Mononuclear Platinum(II) Complex with 2-Phenylpyridine Ligands Showing High Cytotoxicity against Mouse Sarcoma 180 Cells Acquiring High Cisplatin Resistance

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Mouse sarcoma 180 cell with a 25-fold higher cisplatin (CDDP) resistance, termed S-180cisR, is newly established. S-180cisR cells grow quite slowly in the presence of CDDP with high concentration. This may show that S-180cisR cells modulate the cell cycle to acquire CDDP resistance. P-Glycoprotein is selectively expressed on the surface of S-180cisR, which is not on CDDP-sensitive S-180 parent cells. In an experiment using an inhibitor (verapamil) of P-glycoprotein, cytotoxicity of CDDP against S-180cisR is significantly increased (viz., IC_{50} value is decreased) and accumulation of CDDP in S-180cisR cells is also increased. These results indicate that enhanced pumping-out of CDDP by P-glycoprotein should be one of the reasons for the CDDP resistance of S-180cisR. A platinum(II) complex with a cyclometalated 2-phenylpyridine ligand and a nonchelated one (complex **5**) is synthesized, and its structure is determined by X-ray structural analysis. Complex **5** has a cyctotoxicity against S-180cisR higher than that of CDDP and its derivatives with 2- or 3-substituted pyridine ligands (complexes **2**−**4**, **6**, **7**). Complex **5** is incorporated in S-180cisR to an enormously greater extent than CDDP; that is, the ratio of accumulated platinum amount after 3 h is 61.9. In S-180 parent cells, on the other hand, the ratio remains 8.1. This high accumulation of complex **5** into S-180cisR must account for the higher activity of complex **5** against S-180cisR compared to CDDP.

Introduction

There has been only a little success in designing platinum(II) complexes that can overcome acquired *cis*-diamminedichloroplatinum(II) (CDDP, cisplatin) resistance. Reduced accumulation inside cells, increased cytoplasmic detoxification via elevated glutathione and/or metallothionein levels, and enhanced DNA repair and/or increased cellular tolerance to Pt−DNA adducts are proposed as the mechanisms underlying CDDP resistance.[1] It was reported more recently that the expression of glutathione S-transferase π (GST-π) and multidrug-resistance-associated protein (MRP) were enhanced in a dose−response manner as cells acquired progressive CDDP resistance.[2] *cis*-Amminedichloro(2-methylpyridine)platinum(II) (AMD473) was rationally designed to circumvent resistance by sterically inhibiting cellular detoxification by glutathione and other cellular thiols, whereas there still remains the ability to form cytotoxic lesions with DNA.[3] It is known that axial steric hindrance decreases the rate of substitution reactions in square planar complexes. AMD473 was indeed evaluated preclinically in various cell lines such as 41M, CH1, and A2780. It is also interesting that AMD473 can circumvent acquired CDDP resistance in cell lines (41McisR, CH1cisR) where detoxification is not included in platinum drug resistance.

In general, there are two main factors, chemical reactivity and hydrophobicity, that affect the effectiveness of medicines. We therefore planned to investigate a series of AMD473 derivatives with different hydrophobicity, *cis*-$[PtCl_2(NH_3)R]$ (R = 2-substituted pyridine), to develop more effective platinum drugs by elevating the ability for membrane transport. To evaluate the effect of steric hindrance in substitution reactions, analogous platinum(II) complexes with 3-substituted pyridines were also prepared for comparison. During this study, we accidentally found that a platinum(II) complex cyclometalated by a 2-phenylpyridine ligand has high cytotoxicity against mouse sarcoma 180 CDDP-resistant cell line (S-180cisR) compared to CDDP and its derivatives.[4] It should be specially noted that establishment of S-180cisR acquiring high resistant activity is indispensable for the above experiment. Indeed, we have been selecting S-180cisR for 3 years. In the present paper, we concentrate on chemical properties and structures of the platinum(II) complexes, biological properties of S-180cisR, cytotoxicity of the platinum(II) complexes against the original CDDP-sensitive cells (S-180) and S-180cisR, and accumulation of the platinum(II) complexes into S-180 and S-180cisR cells.

Results

Synthesis of Platinum(II) Complexes. Synthetic routes of the platinum(II) complexes are shown in

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Scheme 1. Synthetic Route of Platinum Complexes

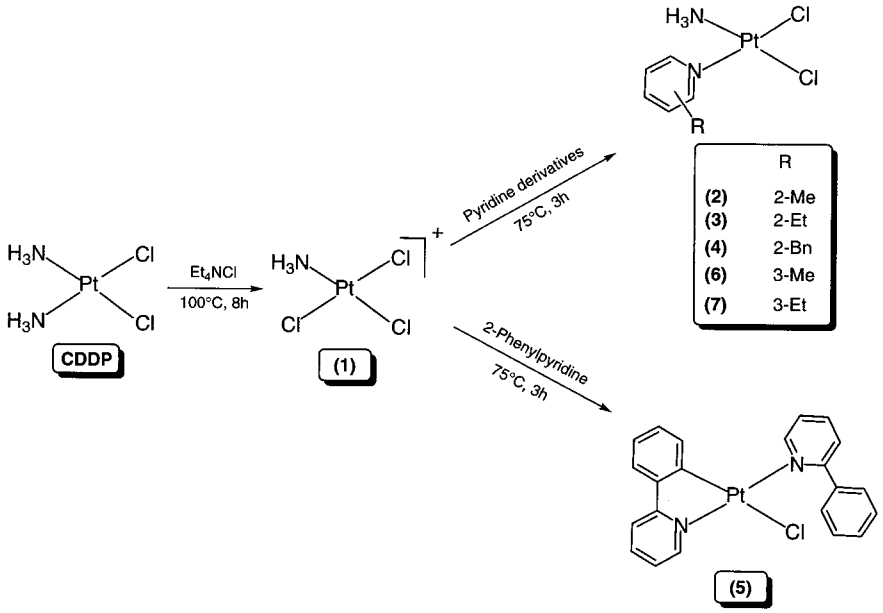

Table 1. Crystallographic Data

	3	5
formula	$C_7H_{12}N_2PtCl_2$	$C_{22}H_{17}N_2PtCl$
fw	390.18	539.93
cryst syst	monoclinic	triclinic
space group	$P2_1/c$ (No. 14)	$P\bar{1}$ (No. 2)
a (Å)	9.771(2)	10.648(2)
b (Å)	9.712(2)	17.588(3)
c (Å)	11.192(2)	10.399(2)
α (deg)		98.45(2)
β (deg)	103.30(1)	94.76(2)
γ (deg)		99.44(1)
V (Å^3)	1033.6(3)	1888.8(7)
Z	4	4
D_c (g cm^{-3})	2.507	1.899
R	0.032	0.034
R_w	0.039	0.041

Scheme 1. Complex **1** was produced by the reaction of CDDP with the chloride anion (Cl^-) because the ammine ligand of CDDP is preferentially substituted by Cl^- because of the trans effect of the chloride ligand. In similar substituted reactions, complexes **2**−**4**, **6**, and **7** were produced by the reaction of complex **1** with 2- or 3-substituted pyridines. In the reaction with 2-phenylpyridine, however, an unexpected complex **5**, in which one of two 2-phenylpyridines coordinates as bidentate and the other does as a monodentate, was obtained.

Crystallographic Data. We succeeded in the crystallization of complexes **3** and **5**, which were subjected to X-ray structural analysis. The crystallographic data are summarized in Table 1. The perspective drawing of complex **3**, whose structure is similar to those of complexes **2**, **4**, **6**, and **7**, is illustrated in Figure 1, and the selected bond distances and angles are listed in Table 2. The platinum atom is surrounded by two chloride atoms, an ammine, and a nitrogen atom of 2-ethylpyridine, forming a square planar geometry. As anticipated from its chemical structure, the pyridyl ring is almost perpendicular to the platinum coordination plane. The Pt−Cl distances are av 2.305(2) Å and the Pt−N distances are av 2.032(7) Å. A similar trend was observed for the corresponding distances of [$PtCl_2$(2,2′-bipyridine)] (av 2.291(4) Å for Pt−Cl and av 2.009(10) Å for Pt−N).[5]

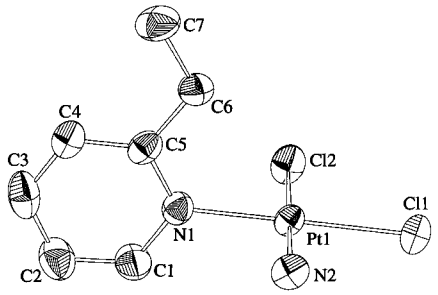

Figure 1. Perspective view of complex **3**.

Table 2. Selected Geometric Features of Complex **3**

distances (Å)		angles (deg)	
Pt(1)−Cl(1)	2.293(2)	Cl(1)−Pt(1)−Cl(2)	91.50(7)
Pt(1)−Cl(2)	2.316(2)	C1(1)−Pt(1)−N(1)	179.1(2)
Pt(1)−N(1)	2.022(6)	Cl(1)−Pt(1)−N(2)	88.5(2)
Pt(1)−N(2)	2.041(7)	Cl(2)−Pt(1)−N(1)	89.4(2)
		Cl(2)−Pt(1)−N(2)	178.5(2)
		N(1)−Pt(1)−N(2)	90.6(3)

For complex **5**, there are two crystallographically independent complexes in an asymmetric unit, though their structures are quite similar to each other (Figure 2 and Tables 1 and 3). These crystallographic data are entirely different from those reported by Mdleleni et al.,[6] whose complex was synthesized in a method different from that for complex **5**. Two 2-phenylpyridine ligands in complex **5** coordinate to a platinum atom in a square planar geometry, where one is cyclometalated through nitrogen and carbon atoms and the other is nonchelated with the nitrogen donor atom. The dihedral angles between the two aromatic rings of cyclometalated 2-phenylpyridine are close to 0°, while the corresponding dihedral angles of nonchelated 2-phenylpyridine are about 45°. The pyridyl ring of the nonchelated 2-phenylpyridine ligand is almost perpendicular to the platinum coordinated plane. The C(m12)−C(m17) distances (1.45(1) Å for m = 1 and 1.44(1) Å for m = 2) in the cyclometalated 2-phenylpyridines lie between a C−C

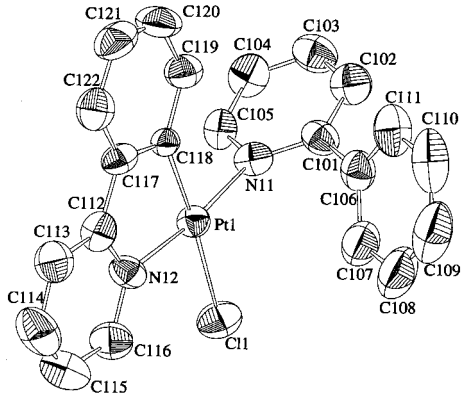

Figure 2. Perspective view of complex **5**. Though there are two crystallographically independent complexes (denoted by $m = 1$ and $m = 2$) in an asymmetric unit whose structures are quite similar to each other; only the complex corresponding to $m = 1$ is illustrated here.

Table 3. Selected Geometric Features of Complex **5**

	distances (Å)		angles (deg)
Pt(1)−Cl(1)	2.402(3)	Cl(1)−Pt(1)−N(11)	90.2(2)
Pt(1)−N(11)	2.055(7)	C1(1)−Pt(1)−N(12)	96.1(2)
Pt(1)−N(12)	2.020(7)	Cl(1)−Pt(1)−C(118)	175.9(2)
Pt(1)−C(118)	1.988(8)	N(11)−Pt(1)−N(12)	173.0(3)
Pt(2)−Cl(2)	2.410(2)	N(11)−Pt(1)−C(118)	93.2(3)
Pt(2)−N(21)	2.039(7)	N(12)−Pt(1)−C(118)	80.4(3)
Pt(2)−N(22)	2.013(7)	Cl(2)−Pt(2)−N(21)	88.2(2)
Pt(2)−C(218)	1.973(9)	C1(2)−Pt(2)−N(22)	96.2(2)
		Cl(2)−Pt(2)−C(218)	177.0(3)
		N(21)−Pt(2)−N(22)	174.6(3)
		N(21)−Pt(2)−C(218)	93.5(3)
		N(22)−Pt(2)−C(218)	82.0(4)

single bond (1.54 Å) and an aromatic bond (1.39 Å), which obviously reflects a conjugation between the two aromatic rings. In the nonchelated 2-phenylpyridines, the C(m01)−C(m06) distances are 1.46(1) Å ($m = 1$) and 1.47(1) Å ($m = 2$), whose values are 0.01−0.03 Å larger than those of the cyclometalated ligands. The Pt−Cl distances are 2.402(3) and 2.410(2) Å, which are about 0.1 Å larger than the Pt−Cl distances of complex **3** (2.305(2) Å) and [$PtCl_2$(2,2′-bipyridine)] (2.291(4) Å),[5] suggesting the trans influence of the C(m18) atom. These facts may be correlated to the high cytotoxicity of complex **5** against S-180cisR.

Cytotoxicity. Cytotoxicity was measured by MTT assay, and the IC_{50} values were determined from the dose-dependence of surviving cells after 3 days of exposure to the platinum(II) complexes (Figure 3). The most prominent result is that complex **5** gives a cytotoxicity ($IC_{50} = 8.6 \pm 2.8$ μM) considerably higher than CDDP ($IC_{50} = 59 \pm 5$ μM) and the other complexes (**2**−**4**, **6**, **7**) ($IC_{50} = 37-49$ μM) against S-180cisR. Indeed, the IC_{50}(CDDP)/ IC_{50}(**5**) ratio is 6.9 for S-180cisR. In S-180 parent cells, however, the difference between complex **5** ($IC_{50} = 3.1 \pm 1.0$ μM) and CDDP ($IC_{50} = 2.4 \pm 0.4$ μM) is much smaller. The resistance factor, IC_{50}(S-180cisR)/ IC_{50}(S-180), is 25 for CDDP, whereas it is only 2.8 for complex **5**.

Proliferative Property. Though S-180 parent cells grow very rapidly, S-180cisR cells grow quite slowly in the presence of CDDP with high concentration. Interestingly, when CDDP was removed from the culture medium, S-180cisR cells started growing much more quickly. These results may suggest that S-180cisR cells

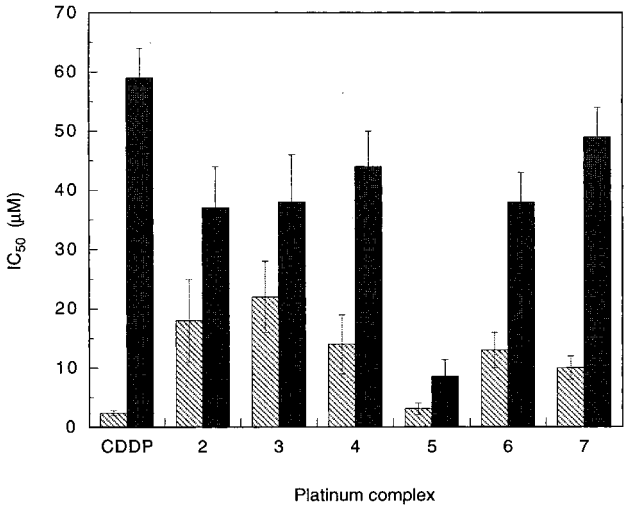

Figure 3. IC_{50} values of platinum complexes: (full bars) CDDP-resistant S-180 cells (S-180cisR); (hatched bars) S-180 parent cells.

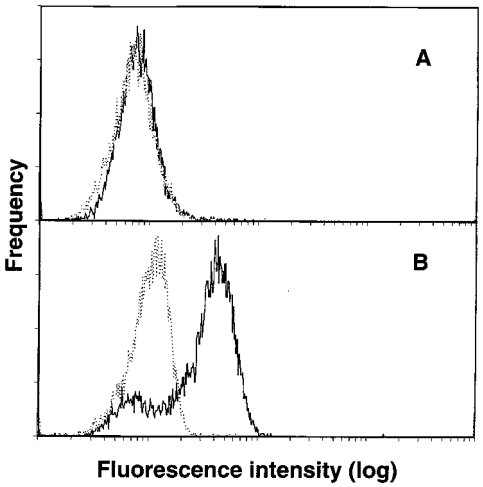

Figure 4. Expression of P-glycoprotein on S-180 parent cells (A) and S-180cisR cells (B): (solid lines) cells were stained with anti-P-glycoprotein and FITC-anti-rabbit IgG (second antibody); (dotted lines) control (second antibody alone).

modulate the cell cycle to acquire the CDDP resistance. It was reported that CDDP binds to DNA and damages it[7] and that the damage of DNA induces G1 cell cycle arrest.[8] Actually, we previously performed cell cycle analysis of Daudi cells and showed that platinum complexes caused G1 arrest.[9] If the cells lower the rate of DNA synthesis, the inhibitory effect by CDDP on the DNA synthesis would be decreased. S-180cisR has been cultured in the presence of 43.6 μM CDDP, whose concentration is much higher than IC_{50} (2.4 μM) for CDDP against S-180 parent cells. During the stepwise selection with increasing concentration of CDDP, the cells that were capable of growing slowly or of rendering the cell cycle slow survived and were established as S-180cisR.

Expression of P-Glycoprotein. To further study the resistance mechanism against CDDP and its derivatives, the expression of P-glycoprotein (P-gP) was analyzed by flow cytometry. As shown in Figure 4, the expression of P-gP by S-180 parent cells was completely negative compared to the negative control (stained with second antibody alone). On the other hand, S-180cisR

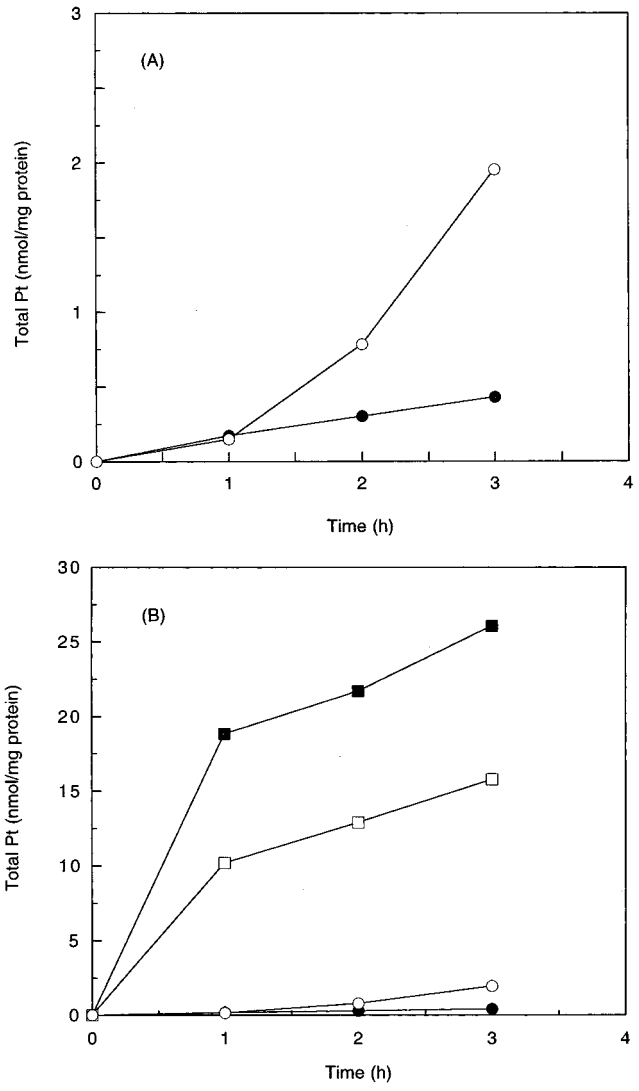

Figure 5. (A) Cellular accumulation of platinum as a function of exposure time of CDDP to S-180 parent cells (○) and S-180cisR cells (●). (B) Cellular accumulation of platinum as a function of exposure time of CDDP to S-180 parent cells (○) and S-180cisR cells (●), 2-phenylpyridine to S-180 parent cells (□) and S-180cisR cells (■).

cells appreciably expressed P-gP, which apparently suggests that one of the possible mechanisms of CDDP resistance in S-180cisR is the activated CDDP efflux induced by P-gP. However, because P-gP is the product of the multidrug-resistant gene, it should pump out all kinds of drugs including CDDP and the other platinum-(II) complexes.[10] Therefore, P-gP cannot account for the high sensitivity of S-180cisR cells to complex **5**.

Cellular Platinum Accumulation. To clarify what factors make S-180cisR cells sensitive to complex **5**, we further examined the accumulation of the plainum(II) complexes in S-180 parent and S-180cisR cells. It has been found that S-180 parent cells incorporate CDDP more quickly than S-180cisR (Figure 5A). In fact, platinum amounts accumulated in S-180 and S-180cisR after 3 h are, respectively, 2.0 (nmol/mg protein) and 0.43 (nmol/mg protein). In comparison with CDDP, complex **5** is incorporated into both cell lines much more quickly (Figure 5B), and the platinum amounts accumulated in S-180 and S-180cisR after 3 h are, respectively, 15.8 (nmol/mg protein) and 26.1 (nmol/mg protein). The platinum accumulation of complex **5** in S-180cisR cells was clearly greater than that in S-180 parent cells.

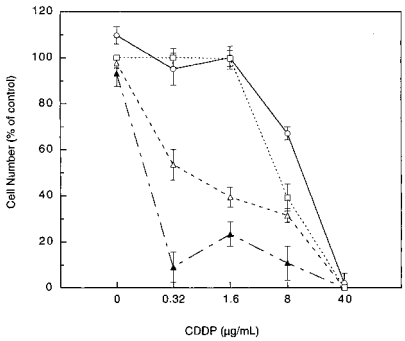

Figure 6. Inhibitory effect of verapamil on cytotoxicity of CDDP against S-180cisR resistant cells. The verapamil concentrations were 0 nM (□), 25 nM (○), 50 nM (△), and 100 nM (▲).

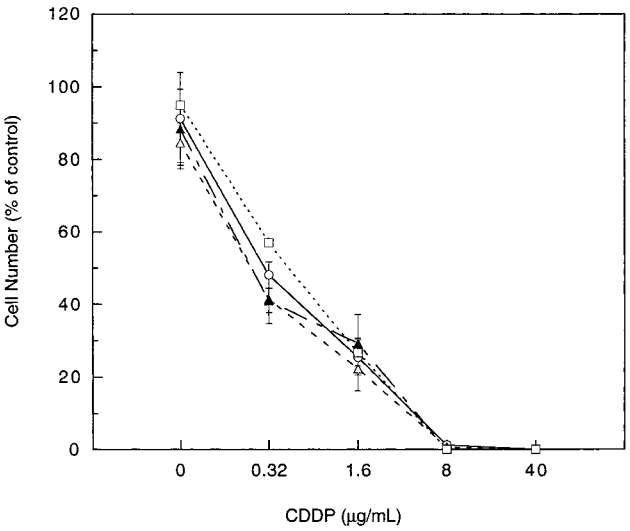

Figure 7. Inhibitory effect of verapamil on cytotoxicity of CDDP against S-180 parent cells. The verapamil concentrations were 0 nM (□), 25 nM (○), 50 nM (△), and 100 nM (▲).

Cytotoxicity against CDDP and Platinum Accumulation after Treatment of Cells with Verapamil. To evaluate the efflux of the platinum complex by P-gP, cells were treated with verapamil,[11] a known inhibitor of P-gP, before the cytotoxicity assay. As shown in Figure 6, the cytotoxicity of CDDP against S-180cisR cells was significantly increased by the treatment with verapamil. In contrast to S-180cisR, treatment of S-180 parent cells with verapamil had no effect on the cytotoxicity (Figure 7). This indicates that efflux of the platinum complex by P-gP is one of the causes of the CDDP resistance in S-180cisR.

We further examined the effect of the treatment with verapamil on platinum accumulation. The platinum accumulation in S-180cisR cells treated with verapamil is actually increased compared to the results of the control experiment (Figure 8). This also means that efflux of the platinum complex by P-gP contributes to the CDDP resistance in S-180cisR cells.

Discussion

It is known that 2-phenylpyridine can form cyclometalated complexes with platinum(II),[6] rhodium(III),[12]

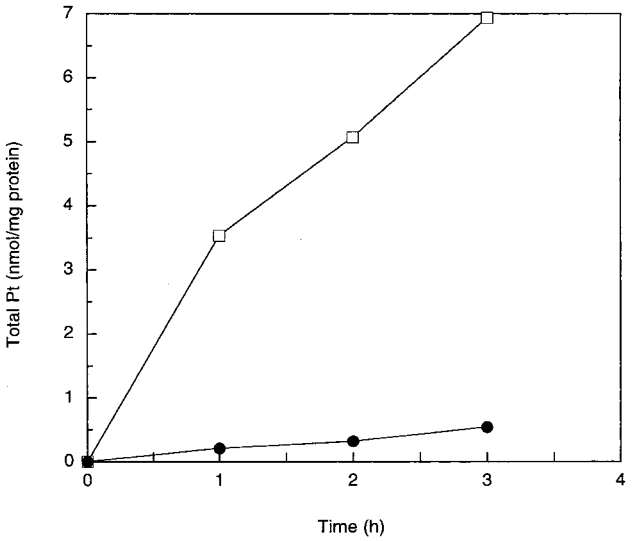

Figure 8. Inhibitory effect of verapamil (100 nM) on cellular accumulation of platinum as a function of exposure time of CDDP to S-180cisR resistant cells. The verapamil concentrations were 0 nM (●) and 100 nM (□).

and so on[13] at the carbon and the nitrogen atoms and that platinum−carbon σ-bonds are fairly stable.[14] One of the noteworthy characteristics of the crystal structure of complex **5** is that the Pt−C distances are unusually short [1.988(8) and 1.973(9) Å] compared to other types of Pt(II)−carbon bond. Indeed, ordinary Pt−C distances are longer than those in complex **5**, e.g., 2.01(3)−2.03(2) Å for [Pt(bph)(1,5-cyclooctadiene)],[15] 2.068(5)−2.092(5) Å for [Pt(η^1: η^1-$C_6H_4C_6H_4$)(PPh_3)$_2$],[16] and 2.052(7)−2.057-(6) Å for *cis*-[Pt{$C_6H_4(PPh_2)$-2}(η^1-C_6H_5)(PPh_3)].[16] This tendency may be due to the effect of the chlorine atom situated at the trans position and/or the intrinsic property of the cyclometaled 2-phenylpyridine ligand. It should be noted that bond distances are well correlated to the stability of the bond; that is, shorter bonds are apt to be more stable. According to this rule and the reported data[14] that Pt−C bonds are generally stable, the Pt−C bond of complex **5** must be also fairly stable. The unusually longer Pt−Cl distances of complex **5**, 2.402(3) and 2.410(2) Å, compared to those of complex **3** (2.305(2) Å) and [$PtCl_2$(2,2′-bipyridine)] (2.291(4) Å),[5] indicate that there is an influence of the C(*m*18) atom situated at the trans position. The weaker Pt−Cl bonds may accelerate the ligand exchange and cause the specific cytotoxicity of complex **5**, whereas detailed mechanisms are under investigation. We also succeeded in the X-ray structural analysis of complex **3**. Similar to complex **2**,[17] the ethyl group of the pyridine ligand is situated at a suitable position to inhibit nucleophilic attack by thiol compounds toward the platinum atom.

We established the novel S-180cisR cell line for examining the cytotoxicity of the platinum complexes. As shown in Figure 3, complex **5** with cyclometalated and nonchelated 2-phenylpyridine ligands gives a high cytotoxicity, while the resistance factor of complex **5** (2.8) is not so different from that of complex **2** (2.1). The following three typical mechanisms have been proposed by Kelland et al. for CDDP resistance:[1] (i) reduced accumulation inside cells, (ii) increased cytoplasmic detoxification via elevated glutathione and/or metallothionein levels, and (iii) enhanced DNA repair and/or increased cellular tolerance to Pt−DNA adducts. Recently, Hour et al. reported that transitional carcinoma cell may gain CDDP resistance through multiple pathways including up-regulation of GST-π, MRP, and probably mdr-1.[2] Concerning mechanism iii, a more attractive idea may be that CDDP resistance is caused by modulation of cell apoptosis. Because complex **2** was supposed to be less reactive than CDDP against nucleophilic attacks by thiols such as gluthatione (mechanism ii), we expected that complexes **3** and **4** bearing more bulky substituent groups should be more effective toward S-180cisR. Contrary to our expectation, however, all these complexes including **2** were actually inactive, which obviously suggests that mechanisms other than mechanism ii should dominate the CDDP resistance of S-180cisR. With respect to mechanism ii, it is instructive to refer to the conclusion that glutathione content may play a less significant role in CDDP resistance in a series of bladder transitional carcinoma cells.[2]

Table 4. Comparison between Ratio of $1/IC_{50}$ and Ratio of Accumulated Pt Amount after 3 h

	$\frac{1/IC_{50}(\mathbf{5})}{1/IC_{50}(CDDP)}$	$\frac{\text{accumulated Pt}(\mathbf{5})}{\text{accumulated Pt(CDDP)}}$
S-180	0.77	7.9
S-180cisR	6.9	61

As suggested in the section of Results, there is a possibility that cell cycle modification may be one of the mechanisms of CDDP resistance. To find another mechanism of CDDP resistance, we measured the amount of P-gP expressed on the cell surface. The selective expression of P-gP in S-180cisR means that the mechanism i can partly explain the acquired CDDP resistance. However, since P-gP pumps out all kinds of drug unselectively, the expression of P-gP cannot explain the large difference in the cytotoxicity of CDDP and complex **5** against S-180cisR. To elucidate this difference, we further estimated the accumulated amount of CDDP and complex **5** in S-180 parent cells and S-180cisR cells and found that complex **5** is accumulated in both S-180 cells and S-180cisR remarkably more quickly than CDDP. When S-180 and S-180cisR cells are compared, complex **5** is more efficiently incorporated into S-180cisR while CDDP is incorporated into S-180 more quickly. It is intriguing to compare the ratio of IC_{50} values and the ratio of accumulation rates as listed in Table 4. It should be noted that the high cytotoxicity of complex **5** against S-180cisR compared to S-180 can be reasonably elucidated by the high cellular accumulation of complex **5**. Hydrophobicity seems to be one of the reasons for this efficient accumulation because transportation of drugs through the cell membrane is generally influenced by their hydrophobicity.[18−20] In fact, complex **5** is more hydrophobic than CDDP in view of the fact that the retention time of complex **5** is longer than that of CDDP in HPLC using an ODS (octadecylsilane) column (data not shown). In S-180 parent cells, the large hydrophobicity of complex **5** is actually reflected in the high ratio (7.9) of platinum accumulation (Table 4). The influence of hydrophobicity seems to emerge more susceptibly in S-180cisR cells; the ratio of platinum accumulation is increased to 61 owing to the elevated incorporation of complex **5** and the suppressed incorporation of CDDP into S-180cisR (Figure 5).

In conclusion, the unusually high CDDP resistance of S-180cisR is supposed to be induced by CDDP efflux by P-gP, suppression of CDDP influx into the cells, and cell cycle modification. Contrary to CDDP, complex **5** is active against S-180cisR as well as S-180 parent cells. We are also considering the interaction with DNA in connection with mechanism iii, viz., enhanced DNA repair and/or increased cellular tolerance to Pt−DNA adducts or suppression of cell apoptosis. Because complex **5** has only one chloride ligand, it cannot form the cross-link intrastrand structure with DNA. Precise mechanisms of the cytotoxicity of complex **5**, including a search of involved proteins, will be the next target.

Experimental Section

Materials and Measurements. 2-Methylpyridine, 3-methylpyridine, 2-ethylpyridine, and tetraethylammonium chloride were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 3-Ethylpyridine and 2-benzylpyridine were commercially available from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). 2-Phenylpyridine was from Sigma-Aldrich Japan Co. (Tokyo, Japan). *cis*-Diamminedichloroplatinum(II) (CDDP, cisplatin) was a product of Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Bradford reagent were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest grade and used without further purification.

Mouse sarcoma 180 cells were obtained from Japanese Cancer Research Resources Bank and maintained in 5% fetal calf serum (FCS), 1% MEM nonessential amino acid (GIBCO BRL, Rockville, MD), and 20 mM HEPES in MEM (Nissui, Tokyo, Japan). Monoclonal rabbit anti-P-glycoprotein polyclonal antibody (mdr Ab-1) was obtained from Oncogene research products (Cambridge, MA). Fluorescein isothiocyanate (FITC)-conjugated goat affinity purified $F(ab')_2$ fragments was purchased from Cappel ICN Pharmaceuticals (Aurora, OH).

Infrared spectra were recorded on a Beckman 4240 IR spectrometer using KBr pellets in the range 200−4000 cm^{-1}. Melting points were measured without calibration using a digital Gallenkamp instrument. Platinum concentration was measured by a Zeeman atomic absorption spectrometer (Spectr AA 400, Varian). Sonication was undertaken with a sonifier (250S, Branson). Flow cytometry was performed by an EPICS ELITE flow cytometer (Beckman Coulter).

Preparation of Platinum Complexes. $[Et_4N][PtCl_3(NH_3)]$ (1). Complex **1** was prepared by a modified method of Abrams et al.[21] as follows. A solution of CDDP, *cis*-$[PtCl_2(NH_3)_2]$, (2 g, 6.67 mmol), and tetraethylammonium chloride (1.2 g, 7.26 mmol) in dimethylacetamide (320 mL) in a two-neck flask was heated to 100 °C (±2 °C) for 8 h (prolonged heating above 105 °C results in extensive decomposition of the reaction mixture). During this time a slow stream of N_2 via the main neck of the flask was bubbled through the reaction mixture using a gas dispersion tube while the side neck of the flask was fitted with a horizontal condenser to collect the dimethylacetamide vapors. At the end of this 8 h period, the reaction volume became approximately 40 mL. After the orange solution was cooled to room temperature, ethyl acetate/hexane (450 mL, 1:1 v/v) was added and the cloudy reaction mixture was kept overnight at −15 °C. This procedure yielded a clear, colorless solution and a thick, orange oil compound. The clear solution was discarded, and the oil was dissolved in doubly distilled water (40 mL). The unreacted CDDP was removed by filtration, and the solution containing complex **1** was evaporated under reduced pressure till dryness to afford the pure orange oil. The orange oil was solidified to give an orange precipitate by treating with ethanol (99.5%, 50 mL) and stirring for 3 h, which was then collected by filtration, washed with ethanol (30 mL) followed by ether, and dried over anhydrous silica gel (1.4 g, 46.8%). The crude product of complex **1** was recrystallized from a water/ethanol/ether mixture (1:1:1 by volume), and within 4 days transparent yellow crystals were formed: mp 124−126 °C; IR (KBr) ν(Pt−Cl) 319 cm^{-1} (s). ^1H NMR (D_2O): δ 1.107 (tt, 3H, J = 3.7 Hz, CH_3), 3.11 (q, 2H, J = 22.0 Hz, CH_2). Anal. ($C_8H_{23}N_2Cl_3Pt\cdot 0.7H_2O$) C, H, N.

***cis*-$[PtCl_2(NH_3)(2\text{-}CH_3C_5H_4N)]$ (2).** Complex **2** was prepared according to a replacement reaction of complex **1**. Complex **1** (449 mg, 1 mmol) was dissolved in water (20 mL) into which 2-methylpyridine (110 mg, 1.11 mmol) was added. The reaction mixture was stirred and heated to 75 °C under reflux for 3 h. A pale-yellow precipitate was formed during this period, and the reaction mixture was further stirred for 2 h at room temperature to improve the chemical yield. The precipitate was collected, washed subsequently with water (4 × 5 mL), ethanol (3 × 5 mL), and ether (20 mL), and dried in vacuo. Complex **2** was recrystallized from ethanol/methanol (6/5 v/v). Yield 45.2% (170 mg); mp 230 °C; IR(KBr) ν(Pt−Cl) 318 cm^{-1} (s); MS *m*/*z* (FAB) 399 [(M +Na)$^+$]. Anal. ($C_6H_{10}N_2Cl_2Pt$) C, H, N.

***cis*-$[PtCl_2(NH_3)(2\text{-}CH_3CH_2C_5H_4N)]$ (3).** Complex **3** was prepared by a procedure similar to that used for complex **2**. The crude product of complex **3** was recrystallized from ethanol/methylene chloride/water (5/4/3 by volume). Transparent green crystals of adequate size and quality for X-ray structural analysis were formed within 1 week. Yield 57.7%; mp 204−206 °C; MS *m*/*z* (FAB) 391 [(M + H)$^+$]. Anal. ($C_7H_{12}N_2Cl_2Pt\cdot 0.38C_2H_6O$) C, N; H: calcd, 3.53; found, 3.11.

***cis*-$[PtCl_2(NH_3)(2\text{-}C_6H_5CH_2C_5H_4N)]$ (4).** Complex **4** was prepared by a procedure similar to that used for complex **2** and was recrystallized from ethanol. After 10 days, the solvent was evaporated completely till dryness at room temperature, and bright-yellow crystals were left. Yield 37.4%; mp 196−198 °C; IR(KBr) ν(Pt−Cl) 322 cm^{-1} (s); MS *m*/*z* (FAB) 452 [(M + H)$^+$]. Anal. ($C_{12}H_{14}N_2Cl_2Pt\cdot 0.59C_2H_6O$) C, N; H: calcd, 3.69; found, 3.13.

***cis*-$[PtCl(2\text{-}C_6H_4C_5H_4N)(2\text{-}C_6H_5C_5H_4N)]$ (5).** We first tried to synthesize *cis*-$[PtCl_2(NH_3)(2\text{-}C_6H_5C_5H_4N)]$, which is an analogue of complex **2**, in a manner similar to that used for complex **2**. One week after the reaction, transparent orange crystals were removed from the mother liquor and were suitable for X-ray studies. The structure of the complex was, however, beyond our expectation; the product turned out to be complex **5**. The crystals were collected by filtration and washed with ethanol and ether. Yield 40%; mp 225−227 °C; IR(KBr, cm^{-1}) ν(Pt−Cl) 328 (w); MS *m*/*z* (FAB) 540 [(M + H)$^+$]. Anal. ($C_{22}H_{17}N_2ClPt\cdot 2.73H_2O$) C, N; H: calcd, 3.84; found, 3.39.

***cis*-$[PtCl_2(NH_3)(3\text{-}CH_3C_5H_4N)]$ (6).** Complex **6** was prepared by a procedure similar to that used for complex **2**. A pale-yellow solid precipitate of complex **6** was formed in 34.3% yield: mp 201 °C; IR(KBr) ν(Pt−Cl) 327 cm^{-1} (s); MS *m*/*z* (FAB) 376 [(M + H)$^+$]. Anal. ($C_6H_{10}N_2Cl_2Pt$) C, H, N.

***cis*-$[PtCl_2(NH_3)(3\text{-}CH_3CH_2C_5H_4N)]$ (7).** Complex **7** was prepared by a procedure similar to that used for complex **2**. Pale-yellow crystals were obtained from ethanol/methanol (1:1) after 2 weeks (32%): mp 182−184 °C; IR(KBr) ν(Pt−Cl) 326 cm^{-1}(s); MS *m*/*z* (FAB) 390 [(M + H)$^+$]. Anal. ($C_7H_{12}N_2Cl_2Pt\cdot 0.1C_2H_6O$) C, H, N.

X-ray Structural Analysis. Single-crystal X-ray diffraction experiments were performed on a Rigaku AFC-7S diffractometer with graphite-monochromated Mo Kα radiation (λ = 0.710 69 Å). Unit cell parameters for a single crystal of complex **3** (0.08 mm × 0.20 mm × 0.25 mm) or complex **5** (0.03 mm × 0.18 mm × 0.20 mm) were determined by a least-squares refinement of 25 reflections in the range 25.3° < 2θ < 29.6° or 29.4° < 2θ < 30.0°, respectively. The intensity data were collected by the ω−2θ scan mode up to 2θ = 50°. The intensities were corrected for Lorentz and polarization effects. Empirical absorption corrections were also applied. The structures were solved by a direct method and refined by full-matrix least-squares techniques on F using an anisotropic thermal parameter for non-H atoms. Hydrogen atoms were included but not refined. In complex **3**, R = 0.032 and R_w = 0.039 were finally obtained for 1489 observed reflections [$I > 3.00\,\sigma(I)$],

and in complex **5**, $R = 0.034$ and $R_w = 0.041$ were also obtained for 4405 observed reflections [$I > 2.00\ \sigma(I)$]. All the calculations were performed using the teXsan crystallographic software package of Molecular Structure Corporation.

Establishment of S-180cisR. A resistant subline, S-180cisR, was established from the parent S-180 by stepwise selection with increasing concentrations of CDDP over a period of 3 years. When the resistance was obtained, cells were chronically exposed to 43.6 μM CDDP concentration.

Cytotoxicity Assay. The tetrazolium-based semiautomated colorimetric assay (MTT assay) developed by Carmichael et al.[22] was modified and used for cytotoxicity assay. Briefly, S-180 or S-180cisR cells (2×10^4/mL, 180 μL/well) in a culture medium were seeded in a 96-well flat-bottom microplate (Falcon) and 20 μL of drug solutions of graded concentrations was added to each well. The plate was incubated for 3 days at 37 °C in 5% CO_2. The MTT reagent was prepared at a concentration of 2 mg/mL in Dulbecco's PBS without calcium and magnesium and stored at 4 °C. After 3 days, MTT reagent (25 μL/well) was added to each well. After another incubation for 4 h at 37 °C, the plate was centrifuged at 3000 rpm for 10 min and the medium was removed. To solubilize the resulting MTT−formazan, 200 μL/well of dimethyl sulfoxide (DMSO) was added to each well followed by thorough mixing with a mechanical plate mixer. Absorbance at 540 nm was measured on a Shimadzu CS-9300PC plate reader. In the inhibitory experiment, a known inhibitor of P-gP, verapamil, was dissolved in ethanol to give a stock solution of 10 mM (reference). Verepamil (final concentration, 100, 50, 25 nM) was added to S-180cisR or S-180 parent cells in microplates and cultured for 1 h before the normal cytotoxicity assay.

Proliferation Assay. S-180 or S-180cisR cells (2×10^4/mL, 1 mL/well) in a culture medium (with 43.6 μM CDDP for S-180cisR) were seeded in a 24-well plate (Corning Costar). Cells were harvested from wells by treatment with trypsin−EDTA, washed, and counted under a microscope.

Flow Cytometry Analysis. S-180 or S-180cisR cells (1×10^6) were preincubated on ice for 30 min with monoclonal rabbit anti-P-glycoprotein polyclonal antibody (1/100, mdr Ab-1), washed three times with CMF−PBS containing 0.1% BSA and 0.1% NaN_3 followed by incubation with fluorescein isothiocyanate (FITC)-conjugated goat affinity purified $F(ab')_2$ fragments to rabbit IgG (1/100, whole molecule) for 30 min on ice. Cells were then washed three times and analyzed by flow cytometry.

Cellular Platinum Accumulation. Cellular platinum accumulation was examined according to the previous method.[19] S-180 or S-180cisR cells (3×10^6) in the exponential growth phase were exposed to the samples (100 μM) with various incubation times (1−3 h) at 37 °C in 5% CO_2. Immediately after the exposure, the medium was removed and the cells were washed twice with CMF−PBS. The cells were harvested by trypsin−EDTA and washed three times more and suspended in 0.5 mL of PBS and sonicated for 3 min on ice. When cells were treated with verapamil, verapamil (final concentration, 100 nM) was added to a culture medium 1 h prior to the addition of CDDP. Bradford reagent was used for a protein determination of the sonicate. BSA solution (2 mg/mL) was used as a standard. Cellular platinum concentrations were measured directly from the cell sonicate by Zeeman atomic absorption spectroscopy. The platinum concentration in the sample was measured by the internal addition method using platinum standard solutions (1 ppm) in 0.2% nitric acid. The amount of accumulated Pt was expressed as nmol/mg of protein.

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References

(1) Kelland, L. R.; Abel, G.; McKeage, M. J.; Jones, M.; Goddard, P. M.; Valenti, M.; Murrer, B. A.; Harrap, K. R. Preclinical anticancer evaluation of bis-acetato-amminedichloro-cyclohexylamine platinum(IV). *Cancer Res.* **1993**, *53*, 2581−2586.
(2) Hour, T. C.; Chen, J.; Huang, C. Y.; Guan, J. Y.; Lu, S. H.; Hsieh, C. Y.; Pu, Y. S. Characterization of chemoresistance mechanisms in a series of cisplatin-resistant transitional carcinoma cell liens. *Anticancer Res.* **2000**, *20*, 3221−3225.
(3) Holford, J.; Sharp, S. Y.; Murrer, B. A.; Abrams, M.; Kelland, L. R. In vitro circumvention of cisplatin resistance by the novel sterically hindered platinum complex AMD473. *Br. J. Cancer* **1998**, *77*, 366−373.
(4) El-Mehasseb, I. M.; Kodaka, M.; Okada, T.; Tomohiro, T.; Okamoto, K.; Okuno, H. Platinum(II) complex with cyclometalating 2-phenylpyridine ligand showing high cyctotoxicity against cisplatin-resistant cell. *J. Inorg. Biochem.* **2001**, *84*, 157−158.
(5) Herber, R. H.; Croft, M.; Coyer, M. J.; Bilash, B.; Sahiner, A. Origin of polychromism of cis square-planar platinum(II) complexes: comparison of two forms of $[Pt(2,2'\text{-bpy})(Cl)_2]$. *Inorg. Chem.* **1994**, *33*, 2422−2426.
(6) Mdleleni, M. M.; Bridgewater, J. S.; Watts, R. J.; Ford, P. C. Synthesis, structure, and spectroscopic properties of ortho-metalated platinum(II) complexes. *Inorg. Chem.* **1995**, *34*, 2334−2342.
(7) Jamieson, E. R.; Lippard, S. J. *Chem. Rev.* **1999**, *99*, 2467−2498.
(8) Agami, R.; Bernards, R. Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. *Cell* **2000**, *102*, 55−66.
(9) Okada, T.; Shimura, T.; Nakanishi, H.; Okuno, H. Inhibition of DNA synthesis in vitro by anticancer platinum pyrimidine greens against Daudi cells. *Chem. Pharm. Bull.* **1992**, *40*, 264−266.
(10) Endicott, J. A.; Ling, V. The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu. Rev. Biochem.* **1989**, *58*, 137−171.
(11) Mistry, P.; Stewart, A. J.; Dangerfield, W.; Okiji, S.; Liddle, C.; Bootle, D.; Plumb, J. A.; Templeton, D.; Charlton, P. In vitro and in vivo reversal of P-glycoprotein-mediated multidrug resistance by a novel potent modulator, XR9576. *Cancer Res.* **2001**, *61*, 749−758.
(12) Nonoyama, M.; Yamasaki, K. Rhodium(II) complexes of benzo[*h*]quinoline and 2-phenylpyridine. *Inorg. Nucl. Chem. Lett.* **1971**, *7*, 943−946.
(13) Bruce, M. I.; Goodall, B. L.; Matsuda, I. Cyclometallation reactions. XIII Reactions of phenyl-substituted heterocyclic nitrogen-donor ligands. *Aust. J. Chem.* **1975**, *28*, 1259−1264.
(14) Ashcroft, S. J.; Mortimer, C. T. The strength of the platinum−carbon σ-bond in the complex $(Et_3P)_2PtPh_2$. *J. Chem. Soc. A* **1967**, 930−931.
(15) Chen, Y.; Woods, C.; Perkovic, M. W.; Rillema, D. P. Crystal structure, physical and photophysical properties of a platinum(II) complex coordinated to the biphenyl dianion and cyclooctadiene. *J. Chem. Crystallogr.* **1996**, *26*, 527−531.
(16) Bennett, M. A.; Dirnberger, T.; Hockless, D. C. R.; Wenger, E.; Willis, A. C. Generation of (η^2-benzyne)bis(triphenylphosphine)-platinum(0): orthometallation of the $Pt(PPh_3)_2$ complexes of benzyne (C_6H_4) and cyclohexyne (C_6H_8). *J. Chem. Soc., Dalton Trans.* **1998**, 271−277.
(17) Chen, Y.; Guo, Z.; Parsons, S.; Sadler, P. J. Stereospecific and kinetic control over the hydrolysis of a sterically hindered platinum picoline anticancer complex. *Chem.—Eur. J.* **1998**, *4*, 672−676.
(18) Dohta, Y.; Browning, C. S.; Rekonen, P.; Kodaka, M.; Okada, T.; Okamoto, K.; Natale, R.; Yip, C.; Farrar, D. H.; Okuno, H. Preparation, structure and cytotoxicity of *cis*-diammineplatinum-(II) dinuclear complexes with 1-alkyluracil and imidate ligands. *Inorg. Chim. Acta* **1997**, *263*, 69−79.
(19) Rekonen, P.; Dohta, Y.; Kodaka, M.; Okada, T.; Okamoto, K.; Okuno, H. Cytotoxicity of platinum(II) dinuclear complexes with 1-alkylthymine ligands against mouse sarcoma 180 cells. *J. Med. Chem.* **1997**, *40*, 515−519.
(20) Kodaka, M.; Dohta, Y.; Rekonen, P.; Okada, T.; Okuno, H. Physicochemical factors for cytotoxic activity in platinum dinuclear complexes with pyrimidine and imide ligands. *Biophys. Chem.* **1998**, *75*, 259−270.
(21) Abrams, M. J.; Giandomenico, C. M.; Vollano, J. F.; Schwartz, D. A. A convenient preparation of the amminetrichloroplatinate-(II) anion. *Inorg. Chim. Acta* **1987**, *131*, 3−4.
(22) Carmichael, J.; Degraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res.* **1987**, *47*, 936−942.

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