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Synthesis and antitumor activity of novel polyphosphazene-(diamine)platinum(II) conjugates

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Abstract

A novel class of water-soluble polyphosphazene-(diamine)platinum(II) conjugate drugs $[N=P(S)Am\cdot PtA_2)]_n$ have been designed and synthesized by incorporating the antitumor (diamine)platinum(II) moiety (A_2Pt^{2+}) to a polyphosphazene back-bone along with a solubilizing groups (S) employing dicarboxylic amino acid (Am) as a spacer group. After characterization of these polymer conjugates by means of multinuclear (^1H, ^{31}P, ^{195}Pt) NMR and IR spectroscopies, elemental analysis and GPC, their antitumor activity were evaluated both in vitro and in vivo against murine leukemia L1210 cell lines and in vitro against five human tumor cell lines. Most of the title polymer conjugates have shown higher in vivo antitumor activity than cisplatin, and in particular $[N=P(OH)(Glu\cdot Pt(DACH))]_n$ (Glu = glutamate, DACH = *trans*(±)-1,2-diaminocyclohexane) exhibit extraordinary high activity (ILS(%) > 500) without cross-resistance to cisplatin as well as good water solubility, and therefore, was subjected to preclinical studies for human clinical trials. © 1997 Elsevier Science B.V.

Keywords: Anticancer drug; Polyphosphazene-(diamine)platinum conjugate; Platinum prodrug; Cisplatin; Drug delivery system; Conjugate drug

1. Introduction

Cisplatin, *cis*-diamminedichloroplatinum(II), is the most effective anticancer drug against testicular, ovarian, bladder, and head and neck cancers (Duran, 1980; Carter, 1984; Loehrer and Einhorn, 1984). However, its usefulness is limited due to its severe toxicities such as nephrotoxicity and neurotoxicity (Krakoff, 1979; Von Hoff et al., 1979). On the other hand the second generation platinum compound, carboplatin (*cis*-diammine (1,1-cyclobutanedicarboxylato)platinum(II)), exhibits

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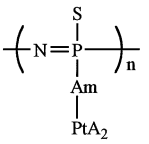
a remarkably reduced toxicity, but it is inferior to cisplatin in efficacy and spectrum of antitumor activity. Furthermore, both cisplatin and carboplatin cause development of resistance to tumor cells (Ozols and Young, 1984; Hong et al., 1988). Accordingly, a great deal of efforts have been made to overcome these drawbacks and to develop a new third generation drug. Currently, more than ten candidate platinum complexes are in clinical studies of phase I or phase II, but most recently, many of them have been abandoned due to serious toxicities, which indicates the probable limit of intrinsic capability of simple mononuclear platinum complexes. Attempts to reduce the toxicity of platinum(II) complexes have been made by combination of the unaltered drug with various organic polymers as carriers (Schechter et al., 1989; Bogdanov et al., 1996), but many of the carriers were found to be not very effective particularly in terms of efficacy of the drug.

We have designed a new polymeric platinum drug affording to controlled-release active platinum moiety in vivo using a biodegradable and/or hydrolytically degradable polyphosphazene of a low molecular weight ($Mw = 10^4–10^5$), which was developed for drug delivery system recently by the authors (Sohn et al., 1995). Polyphosphazene is a linear polymer with an inorganic back-bone made of phosphorus and nitrogen atoms linked by alternating single and double bonds, which is represented by $[N = P(R)(R')]_n$. A great variety of polymers with different physico-chemical properties have been made depending on the substituents R and R′. In particular, if an amino acid is used as substituent, the polymer is known to be biodegradable or hydrolytically degradable (Allcock et al., 1982, 1994a,b). For instance, the ethylglycine-substituted polymer is known to undergo degradation into nontoxic glycine, phosphoric acid and ammonium ion in aqueous solution (Allcock et al., 1994b).

We have employed dicarboxylic amino acids (Am) such as aspartic and glutamic acids as a spacer in order to chelate the bivalent cationic (diamine)platinum(II) moiety (A_2Pt^{2+}) by covalent bonding. Also a solubilizing group (S) was introduced into the polymer back-bone as another substituent in order to control the solubility and degradability of the final polymer conjugates as shown in the following molecular structure



S=OH, OCH_3, $NHCH_3$, $N(CH_3)$

Am=Aspartate(Asp), glutamate(Glu)

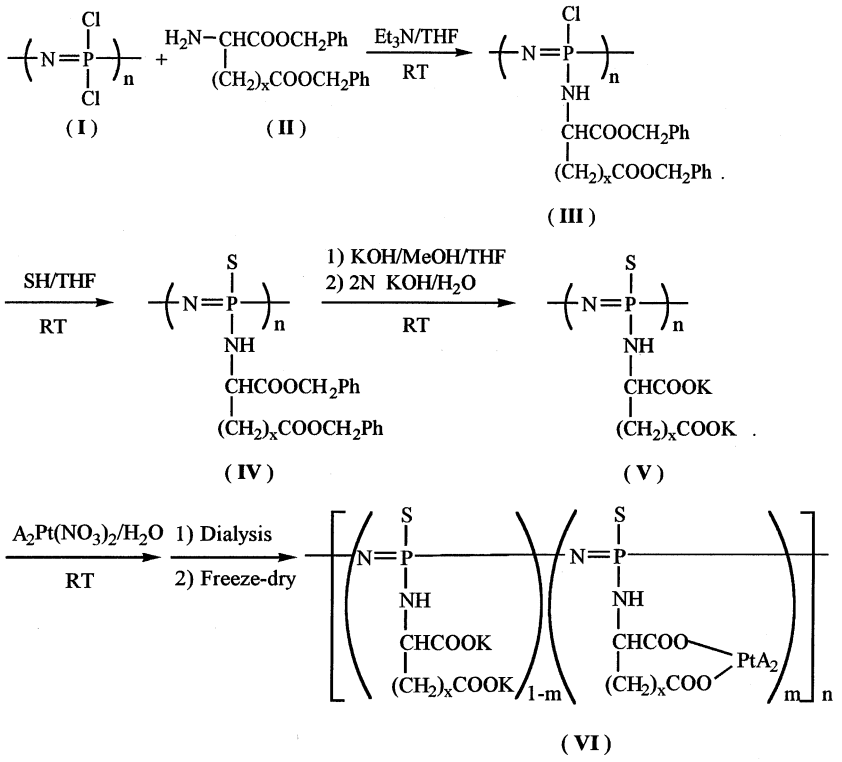
A = NH_3, CH_3NH_2; A_2=*trans*(±)-1,2-diaminocyclohexane(DACH), 1,1-diaminomethylcyclobutane (DAMCB), tetrahydro-4H-pyran-4,4-dimethaneamine(THPDMA).

2. Materials and methods

2.1. Chemistry

The polyphosphazene-(diamine)platinum(II) conjugates were synthesized according to the following reaction Scheme 1.

The low molecular weight ($Mw = 10^4–10^5$) poly(dichlorophosphazene) obtained by the author's procedure was used without purification for the substitution reactions. The two chlorine atoms on the phosphazene back-bone were substituted stepwise with an equimolar dicarboxylic amino acid and then with a solubilizing group. All these substitution reactions were performed in throughly dried solvents and under inert atmosphere, until the completely substituted product (**IV**) was obtained. In order to incorporate the (diamine)platinum moiety into the polymer by complexing with the amino dicarboxylate via simple metathesis reaction, the copolymer (**IV**) was subjected to complete hydrolysis reactions in two steps to obtain a water soluble alkali metal salt form (**V**). The first hydrolysis reaction of the copolymer (**IV**) was performed in the presence of alkali in a slovent mixture of methanol and THF, in which the hydrolyzed product (**V**) precipitated out. The final hydrolysis reaction was carried out in aqueous alkali solution. The water soluble copolymer (**V**) was finally reacted with an aqueous



Scheme 1.

solution of (diamine)platinum nitrate prepared by the literature method (Harrison et al., 1980) in a desired mole ratio. The final reaction mixture was subjected to dialysis using a membrane (molecular weight cutoff: 1200) to remove the byproduct (KNO_3) and other low molecular weight impurities. The platinum content of the final products (**VI**) was determined by controlling the reactant mole ratio, but 80–90% of the theoretical amount of platinum(II) ($m = 0.8$–0.9) was optimal for antitumor activity and solubility.

2.2. Synthesis

All the reactions in organic solvents were carried out under a dry nitrogen atmosphere using standard Schlenk line techniques. Organic solvents used in the reaction were dried thoroughly by distilling over sodium/benzophenone or sodium hydride under a dry nitrogen atmosphere.

2.2.1. General procedure for the synthesis of the title complexes $[NP(S)(Am \cdot PtA_2)]_n$

L-Aspartoyl or L-glutamoyl dibenzyl ester *p*-toluenesulfonate (38.9 mmol) and triethylamine

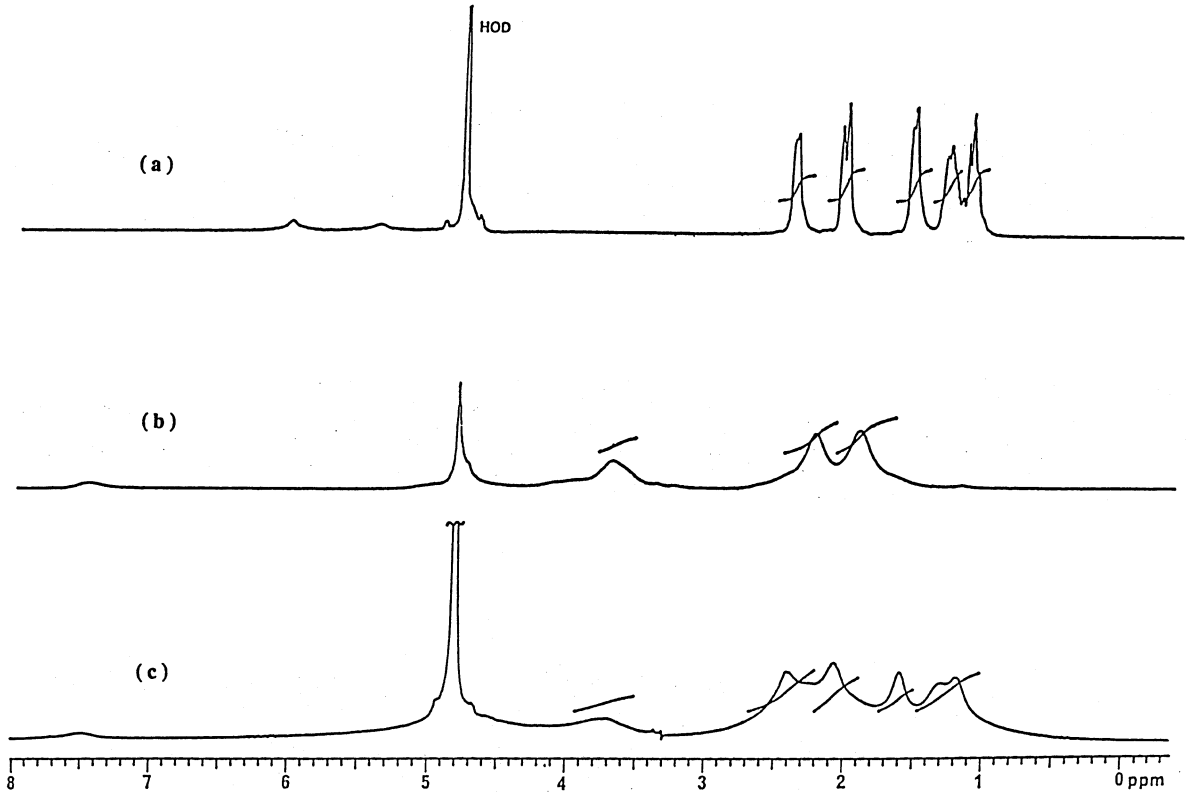


Fig. 1. ^{1}H NMR spectra of (a) (DACH)Pt(NO_3)$_2$, (b) [N = P(OH)(Glu·K_2)]$_n$ and (c) {N = P(OH)[Glu·Pt(DACH)]}$_n$.

(77.8 mmol) were dissolved in THF (400 ml), and after the solution is cooled to 0°C, low molecular weight ($Mw = 10^4$–10^5) poly(dichlorophosphazene) (3.0 g, 25.9 mmol) in THF (150 ml) was slowly added. The reaction mixture was stirred for 24 h at room temperature and the resultant precipitate ($Et_3N \cdot HCl$) was filtered out. Solubilizing agent (SH = H_2O, CH_3OH, CH_3NH_2, $(CH_3)_2NH$) (27.2 mmol) and triethylamine (27.2 mmol) were added to the filtrate, and the reaction mixture was stirred for 12 h at room temperature. After the precipitate was filtered out, the filtrate was subjected to vacuum evaporation to dryness at 30°C. The crude copolymer (**IV**) was purified twice using solvent pairs of THF/*n*-hexane and THF/H_2O (yield, 85%). To a solution of the copolymer (10.0 mmol) dissolved in a solvent mixture (80 ml) of THF and methanol (1:1, vol.%) was added a solution of an excess potassium hydroxide (30.0 mmol) in the same solvent mixture (50 ml), and the reaction mixture was stirred for 1 h. The precipitated product was filtered and washed with the same solvent mixture, and then dissolved in 2N KOH aqueous solution (40 ml) to complete hydrolysis. After the solution was stirred for 1 h, the same solvent mixture of THF/MeOH (500 ml) was added to precipitate the hydrolyzed product (**V**), which was filtered and washed with ethyl ether and then vacuum dried (yield, 90%). To a solution of the hydrolyzed product (4.0 mmol) in distilled water (50 ml) was added an equimolar aqueous solution (40 ml) of a (diamine)platinum(II) nitrate. The reaction mixture was stirred for 1 h at room temperature, and then subjected to dialysis for 24 h using a membrane (dialysis tubing benzolylated from Sigma; Molecular weight cutoff, 1200 (D7884)) to remove the byproduct (KNO_3) and low molecular weight impurities. The dialyzed solution was freeze-dried and then vacuum dried.

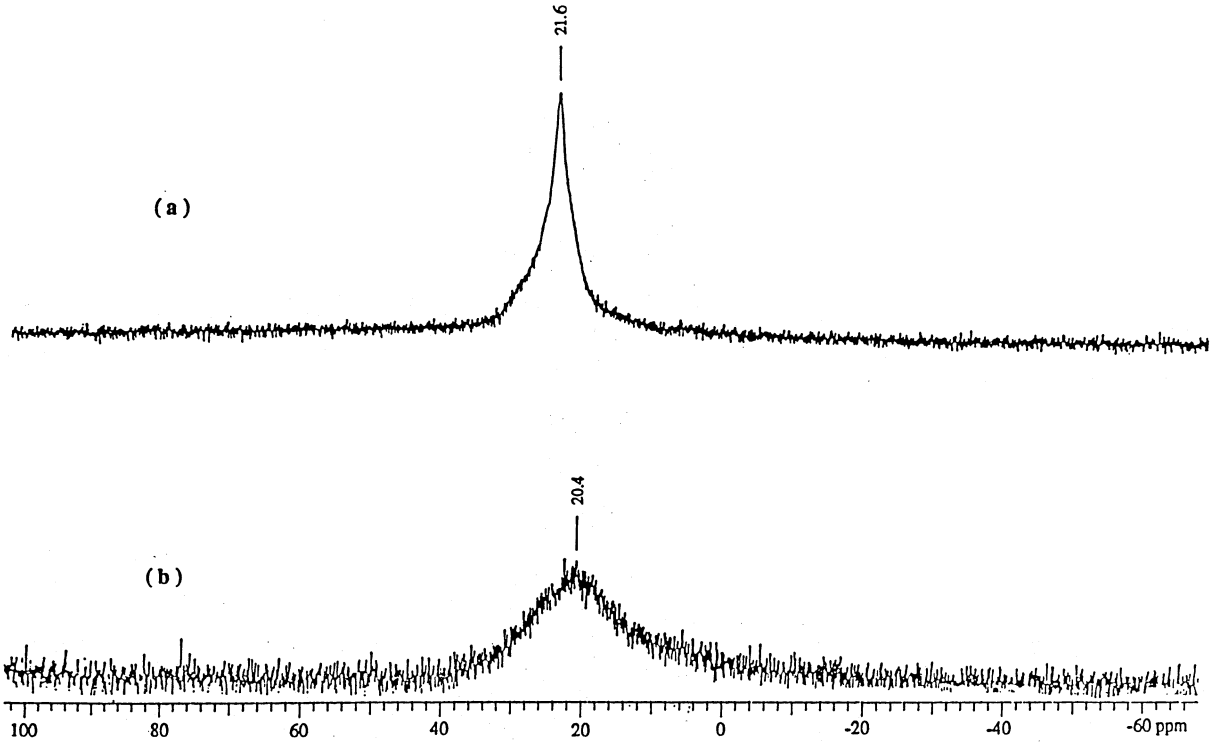


Fig. 2. ^{31}P NMR spectra of (a) $[N=P(OH)(Glu\cdot K_2)]_n$ and (b) $\{N=P(OH)[Glu\cdot Pt(DACH)]\}_n$.

*2.2.2. Poly(hydroxy)[L-asparto-((±)trans-1,2-diaminocyclohexane)platinum]phosphazene (**1**)*

Overall yield, 61%; ^1H NMR (D_2O): δ 1.2–1.3 (b, 4H, diaminocyclohexane C–4, C–5 protons), 1.6 (b, 2H, diaminocyclohexane C–3, C–6 protons), 2.1 (b, 2H, diaminocyclohexane C–3, C–6 protons), 2.4 (b, 2H, CH_2–CO_2), 2.7 (b, 2H, diaminocyclohexane C–1, C–2 protons), 3.8 (b, 1H, CH–CH_2); IR (film, cm^{-1}): 3428 (broad, OH), 3213 (broad, NH), 1618 (assymmetric C=O), 1384 (symmetric C=O); $Mw/Mn = 9.0\times10^3/5.0\times10^3$.

Anal. – Calc. for $\{NP(OH)[C_4H_4NO_4\cdot Pt(C_6H_{14}N_2)]_{0.9}\ (C_4H_4NO_4K_2)_{0.1}\}\cdot 2H_2O$: C, 21.6; H, 4.50; N, 10.1; Pt, 35.2. Found C, 22.3; H,4.27; N, 9.58; Pt, 34.3.

*2.2.3. Poly(hydroxy)[L-asparto-(diammine)platinum]-phosphazene (**2**)*

Overall yield, 68%; ^1H NMR (D_2O): δ 2.7 (b, 2H, CH_2–CO_2), 3.9 (b, 1H, CH–CH_2); IR (film, cm^{-1}): 3443 (broad, OH), 3259 (broad, NH), 1639 (assymmetric C=O), 1369 (symmetric C=O); $Mw/Mn = 9.0\times10^3/6.2\times10^3$.

Anal. – Calc. for $\{NP(OH)[C_4H_4NO_4\cdot Pt(NH_3)_2]\}\cdot 3H_2O$: C, 10.1; H, 3.37; N, 8.84; Pt, 41.1. Found C, 10.4; H, 2.88; N, 9.54; Pt, 40.4.

*2.2.4. Poly(hydroxy)[L-asparto-(2,2-dimethyl-1,3-diaminopropane)platinum]phosphazene (**3**)*

Overall yield, 64%; ^1H NMR (D_2O): δ 1.4 (b, 6H, 2,2-dimethyl protons), 2.3–2.5 (b, 6H, CH_2-CO_2 + diaminopropane C–1, C–3 protons), 3.8 (b, 1H, CH–CH_2); IR (film, cm^{-1}): 3442 (broad, OH), 3224 (broad, NH), 1632 (assymmetric C=O), 1400 (symmetric C=O).

Anal. – Calc. for $\{NP(OH)[C_4H_4NO_4\cdot Pt(C_5H_{14}N_2)]_{0.7}\ (C_4H_4NO_4K_2)_{0.3}\}$: C, 21.3; H, 3.52; N, 11.2; Pt, 32.4. Found C, 21.4; H, 4.26; N, 10.5; Pt, 32.8.

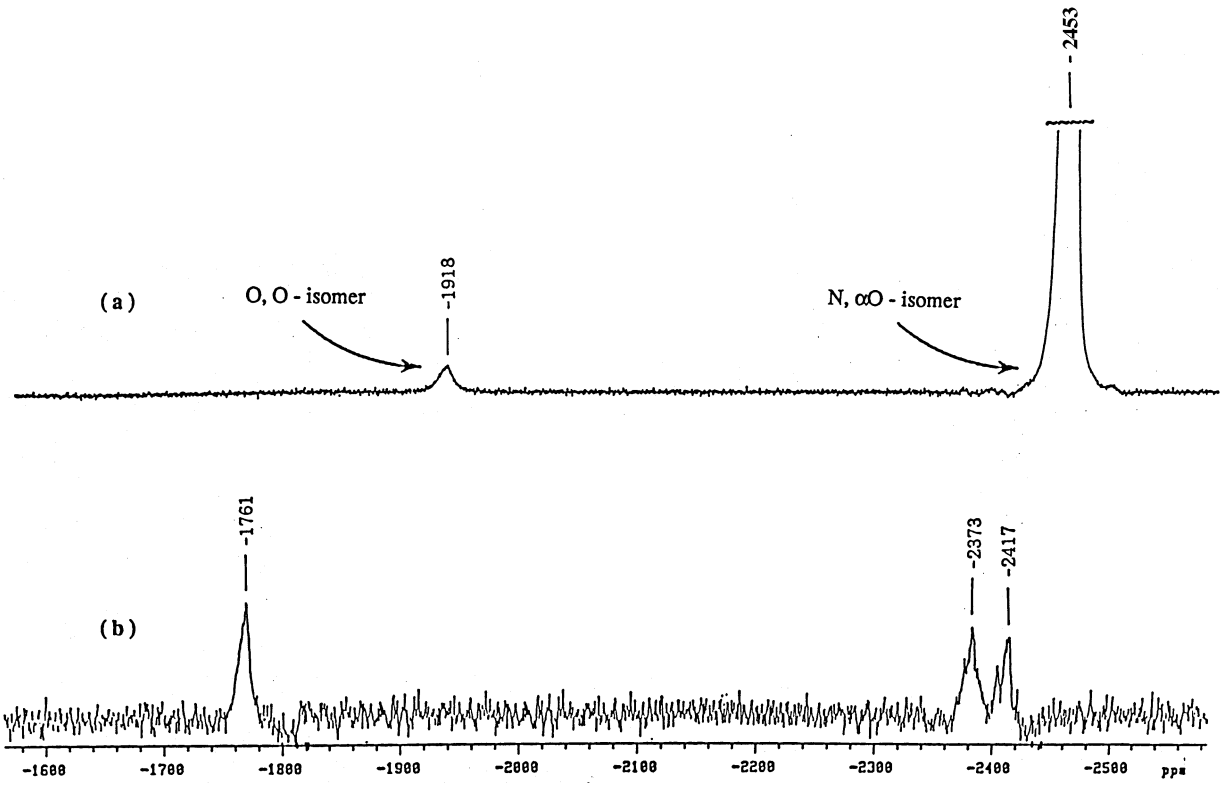


Fig. 3. ^{195}Pt NMR spectra of unconjugated free (a) (Glu)Pt(DACH) and (b) $\{N=P(OH)[Glu\cdot Pt(DACH)]\}_n$.

*2.2.5. Poly(hydroxy)[L-asparto-(1,1-diaminomethylcyclobutane)platinum]phosphazene (**4**)*

Overall yield, 68%; ^{1}H NMR (D_2O): δ 0.8–1.1 (b, 6H, cyclobutane protons), 2.2–2.4 (b, 6H, CH_2-CO_2 + 1,1-diaminomethyl protons), 3.8 (b, 1H, $CH-CH_2$); IR (film, cm^{-1}): 3442 (broad, OH), 3224 (broad, NH), 1632 (assymmetric C=O), 1400 (symmetric C=O).

Anal. – Calc. for $\{NP(OH)[C_4H_4NO_4\cdot Pt(C_6H_{14}N_2)]_{0.8}\ (C_4H_4NO_4K_2)_{0.2}\}\cdot 2H_2O$: C, 21.5; H, 4.23; N, 10.3; Pt, 31.8. Found C, 22.1; H, 4.51; N, 9.84; Pt, 32.1.

*2.2.6. Poly(methoxy)[L-asparto-((±)trans-1,2-diaminocyclohexane)platinum]phosphazene (**5**)*

Overall yield, 61%; ^{1}H NMR (D_2O): δ 1.2–1.3 (b, 4H, diaminocyclohexane C–4, C–5 protons), 1.6 (b, 2H, diaminocyclohexane C–3, C–6 protons), 2.3 (b, 4H, CH_2-CO_2 + diaminocyclohexane C–3, C–6 protons), 2.7 (b, 2H, diaminocyclohexane C–1, C–2 protons), 3.4 (b, 3H, CH_3-O), 3.8 (b, 1H, $CH-CH_2$); IR (film, cm^{-1}): 3426 (broad, OH), 3218 (broad, NH), 1594 (assymmetric C=O), 1398 (symmetric C=O); Mw/Mn = $8.2\times10^3/6.0\times10^3$.

Anal. – Calc. for $\{NP(CH_3O)[C_4H_4NO_4\cdot Pt(C_6H_{14}N_2)]_{0.8}\ (C_4H_4NO_4K_2)_{0.2}\}\cdot 2H_2O$: C, 23.3; H, 4.51; N, 9.98; Pt, 30.9. Found C, 23.4; H, 4.45; N, 9.67; Pt, 31.8.

*2.2.7. Poly(ethoxy)[L-asparto-((±)trans-1,2-diaminocyclohexane)platinum]phosphazene (**6**)*

Overall yield, 62%; ^{1}H NMR (D_2O): δ 1.1–1.3 (b, 4H, diaminocyclohexane C–4, C–5 protons), 1.4–1.6 (b, 5H, CH_3-CH_2 + diaminocyclohexane C–3, C–6 protons), 2.2 (b, 2H, diaminocyclohexane C–3, C–6 protons), 2.4 (b, 2H, CH_2-CO_2), 2.8 (b, 2H, diaminocyclohexane C–1, C–2 protons), 3.4 (2H, CH_2-O), 3.8 (b, 1H, $CH-CH_2$); IR (film, cm^{-1}): 3426 (broad, OH), 3212 (broad,

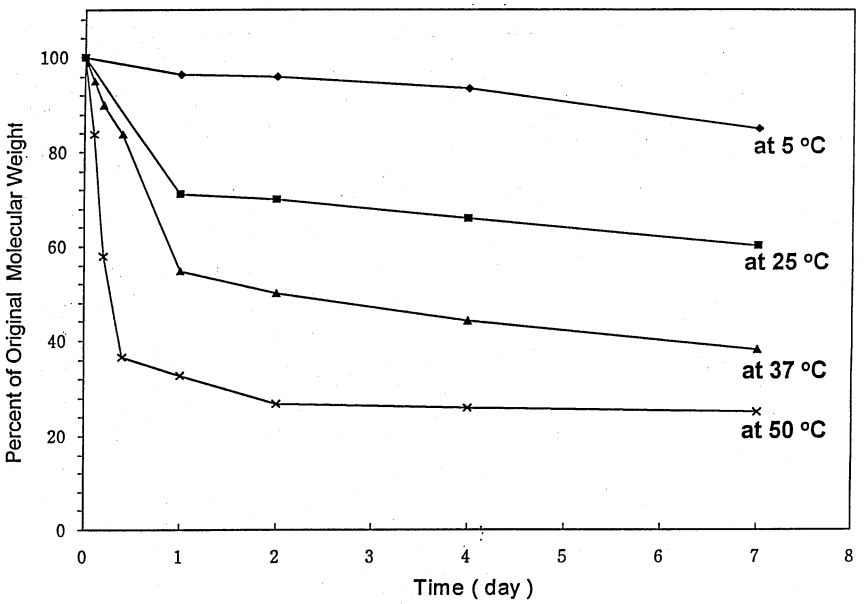


Fig. 4. Rate of hydrolytic degradation of $\{N=P(OH)[Glu\cdot Pt(DACH)]\}_n$ in aqueous solution at various temperatures.

NH), 1624 (assymmetric C=O), 1390 (symmetric C=O); Mw/Mn = $1.0\times10^4/7.4\times10^3$.

Anal. – Calc. for $\{NP(C_2H_5O)[C_4H_4NO_4\cdot Pt(C_6H_{14}N_2)]_{0.8}\ (C_4H_4NO_4K_2)_{0.2}\}\cdot 2H_2O$: C, 28.6; H, 4.69; N, 9.69; Pt, 30.0. Found C, 28.7; H, 5.10; N, 9.01; Pt, 29.1.

2.2.8. Poly(methylamino)[L-asparto-((±)trans-1,2-diaminocyclohexane)platinum]phosphazene (7)

Overall yield, 65%; ^{1}H NMR (D_2O): δ 1.2–1.4 (b, 4H, diaminocyclohexane C–4, C–5 protons), 1.6 (b, 2H, diaminocyclohexane C–3, C–6 protons), 2.1 (b, 4H, CH_2–CO_2 + diaminocyclohexane C–3, C–6 protons), 2.4–2.6 (b, 5H, diaminocyclohexane C–1, C–2 + methylamino protons), 3.8 (b, 1H, CH–CH_2); IR (film, cm^{-1}): 3385 (broad, OH), 3245 (broad, NH), 1645 (assymmetric C=O), 1387 (symmetric C=O); Mw/Mn = $1.1\times10^4/8.2\times10^3$.

Anal. – Calc. for $\{NP(CH_4N)[C_4H_4NO_4\cdot Pt(C_6H_{14}N_2)]_{0.8}\ (C_4H_4NO_4K_2)_{0.2}\}\cdot 2H_2O$: C, 24.3; H, 4.64; N, 12.8; Pt, 31.0. Found C, 24.8; H, 4.62; N, 12.4; Pt, 30.8.

2.2.9. Poly(hydroxy)[L-glutamato-((±)trans-1,2-diaminocyclohexane)platinum]phosphazene (8)

Overall yield, 65%; ^{1}H NMR (D_2O): δ 1.1–1.3 (b, 4H, diaminocyclohexane C–4, C–5 protons), 1.5 (b, 2H, diaminocyclohexane C–3, C–6 protons), 2.0 (b, 4H, CH_2–CH_2 + diaminocyclohexane C–3, C–6 protons), 2.3 (b, 4H, CH_2–CO_2 + diaminocyclohexane C–1, C–2 protons), 3.7 (b, 1H, CH–CH_2); IR (film, cm^{-1}): 3422 (broad, OH), 3234 (broad, NH), 1634 (assymmetric C=O), 1400 (symmetric C=O); Mw/Mn = $1.2\times10^4/6.9\times10^3$.

Anal. – Calc. for $\{NP(OH)[C_5H_6NO_4\cdot Pt(C_6H_{14}N_2)]_{0.9}\ (C_5H_6NO_4K_2)_{0.1}\}\cdot 2H_2O$: C, 23.6; H, 4.50; N, 10.1; Pt, 33.2. Found C, 23.6; H, 4.21; N, 9.59; Pt, 34.0.

2.2.10. Poly (hydroxy)[L-glutamato-(diammine)-platinum]-phosphazene (9)

Overall yield, 67%; ^{1}H NMR (D_2O): δ 2.2 (b, 2H, CH_2–CH_2), 2.5 (b, 2H, CH_2–CO_2), 3.8 (b, 1H, CH–CH_2); IR (film, cm^{-1}): 3460 (broad, OH), 3250 (broad, NH), 1628 (assymmetric C=O), 1386 (symmetric C=O); Mw/Mn = $1.3\times10^4/7.1\times10^3$.

Anal. – Calc. for $\{NP(OH)[C_5H_6NO_4 \cdot Pt(NH_3)_2]\} \cdot 3H_2O$: C, 12.3; H, 3.88; N, 11.5; Pt, 39.9. Found C, 12.0; H, 3.21; N, 10.1; Pt, 38.4.

*2.2.11. Poly(hydroxy)[L-glutamato-(tetrahydro-4H-pyran-4,4-dimethaneamine)platinum]-phosphazene (**10**)*

Overall yield, 65%; ^{1}H NMR (D_2O): δ 1.5 (b, 4H, pyran C–3, C–5 protons), 2.4–2.6 (8H, $CH_2-CH_2 + CH_2-CO_2$ + 4,4-dimethaneamine protons), 3.7 (b, 5H, CH-CH_2, + pyran C–2, C–6 protons); IR (film, cm^{-1}): 3446 (broad, OH), 3234 (broad, NH), 1638 (assymmetric C=O), 1384 (symmetric C=O); Mw/Mn = $1.5 \times 10^4/8.9 \times 10^3$.

Anal. – Calc. for $\{NP(OH)[C_5H_6NO_4 \cdot Pt(C_7H_{16}N_2O)]_{0.8} (C_5H_6NO_4K_2)_{0.2}\} \cdot 2H_2O$: C, 24.1; H, 4.53; N, 9.53; Pt, 29.5. Found C, 23.8; H, 4.33; N, 10.7; Pt, 31.5.

*2.2.12. Poly(methoxy)[L-glutamato-((±)trans-1,2-diaminocyclohexane)platinum]phosphazene (**11**)*

Overall yield, 60%; ^{1}H NMR (D_2O): δ 1.1–1.3 (b, 4H, diaminocyclohexane C–4, C–5 protons), 1.5 (b, 2H, diaminocyclohexane C–3, C–6 protons), 2.1 (b, 4H, CH_2-CH_2 + diaminocyclohexane C–3, C–6 protons), 2.4 (b, 4H, CH_2-CO_2 + diaminocyclohexane C–1, C–2 protons), 3.3 (b, 3H, CH_3-O), 3.8 (b, 1H, CH–CH_2); IR (film, cm^{-1}): 3420 (broad, OH), 3254 (broad, NH), 1618 (assymmetric C=O), 1387 (symmetric C=O); Mw/Mn = $8.2 \times 10^3/6.5 \times 10^3$.

Anal. – Calc. for $\{NP(CH_3O)[C_5H_6NO_4 \cdot Pt(C_6H_{14}N_2)]_{0.8} (C_5H_6NO_4K_2)_{0.2}\} \cdot 2H_2O$: C, 25.0; H, 4.70; N, 9.71; Pt, 30.1. Found C, 25.0; H, 4.66; N, 9.26; Pt, 29.4.

*2.2.13. Poly(methylamino)[L-glutamato-((±)trans-1,2-diaminocyclohexane)platinum]phosphazene (**12**)*

Overall yield, 68%; ^{1}H NMR (D_2O): δ 1.1–1.3 (b, 4H, diaminocyclohexane C–4, C–5 protons), 1.6 (b, 2H, diaminocyclohexane C–3, C–6 protons), 2.0 (b, 4H, CH_2-CH_2 + diaminocyclohexane C–3, C–6 protons), 2.3–2.6 (b, 7H, CH_2-CH_2 + diaminocyclohexane C–1, C–2 + methylamino protons), 3.8 (b, 1H, CH–CH_2); IR (film, cm^{-1}): 3418 (broad, OH), 3194 (broad, NH), 1620 (assymmetric C=O), 1403 (symmetric C=O); Mw/Mn = $1.4 \times 10^4/8.6 \times 10^3$.

Anal. – Calc. for $\{NP(CH_4N)[C_5H_6NO_4 \cdot Pt(C_6H_{14}N_2)]_{0.8} (C_5H_6NO_4K_2)_{0.2}\} \cdot 2H_2O$: C, 25.0; H, 4.90; N, 12.4; Pt, 30.1. Found C, 25.3; H, 5.53; N, 12.0; Pt, 30.1.

*2.2.14. Poly(dimethylamino)[L-glutamato-((±)trans-1,2-diaminocyclohexane)platinum]phosphazene (**13**)*

Overall yield, 67%; ^{1}H NMR (D_2O): δ 1.1–1.3 (b, 4H, diaminocyclohexane C–4, C–5 protons), 1.6 (b, 2H, diaminocyclohexane C–3, C–6 protons), 2.0 (b, 4H, CH_2-CH_2 + diaminocyclohexane C–3, C–6 protons), 2.4–2.6 (b, 10H, CH_2-CO_2 + diaminocyclohexane C–1, C–2 + dimethylamino protons), 3.8 (b, 1H, CH–CH_2); IR (film, cm^{-1}): 3432 (broad, OH), 3198 (broad, NH), 1628 (assymmetric C=O), 1397 (symmetric C=O); Mw/Mn = $1.4 \times 10^4/7.6 \times 10^3$.

Anal. – Calc. for $\{NP(C_2H_6N)[C_5H_6NO_4 \cdot Pt(C_6H_{14}N_2)]_{0.8} (C_5H_6NO_4K_2)_{0.2}\} \cdot 2H_2O$: C, 26.6; H, 5.15; N, 12.1; Pt, 29.3. Found C, 27.4; H, 5.80; N, 11.8; Pt, 29.0.

2.3. Instrumentation

^{1}H NMR spectra were recorded on a Varian Gemini-300 spectrometer operating at 300 MHz in the Fourier transform mode with 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 triphenyl phosphate (^{31}P) and Na_2PtCl_6 (^{195}Pt) as an external standard. IR spectra were recorded on Midac 101025 FT-IR spectrophotometer. Molecular weight measurements were performed by gel permeation chromatography using a Waters Associates HPLC/GPC 150C unit and ultrastyragel or ultrahydragel column. Polystyrene or poly (ethylene oxide) were used to calibrate the columns. Sample concentrations were approximately 1.5% (w/v) in THF or deionized/distilled water. Elemental analysis were carried out by the Chemical Analysis Center at KIST.

2.4. Evaluation of antitumor activity

2.4.1. In vitro assay

SRB (sulforhodamine B) assay, developed for

measuring the cellular protein content of the cultures, was applied for the measurement of the cytotoxicity of the compounds against the human tumor cells. The SK-MEL-2 melanoma, A-549 non-small cell lung, SKOV-3 ovarian, HCT-15 colon and XF-498 CNS tumor cell lines were maintained as stocks in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco). The rapidly growing cells were harvested, counted, and inoculated at the appropriate concentrations ($1\text{-}2 \times 10^4$ cells/well) into 96 well microtiter plates. After incubation for 24 h, the compounds dissolved in culture medium were applied to the culture wells in triplicate followed by incubating for 48 h at 37°C under 5% CO_2 atmosphere. The cultures fixed with cold TCA were stained by 0.4% SRB dissolved in 1% acetic acid. After solubilizing the bound dye with 10 mM unbuffered tris base by gyratory shaker, the absorbance at 520 nm was measured with a microplate reader (Dynatech Model MR 700). Fifty percent inhibitory concentration (ID_{50}) was defined as the concentration which reduced absorbance by 50% of untreated wells as of the control in the SRB assay.

For in vitro assay against the murine luekemia L1210 line, cells maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum were adjusted to 1×10^6 cells/ml and distributed to 24 well tissue culture plates (0.5 ml/well). Test compounds were serially diluted and added to wells (0.5 ml/well). Following 48 h incubation in a 5% CO_2 atmosphere at 37°C, cell counts were determined with a Coulter Model ZM cell counter. Cell growth in the presence of test compounds was expressed as a percentage of growth in untreated control wells and the concentration of compound producing 50% inhibition of cell growth was determined (ED_{50}).

2.4.2. In vivo assay

These tests were carried out using the ascites cell form of L1210 lymphoid leukemia, which was obtained from BDA/2 donor mice bearing 3- to 5-day tumor growth. L1210 leukemia cells (10^6) were inoculated i.p. in BDF mice (6–8 week old, 20-25 g; 8 mice per group), and 24 h later, compounds were administered i.p. on days 1, 5, and 9. Mortality was recorded and the mean survival time was calculated for each group. The percentage of increased life span (ILS) was calculated as follows:

$$\%\mathrm{ILS} = (T - C)/C\ 100$$

where T is the mean survival time of the drug treated mice and C is that of control mice.

3. Results and discussion

3.1. Synthesis and properties

Most of the polyphosphazene-(diamine) platinum conjugates prepared by the synthetic Scheme 1 are hardly obtained as copolymers fully substituted by (diamine)platinum moiety. Equimolar reaction of the (diamine)platinum salt with the potassium salt of the phosphazene polymer (**V**) usually resulted in 80–90% platinum complexation of the amino dicarboxylate ligands on the polymer back-bone. Use of excess (diamine)platinum salt gave rise to increase of platination mole ratio above 90%, but the resultant polymers nearly fully complexed by (diamine)platinum show considerably lower solubility in water without improved antitumor activity. All the polyphosphazene-(diamine)platinum conjugates are obtained in light yellow powder form without melting point. They are all very soluble in water (> 20 mg/ml), but almost insoluble in most organic solvents including alcohol and other polar solvents. All the polyphosphazene-(diamine)platinum conjugates thus prepared have been characterized by means of multinuclear (^1H, ^{31}P, ^{195}Pt) NMR and IR spectroscopies and elemental analysis. Most of the polymer conjugates were also subjected to molecular weight measurement.

A typical ^1H NMR spectrum of one of the representative conjugates $\{NP(OH)[Glu \cdot Pt(DACH)]\}_n$ is shown along with the spectra of its separate reactants in Fig. 1. The cyclohexyl protons of unreacted $(DACH)Pt(NO_3)_2$ show five resonances due to the ring conformation. When the platinum moiety is coordinated by the dicarboxylate ligand on the polymer back-bone, these cyclohexyl proton peaks are not shifted but

Table 1
In vitro and in vivo activities against the leukemia L1210 cell line of polyphosphazene-(diamine)platinum conjugates

Compounds	In vitro	In vivo		
	ED_{50} (μg/ml)	Dose (mg/kg)	ILS (%)	60 day survivors
1	1.2	60	>542	8/8
		30	>288	3/8
2	9.3	30	44	0/8
3	2.0	30	>279	3/8
4	2.1	30	>460	6/8
5	2.3	30	>142	1/8
6	2.1	30	>143	1/8
7	1.2	30	>195	1/8
8	1.9	60	>435	6/8
		30	>157	1/8
9	10	30	45	0/8
10	3.3	30	>252	2/8
11	3.8	30	>104	1/8
12	0.8	30	>260	2/8
13	2.3	60	>378	5/8
		30	174	0/8
Cisplatin	0.3	4	80	0/8

broadened with partial overlapping with those of the amino dicarboxylate protons. Fig. 2 shows the ^{31}P NMR spectrum of the same conjugate along with that of the unconjugated polymer exhibiting a broad singlet at 21.6 ppm, which is frequently observed for copolymers (Allcock and Coggio, 1990). It is not surprising that the platinated conjugate polymer shows even broader resonance without significant chemical shift. More interesting and important are the ^{195}Pt NMR spectra of the polymer conjugates. It is well known that ^{195}Pt resonance is very sensitive to its chemical environment (Gibson et al., 1990). Unfortunately, it has not been successful to measure the ^{195}Pt NMR of the present conjugate polymer 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 concentrated aqueous solution. However, the ^{195}Pt NMR spectrum of the representative conjugate $NP(OH)[Glu \cdot Pt(DACH)]_n$ in aqueous solution shown in Fig. 3 is informative on the solution behavior of the platinum conjugate. From the comparison of the ^{195}Pt NMR spectrum of the platinum conjugate with that of unconjugated (diamine)platinum glutamate previously studied (Lee et al., 1994) it may be presumed that the resonance at 1761 ppm is due to the (diamine)platinum moiety conjugated through (*O*, *O*) coordination mode to the polymeric amino dicarboxylate whereas the two resonances at 2373 and 2417 ppm due to the (diamine)platinum glutamate moiety detached or fragmented from the polymer back-bone. In our previous study we have confirmed that (*O*, *O*) isomer of the (diamine)platinum glutamate is subjected to isomerization to the more thermodynamically stable (*N*, *O*) isomer. Such an observation of the detached or fragmented (diamine)platinum moiety in aqueous solution clearly indicates that the polyphosphazene-(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. We have performed a separate study on the degradation of the polymer conjugate in aqueous solution at various temperatures and the results are exhibited in Fig. 4. The average molecular weights (Mw) of the present polymer conjugates measured in fresh aqueous solution are in the range of 8000 to 15 000. It is seen in the figure that the polymer

Table 2
In vivo activity of $\{NP(OH)[Glt \cdot Pt(DACH)]\}_n$ against murine cell lines

Cell lines	Samples	Dosage (mg/kg)	Mean animal weight (grams on days indicated)				60 day survivors	ILS (%)
			1	5	9	13		
L1210/CDDP								
	8	55	20	19	19	18	5/10	>336
		37	20	20	20	20	7/10	>531
		25	20	20	21	21	2/10	68
	cisplatin	8	20	20	16	15	0/10	15
		5.3	20	20	20		0/10	5
		3.5	20	21	22		0/10	5
L1210/0								
	8	55	20	19	19	18	8/10	>566
		37	20	19	19	19	3/9	>166
		25	20	20	20	20	5/10	>461
	cisplatin	8	20	19	18	16	1/9	125
		5.3	20	20	21	19	0/10	93
		3.5	20	20	21	20	0/10	75

conjugate is stable for more than a week at 5°C but at above room temperature molecular degradation occurs rapidly. The study on the detailed mechanism of degradation is underway.

3.2. Antitumor activity

All the polyphosphazene-(diamine)platinum conjugates prepared in this study have been subjected to both in vitro and in vivo assay using murine leukemia L1210 cell line and the results are listed in Table 1. The in vitro cytotoxicity of the polymer conjugates was found to be time-dependent but mostly in a couple of days to reach steady values, which is understandable in terms of their degradation rate as was mentioned in the previous section. However, no quantitative relationship could be found between the in vitro and in vivo activities measured in our screening system. All the conjugates except for the compounds 2 and 9 in which the carrier amine ligand is monodentate ammine (NH_3) exhibit outstanding antitumor activity. In particular, compounds 1, 4, 8 and 13 have shown extraordinary high in vivo activity even compared with cisplatin. Although it is not easy to explain why the present polymer conjugates exhibit so high in vivo antitumor activity against the murine cell, it may be presumed that the activity is probably ascribed to the controlled release of the (diamine)platinum moiety from the polyphosphazene back-bone as was explained in the multinuclear NMR study of the previous section. However, it is not clearly understood why only diammineplatinum complexes 2 and 9 exhibit low activity.

More detailed examination of the antitumor activity in relation to the local molecular structure of the polymer conjugates reveals that the spacer groups linking the bioactive (diamine)platinum moiety to the polymer back-bone, that is, aspartate and glutamate do not give rise to difference in activity, but solubilizing groups (OH, OCH_3, OC_2H_5, $NHCH_3$, $N(CH_3)_2$) and carrier amine ligands seem to affect largely the antitumor activity of the conjugates. The hydrophilic solubilizing ligands were introduced in order to solubilize the final conjugate products in water, but they may also influence on the rate and pattern of degradation of the polymer conjugates, since it is known that hydrolytic degradation of the polyphosphazene is greatly dependent on the side groups substituted on the phosphorus atom of the back-bone (Allcock et al., 1982). Among the carrier amine ligands used in this study, DACH and DAMCB have shown the best activity.

Table 3
Growth inhibitory effect of selected polymer conjugates against various human cell lines in vitro

Compounds	ID_{50} (μg/mL)				
	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
1	0.84	4.3	2.1	1.1	0.43
4	5.9	3.4	8.7	2.1	5.3
8	0.85	2.5	1.5	0.65	0.64
10	5.7	7.2	8.4	5.3	6.7
13	2.8	2.8	3.1	2.6	3.1
Cisplatin	1.0	0.53	1.5	0.34	2.5
Carboplatin	16.4	13.1	31.0	14.0	42.2

One of the most important criteria to be a 3rd generation platinum anticancer drug is to overcome cross-resistance. Therefore, one of the best compounds among the present polymer conjugates, that is, compound **8** was subjected to in vivo assay using both parent murine leukemia L1210 (L1210/0) and cisplatin-resistant L1210 (L1210/CDDP) cell lines and compared with cisplatin in Table 2. It is clearly seen that cisplatin is not active against its resistant cell line but the present compound **8** is equally active against cisplatin-resistant cell line and therefore, there is no cross-resistance for the present compound. This fact is not surprising, since it is known that cross-resistance of platinum drugs is related with the structure of the carrier amine ligands (Kelland et al., 1992) and in particular DACH ligand is one of the best ligands to overcome cross-resistance.

In order to see cytotoxicity of the present polymer conjugates against human tumor cells, some selected compounds were assayed against five human tumor cell lines and the results are tabulated in Table 3. All the present polymer conjugates show also high cytotoxicity against all the human tumor cell lines tested.

In conclusion, the present polyphosphazene-(diamine)platinum conjugates meet fully all the requirements for the third generation platinum anticancer drug: high antitumor activity, low toxicity, no cross-resistance and good water solubility. Therefore, among the present conjugates, the representative compound **8** was selected as a candidate compound for human clinical trials and preclinical studies including various toxicity studies are nearly in completion.

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