

In Vitro Cytotoxicity of a Novel Platinum(II) Coordination Complex Containing Diaminocyclohexane and Dichloropropane

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We have synthesized a novel platinum(II) coordination complex containing *cis*-1,2-diaminocyclohexane (DACH) as a carrier ligand and 1,3-dichloropropane (DCP) as a leaving group. A new series of [Pt(*cis*-DACH)(DCP)](PC) was evaluated for its cytotoxic activity on MKN-45 human gastric adenocarcinoma cells and normal primary cultured kidney cells. The new platinum complex has demonstrated high efficacy in the cytotoxicity against MKN-45/P, MKN-45/ADM and MKN-45/CDDP cell-lines. The cytotoxicity of PC against rabbit proximal renal tubular cells, human renal cortical cells and human renal cortical tissues, determined by MTT assay, the [³H]-thymidine uptake and glucose consumption tests, was found to be quite less than those of cisplatin. Based on these results, this novel platinum(II) coordination complex appears to be better for improving antitumor activities with low nephrotoxicity and is a valuable lead in the development of new, clinically available anticancer chemotherapeutic agents.

Key Words: Selective cytotoxicity, Human gastric adenocarcinoma cells, Nephrotoxicity, Platinum coordination complex

INTRODUCTION

The introduction of the square-planar complex cisplatin into the clinical treatment of cancer has resulted in the excellent response rates for some tumor types, especially testicular and ovarian cancers (Rosenberg et al, 1969). While the unfavorable nephrotoxicity profile of cisplatin has been overcome by the development of the second-generation agent, carboplatin, there remains an unquestionable need for additional platinum(II) coordination complexes which have more favorable therapeutic indices and circumvent resistance (Calvert et al, 1985). As with other cancer chemotherapeutic agents, cellular resistance to the clinically used platinum agents, cisplatin and carboplatin, represents a major clinical limitation to the efficacy (Mangioni et al, 1989; Einhorn, 1990).

It is known that renal cortical accumulation of cisplatin causes necrosis of the proximal tubule and late development of internal cysts (Litterest et al, 1981). The major toxicological limitation is its dose-related accumulative and irreversible side effects of severe nephrotoxicity, nausea and vomiting (Krakoff, 1979; Andrews & Howel, 1990).

The antitumor activity of platinum complexes containing 1,2-diaminocyclohexane (DACH) was investigated as a carrier ligand (Connors et al, 1972, Gale et al, 1974). Kidani (1985) synthesized Pt(oxalato)(*trans*-*l*-DACH)[*l*-OPH] using oxalic acid with selected *trans*-*l*-DACH among *trans*-*l*-, *trans*-*d*- and *cis*-isomers.

Consequently, there is much interest in obtaining agents that have less toxicity and more favorable anticancer chemotherapeutic indices. To accomplish this aim, we have synthesized a new platinum(II) coordination complex containing *cis*-DACH as a carrier ligand and 1,3-dichloropropane (DCP) as a leaving group.

In the present study we evaluated *in vitro* antitumor

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activity of the new platinum complex on the various human gastric adenocarcinoma cell lines and its cytotoxicity on rabbit proximal tubular cells, human renal cortical cells and on histocultured human renal cortical tissues.

METHODS

Materials

Dulbecco's Modified Eagle's (DMEM), Ham's Nutrient Mixture F12 (F12) and RPMI-1640 media were purchased from GIBCO (Grand Island, NY, USA). Cisplatin, hormones, transferrin, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Powdered medium, EDTA-trypsin and soybean trypsin inhibitor were obtained from Life Technologies (Grand Island, NY, USA). Class IV collagenase was obtained from Worthington Co. (Freedhold, NY, USA). Iron oxide was prepared by the method described by Cook & Pickering (1958). Stock solutions of iron oxide in 0.9% NaCl were sterilized in an autoclave and diluted with phosphate-buffered saline (PBS) prior to the application. The novel platinum coordination complex, Pt(II)(*cis*-DACH) (DCP)[PC] (Fig. 1); the PC utilized was synthesized in the Department of Pharmacochemistry, Kyung Hee University College of Pharmacy. This agent was dissolved in sterilized cell culture media prior to usage.

Cell cultures

Primary rabbit kidney proximal tubular cells: Each

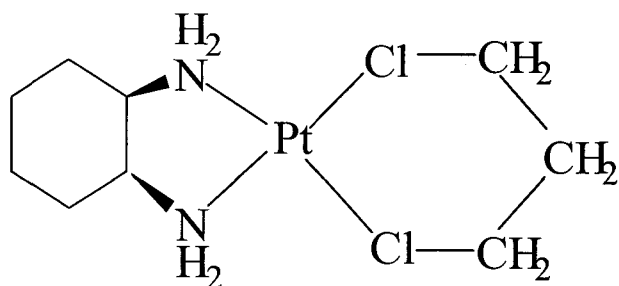


Fig 1. Chemical structure of a new platinum(II) coordination complex. (1,3-Dichloropropane)-*cis*-1,2-diaminocyclohexaneplatinum(II) [Pt(II)(*cis*-DACH)(DCP)]. DCP: 1,3-Dichloropropane, DACH: 1,2-Diaminocyclohexane.

growth study was conducted with an individual primary culture set derived from purified proximal tubules obtained from a single rabbit kidney. Individual kidneys were obtained from male New Zealand White rabbits (2~2.5 kg). Primary cultures were initiated from purified rabbit kidney proximal tubules by a modification of the method described by Chung et al (1982) (Jung et al, 1992; Taub, 1997). To summarize, each kidney was perfused via the renal artery, first with PBS to remove the blood, and then with a 0.5% (w/v) solution of iron oxide. The renal cortex was removed by slicing and renal cortical slice were homogenized in a sterile Dounce homogenizer. The tubules in the homogenate were then filtered through a 253 μ mesh screen, and then through an 83 μ mesh screen. The material which remained on the 83 μ mesh (proximal tubules and glomeruli) was transferred to a 50 ml tube containing basal medium (DME/F12 medium). The glomeruli (which were covered with iron oxide) were then removed with a stir bar. The remaining material, the renal proximal tubules, was incubated for two minutes at 23°C in basal medium containing 0.125 mg/ml collagenase (class IV) and 0.025% soybean trypsin inhibitor. The tubules were washed by centrifugation, and the primary cultures were inoculated into 35 mm culture dishes containing basal medium further supplemented with 5 μ g/ml insulin, 5×10^{-8} M hydrocortisone and 5 μ g/ml transferrin. Approximately 100 culture dishes of proximal tubule cells could be obtained from a single rabbit kidney preparation. The cultures were maintained in a humidified 5% CO₂/95% air environment at 37°C. The purified rabbit kidney proximal tubules attached to the culture dish within a day of plating. The medium was replaced the day after plating so as to remove unattached tubules. The medium was replaced every two days thereafter. During the initial 4 days, cell outgrowth occurred from the attached tubules in culture.

Primary human kidney cortical cells: Normal human kidney tissues were obtained at the time of radical nephrectomy in patients with renal cell carcinoma. Human kidney cortical tissues were washed 3 or 4 times with DME/F12 medium (1 : 1 ratio) supplemented with penicillin G/streptomycin. A single-cell suspension was obtained by mechanical disaggregation with a sterilized surgical knife and subsequent incubation with collagenase (0.124 mg/ml) and trypsin inhibitor (2.5 mg/ml) for 2 minutes. The process was stopped by centrifugation (1,000 rpm for

5 minutes) and the particles of the kidney cortical tissues were suspended in DME/F12 medium supplemented with insulin ($5 \mu\text{g/ml}$), transferrin ($5 \mu\text{g/ml}$), hydrocortisone ($5 \times 10^{-8} \text{ M}$), triiodothyronine ($5 \mu\text{g/ml}$), prostaglandin E_1 ($5 \times 10^{-8} \text{ M}$) and 1% fetal bovine serum (FBS) (GIBCO, Grand Island, NY). These suspended cells were seeded in a culture dish and kept in an incubator at 37°C with a highly humidified 5% $\text{CO}_2/95\%$ air. After 2 weeks of incubation, the cells were confluent and used for experiments (Jung et al, 1998).

Cancer cell lines: Human gastric adenocarcinoma cell lines (MKN-45/P, MKN-45/ADM and MKN-45/CDDP) were obtained from Cell-Biology Lab. of Atomic Power Hospital, Seoul, Korea. MKN-45 cells were cultured in RPMI 1,640 medium supplemented with 10% FBS, 20 mM sodium bicarbonate, 15 mM HEPES, 92 units/ml penicillin G and $200 \mu\text{g/ml}$ streptomycin. All the cells were incubated in humidified 5% $\text{CO}_2/95\%$ air at 37°C .

Histoculture: Normal kidney tissues, identified by frozen section at the time of radical nephrectomy, were transported in a container to the laboratory which is near the operating room. The normal human kidney tissues were divided into 2~3 mm diameter pieces and five pieces were placed on the top of each previously hydrated Spongostan gel ($1 \times 1 \times 1 \text{ cm}$) (Health Design Industries, Rochester, NY, USA). One gel was put in each well of a six-well plate. 3 ml of MEM supplemented with 10% FBS, $50 \mu\text{g/ml}$ gentamicin and $1 \mu\text{g/ml}$ cefotaxime were added to each well of the plate. The final volume of the medium was sufficient to reach the upper gel surface without immersing it. Culture plates were covered and maintained in humidified 5% $\text{CO}_2/95\%$ air atmosphere at 37°C . The histoculture was incubated in medium containing $50 \mu\text{M}$ of newly synthesized platinum (II) coordination complex and cisplatin, and the specimens were washed with PBS and transferred to fresh medium (Freeman & Hoffman, 1986; Chang et al, 1992).

In vitro antitumor activity

Cancer cell lines were cultured in the growth media for each cell line in an incubator with a highly humidified 5% $\text{CO}_2/95\%$ air atmosphere at 37°C . After 3 days of incubation, all cell lines were dissociated with 0.025% trypsin-EDTA for dispersal and centrifuged at 1,000 rpm for 5 minutes. The pellets

were suspended with fresh medium. Individual wells of a 96-well tissue culture microtiter plate were inoculated with 0.1 ml of the appropriate medium containing 10^5 cells. New platinum(II) coordination complex and cisplatin were added in various concentrations. After 48 hours of incubation, 0.05 ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each well. After 4 hours of exposure, 0.05 ml of DMSO was added to each well and absorption rates at 630 nm were measured using an ELISA reader (Shimoyama et al, 1989).

Cytotoxicity on normal kidney cells

The confluent primary rabbit kidney proximal tubular and human kidney cortical cells were disaggregated by using 0.02% EDTA in 0.05% trypsin. Single-cell suspension was then produced by centrifugation (1,000 rpm, 10 minutes) and resuspension in DME/F12 medium. This suspension was seeded at a cell density of 10^5 cells per well on a 96-well plate, with $100 \mu\text{l}$ of medium per well. Drugs were added in various concentrations (final concentrations: 5, 25, 50, 250 and $500 \mu\text{M}$) and the cell cultures were incubated for 48 hours in an incubator a highly humidified atmosphere of 5% $\text{CO}_2/95\%$ air at 37°C . Thereafter, $50 \mu\text{l}$ of the medium containing MTT (5 mg/ml) was removed and the wells were washed with PBS, and then $50 \mu\text{l}$ of DMSO was added to each well to solubilize the precipitates. Then the plates were transferred to an ELISA reader to measure the absorbance of the extracted dye at 630 nm. All experiments were performed at least 3 times, with 6 wells for each concentration of the new platinum (II) coordination complex and cisplatin (Mossman, 1973).

Thymidine uptake

Primary cultured proximal tubular and cortical cells were seeded in each well of a 24-well plate at a cell density of 10^5 cells per well. After 1 hour of incubation, platinum(II) coordination complexes were added, and the cells were incubated for 48 hours in a humidified incubator containing an atmosphere of 5% $\text{CO}_2/95\%$ air at 37°C . Thereafter, [^3H]-thymidine ($1 \mu\text{Ci/ml}$ specific radioactivity) was added to each well, and cells were incubated for 24 hours in the same humidified incubator.

After trypsin-EDTA treatment, all cells were collected and washed twice with 10% TCA and phosphate buffer. The cells were then solubilized with 0.5 M NaOH for 2 hours at 37°C. The cells were neutralized with 0.5 M HCl and a scintillating cocktail was added (Scint-AXF, Packard, CT). Then the amount of radioactivity uptake by the cells was determined in a β -counter (Beckman, LS 5000 TD).

Glucose consumption by histocultured specimens

This study was performed essentially as described by Chang et al (1992). In brief, 50 μ l of culture medium was removed every 24 hours for determination of medium glucose content in triplicate using the HK 20 assay kit from Sigma (St. Louis, MO, USA). Measurements were made by monitoring the change in optical density at 340 nm due to the reduction of NAD catalyzed by hexokinase with the glucose content of the medium plotted in semilog form against time using the Sigma plot program (Janel Scientific, Corte, Madera, CA, USA). A simple exponential model of glucose consumption was then fitted to the data with the Systat program (Systat., Inc, Evanston, IL, USA). The half-life of glucose was calculated from the slope parameter of this model using the equation $t=0.693/s$, where s =slope of the best fit linear regression line or the natural log of the glucose concentration plotted against time. The glucose content of the medium was measured daily for 3 days. The log values over 3 days were plotted against time and the slope of the best-fit line was taken as a glucose consumption rate during the 3-day measurement period (one period).

RESULTS

Selective cytotoxicity

The sensitivity of the MKN-45 human gastric cancer cell-lines to the 2 platinum-containing compounds are shown in Table 1. Drug concentration (CC_{50}), cytotoxic index by 50%, was determined by MTT assay after treatment with the compounds for 48 hr. CC_{50} of cisplatin for the MKN-45/P and MKN-45/ADM cells did not differ significantly.

To investigate renal cytotoxicity, primary cultured rabbit and human normal kidney cells were incubated with various concentrations of cisplatin and the new

compound for 48 hr. The cytotoxicities of PC on rabbit kidney proximal tubular cells and human kidney cortical cells were markedly lower, by about 7- and 8-fold, compared with cisplatin, respectively (Table 2 and 3).

Effect on [3H] thymidine uptake by normal kidney cells

To evaluate the nephrotoxicity of the new Pt(II) complex, we measured [3H] thymidine uptake by rabbit proximal tubular cells and human renal cortical cells.

Rabbit renal proximal tubular cells: The cytotoxicities of cisplatin and PC against primary cultured rabbit renal proximal tubular cells are shown in Table 2. PC (CC_{50} : 217.3 μ M) showed a lower level cytotoxicity compared to cisplatin. In addition to the MTT assay, cytotoxicity was determined using [3H] thymidine uptake assay. Results are shown in Table 4. At

Table 1. *In vitro* cytotoxicities of Pt(II) complexes on MKN-45 human gastric adenocarcinoma cell lines

Compounds	CC_{50} (μ M)		
	MKN-45/P ^{a)}	MKN-45/ADM ^{b)}	MKN-45/CDDP ^{c)}
Cisplatin	20.5 \pm 4.13 ^{d)}	22.7 \pm 3.85	119.6 \pm 17.28
PC ^{e)}	24.0 \pm 4.55	27.4 \pm 5.19	47.9 \pm 6.54*

CC_{50} indicates mean cytotoxic concentration with MTT assay, a) Wild type cell, b) Adriamycin-resistant cell, c) Cisplatin-resistant cell, d) Each value is the mean \pm S.E of three experiments, e) [Pt(II)(*cis*-DACH)(DCP)].

*Significantly different from cisplatin-control ($P < 0.05$).

Table 2. *In vitro* cytotoxicities of platinum complexes on proximal tubular cells of rabbit kidney

Compounds	CC_{50} (μ M)	Ratio ^{a)}
Cisplatin	29.1 \pm 4.25 ^{b)}	—
PC ^{c)}	217.3 \pm 34.63*	7.47

a) The ratio is the CC_{50} of the PC divided by that of the cisplatin, b) Each value is the mean \pm S.E of at least three experiments, c) [Pt(II)(*cis*-DACH)(DCP)].

*Significantly different from cisplatin-control ($P < 0.05$).

a concentration of 5×10^{-5} M, cells treated with PC showed a [3 H] thymidine uptake of 52.9%, whereas those treated with cisplatin showed a value of 7.6%. These results indicate that the cytotoxicity of PC was significantly less than that of cisplatin, and the [3 H] thymidine uptake assay is more sensitive than the MTT assay.

Table 3. *In vitro* cytotoxicities of platinum complexes on cortical cells of human kidney

Compounds	CC ₅₀ (μ M)	Ratio ^{a)}
Cisplatin	27.6 \pm 3.95 ^{b)}	—
PC ^{c)}	225.8 \pm 37.07*	8.18

a) The ratio is the CC₅₀ of the PC divided by that of the cisplatin, b) Each value is the mean \pm S.E of at least three experiments, c) [Pt(II)(*cis*-DACH)](DCP).

*Significantly different from cisplatin-control (P < 0.05).

Table 4. Effect of platinum(II) coordination complexes on 3 H-thymidine incorporation into primary cultured proximal tubular cells of rabbit-kidney

Compounds	[3 H]-thymidine uptake (cpm/10 ⁵ cells)	Uptake rate (%)
Control	587.3 \pm 71.45	100.0
Cisplatin	44.5 \pm 6.07	7.6
PC	310.8 \pm 40.29	52.9

Concentration of platinum (II) coordination complexes in culture medium; 5×10^{-5} M, PC: [Pt(II)(*cis*-DACH)](DCP), Values are means \pm S.E. All the incorporations were determined in triplicate.

Human renal cortical cells: PC showed significantly lower cytotoxicity (CC₅₀ : 225.8 μ M) than cisplatin (CC₅₀ : 27.6 μ M) (Table 3). Table 5 shows that [3 H] thymidine incorporation is more significantly inhibited by cisplatin (91.8%) than by PC (46.4%) in human renal cortical cells.

Effect on glucose consumption by human kidney tissues

The glucose consumption rate was calculated as the half-life of the glucose in which may vary with the drug tested and with the drug concentration (Table 6). In measuring the glucose consumption, one period was defined as more than 3 measurements per day in a 4-week culturing period of human renal cortical tissue. The glucose half-life before adding PC was approximately 60~65 hr and did not show any significant increase for 4 periods, and then increased to over 74 hr from the fifth period up to the 7th

Table 5. Effect of platinum(II) coordination complexes on 3 H-thymidine incorporation into primary cultured renal cortical cells of human-kidney

Compounds	[3 H]-thymidine uptake (cpm/10 ⁵ cells)	Uptake rate (%)
Control	601.7 \pm 67.20	100.0
Cisplatin	49.1 \pm 5.75	8.2
PC	322.4 \pm 38.51	53.6

Concentration of platinum(II) coordination complexes in culture medium; 5×10^{-5} M, PC: [Pt(II)(*cis*-DACH)](DCP), Values are means \pm S.E. All the incorporations were determined in triplicate.

Table 6. Change of glucose half-life on histocultured renal cortical tissue specimens that were treated with 10^{-4} M cisplatin and 10^{-4} M experiment drug (PC) for 72 hours exposure.

Compounds	Glucose half-life (hours, M \pm S.E)						
	1	2	3	4	5	6	7 (period)
Cisp	60.3 \pm 7.06	62.7 \pm 8.25	64.2 \pm 7.14	329.6 \pm 42.13	370.5 \pm 47.06	340.4 \pm 41.22	294.8 \pm 35.09
PC	61.5 \pm 6.51	60.9 \pm 7.57	62.6 \pm 8.03	64.8 \pm 8.15**	69.3 \pm 8.09**	71.8 \pm 9.45*	74.4 \pm 9.78*

One period constitutes 3 days, All experimental histocultures had their own control that was treatments three period. Glucose half-life on control specimen was very steady, Cisp: Cisplatin, PC: [Pt(*cis*-DACH)](DCP).

*Significantly different from cisplatin-control (*P < 0.05, **P < 0.01)

period. However, the effect of PC was less marked than that of cisplatin. The half-life of the medium glucose content was longer in cisplatin-treated cultures than in those treated with PC. Cultures treated with PC showed a slight increase in glucose half-life compared to the control. In the case of cisplatin the glucose half-life was lengthened after treatment compared with the pre-treatment control. The specimens treated with 10^{-4} M of cisplatin still exhibited prolonged glucose half-life in the post-treatment period.

DISCUSSION

Since Rosenberg et al (1969) first described the antitumor activity of cisplatin, it has become an important drug in the treatment of selected human malignant tumors. However, its clinical uses are limited by its dose-related renal toxicity. While the unfavorable nephrotoxicity has been overcome by the development of the second-generation agent, carboplatin, there remains an unquestionable need for further improved platinum-containing compounds which have more favorable therapeutic indices and circumvent resistance.

The advanced knowledge of structure-activity relationship of platinum(II) complexes has clarified that the carrier ligands and its leaving groups are essential for their *in vivo* antitumor activities. The contribution of the carrier ligand may be related to the potency and spectrum of antitumor activity, and that of the leaving group may be related to the dissociation rate from the platinum complex. One of the structural modifications that is widely accepted as having resulted in an increased therapeutic index is the attachment of DACH (Connors et al, 1972; Clear & Hoeschele, 1973; Gale et al, 1974; Ridgway et al, 1977; Jung et al, 1998). Several forms of DACH compounds, such as cis-DACH, trans-*l*-DACH, and trans-*d*-DACH, exist. Among these DACH derivatives, trans-*l*-DACH has been known to have more significant antitumor activity (Inagaki & Kidani, 1986). Moreover, it is essential to consider that the leaving group is an important factor influencing the activity of the platinum coordination complexes. The platinum(II) coordination complexes appear to penetrate cell membranes by diffusion, and then the leaving group is displaced directly by hydrolysis with nucleic acid groups. These studies indicated that the

dissociation of the leaving groups is an important factor in antitumor and toxic activities. However, when the rate of dissociation is much higher, it causes toxic effects since the platinum complexes react with normal protein instead of DNA in cancer cells. On the other hand, when the dissociation rate is too low, it is excreted into the extracellular compartment before showing any antitumor activity.

The mechanism of the nephrotoxicity induced by platinum(II) coordination complexes is not completely understood. To date, investigators have demonstrated that cytotoxicity induced by a variety of platinum(II) coordination complexes may be attributed at least in part to the inhibition of blood flow in the kidney or the depletion of intracellular glutathione (Meijer et al, 1982). Dobyhan et al (1980) have reported that the site-specific injury was in the pars recta (S3) segment of the proximal tubules. Gonzalez-Vitale et al (1980) noted that the distal tubule was the most consistently damaged region in the human kidney. Furthermore, a number of investigators (Jones et al, 1986) suggested that both the proximal and distal tubules have been damaged. The nephrotoxicity of cisplatin found in this study is very similar to that reported previously, in terms of both the histopathology and the effects on various measures of renal function (Dobyhan et al, 1980).

The new platinum(II) coordination complex produced and used in this study has generally been screened for antitumor activity and nephrotoxicity using human bladder carcinoma cell lines and normal human and rabbit kidney cells, respectively. The new synthetic platinum(II) coordination complex, PC, exhibited significant *in vitro* antitumor activity against human gastric adenocarcinoma cell lines.

A criteria for *in vitro* antitumor activity is generally expressed in the cytotoxicity index and is accepted as possible antitumor drugs. PC showed antitumor activity comparable to cisplatin. The comparison of antitumor activities of PC and cisplatin exhibited similarity in levels of activity against MKN-45 human gastric cancer cell lines. Since the only difference in chemical structure between PC and cisplatin is the "DACH-isomer", it can be inferred that the dissociation times or rates are different after absorption of the compounds in the cells due to the different leaving groups.

In this study, PC was less cytotoxic than cisplatin in renal tissues. It is conceivable that the modifications of the carrier ligand as a DACH and the

leaving group as a DCP derived from cisplatin significantly decreased nephrotoxicity. Mortine & Borch (1998) reported that the LLC-PK₁ (pig proximal tubule epithelial cell line) was a good model in evaluating the nephrotoxicity induced by cisplatin *in vitro*. My present study using primary cultured cells showed reliable data although the LLC-PK₁ cell line was not used.

The appearance of glucose in urine is one of the early signs of proximal tubular dysfunction *in vivo*, and therefore we chose the glucose consumption test as a parameter to assess the nephrotoxicity in human renal cortical tissue. Chang et al (1992) have reported that histocultured renal cortical tissues evaluated using the glucose consumption test provided a good association for cisplatin toxicity. Furthermore, glucose consumption measurements in histocultured human renal cortical tissues were more sensitive than the thymidine-incorporation endpoint (Chang et al, 1994). The results revealed that the newly developed platinum complex has similar or greater anticancer efficacy compared to cisplatin, especially high concentrations. As mentioned above, however, this new complex has very low nephrotoxicity. Therefore, this new complex may possibly be clinically useful for high-dose chemotherapy, with reduced side effects. Based on these results, this novel platinum complex represents a valuable lead and justifies clinical studies in the development of a new anticancer chemotherapeutic agent capable of improving anticancer efficacy with low toxicity.

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