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In Vitro Cytotoxicity of Novel Platinum(II) Coordination Complexes Containing Diaminocyclohexane and Diphenylphosphines

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We have synthesized new platinum(II) analogs containing 1,2-diaminocyclohexane (dach) as a carrier ligand, 1,3-bis(diphenylphosphino) propane (DPPP) /1,2-bis(diphenylphosphino)ethane (DPPE) as a leaving group and nitrates to improve solubility. In the present study, the cytotoxicity of [Pt(trans-*l*-dach)(DPPP)] · $2NO_3$ (KHPC-001) and [Pt(trans-*l*-dach)(DPPE)] · $2NO_3$ (KHPC-002) was evaluated and compared on various P-388 cancer cell lines and porcine kidney cell line (LLC-PK_1). The new platinum complexes demonstrated high efficacy on P-388 mouse leukemia cell line as well as cisplatin-resistant (P-388/CDDP) and adriamycin-resistant (P-388/ADR) P-388 cell lines. The intracellular platinum content was measured by a flame atomic absorption spectrophotometer (FAAS), and it was comparable to the results of IC_{50} of the three complexes on LLC-PK_1 and P-388/S cells, while only DPPE compound was accumulated in high volume in P-388/ADR and P-388/CDDP cells. While the DNA-interstrand cross-links of KHPC-001, KHPC-002 and cisplatin were similar on P-388/S leukemia cells, these new platinum complexes were much less DNA cross-linking to a kidney derived cell line, LLC-PK_1. These results indicate that KHPC-001 and KHPC-002 are a third-generation platinum complexes with potent antitumor activity and low nephrotoxicity.

Key Words: Nephrotoxicity, Platinum coordination complex, Antitumor activity

INTRODUCTION

The introduction of the square-planar complex cisplatin into the clinical treatment of cancer has resulted in excellent response rates for some tumor types, especially testicular and ovarian cancer (Lippman et al, 1973; Rosenberg et al, 1969). While the unfavorable nephrotoxicity profile of cisplatin has been overcome by the development of the second-generation agent, carboplatin (Calvert et al, 1985; Wiltshaw 1985; Harrap et al, 1985), there remains an unquestionable need for new platinum drugs which circumvent acquired cisplatin-resistance. As with other cancer chemotherapeutic agents, cellular resistance to the clinically used platinum agents, cisplatin and carboplatin, represents a major clinical limitation to their efficacy (Einhorn 1990; Mangioni et al, 1989). It is known that renal cortical accumulation of cisplatin leads to necrosis of the proximal tubule and late development of internal cysts (Hardaker et al, 1974; Krakoff 1979; Litterest et al, 1977). The major toxicological limitation is its dose related accumulative and irreversible side effects of severe nephrotoxicity, nausea and vomiting (Kociba & Sleight 1971; Schaeppi et al, 1973). Development of resistance in initially responded tumors leads to treatment failure (Andrews & Howell 1990; Timmer-Bosscha et al, 1992).

The antitumor activity of platinum complexes containing 1,2-diaminocyclohexane (dach) as a carrier

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ligand was investigated by Connors et al (1972) and Gale et al (1974). Kidani (1977) synthesized Pt (oxaloto)(trans-*l*-dach)[1-OPH] and Pt(malonato) (trans-*l*-dach)[1-PHM] using oxalic acid/ malonic acid with selected trans-*l*-dach among trans-*l*, trans-*d* and cis-isomers.

Our platinum-based drug discovery program is aimed at enhancing the anticancer effect and reducing side effects of platinum complexes. To accomplish this goal, we have synthesized new platinum analogs containing dach as a carrier ligand and DPPE/DPPP as leaving groups. Previously, we showed that these new platinum complexes were much less nephrotoxicity than cisplatin (Jung et al, 1993). This study examine the in vitro anticancer activity of new platinum complexes on several types of P-388 mouse leukemia cells, and the difference between cytotoxicities of these compounds on P-388 and porcine kidney cells LLC-PK_1, in comparison with cisplatin.

METHODS

Materials

Dulbeco's Modified Eagle's (DME) and RPMI-1640 media were purchased from Gibco (Grand Island, NY). S_1-nuclease was purchased from Takara Shuzo (Otsu, Japan). Proteinase K, RNase A, cisplatin and other agents were obtained from Sigma (St. Louis, MO). KHPC-001 and KHPC-002 were synthesized in the Department of Pharmacochemistry, College of Pharmacy, Kyung Hee University as previously reported (Jung et al, 1993). These agents were dissolved in sterilized phosphate-buffered saline (PBS).

Cell culture

A procine kidney cell line, LLC-PK_1, was cultured in DME medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). P-388/S, P-388/ADR and P-388/CDDP cells were passaged weekly through female BALB/cX DBA/2(CDF_1) mice (Nippon SLC, Hamamtsu, Japan) and harvested from tumor-bearing mice 6-7 days after transplantation. These cells were maintained *in vitro* culture in RPMI 1640 medium supplemented with 10% FBS.

In vitro cytotoxicity assay

All cell lines were suspended in DME or RPMI-1640 media supplemented with 10% FBS, and 1 ml/well of diluted cell suspension ($5\text{-}10\times10^4$ cells/ml) was seeded to a 24-well plate (Falcon 3047) and incubated with varying concentrations of a platinum complexes at 37°C with a humidified atmosphere of 5% CO_2 and 95% air for 48 hours. After the end of culture, cells were stained with trypan blue and counted under a microscope.

DNA interstrand cross-link assay

DNA interstrand cross-linking (ISC) by platinum complexes was measured by the ethidium bromide-staining assay (Siram & Ali-Osman, 1990; Yoshida et al, 1994). To summarize, 10 μg/0.1 ml of DNA purified from cells treated with new platinum complexes was added to 0.4 ml of 20 mM potassium phosphate-2 mM EDTA buffer (pH 11.8) and one of a pair tube was denatured by heating for 10 min at 100°C. After immediate cooling, S_1-nuclease digestion mixture(39 mM sodium acetate, 280 mM sodium chloride, 1 mM zinc sulfate, 500 units of S_1-nuclease) was added to the denatured DNA and incubating at 37°C for 30 min. Both the S_1 nuclease treated tube and unheated control tube were mixed with 1.0 ml of 10 mM Tris-0.1 mM EDTA buffer (pH 8.0) and 1.5 ml of ethidium bromide solution (2 μg/ml), and the fluorescence was measured using a fluorospectrophotometer (Hitachi M-850) at 305 nm excitation and 590 nm emission wavelengths at 15°C. The DNA-ISC index was calculated as follows:

$$\text{ISC index} = [-\ell n\ X\ (\text{drug-treated})]-[-\ell n\ X\ (\text{control})]$$

$$X = \frac{\text{Fluorescence}_{\text{unheated}} - \text{Fluorescence}_{\text{heated}}}{\text{Fluorescence}_{\text{unheated}}}$$

Measurement of platinum content in the cells

Intracellular platinum content was measured by the previously reported method (Tashiro & Sato, 1992). Cells were incubated with 20 μM of each platinum complex in the respective growth media for multiple times. At the end of incubation, cells were collected

Table 1. *In vitro* cytotoxicities of Pt(II) complexes on P-388/S and P-388/ADR, P-388/CDDP cells

Compound	IC_{50} (μM)		
	P-388/S[a)]	P-388/ADR[b)]	P-388/CDDP[c)]
Cisplatin	0.21±0.01[d)] (1.0)	0.12±0.03 (0.57)	16.93±3.60 (80.6)
KHPC-001	0.32±0.02 (1.0)	1.65±0.25 (5.16)	1.21±0.19 (3.78)
KHPC-002	0.29±0.04 (1.0)	4.87±0.15 (16.8)	2.44±0.27 (8.41)

Various concentrations of Pt(II) complexes were administered on each cells for 48 hr.
a) wild type cells, b) adriamycin-resistant cell, c) cisplatin-resistant cell, d) Each value is the mean±S.E. of three experiments. Data in parentheses are the resistance ratio to the P-388/S.

by centrifugation and washed twice with ice cold PBS. The cell pellets were digested with 60% nitric acid at 70°C for 5 hours. After cooling, the solution was neutralized with 40% sodium hydroxide and 25% sodium carbonate, and 100 mM sodium diethyldithiocarbamate (DDTC) was added. Then, the solution was heated at 80°C for 1 hour. The $Pt(DDTC)_2$ formed was extracted by chloroform three times, and then evaporated. After dissolving it in 0.1 ml methanol, it was analyzed for platinum at 269.5 nm using a flameless atomic absorption spectrophotometer (Nippon Jarrel Ash, Model AA-8500). The platinum content in the cells was measured in terms of nmol/mg protein. Protein concentrations were determined by Bradford method (1976).

RESULTS

In vitro cytotoxicity

The sensitivity of the wild type P-388/S leukemia cells, a resistant phenotype to adriamycin(P-388/ADR) and a resistant phenotype to cisplatin (P-388/CDDP) to the 3 platinum containing compounds are shown in Table 1. Drug concentration that inhibits cell proliferation by 50% (IC_{50}), was determined through direct cell counting after treatment with compounds for 48 hours. Cisplatin did not show significantly different IC_{50} between P-388/S and P-388/ADR cells, whereas the new compounds, KHPC-001 and KHPC-002, showed resistance 5- and 17-fold more, respectively, against P-388/ADR cells. On P-388/CDDP cell-line with acquired cisplatin resistance, cytotoxicity of cisplatin was prominently weak compared with other type cells, while new compounds showed much higher cytotoxicity.

Table 2. *In vitro* cytotoxicities of Pt(II) complexes on LLC-PK1 cells

Compound	IC_{50} (μM)	Ratio[a)]
Cisplatin	0.62±0.04[b)]	
KHPC-001	6.3±0.30	10.2
KHPC-002	11.1±0.78	17.9

a) The ratio is the IC_{50} of the Pt(II) complexes divided by that of the cisplatin, b) Each value is the mean±S.E. of at least three experiments.

To investigate renal cytotoxicity, $LLC\text{-}PK_1$ porcine normal kidney cells were incubated with cisplatin and new compounds for 48 hours in various concentrations (Table 2). The cytotoxicity of both new platinum compounds (KHPC-001 and KHPC-002) on $LLK\text{-}PK_1$ cells was markedly lower (10- and 18-fold, respectively) compared with cisplatin. Also with regard to the leaving ligand, DPPE was less cytotoxic than DPPP on the $LLK\text{-}PK_1$ cells.

Platinum accumulation

In order to investigate a relationship between the cytotoxicity and cellular accumulation of platinum compounds, platinum contents in the cells were measured by FAAS. Fig. 1 shows the progress of accumulation of platinum in $LLC\text{-}PK_1$ and P-388/S cells. In view of the platinum contents in $LLC\text{-}PK_1$ cells, cisplatin-treated cells showed increased platinum uptake depending on the incubation time, whereas the new compounds-treated cells increased platinum quantity much less than cisplatin even after 8 hours of

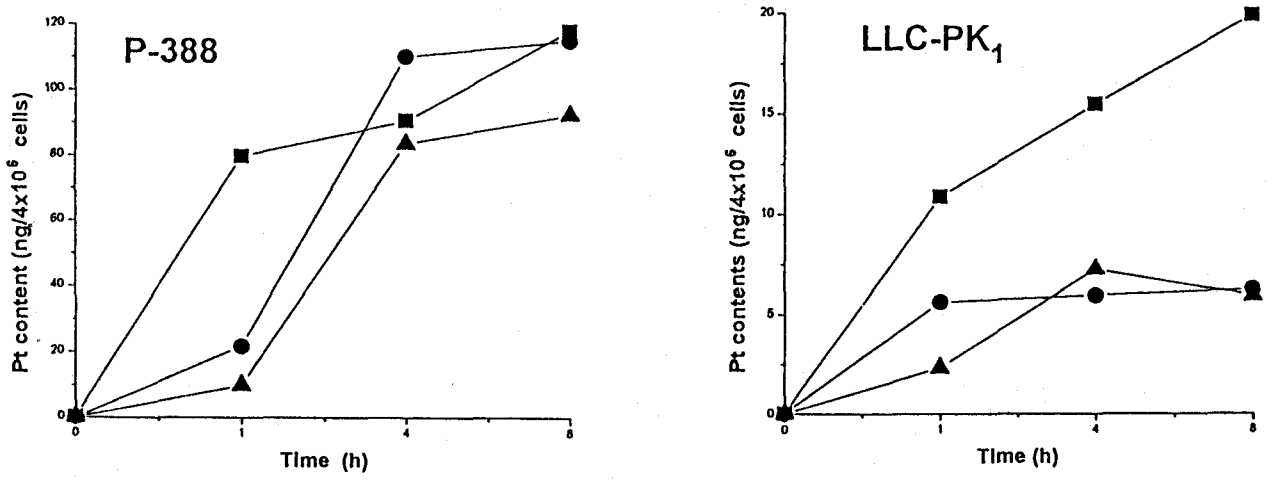

Fig. 1. Accumulation of platinum in P-388 cells and LLC-PK1 cells exposed to 10 μM cisplatin (■), KHPC- 001 (●) or KHPC-002 (▲) for indicated times.

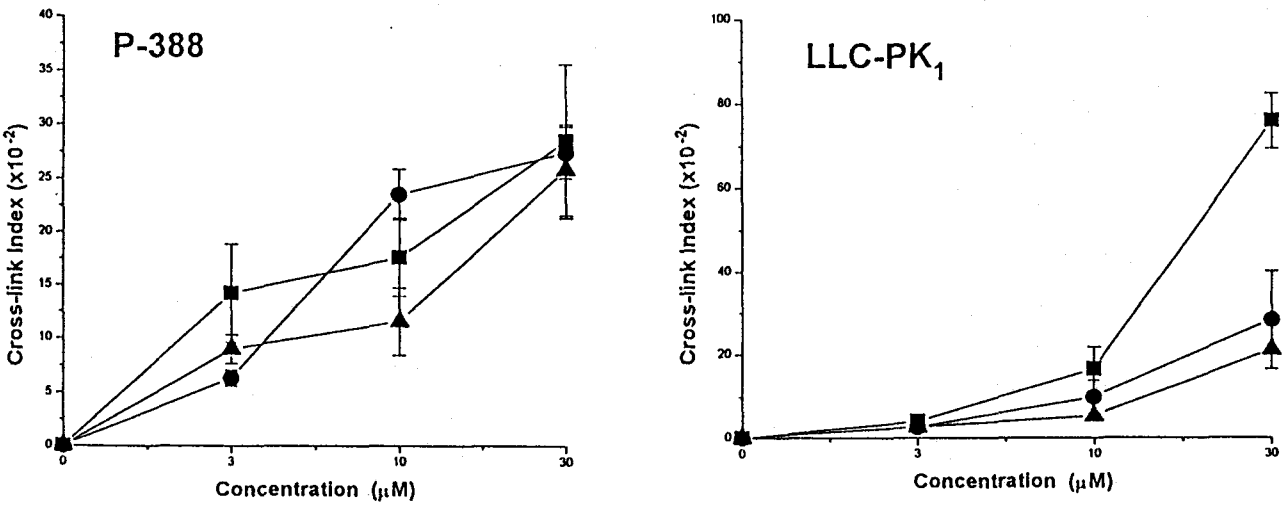

Fig. 2. DNA intrastrand cross-link index in P-388 and LLC-PK$_1$ cells treated with varying concentrations of cisplatin (■), KHPC-001 (●) or KHPC-002(▲) for 2 hrs.

incubation. The initial accumulation rate of KHPC-001 and KHPC-002 was about 5-fold less than that of cisplatin after 2 hr of exposure to these compounds. The platinum contents in P-388/S leukemia cell lines treated with KHPC-001 or KHPC-002 increased up to 4 hr and formed plateau, and the course was similar to that with cisplatin.

DNA interstrand cross-link of platinum complexes

The cytotoxic activities of platinum compounds are attributed to their ability to form DNA-platinum adducts. DNA-interstrand cross-link (ISC) was analyzed to estimate a level of platinum complexes in the target DNA macromolecule. Fig. 2 shows that DNA-ISC was increased in a dose-dependent manner by all of the platinum compounds on P-388/S and LLC-PK$_1$ cells. DNA-ISC in P-388/S cells treated with KHPC-001 or KHPC-002 was comparable to that with cisplatin, and these results are paralleled with those of cytotoxicity tests. On the other hand, DNA-ISC induced by cisplatin was about 3 times higher than that by new platinum compounds on LLC-PK$_1$ cells.

DISCUSSION

This study indicates that new platinum complexes,

KHPC-001 and KHPC-002, showed potent antitumor and cytotoxic effects on P-388 mouse leukemia cells but much low cytotoxicity on a kidney derived cells, LLC-PK_1. This low toxicity of KHPC-001 and KHPC-002 on the LLC-PK_1 cells was due to its lower accumulation in the cells than cisplatin. Cisplatin is an important antineoplastic drug in clinical treatment of various tumors, but its cytotoxicity on normal tissues and cross resistance has contributed to failure in treatment. Various platinum analogues have been synthesized so as to overcome the toxicity and cross resistance, such chemical endeavors have led to the release of second generation platinum complexes, such as carboplatin and tetraplatin. Although these drugs are being used in anticancer chemotherapy, their toxicities on normal tissues and expression of resistance to these drugs still remains at issue.

The goal of these chemical efforts to synthesize new platinum analogues is to find a drug that is both more active and less toxic than cisplatin or that is comparable in activity but less toxic than the parent compound.

The structure-activity relationships were clarified by the effect of carrier ligands and leaving groups on *in vivo* antitumor activity. 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 platinum complex.

One of the structural modification that is widely accepted as having resulted in an increased therapeutic index is the attachment of 1,2-diaminocyclohexane (dach) (Connors et al, 1972; Gale et al, 1974). Some of the dach compounds which exist today are cis-dach, trans-*l*-dach and trans-*d*-dach. Among these dach derivatives, trans-*l*-dach has been known to have significant antitumor activity (Inagaki & Kitani, 1986). Moreover, it is important to take into recurrent the leaving group which greatly influences the activity of platinum compounds. The platinum compounds appear to penetrate cell membrane by diffusion, and the leaving group is displaced directly by hydrolysis. This is responsible for the formation of activated species of drug which reacts with DNA (i.e. with the guanine N7 forms), causing the inhibition of DNA replication and cytotoxic effect (Tashiro, 1989; 1992). In addition to their reactivity with DNA, platinum complexes can react with protein-bound sulfhydryl groups of the proximal tubules, with resulting in significant toxic action on renal function (Allan et al, 1988). These studies a indicate that the dissociation of leaving group is an important factor in antitumoral and toxic activities. However, when the rate of dissociation is much higher, it causes toxicological effects since the platinum complexes react with normal protein instead of DNA in cancer cells. Contrastly, when the dissociation rate is too low, it is excreted to extracellular compartment before showing any antitumor activity.

The new complexes showed anticancer activity comparable to cisplatin on P-388 wild type cells. Surprisingly, these new complexes exhibited potent cytotoxicity even on P-388/CDDP cells, whose mechanism of resistance may be caused primarily by reduced drug uptake (Tashiro et al, 1989). Therefore, these new platinum complexes seems to be not inactivated by metallothionein, and the mechanism of intracellular platinum uptake seems to be different between cisplatin and the new platinum complexes.

The renal toxicity of cisplatin has been reported to be resulted from the damage in proximal and distal tubule (Tay et al, 1988). The sensitivity of KHPC-001 and KHPC-002 was decreased by 10.2 times and 17.9 times, respectively, compared with cisplatin using LLC-PK_1 porcine kidney cells.

In order to investigate the correlation between intracellular accumulation of platinum and anticancer activity, platinum content was measured in LLC-PK_1 cells and P-388 leukemia cells. In view of LLC-PK_1 cells, it was decreased approximately three-fold compared with cisplatin, and platinum contents were increased time-dependently in P-388/S cells. KHPC-002 showed an exeptionally high accumulation of platinum for 1 hour treatment.

The activity of bifunctional alkylating agents such as the Pt(II) complexes has been reported to be attributed to DNA interstrand cross-links in no more than 1% of total DNA-Pt adducts in relation to their antitumor effectiveness (Roberts & Pascoe, 1972). In this study, DNA-interstrand cross-linking index was examined to investigate the action of platinum complexes to target site by the ethidium bromide (EtBr) binding assay. This assay is based on the rapid reannealing of crosslinked DNA to its double helical configuration after a denaturation and immediate renaturation cycle. Because of the high EtBr binding capacity of double helical DNA, the value of subtracted residual EtBr fluorescence after the heating/cooling cycle from that of non-heated native DNA provides the extent of interstrand cross-linking in the

DNA induced by the Pt(II) complexes. S_1-nuclease was treated after heating/cooling cycle to digest RNA and single-strand DNA, and thereby minimizing their non-crosslink-related contribution to the residual fluorescence.

Table 1 and 2 show the effects of the platinum complexes on the P-388/S and LLC-PK_1 cells, respectively, as the mean of anticancer activity and renal toxicity. One aspect of the results was closely correlated to the results of cytotoxicity (IC_{50}) and intracellular platinum contents, indicating the cytotoxicity in P-388/S leukemia cells was very sensitive except in LLC-PK_1 cells.

In conclusion, this study indicates that newly synthesized platinum complexes, [Pt(trans-*l*-dach) (DPPP)] · $2NO_3$ and [Pt(trans-*l*-dach) (DPPE)] · $2NO_3$, showed the comparable cytotoxicity on different types P-388 mouse leukemia cell lines to that of cisplatin, and especially these compounds exhibited selective cytotoxicity on the P-388/CDDP cells. Moreover these compounds show less cytotoxicity on LLC-PK_1 cells. Based on these results, these newly synthesized platinum complexes represent a valuable lead in the development of new anticancer chemotherapeutic agents capable of improving antitumor activity and reducing nephrotoxicity.

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