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Preclinical report

Synthesis and antitumor activity of cyclotriphosphazene–(diamine)platinum(II) conjugates

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A new class of water-soluble cyclotriphosphazene–(diamine)platinum(II) conjugate drugs [NP(Am·Li$_2$)(Am·PtA$_2$)]$_3$ (Am: dicarboxylic amino acid; A$_2$: diamine) has been synthesized and characterized by means of elemental analysis, multinuclear (^{1}H, ^{31}P, ^{13}C, ^{195}Pt) NMR and IR spectroscopies. All the title compounds were subjected to both *in vitro* and *in vivo* assays against the murine leukemia L1210 cell line and selected human tumor cells. Most of the title compounds have shown higher *in vivo* antitumor activity than cisplatin and carboplatin, and, in particular, [NP(L-Glu·Li$_2$)(L-Glu·Pt(-dach)]$_3$ (Glu=glutamate, dach=*trans*(±)-1,2-diaminocyclohexane) showed extraordinary high activity (ILS > 500%) equally against both parent and cisplatin-resistant leukemia L1210 cell lines. Furthermore, this candidate compound (KI 60606) exhibited a wider spectrum of *in vitro* activity by showing higher cytotoxicity against all the selected human tumor cells than cisplatin and, therefore, was subjected to preclinical studies which are now near completion. [© 2000 Lippincott Williams & Wilkins.]

Key words: Anticancer drug, antitumor agent, conjugate drug, cyclotriphosphazene, platinum.

Introduction

Since cisplatin was approved as an anticancer drug by the FDA in 1979, a great deal of effort has been made to find better platinum drugs with less toxicity and better activity. Consequently, in addition to carboplatin, oxaliplatin and Nedaplatin have been approved as new platinum drugs even for limited use in recent years. All these approved platinum drugs are mononuclear platinum(II) complexes, but no polymeric or oligomeric platinum compound has been commercialized yet. We have recently reported a new class of water-soluble polyphosphazene–(diamine)platinum(II) conjugates affording controlled-release of the antitumor (diamine)platinum(II) moiety *in vivo*.[1–3] These polymeric conjugates have shown higher *in vivo* antitumor activity than cisplatin against the murine leukemia L1210 cell line. In particular, the representative compound, [N=P(OH)(L-Glu·Pt(dach))]$_n$ (Glu=glutamate, dach=*trans*(±)-1,2-diaminocyclohexane), exhibited high *in vivo* activity [ILS(%) > 500] with no cross-resistance to cisplatin and, therefore, was subjected to preclinical studies for human clinical trials. However, it has been found that this polymeric platinum(II) compound caused severe anaphylactic reaction in guinea pig, probably due to its polymeric nature. Therefore, we have changed the drug delivery system from the polymeric phosphazene backbone to oligomeric cyclotriphosphazene.

Cyclotriphosphazene has a six-membered ring structure composed of phosphorus and nitrogen atoms linked by alternating single and double bonds represented by $-(N{=}P)-_3$. A great variety of trimeric derivatives with different physico-chemical properties were designed and synthesized by nucleophilic substitution of the chlorotrimers, $(N{=}PCl_2)_3$, with various nucleophiles.[4–13] In particular, functionalization of cyclotriphosphazene by substitution with various amino acid esters was reported by the authors.[14] If an amino acid is used as a substituent, its trimeric derivative is known to be degradable into non-toxic amino acid, phosphate and ammonium ions in aqueous solution.[15–17]

We have employed a dicarboxylic amino acid (Am) such as aminomalonic (Aml), L-aspartic (L-Asp) and L-

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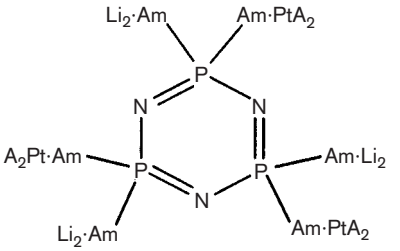
glutamic (L-Glu) acids not only as a spacer to chelate the bivalent cationic (diamine)platinum (II) moiety by covalent bonding but also as a solubilizing group. Here we report synthesis and antitumor activity of the trimeric phosphazene–(diamine)platinum(II) conjugates of the following structure:



where Am is a dicarboxylic amino acid and A_2 is a diamine.

Materials and methods

Materials

Hexachlorocyclotriphosphazene, $(NPCl_2)_3$ (Aldrich, Milwaukee, WI) was purified by sublimation at 55°C under vacuum (about 0.1 mmHg). Benzene, toluene and xylene were dried and distilled over sodium benzophenone, and triethylamine was distilled over BaO under dry nitrogen. *Trans*(±)-1,2-diaminocyclohexane (dach), 2,2-dimethyl-1,3-propanediamine (dmpda), ethylenediamine (en) and all other reagents purchased from Aldrich were used without further purification. Diethyl aminomalonate hydrochloride, diethyl aspartate hydrochloride and diethyl glutamate hydrochloride were prepared according to the literature,[18] and their cyclotriphosphazene derivatives were synthesized by our previous method.[14] The intermediate compounds (diamine)PtI_2 and their water-soluble sulfates (diamine)$PtSO_4$ were prepared according to the literature.[19,20]

Measurements

All reactions were carried out under an atmosphere of dry nitrogen by using standard Schlenk-line techniques. IR spectra were recorded on an Analect FX-6160 FT-IR spectrometer as KBr pallets. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Gemini-300 NMR spectrometer relative to tetramethylsilane as an internal standard. Proton-decoupled ^{31}P- and ^{195}Pt-NMR spectra were measured with the same spectrometer operated at 121.4 MHz (^{31}P) and 64.39 MHz (^{195}Pt) using 85% aqueous phosphoric acid and Na_2PtCl_6 as external standards, respectively. Liquid chromatography was carried out on a column packed with 200–400 mesh silica gel (Aldrich). Elemental analysis was performed by the Advanced Analysis Center at KIST.

Evaluation of antitumor activity

The *in vitro* and *in vivo* assays of the title compounds were performed according to our previous method.[1]

Synthesis of cyclotriphosphazene–(diamine)platinum(II) conjugates

{N_3P_3(L-Glu·Li_2)$_3$[L-Glu·Pt(dach)]$_3$} ***(1)***. Hexakis (-di-ethyl glutamate)cyclotriphosphazene, [NP(L-Glu·Et_2)$_2$]$_3$, (1.04 g, 0.77 mmol) was suspended in an aqueous solution of LiOH·H_2O (0.42 g, 10.0 mmol) and the reaction mixture was stirred at room temperature for 1 day until a clear solution was obtained. This solution was freeze-dried. The dried product was suspended in excess methanol, which was stirred for 6 h to wash out excess LiOH. The insoluble product was filtered and washed with acetone and diethyl ether, and then dried in vacuum to obtain a white solid product [NP(L-Glu·Li_2)$_2$]$_3$·12-H_2O in 95% yield.

The intermediate (dach)PtI_2 (1.94 g, 3.44 mmol) was suspended in an aqueous solution (50 ml) containing silver sulfate (1.14 g, 3.44 mmol) and the reaction mixture was vigorously stirred under darkness for 5–6 h. The resulting precipitate (AgI) was filtered out and the filtrate solution was used for the following platination reaction.

To a solution of [NP(L-Glu·Li_2)$_2$]$_3$·12H_2O (1.0 g, 0.77 mmol) dissolved in distilled water (30 ml) was slowly added dropwise an aqueous solution of (dach)$PtSO_4$ (50 ml) at 0°C under controlled pH of the solution at above 7.0 (the pH of the reaction solution should not be lower than 7.0 during the addition of the trimer solution and, if necessary, it should be adjusted to 7.0–7.5 using lithium hydroxide). The reaction solution was warmed up slowly to 20°C and further stirred for 10–20 min to complete platination. After $BaCl_2$·2H_2O (0.84 g, 3.44 mmol) was added to the solution with stirring, the reaction mixture was further stirred for 5–10 min and then the precipitated $BaSO_4$ was quickly filtered out. The filtrate solution was immediately dropped to excess of acetone (800 ml) to precipitate the final product. The resultant solid product was filtered and washed with acetone, and vacuum-dried at room temperature over P_2O_5.

Overall yield 70%. Found (Calcd for $C_{48}H_{78}N_{15}O_{24}P_3Li_6Pt_3$·12$H_2O$): C, 26.6 (26.4); H, 4.21 (4.15); N, 9.59 (9.62); P, 3.68 (4.25); Li, 1.86 (1.91); Pt, 27.5 (26.9), ^{1}H-NMR (D_2O, p.p.m.): 1.1–1.4

(4H, diaminocyclohexane C-4, C-5 protons), 1.6 (2H, diaminocyclohexane C-3, C-6 protons), 2.1 (4H, C**H**$_2$-CH_2-CO_2+diaminocyclohexane C-3, C-6 protons), 2.5 (4H, C**H**$_2$-CO_2+diaminocyclohexane C-1, C-2, protons), 3.7 (1H, C**H**-CH_2), IR (KBr, cm^{-1}): *v*(P=N) 1170 1124; *v*(COO) 1582, 1410.

{N$_3$P$_3$(L-Glu·Li$_2$)$_3$[L-Glu·Pt(dmpda)]$_3$} ***(2)***. This compound was prepared by the same procedure for **1** using $[NP(L\text{-}Glu\cdot Li_2)_2]_3\cdot 12H_2O$ (1.0 g, 0.77 mmol), (dmpda)PtI_2 (2.73 g, 3.44 mmol), Ag_2SO_4 (1.14 g, 3.44 mmol) and $BaCl_2\cdot 2H_2O$ (0.84 g, 3.44 mmol).

Overall yield, 67%. Found (Calcd) for $C_{45}H_{78}N_{15}O_{24}P_3Li_6Pt_3\cdot 12H_2O$): C, 24.8 (25.2); H, 4.33 (4.78); N, 10.7 (9.78); P, 3.65 (4.32); Li, 1.86 (1.94); Pt, 28.5 (27.2), ^{1}H-NMR (D_2O, p.p.m.): 1.1–1.5 (6H, 2,2-dimethyl protons), 2.2–2.6 (8H, C**H**$_2$-C**H**$_2$-CO_2+1,3-propandiamine protons), 3.7 (1H, C**H**-CH_2), IR (KBr, cm^{-1}): *v*(P=N) 1170 1124; *v*(COO) 1587, 1384.

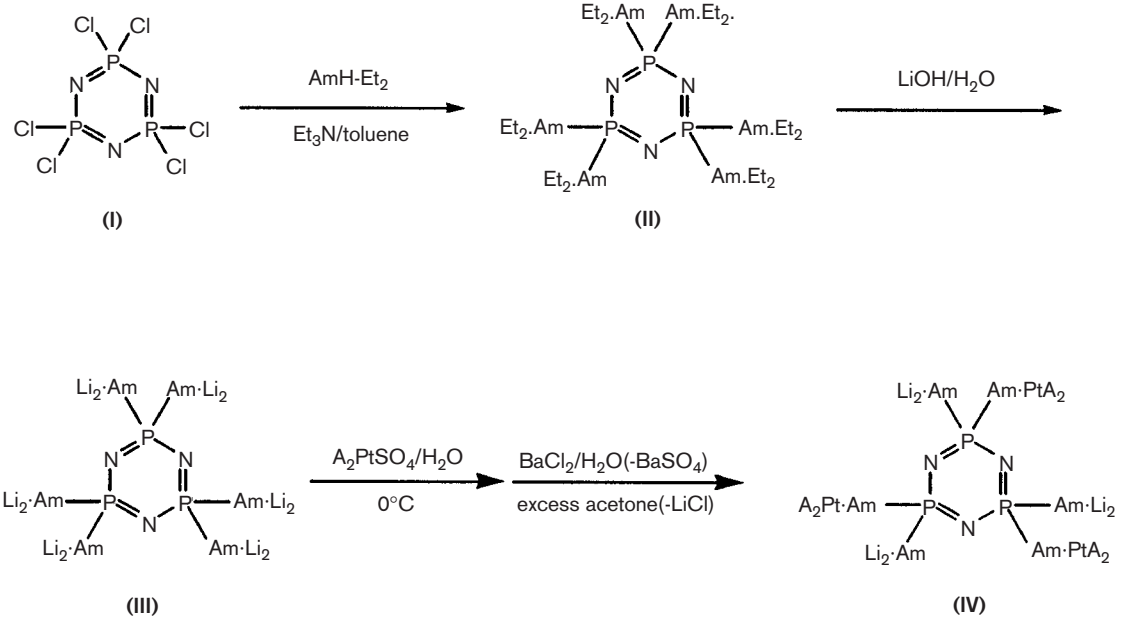
{N$_3$P$_3$(L-Glu·Li$_2$)$_3$[L-Glu·Pt(en)]$_3$} ***(3)***. This compound was prepared by the same procedure for **1** using $[NP(L\text{-}Glu\cdot Li_2)_2]_3\cdot 12H_2O$ (1.0 g, 0.77 mmol), (en)PtI_2 (2.52 g, 3.44 mmol), Ag_2SO_4 (1.14 g, 3.44 mmol) and $BaCl_2\cdot 2H_2O$ (0.84 g, 3.44 mmol).

Overall yield, 65%. Found (Calcd for $C_{36}H_{60}N_{15}O_{24}P_3Li_6Pt_3\cdot 12H_2O$): C, 20.5 (21.4); H, 4.01 (4.19); N, 10.9 (10.4); P, 3.95 (4.59); Li, 1.91 (2.06); Pt, 28.4 (28.9), ^{1}H-NMR (D_2O, p.p.m.): 2.1–2.6 (8H, C**H**$_2$-C**H**$_2$-CO_2+ethylene diamine protons), 3.8 (1H, C**H**-CH_2), IR (KBr, cm^{-1}): *v*(P=N) 1170 1124; *v*(COO) 1638, 1400.

{N$_3$P$_3$(L-Asp·Li$_2$)$_3$[L-Asp·Pt(dach)]$_3$} ***(4)***. This compound was prepared by the same procedure for **1** using $[NP(L\text{-}Asp\cdot Li_2)_2]_3\cdot 12H_2O$ (1.0 g, 0.82 mmol), (dach)PtI_2 (3.17 g, 3.64 mmol), Ag_2SO_4 (1.28 g, 3.64 mmol) and $BaCl_2\cdot H_2O$ (0.94 g, 3.64 mmol).

Overall yield, 67%. Found (Calcd for $C_{42}H_{68}N_{15}O_{24}P_3Li_6Pt_3\cdot 12H_2O$): C, 24.3 (24.0); H, 4.27 (4.32); N, 9.58 (10.0); P, 3.78 (4.42); Li, 1.86 (1.98); Pt, 28.3 (27.9), ^{1}H-NMR (D_2O, p.p.m.): 1.2–1.4 (4H, diaminocyclohexane C-4, C-5 protons), 1.6 (2H, diaminocyclohexane C-3, C-6 protons), 2.2 (2H, diaminocyclohexane C-3, C-6 protons), 2.5 (2H, diaminocyclohexane C-1, C-2 protons), 2.8 (2H, C**H**$_2$-CO_2), 3.7 (1H, C**H**-CH_2), IR (KBr, cm^{-1}): *v*(P=N) 1172 1114; *v*(COO) 1578, 1448.

{N$_3$P$_3$(L-Asp·Li$_2$)$_3$[L-Asp·Pt(dmpda)]$_3$} ***(5)***. This com-



Scheme 1.

pound was prepared by the same procedure for **1** using $[NP(L\text{-}Asp\cdot Li_2)_2]_3\cdot 12H_2O$ (1.0 g, 0.82 mmol), (dmpda) PtI_2 (2.94 g, 3.28 mmol), Ag_2SO_4 (1.28 g, 3.64 mmol) and $BaCl_2\cdot H_2O$ (0.94 g, 3.64 mmol).

Overall yield, 65%. Found (Calcd for $C_{39}H_{66}N_{15}O_{24}P_3Li_6Pt_3\cdot 12H_2O$): C, 22.4 (22.7); H, 5.01 (4.39); N, 9.52 (10.2); P, 3.73 (4.50); Li, 1.92 (2.02); Pt, 29.5 (28.3), ^{1}H-NMR (D_2O, p.p.m.): 1.1 (6H, 2,2-dimethyl protons), 2.2–2.5 (6H, C**H**$_2$-CO_2+1,3-propandiamine protons), 3.7 (1H, C**H**-CH_2), IR (KBr, cm^{-1}): *v*(P=N) 1173 1119; *v*(COO) 1588, 1395.

{N_3P (L-Asp·Li_2)$_3$[L-Asp·Pt (en)]$_3$} ***(6)***. This compound was prepared by the same procedure for **1** using $[NP(L\text{-}Asp\cdot Li_2)_2]_3\cdot 12H_2O$ (1.0 g, 0.82 mmol), (en)PtI_2 (2.71 g, 3.28 mmol), Ag_2SO_4 (1.28 g, 3.64 mmol) and $BaCl_2\cdot H_2O$ (0.94 g, 3.64 mmol).

Overall yield, 65%. Found (Calcd for $C_{30}H_{48}N_{15}O_{24}P_3Li_6Pt_3\cdot 12H_2O$): C, 17.5 (18.6); H, 4.01 (3.74); N, 10.9 (10.8); P, 3.95 (4.79); Li, 2.01 (2.15); Pt, 31.2 (30.2), ^{1}H-NMR (D_2O, p.p.m.): 2.3–2.5 (6H, CH-C**H**$_2$-CO_2+ethylene diamine protons), 3.8 (1H, C**H**-CH_2), IR (KBr, cm^{-1}): *v*(P=N) 1200 1135; *v*(COO) 1620, 1381.

{N_3P_3(Aml·Li_2)$_3$[Aml·Pt(dach)]$_3$} ***(7)***. This compound was prepared by the same procedure for **1** using $[NP(Aml\cdot Li_2)_2]_3\cdot 12H_2O$ (1.0 g, 0.88 mmol), (dach)PtI_2 (3.24 g, 3.91 mmol), Ag_2SO_4 (1.30 g, 3.91 mmol) and $BaCl_2\cdot 2H_2O$ (0.96 g, 3.91 mmol).

Overall yield, 68%. Found (Calcd for $C_{36}H_{54}N_{15}O_{24}P_3Li_6Pt_3\cdot 12H_2O$): C, 21.7 (21.4); H, 3.41 (3.90); N, 9.82 (10.4); P, 3.88 (4.61); Li, 1.79 (2.07); Pt, 29.5 (29.0), ^{1}H-NMR (D_2O, p.p.m.): 1.1–1.3 (4H, diaminocyclohexane C-4, C-5 protons), 1.5 (2H, diaminocyclohexane C-3, C-6 protons), 2.0 (2H, diaminocyclohexane C-3, C-6 protons), 2.3 (2H diaminocyclohexane C-1, C-2 protons), 3.9 (1H, C**H**-CO_2), IR (KBr, cm^{-1}): *v*(P=N) 1173 1107; *v*(COO) 1640, 1451.

{N_3P_3(Aml·Li_2)$_3$[Aml·Pt(en)]$_3$} ***(8)***. This compound was prepared by the same procedure for **1** using

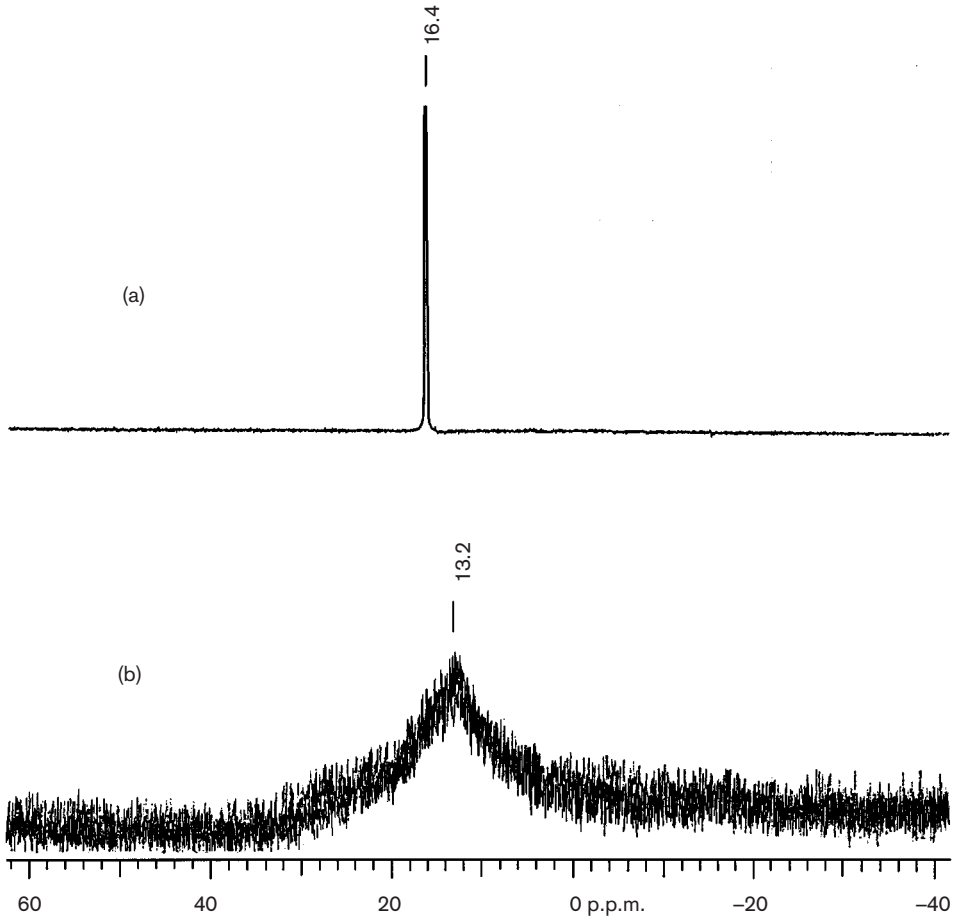


Figure 1. ^{31}P-NMR spectra of $[N_3P_3(L\text{-}Glu\cdot Li_2)]_6$ (a) and $\{N_3P_3(L\text{-}Glu\cdot Li_2)_3[L\text{-}Glu\cdot Pt(dach)]_3\}$ (b).

$[NP(Aml{\cdot}Li_2)_2]_3{\cdot}12H_2O$ (1.0 g, 0.88 mmol), $(en)PtI_2$ (2.93 g, 3.91 mmol), Ag_2SO_4 (1.30 g, 3.91 mmol) and $BaCl_2{\cdot}2H_2O$ (0.96 g, 3.91 mmol).

Overall yield, 65%. Found (Calcd for $C_{24}H_{36}N_{15}O_{24}P_3Li_6Pt_3{\cdot}12H_2O$): C, 14.9 (15.5); H, 3.01 (3.26); N, 11.2 (11.3); P, 4.95 (5.01); Li, 1.90 (2.25); Pt, 32.4 (31.6), ^{1}H-NMR (D_2O, p.p.m.): 2.3 (4H, ethylene diamine protons), 3.8 (1H, C**H**-CO_2), IR (KBr, cm^{-1}): *v*(P=N) 1210 1128; *v*(COO) 1588, 1400.

Results

Synthesis and characterization

The synthetic pathway for the title compounds is summarized in Scheme 1. Hexakis (dicarboxylic amino acid ester)cyclotriphosphazenes (**II**) were prepared by reaction of hexachlorocyclotriphosphazene with dicarboxylic amino acid esters in the presence of triethylamine as a HCl acceptor and then subjected to hydrolysis with lithium hydroxide in aqueous solution to obtain their lithium salts (**III**) according to our previous method.[14] The ^{31}P-NMR spectrum (Figure 1) measured during the hydrolysis reaction shows a sharp singlet at around 16 p.p.m., which indicates that the phosphazene ring remained intact during the hydrolysis reaction. No other signals that might be attributed to decomposition were observed. Complete hydrolysis of the amino acid esters of trimers was also confirmed by disappearance of the ethyl proton resonances of the esters in their ^{1}H-NMR spectra (Figure 2) and also by the shift of the carboxylate stretching frequency from 1700 to 1561–1628 cm^{-1} in their IR spectra (Figure 3). All the compounds showed strong bands at 1180–1200 cm^{-1} in their IR spectra that are characteristic of the PN backbone stretchings of the phosphazene ring. The ^{13}C-NMR spectra (Figure 4) of the lithium salts exhibited characteristic signals for the carboxylate carbons (δ: 184, 186).

The amino acid lithium salts of cyclotriphosphazene (**III**) were reacted with $(diamine)PtSO_4$ at a mole ratio of 1:3–6 in aqueous solution to obtain the cyclotriphosphazene–(diamine)platinum(II) conjugates by a metathesis reaction. However, the maximum degree of platination was 3 mol of (diamine)platinum(II) per cyclotriphosphazene, probably due to the steric hindrance of the relatively bulky (diamine)platinum moiety. To obtain maximally platinated product (**IV**), at least 4 mol of (diamine)platinum(II) per cyclotripho-

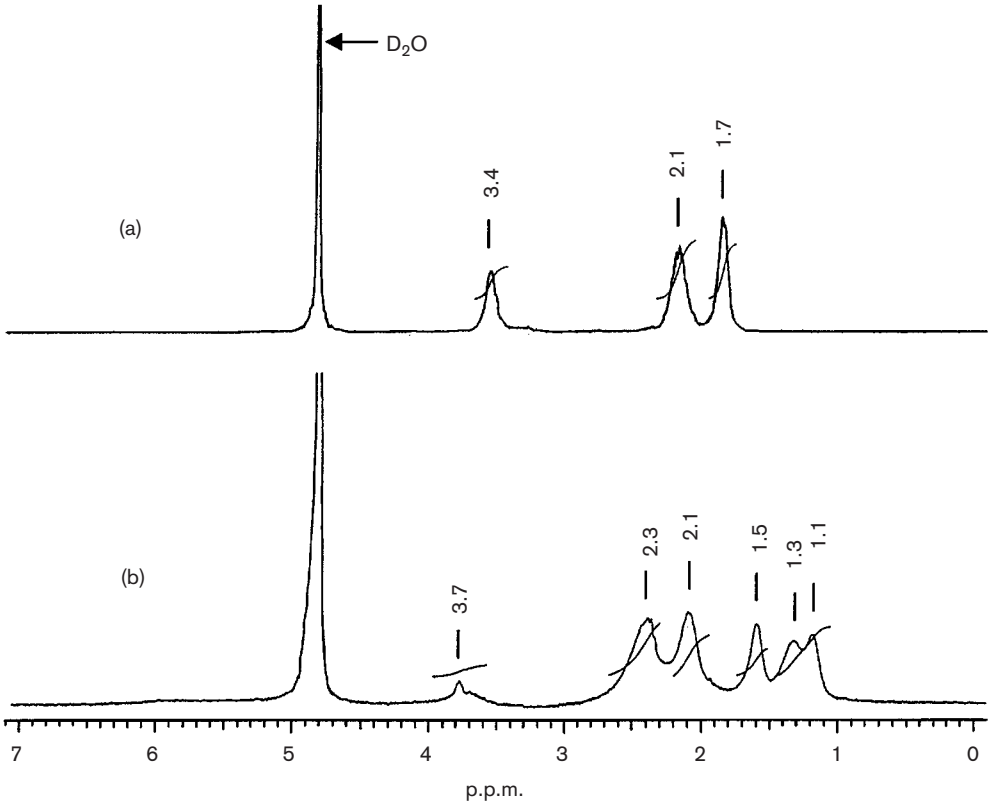


Figure 2. ^{1}H-NMR spectra of $[N_3P_3(L\text{-}Glu{\cdot}Li_2)]_6$ (a) and $\{N_3P_3(L\text{-}Glu{\cdot}Li_2)_3[L\text{-}Glu{\cdot}Pt(dach)]_3\}$ (b).

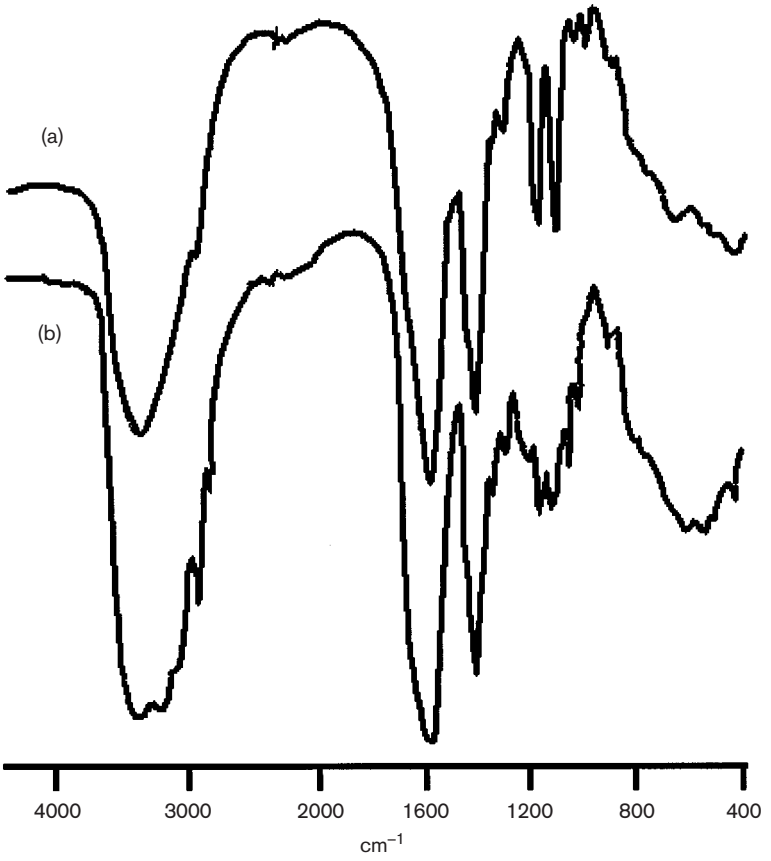


Figure 3. IR spectra of $\{N_3P_3(L\text{-Glu}\cdot Li_2)\}_6$ (a) and $\{N_3P_3(L\text{-Glu}\cdot Li_2)_3[L\text{-Glu}\cdot Pt(dach)]_3\}$ (b).

Table 1. *In vitro* and *in vivo* activities against the leukemia L1210 cell line of cyclotriphosphazene–(diamine)platinum (II) conjugates

Compounds	*In vitro*	*In vivo*		
	ED_{50} (μM/ml)	Dose (mg/kg)	ILS (%)	60 day survivors
1	2.6	60	>577.4	6/8
(KI 60606)		30	>323.8	2/8
2	9.4	60	191.2	
		30	166.2	
3	35.9	60	106.7	0/8
		30	99.5	0/8
4	5.2	60	>448.8	4/8
		40	>243.8	1/8
5	9.4	60	166.2	0/8
		30	160.4	0/8
6	5.8	60	toxic	0/8
		30	250.9	0/8
7	2.2	60	202.9	0/8
		30	174.3	0/8
8	3.3	60	toxic	0/8
		30	>226.9	1/8
Cisplatin	0.33	4	74	0/8
Carboplatin	3.8	40	68	0/8

sphazene were required. To eliminate the byproduct, lithium sulfate and unreacted (diamine)platinum(II) sulfate from the reaction mixture, different solubilities of the platinated complex and alkali or alkaline earth metal sulfate may be used. For instance, amino acid alkaline earth metal salt of cyclotriphosphazene may be reacted with (diamine)platinum sulfate, resulting in an insoluble alkaline earth metal sulfate with the water-soluble (diamine)platinum conjugate in the pregnant solution. If (diamine)platinum sulfate and the amino acid lithium salt of the cyclotriphosphazene are reacted, the resultant platinum conjugate and lithium sulfate are both water soluble and coexist in the aqueous reaction solution. However, when barium chloride was added to the reaction solution, barium sulfate precipitated out quantitatively leaving lithium chloride and the platinum complex (**IV**) in the aqueous solution. After filtering out barium sulfate, the filtrate was added to excess acetone to obtain the pure platinum complex (**IV**) as a powder. All the cyclotriphosphazene–(diamine)platinum conjugates (**IV**) were obtained as light yellow powder without a melting point. They are all very soluble in water (above 20 mg/ml) and slightly soluble in alcohol, but

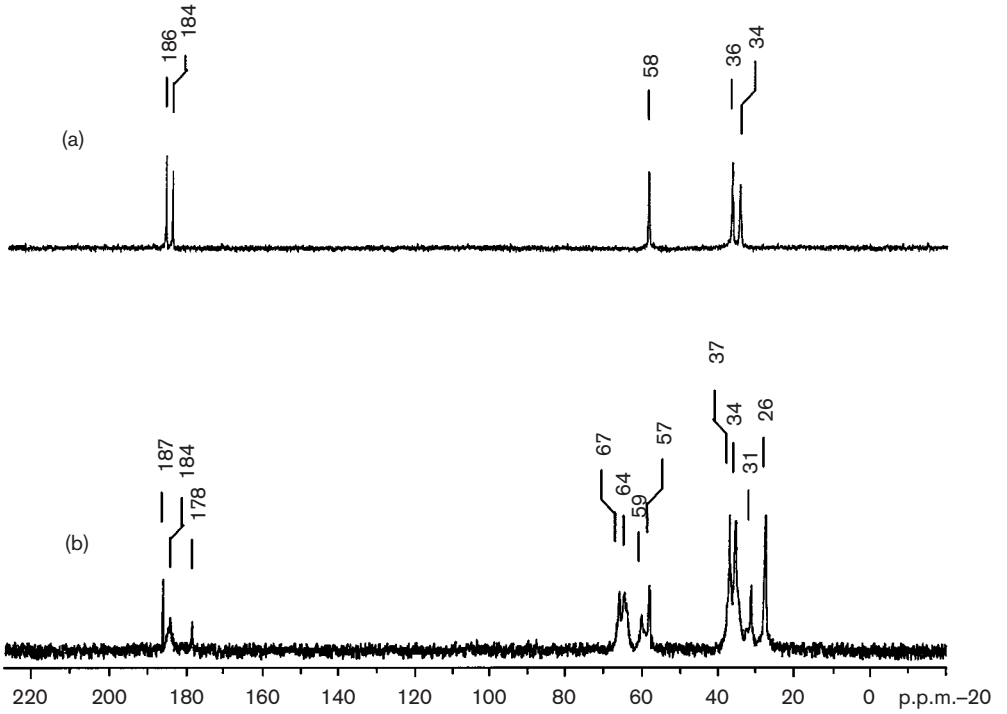


Figure 4. ^{13}C-NMR spectra of $[N_3P_3(\text{L-Glu}\cdot Li_2)]_6$ (a) and $\{N_3P_3(\text{L-Glu}\cdot Li_2)_3[\text{Glu}\cdot Pt(dach)]_3\}$ (b).

almost insoluble in most other organic solvents. All the cyclotriphosphazene–(diamine)platinum conjugates thus prepared were characterized by means of elemental analysis and multinuclear (^{1}H, ^{31}P, ^{195}Pt) NMR and IR spectroscopies.

A typical ^{1}H-NMR spectrum of one of the representative conjugates $\{N_3P_3(\text{L-Glu}\cdot Li_2)_3[\text{L-Glu}\cdot Pt(dach)]_3\}$ (**1**) is shown along with the spectrum of its reactant in Figure 2. The cyclohexyl protons of unreacted $(dach)PtSO_4$ showed five resonances due to the ring conformation. When the platinum atom was coordinated by the dicarboxylate ligand of the trimer, these cyclohexyl proton peaks were not shifted but broadened with partial overlapping with those of the amino acid protons. Figure 1 shows the ^{31}P-NMR spectrum of the same conjugate along with that of the unconjugated trimer exhibiting a broad singlet at 13.2 p.p.m., which is frequently observed for trimers.[21] It is not surprising that the platinated conjugate shows even broader resonance without significant chemical shift. In the ^{13}C-NMR spectra of a platinated trimer **1** (Figure 4), three carbonyl resonances were observed at 187, 184 and 178 p.p.m. The chemical shifts at 187 and 184 p.p.m. are assigned to the uncoordinated carboxylate group, whereas those at 178 and 184 p.p.m. (overlapped) are assigned to the coordinated carboxylate group. These ^{13}C resonances are in accord with those of the amino acid metal complexes in the literature.[22,23]

More interesting and important are the ^{195}Pt-NMR spectra of the trimer conjugates. It is well known that ^{195}Pt resonance is very sensitive to its chemical environment.[22] In our previous study we have confirmed that the (*O*,*O*) chelate isomer of the (diamine)platinum glutamate is subjected to isomerization to the more thermodynamically stable (*N*,*O*) isomer in aqueous solution. Unfortunately, it has not been successful to measure the ^{195}Pt-NMR spectra of the present trimeric conjugates in fresh aqueous solution, since the ^{195}Pt resonance signal could be observed only after scanning for at least half a day, probably due to its band broadening even in its concentrataed aqueous solution. However, the ^{195}Pt-NMR spectrum of the representative conjugate compound **1** in aqueous solution (Figure 5) is informative on the solution behavior of the platinum conjugate. From the comparison of the ^{195}Pt-NMR spectrum of the trimer-platinum conjugate with that of free (diamine)platinum glutamate previously studied,[23] it may be presumed that the resonance at 2417 p.p.m. is due to the (diamine)platinum moiety chelated by the dicarboxylic amino acid group of the trimers through the (*N*,*O*) chelation mode. The uncoordinated carboxylate group may attack a nearby phosphorus atom on the trimer ring skeleton,[16,17] which would eventually lead to ring cleavage and decomposition of the trimers.

Also we have performed a separate study on the degradation of compound **1** in D_2O. Compound **1** in

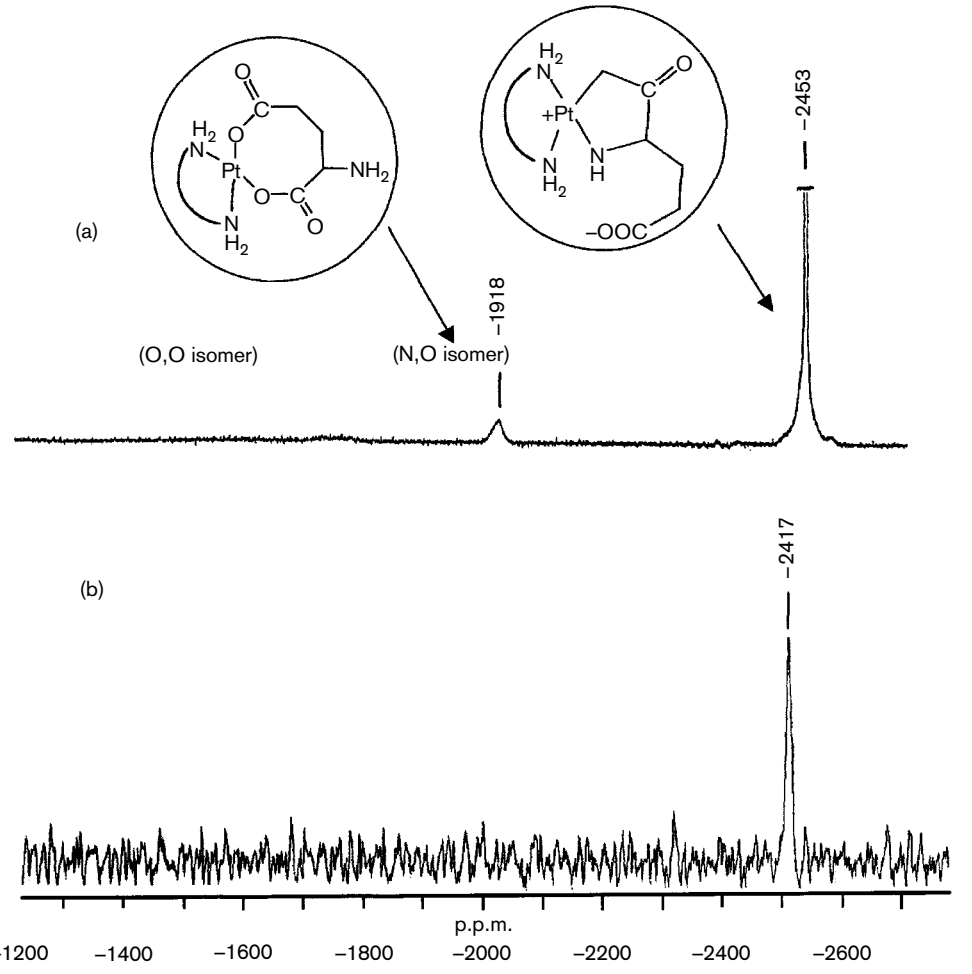


Figure 5. ^{195}Pt-NMR spectra of L-Glu·Pt(dach) (a) and $\{N_3P_3(\text{L-Glu}\cdot Li_2)_3[\text{L-Glu}\cdot\text{Pt(dach)}]_3\}$ (b).

D_2O was monitored as a function of time at room temperature using ^{31}P-NMR spectroscopy (Figure 6). The initial ^{31}P-NMR spectrum of a freshly prepared aqueous solution of compound **1** shows a broad singlet at 13.2 p.p.m., but on standing at room temperature new peaks assignable to the ring-opened phosphazene species appear and increase as the initial peak decreases. Such an observation clearly indicates that the cyclotriphosphazene–(diamine)platinum conjugate is subjected to degradation in aqueous solution, resulting in controlled release of the antitumor platinum moiety, which seems to play an important pharmacokinetic role *in vivo*.

Antitumor activity

All the cyclotriphosphazene–(diamine)platinum conjugates prepared in this study were subjected to *in vitro* and *in vivo* assays using the murine leukemia L1210 cell line and the results are listed in Table 1. All these trimeric conjugates except for compound **3** in which the carrier amine ligand is ethylenediamine exhibit good *in vitro* and *in vivo* antitumor activities comparable to those of the polyphosphazene–platinum(II) conjugates previously reported.[1] In particular, compounds **1** (KI 60606) and **4** have shown extraordinary high *in vivo* activity. One of the most important criteria to be a third-generation platinum anticancer drug is to overcome cross-resistance. Therefore, one of the best compounds among the present trimeric conjugates, i.e. KI 60606, was subjected to a separate trial to compare *in vivo* activity against parent and cisplatin-resistant leukemia L1210 cell lines. The results in Table 2 clearly show that KI 60606 was equally active against both parent and cisplatin-resistant cell lines, and showed no cross-resistance to cisplatin. To examine further the cross-resistance of KI 60606 against different cell lines, *in vitro* assays were also performed using cisplatin-resistant A2780/CP70 and adriamycin-resistant

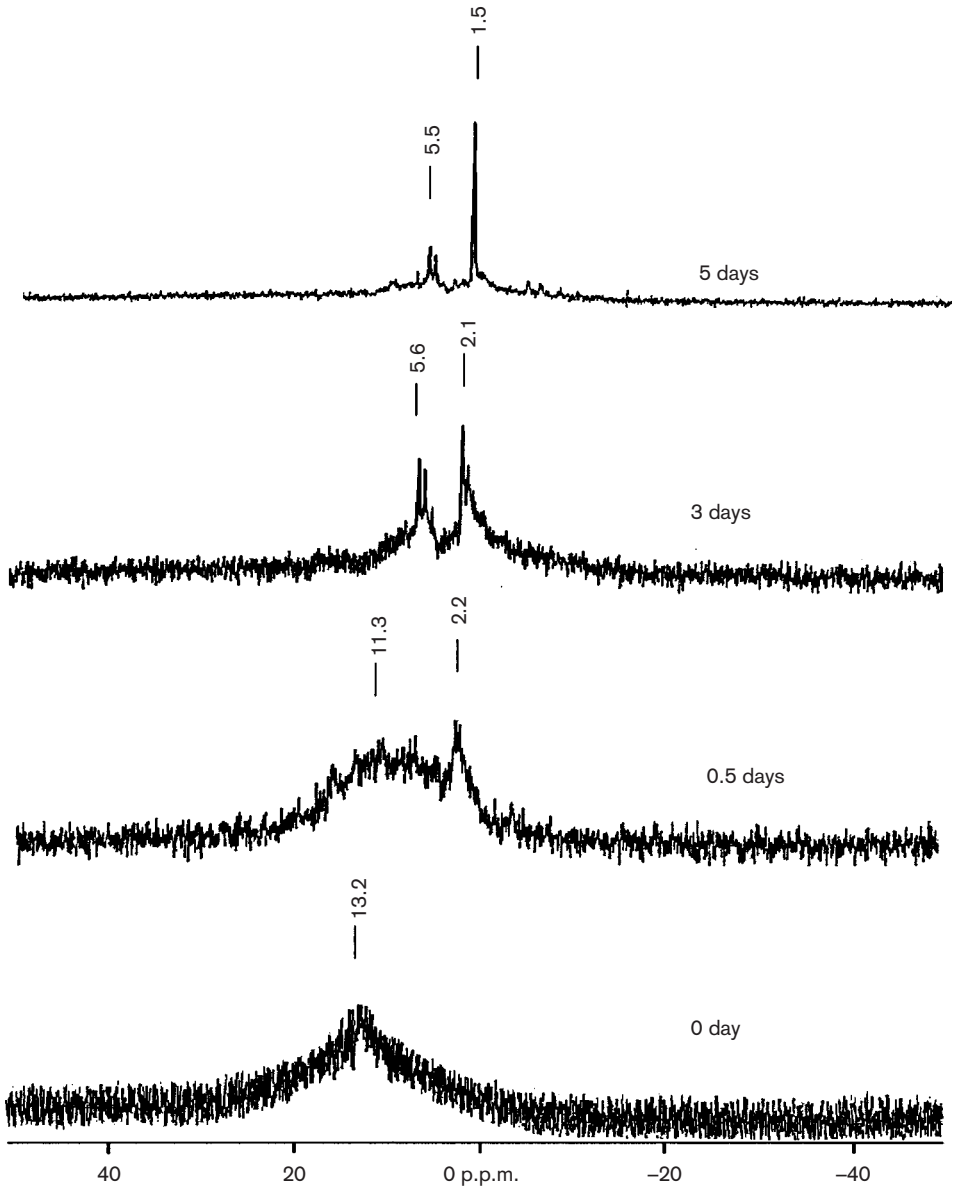


Figure 6. Time-dependent ^{31}P-NMR spectra of compound **1**.

Table 2. *In vivo* cross-resistance of KI 60606 to cisplatin

Cell lines	Samples	Dosage (mg/kg)	Mean animal weight (g on days indicated)				60 day survivors	ILS (%)
			1	5	9	13		
L1210/0	KI60606	60	21	17	17	16	7/10	>600
		40	21	19	19	19	4/10	>268
	cisplatin	8	21	18	17	15	4/10	>218
		5.3	21	20	20	19	1/10	>125
L1210/cisplatin	KI 60606	60	19	16	16	16	6/10	>522
		40	19	17	18	17	8/10	>522
	cisplatin	8	19	18	16	–	0/10	11
		5.3	19	19	18	–	0/10	11

HCT15/CLO2 cell lines. The resistance factors in Table 3 indicate that KI 60606 also shows no cross-resistance to both cell lines. In order to see the spectrum of anticancer activity, KI 60606 was also subjected to *in vitro* assay against selected human tumor cell lines. As shown in Table 4, KI 60606 shows higher cytotoxicity against all the selected human tumor cell lines than cisplatin and carboplatin.[24–28] In particular, KI 60606 shows excellent activity against human non-small cell lung, ovarian and colon cancers.

Discussion

The present cyclotriphosphazene–platinum(II) conjugates are a new class of oligomeric platinum complexes affording controlled release of the antitumor moiety, (diamine)platinum(II), *in vivo* when administered via injection.

In a previous paper,[1] (diamine)platinum(II) complexes conjugated to polyphosphazene through dicarboxylic amino acids exhibited excellent anticancer activity but were found to respond positively to an anaphylaxis test. Therefore, we have changed our drug delivery system to the oligomeric cyclotriphosphazene, to which dicarboxylic amino acids were introduced as a spacer for platination and also as a solubilizing group. The present conjugate drugs were found to respond negatively to anaphylaxis test and have shown excellent antitumor activity with lower toxicity. In particular, compound **1** (KI 60606) exhibited high *in vivo* antitumor activity against both murine and human tumor cell lines. Although it is not easy to explain why the present trimer conjugates exhibit so high *in vivo* antitumor activity against the murine tumor cell, it may be presumed that the high activity is probably ascribed to the controlled release of the (diamine)platinum moiety from the phosphazene trimer skeleton as was explained in the multinuclear NMR study in the previous section.

More detailed examination of the antitumor activity in relation to the molecular structure of the trimeric conjugates reveals that the spacer groups linking the bioactive (diamine)platinum(II) moiety to the trimer ring, i.e. aspartate or glutamate, seem not to give rise to any difference in activity, but the structure of the carrier amine ligands seems to largely affect the antitumor activity of the conjugates. The hydrophilic solubilizing ligand was introduced in order to solubilize the final conjugate products in water, but they may also influence the rate and pattern of degradation of the trimeric conjugates, since it is known that hydrolytic degradation of the phosphazene trimer is

Table 3. *In vitro* cross-resistance of KI 60606 to cisplatin and adriamycin

Compounds	ED_{50} (μM)		R_f (R/S)
	A2780	A2780/CP70	
KI 60606	0.24	0.27	1.1
Cisplatin	11.0	34.8	3.2
Carboplatin	3.24	13.8	4.2
Compounds	ED_{50} (μM)		R_f (R/S)
	HCT15	HCT15/CLO2	
KI 60606	1.74	1.89	1.1
Cisplatin	7.90	11.3	1.4
Carboplatin	16.9	32.1	1.9
Adriamycin	0.12	3.84	32.0

Table 4. *In vitro* activity of KI 60606 against human tumor cells

	Cell lines	ED_{50} (μM)		
		KI 60606	Cisplatin	Carboplatin
Non-small cell lung cancer	A549	1.92	6.09	30.6
	H460	1.89	2.92	9.80
	H26	0.33	3.84	12.6
	LXFA529	5.04	22.7	111
Ovarian cancer	SK-OV-3	1.35	11.9	32.8
	OVCAR-5	1.23	5.79	23.7
	OVCAR-8	2.64	5.82	31.3
	IGROV-1	1.23	1.33	4.36
Colon cancer	HCT116	1.32	12.7	92.3
	DLD-1	8.28	15.4	94.8
	SW620	0.30	13.3	89.7
Melanoma	SKMEL-2	0.45	0.94	14.2
CNS cancer	XF498	1.23	3.08	13.7

greatly dependent on the side groups substituted on the phosphorus atom of the ring.[17,29]

Conclusion

The antitumor moiety (diamine)platinum(II) was conjugated to cyclotriphosphazene using dicarboxylic amino acid as a spacer. Among the present conjugate complexes, compound **1** (KI 60606) fully meets the requirements for third-generation platinum anticancer drugs: high antitumor activity, low toxicity, no cross-resistance and good water solubility. Therefore, this representative compound was selected as a candidate for human clinical trials and the preclinical studies including various toxicity tests are nearly complete. The high *in vivo* antitumor activity of the present conjugates seems to be attributed to controlled release of the active (diamine)platinum(II) moiety, which is induced by hydrolytic degradation of the trimers. This new compound has a high potential for commercialization as a third-generation anticancer drug.

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