysis of activation of caspase-3 and caspase-7 using the substrate DEVD-aminoluciferin from the Caspase-Glo 3/7 Assay kit (Promega) according to the manufacturer's instructions. Briefly, gastric cancer cells (10^4 per well) were plated on a 96-well culture plate with three replicates per treatment. After 24 h of plating, cells were treated for 72 h with graded concentrations of CDDP (0–200 μmol/L), [PtCl$_2$(L)] (0–200 μmol/L), [PdCl$_2$(L)] (0–200 μmol/L), L-OHP (0–100 μmol/L) or CABDA (0–400 μmol/L). Caspase-Glo reagent was added to each well and incubated for 1 h, and luminescence was measured using a LUMAT LB 9507 luminometer (Berthold Technologies). Results were analyzed by Welch's t-test between MKN45 (0) and MKN45 (CDDP).

Assessment of DNA double-strand breaks

Cells were washed with PBS (–) and subsequently dissolved in 1 cell lysis buffer (Cell Signaling Technology) containing 20 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L h-glycerophosphate, 1 mmol/L Na3VO4, and 1 Ag/mL leupeptin with the addition of 1 mmol/L phenylmethylsulfonyl fluoride. After disruption in an ice bath using a Bio-ruptor sonicator (Cosmo Bio) for 15 s, lysates were centrifuged at 15,000 rpm for 10 min at 4°C. Each sample was normalized as equal protein concentrations using a protein assay kit (Bio-Rad Laboratories). An equal quantity of 2 SDS-PAGE sample buffer [0.5 mol/L Tris–HCl (pH 7.2), 1% SDS, 100 mmol/L β-mercaptoethanol, and 0.01% bromophenol blue] was added to each sample, followed by boiling for 5 min at 100°C. Aliquots of sample were fractioned on 8% to 15% SDS-PAGE and were then electroblotted onto nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS (–) for 1 h at room temperature. The membrane was incubated with primary antibodies, anti-γH2AX (Bethyl Laboratories, Inc., 1:2000), overnight at 4°C and was then washed with 0.05% Tween 20 in PBS (–) three times at 5-min intervals. The membrane was incubated with secondary antibody for 1 h at room temperature followed by three washes with 0.05% Tween 20 in PBS (–) three times at 5-min intervals. The membrane was treated with enhanced chemiluminescence detection reagents (Amersham) for 1 min at room temperature and exposed to scientific imaging films (Eastman Kodak), and proteins were visualized as bands. Filters were stripped and re-probed with monoclonal β-actin antibody (Abcam plc) as an internal control.

Animals and tumor models

Pathogen-free female nude mice (BALB/c Slc-nu/nu) aged 4 weeks and weighing 20–25 g were obtained from Japan SLC (Kyoto, Japan). Animals were allowed to acclimatize for 2 weeks in the animal facility before any interventions were initiated. Xenograft tumor models were established by subcutaneously implanting 3×10^6 gastric cancer cells (MKN45 (0), MKN45 (CDDP)) in 200 μL of PBS. Experimental procedures were approved by the Nagoya City University Center for Experimental Animal Science, and mice were raised in accordance with the guideline of the Nagoya City University Center for Animal Experiments.

In vivo treatment

At 7 days after tumor inoculation, mice were given an intraperitoneal injection of CDDP, [PtCl$_2$ (L)] or [PdCl$_2$ (L)] at a dose of 40 μmol/kg. Tumor growth was monitored daily by measuring tumor volume with vernier calipers. Tumor volume was calculated using the following formula: (length × width × depth)/2. Each group

consisted of 5 mice. Results were analyzed by multiple testing (Holm method) between groups.

Statistical analysis

Descriptive statistics and simple analyses were carried out using the statistical package R version 2.4.1 (www.r-project.org/). Apoptosis induction was analyzed by Welch's *t*-test. Antitumor effects were analyzed by the Bonferroni-Holm method. P-values of <0.05 were considered to be statistically significant.

Results

Crystal structure of [PtCl₂ (L)] and [PdCl₂ (L)]

The crystal structures of [PtCl$_2$ (L)] and [PdCl$_2$ (L)] (Cambridge Crystallographic Data Center as supplementary publication no. CCDC-835397) show that each metal atom is surrounded by four donor atoms, two nitrogen atoms and two chloride ions, in a cis-configuration. As expected, the geometry around the metal center is approximately square planar (Figure 1). The pyranoid ring of the sugar unit adopts an unusual α-$^4C_1$ conformation. Thus, both complexes have similar structures.

Conformational analysis of sugar units on [PtCl₂ (L)] and [PdCl₂ (L)] by means of NMR measurements

^{1}H-NMR and ^{13}C-NMR spectra of the two complexes were obtained in DMF-d$_7$/D$_2$O and unambiguously assigned by ^{1}H-^{1}H and ^{13}C-^{1}H COSY two-dimensional NMR spectroscopy. Conformation of the sugar ring in both complexes was investigated by ^{1}H-NMR spectroscopy in DMF-d$_7$/D$_2$O after OH proton exchange, which reveals signals originating from protons that are attached to the carbon atoms of the sugar unit. The vicinal proton-proton coupling constants for [PtCl$_2$ (L)] ($^3J_{1,2}$ = 3.2, $^3J_{2,3}$ = 11.2, $^3J_{3,4}$ = 8.4, $^3J_{4,5}$ = 10.0 Hz) and [PdCl$_2$ (L)] ($^3J_{1,2}$ = 3.4, $^3J_{2,3}$ = 11.2, $^3J_{3,4}$ = 8.6, $^3J_{4,5}$ = 10.0 Hz) correspond to α-$^4C_1$ conformations as observed in the X-ray crystallography, indicating the structural similarity in the sugar unit in the solid and solution states.

Genes up-regulated in CDDP-resistant gastric cancer sublines

The 20-fold changes in gene expression for MKN45 (0) and MKN45 (CDDP) are presented in Table 1. Among 84 genes related to human cancer drug resistance and metabolism, 8 genes were significantly altered with fold changes larger than 20. Genes that were up-regulated by greater than 20-fold were ABCB1, APC, ATM, BRCA2 and CDKN2A, whereas down-regulated genes were CYP2B6, CYP2C19 and PPARγ.

[PdCl₂ (L)] revealed minimum resistance to CDDP-resistant gastric cancer cells

We investigated the cytotoxicity of CDDP, [PtCl$_2$ (L)], [PdCl$_2$ (L)], L-OHP and CABDA in the gastric cancer cell lines MKN28 (0), MKN28 (CDDP), MKN45 (0) and MKN45 (CDDP), and summarize the results in Table 2. In the parent cell lines (MKN28 (0) and MKN45 (0)), [PtCl$_2$ (L)] and [PdCl$_2$ (L)] exhibited lower cytotoxicity than CDDP and L-OHP, and higher cytotoxicity than CABDA. Resistance factor (RF) was calculated as the relative ratio of IC$_{50}$ values in both cell lines (MKN28 (CDDP)/MKN28 (0) or MKN45 (CDDP)/MKN45 (0)).

Figure 1 Structures of complexes used in this study. (**A**) Perspective drawing of [PtCl$_2$ (L)] with atomic numbering scheme in the crystal. Selected bond length (Å) and angles (°), Pt(1)-Cl(1) 2.2985(8), Pt(1)-Cl(2) 2.2922(8), Pt(1)-N(1) 2.016(3), Pt(1)-N(2) 2.006(3); Cl(1)-Pt(1)-Cl(2) 89.77(3), Cl(1)-Pt(1)-N(1) 94.84(7), Cl(2)-Pt(1)-N(2) 95.30(7), N(1)-Pt(1)-N(2) 80.14(10). (**B**) Perspective drawing of [PdCl$_2$ (L)] with atomic numbering scheme in the crystal. Selected bond length (Å) and angles (°), Pd(1)-Cl(1) 2.2940(8), Pd(1)-Cl(2) 2.2832(8), Pd(1)-N(1) 2.033(2), Pd(1)-N(2) 2.025(2); Cl(1)-Pd(1)-Cl(2) 91.04(3), Cl(1)-Pd(1)-N(1) 94.02(7), Cl(2)-Pd(1)- N(2) 94.50(6), N(1)-Pd(1)-N(2) 80.49(8).
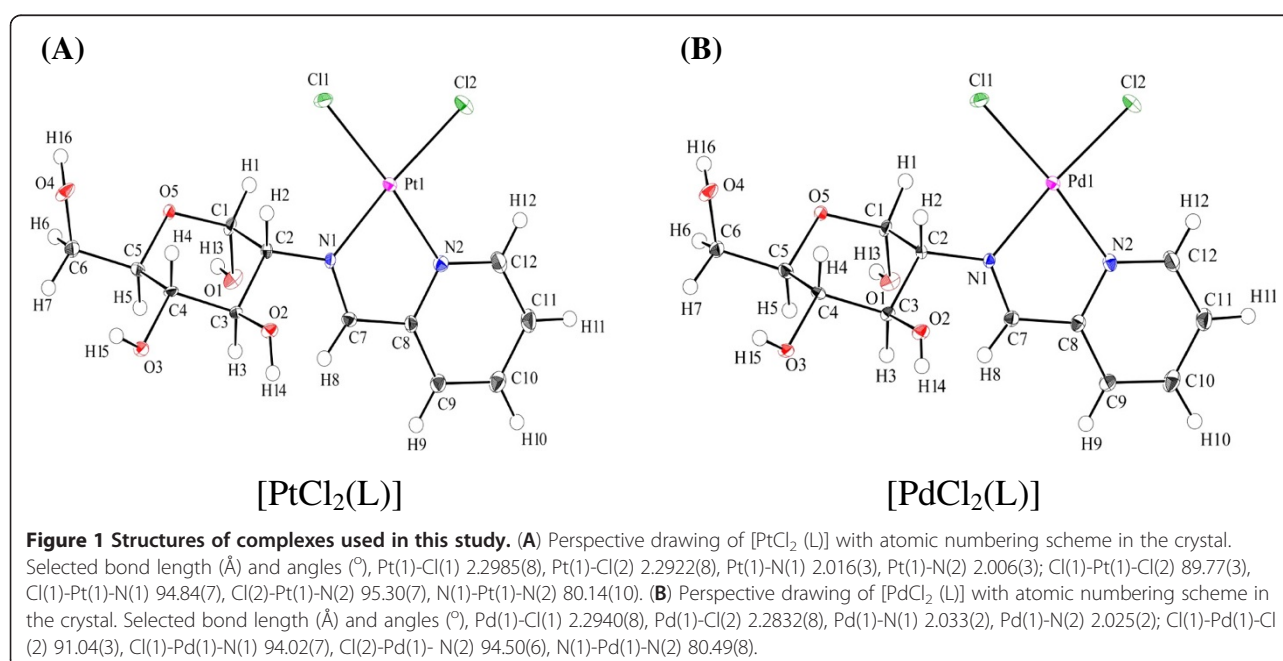
Table 1 Expression profiles of genes related to human cancer drug resistance and metabolism showing at least 20-fold change in expression

Symbol	GenebankID	Incease	Gene name
ABCB1	NM_000927	122.73	ABC20,CD243,CLCS,GP170,MDR1,MGC163296,P-GP,PGY1
APC	NM_000038	27.25	BTPS2,DP2,DP2.5,DP3,GS
ATM	NM_000051	27.35	AT1,ATA,ATC,ATD,ATDC,ATE,DKFZp781A0353,MGC74674,TEL1,TELO1
BRCA2	NM_000059	34.61	BRCC2,BROVCA2,FACD,FAD,FAD1,FANCB,FANCD,FANCD1
CDKN2A	NM_000077	2689.53	ARF,CDK4I,CDKN2,CMM2,INK4,INK4a,MLM,MTS1,TP16,p14,Prop14ARF,p16,p16INK4,p16INK4a,p19
CYP2B6	NM_000767	−39.27	CPB6,CYP2B,CYPIIB6,IIB1,P450
CYP2C19	NM_000769	−145.20	CPCJ,CYP2C,P450C2C,P450IIC19
PPARG	NM_015869	−29.31	CIMT1,NR1C3,PPARG1,PPARG2,PPARgamma

Similarly to CABDA, cells treated with [PtCl$_2$ (L)] showed cross-resistance to CDDP. On the other hand, [PdCl$_2$ (L)] overcame cross-resistance to CDDP, similarly to L-OHP, although [PdCl$_2$ (L)] showed a lower degree of cross-resistance than L-OHP (Table 2).

[PdCl$_2$ (L)] induced apoptosis in CDDP-resistant gastric cancer cell lines

We examined apoptosis induction by CDDP, [PtCl$_2$ (L)], [PdCl$_2$ (L)], L-OHP and CABDA in the gastric cancer cell lines MKN45 (0) and MKN45 (CDDP) (Figure 2A). In the parental cell line (MKN45 (0)), all drugs tended to induce apoptosis in a dose-dependent manner. In the CDDP-resistant subline (MKN45 (CDDP)), induction of apoptosis by CDDP, CABDA and [PtCl$_2$ (L)] was lower than in the parental cell line. On the other hand, [PdCl$_2$ (L)] and L-OHP maintained apoptosis induction against CDDP-resistant gastric cancer cells.

Table 2 *In vitro* cytotoxicity assay in CDDP-sensitive and -resistant gastric cancer cell lines

MKN28	Resistance factor	IC50(μM)	
		MKN28(0)	**MKN28(CDDP)**
[PdCl2(L)]	1.02	78.9 ± 4.0	80.8 ± 6.6
L-OHP	1.19	46.4 ± 4.0	55.2 ± 3.8
[PtCl2(L)]	2.54	111.7 ± 27.1	283.9 ± 19.3
CDDP	3.37	19.4 ± 2.4	65.4 ± 4.6
CABDA	4.33	202.9 ± 17.2	878.3 ± 34.1
MKN45	**Resistance factor**	**IC50(μM)**	
		MKN45(0)	**MKN45(CDDP)**
[PdCl2(L)]	1.14	61.2 ± 6.8	69.7 ± 4.1
L-OHP	1.3	27.3 ± 1.1	35.6 ± 6.7
[PtCl2(L)]	2.18	129.5 ± 14.8	282.6 ± 34.5
CDDP	3.27	23.5 ± 2.2	77.0 ± 8.5
CABDA	3.42	152.8 ± 3.7	522.0 ± 27.4

[PdCl$_2$ (L)] induced DNA double-strand breaks in CDDP-resistant gastric cancer cells

Cells were labeled with an antibody against phosphorylated histone H2AX (γ-H2AX), which detects double-strand breaks caused by drugs such as CDDP [19]. We used Western blotting for evaluation of γ-H2AX protein expression by CDDP and [PdCl$_2$ (L)] in the gastric cancer cell lines MKN45 (0) and MKN45 (CDDP). In the parental cell line (MKN45 (0)) treated with CDDP or [PdCl$_2$ (L)], γ-H2AX protein levels increased and were the same by 24 and 48 h after treatment. In the CDDP-resistant subline (MKN45 (CDDP)), γ-H2AX protein levels increased with [PdCl$_2$ (L)], but did not increase with CDDP (Figure 2B). These results indicated that [PdCl$_2$ (L)], but not CDDP induced DNA double-strand breaks in CDDP-resistant gastric cancer cells.

[PdCl$_2$ (L)] significantly suppressed CDDP-resistant gastric cancer cell proliferation

We examined the effects of CDDP, [PtCl$_2$ (L)] and [PdCl$_2$ (L)] on xenograft tumor models established by subcutaneously implanting the gastric cancer cell lines MKN45 (0) and MKN45 (CDDP). At 7 days after tumor inoculation, mice were given an intra-peritoneal injection of CDDP, [PtCl$_2$ (L)] or [PdCl$_2$ (L)] at a dose of 40 μmol/kg. In MKN45 (0) nude mice, CDDP, [PtCl$_2$ (L)] and [PdCl$_2$ (L)] suppressed tumor growth significantly as compared to controls ($p < 0.01$). In MKN45 (CDDP) nude mice, [PdCl$_2$ (L)] suppressed tumor growth significantly ($p < 0.01$) as compared to CDDP, but [PtCl$_2$ (L)] did not (Figure 3). None of the therapies had any obvious side effects, such as diarrhea or weight loss (data not shown).

Discussion

[PtCl$_2$ (L)] and [PdCl$_2$ (L)] were developed as antitumor drugs with sugar conjugated ligands, and were expected to have a number of advantages, including significant reductions in side effects, improved water solubility, and
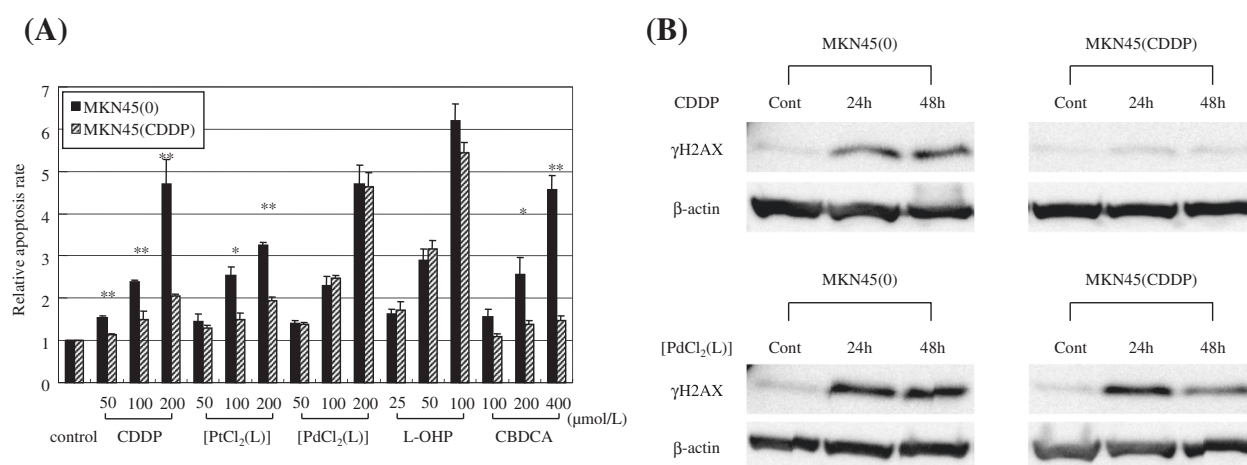
Figure 2 Investigation of cytotoxicity mechanism of [PtCl₂ (L)] and [PdCl₂ (L)]. (**A**) [PdCl₂ (L)] induced apoptosis on CDDP-resistant gastric cancer cell lines. Apoptosis was assessed by analyzing activation of caspase-3 and caspase-7. Mean of three independent experiments in triplicate; bars, SE. Values for apoptosis of cells in FBS alone were used as controls. Significance was determined by Welch's *t*-test. *, P < 0.05, **, P < 0.01 relative to parental cell line. (**B**) [PdCl₂ (L)] induced DNA double-strand breaks in CDDP-resistant gastric cancer cells. Cells were labeled with antibody against phosphorylated histone H2AX (γ-H2AX), which detects double-strand breaks caused by drugs such as CDDP. An evaluation of γ-H2AX protein expression was investigated by Western blotting at 24 or 48 h after treatment.

greater cellular uptake. These complexes were very easily prepared in good yields by one-pot reaction of Pt or Pd salts, amino sugar and pyridine aldehyde derivative without isolation of Schiff base ligand, and were characterized by X-ray crystallography and ^{1}H- and ^{13}C-NMR spectra. One-pot reaction is a strategy to improve the efficiency of a chemical reaction whereby a reactant is subjected to successive chemical reactions. This saves time and resources by avoiding lengthy separation processes and purification of the intermediate chemical compounds while increasing chemical yield.

In this report, we found that gastric cancer cell lines adapted to growth in the presence of 10 μmol/L CDDP (MKN45 (CDDP)) showed enhanced ABCB1 and CDKN2A expression as compared with their CDDP-sensitive parental cell lines (MKN45 (0)) (Table 1). Prolongation of the cell cycle at the G1-S transition allows for DNA repair to occur. It is therefore unsurprising that growth arrest mediated by CDKN2A is able to enhance resistance to drugs whose mechanism of action is dependent on DNA damage, such as CDDP [20]. ABCB1 is the most extensively studied ABC transporter [21].
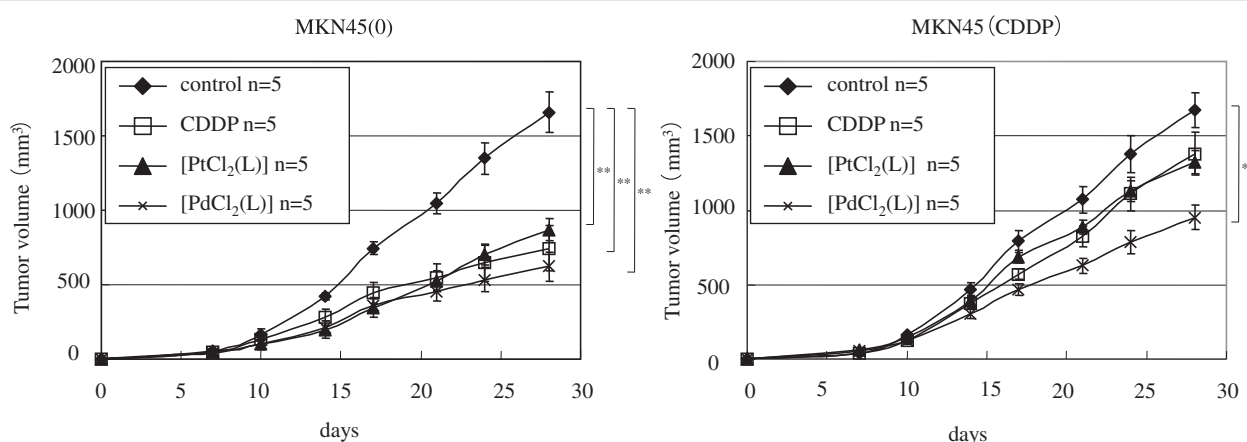

Figure 3 [PdCl₂ (L)] significantly suppressed CDDP-resistant gastric cancer cell proliferation in xenograft model. Cells were inoculated in dorsal skin at a concentration of 3×10^6 gastric cancer cells (MKN45 (0), MKN45 (CDDP)) in 200 μL of PBS. At 7 days after tumor inoculation, tumor-bearing mice were given intraperitoneal injection of CDDP, [PtCl₂ (L)] or [PdCl₂ (L)] at a dose of 40 μmol/kg (n = 5 for each). Tumor volumes were monitored for 28 days in control mice (no treatment), and mice treated with CDDP, [PtCl₂ (L)] or [PdCl₂ (L)]. Data are means ± SE. Significance was determined by the Bonferroni-Holm method. **, P < 0.01 relative to controls.

The expression of P-glycoprotein ABCB1 is implicated in multidrug resistance (MDR). MDR proteins confer drug resistance by reducing intracellular drug accumulation due to active efflux of drugs [22,23]. The CDDP-resistant cell line (MKN45 (CDDP)) was useful for studying the resistance mechanisms of CDDP and for studying the effects of other anticancer drugs for gastric cancer under CDDP resistance.

Many experiments have been performed in order to develop new anti-cancer drugs that show preferential accumulation within the target tumor tissue for various active targeting approaches, such as liposomes [24], polymer microspheres [25-27] and nanoparticles [28-31]. Our results indicate that the glucose-linked anticancer drug is a useful drug delivery system for accumulation in the target tumor.

In order to circumvent CDDP resistance, significant amounts of work have been devoted to preparing anticancer complexes, including amine Pt complexes [32,33], diamine Pt complexes [34,35], *trans*-Pt complexes [36-38], multinuclear Pt complexes [39-41] and Pt (IV) coordination complexes [42-44]. Progress in the field of anticancer chemistry of Pd-based transition metal complexes has been reviewed [45]. [PdCl$_2$ (L)] and L-OHP overcame cross-resistance to CDDP, although [PdCl$_2$ (L)] showed a lower degree of cross-resistance than L-OHP (Table 2). The cytotoxicity of L-OHP in CDDP-resistant cell lines has been considered to be due to the differences of DNA damage and/or recognition processes between CDDP and L-OHP [46]. The DNA damage caused by Pd (II) compounds is reportedly processed in a different manner from that induced by Pt (II) complexes [47]. In the CDDP-resistant subline (MKN45 (CDDP)), [PdCl$_2$ (L)] showed significantly higher antitumor effects *in vitro* (Table 2) and *in vivo* (Figure 3) as compared with CDDP and [PtCl$_2$ (L)]. Apoptosis by [PdCl$_2$ (L)] did not decrease when compared with parental cells, although apoptosis induced by [PtCl$_2$ (L)] decreased (Figure 2A). These results indicate that the resistance mechanism of Pd (II) complexes might be different from those of Pt (II) complexes.

Phosphorylation of histone H2AX (γH2AX) has been used as an indicator of exposure to a variety of DNA-damaging agents such as ionizing radiation [48], gemcitabine [49], topotecan [50], etoposide, bleomycin, and doxorubicin [51]. The stimulus for γH2AX formation after CDDP treatment is replication fork collapse and subsequent double-strand break formation at sites of inter-strand cross-links [52,53] immediately after formation of double-strand breaks [52,54]. The present results revealed that [PdCl$_2$ (L)] induced DNA double-strand breaks in CDDP-resistant gastric cancer cells in which CDDP could not induce DNA double-strand breaks (Figure 2B).

Conclusion

We demonstrated that a new glycoconjugated Pt (II) complex, [PtCl$_2$ (L)], and a new glycoconjugated Pd (II) complex, [PdCl$_2$ (L)], showed significant antitumor effects in CDDP-sensitive gastric cancer and executed their biological effects by inducing apoptosis. In addition, [PdCl$_2$ (L)] overcame cross-resistance to CDDP in CDDP-resistant gastric cancer, while [PtCl$_2$ (L)] did not. When compared with L-OHP, [PdCl$_2$ (L)] showed a lower degree of cross-resistance to CDDP and [PdCl$_2$ (L)] is speculated to be less toxic to the kidney than Pt complexes such as L-OHP and CDDP. Furthermore, glucose conjugation may increase drug solubility and tumor selectivity. From these findings, we conclude that [PdCl$_2$ (L)] is a potentially useful antitumor drug for CDDP-resistant gastric cancer.

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