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Enhanced Antitumor Activitiy of *trans*(±)-1,2-Diaminocyclohexaneglutamatoplatinum(II) Formulated with Stealth Liposome

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Abstract—The antitumor platinum(II) compound, [Pt(dach)(Glu)] (dach = *trans*(±)-1,2-diaminocyclohexane, Glu = glutamate) was formulated with a stealth liposome to improve its biological activity. Liposomes were composed of PC/PEG2000-PE/CH (PC = 1,2-diacyl-glycero-3-phosphocholine; PEG2000-PE = poly(ethylene glycol)2000-1,2-diacyl-glycero-3-phosphoethanolamine; CH = cholesterol) involving different acyl moieties of phospholipids such as DO (dioleoyl), DM (dimyristoyl) or DS (distearoyl) group. Among the different acyl groups in the stealth liposomes, the DM formulation was optimal for the preparation of the liposomal [Pt(dach)(Glu)] at the mole ratio of DMPC/PEG2000-DMPE/CH = 50/5/45 and at the weight ratio of drug/lipid = 1/20, which is represented as L-[Pt(dach)(Glu)]. In vitro cytotoxicity was examined in sensitive A2780 and ME180 and their cisplatin-resistant A2780/PDD and ME180/PDD cancer cells. L-[Pt(dach)(Glu)] was 2∼3 times more cytotoxic than the free complex [Pt(dach)(Glu)] and cisplatin in sensitive cells, and 4∼8 times more cytotoxic in resistant cells. Thus, the resistance index of L-[Pt(dach)(Glu)] was 1.3∼2 while those of the free complex and cisplatin were 5∼6, which indicates that L-[Pt(dach)(Glu)] overcome the cisplatin resistance in both resistant cells. In vivo antitumor activity was assayed against the L1210/S leukemia. The optimal activities (% T/C) of the free complex and L-[Pt(dach)(Glu)] were >459/20 and >442/200 mg/kg, respectively. Considering the amount of the platinum complex in L-[Pt(dach)(Glu)], the liposomal [Pt(dach)(Glu)] displayed 2-fold higher drug potency than the free complex. The biodistribution experiment using LE52 tumor-bearing mouse showed excellent lung targeting property of L-[Pt(dach)(Glu)].

Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II)] is one of the most widely used anticancer agents in cancer chemotherapy, but its continuous use is limited due to its toxic side effects and acquired drug resistance.[1] Among several attempts to reduce cisplatin-induced toxicity and overcome cisplatin resistance are the synthesis of *trans*(±)-1,2-diaminocyclohexane(dach) platinum complexes and the formulations using liposomes. The (dach)platinum complexes have attracted significant attention because they are not cross-resistant to cisplatin,[2] but their clinical use is still difficult due to their drug-induced toxicity and unsatisfactory physicochemical properties. To reduce platinum drug-induced toxicity[3] and modify its phamacokinetics,[4,5] the liposomes have been found to be effectively applied. Liposomes could be used for both hydrophilic[6–8] and hydrophobic platinum complexes[3,9,10] to enable their in vivo administrations. Thus the liposomes encapsulating platinum complexes have been proved to reduce drug toxicity and increase their therapeutic indexes such as antitumor activity, tumor targeting[11,12] and overcoming cisplatin resistance.[4,5] Especially, stealth liposomes using long-circulating pegylated phospholipids have shown to prolong the circulation time, increase tumor localization, and enhance therapeutic efficacy of platinum complexes.[6–8,13,14]

Among the applications of stealth liposomes to platinum complexes, SPI-077 and L-NDDP are well-known representative examples for hydrophilic and hydrophobic platinum complexes, respectively. SPI-077 is a stealth liposomal formulation of cisplatin, which has a prolonged circulation time and increased tumor platinum disposition, and its antitumor efficacy was significantly improved compared to cisplatin in several cancer models.[6–8] L-NDDP [liposomal *cis*-bis-neodeca-

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noato-*trans*-*R*,*R*-1,2-diaminocyclohexaneplatinum(II)] entrapped into polyethylene glycol-coated liposomes was effective in reducing tumor growth in non-reticulo-endothelial system organs, and a high tumor accumulation of NDDP was achieved at the tumor site.[13] Based on these previous reports, we have prepared *trans*(±)-1,2-diaminocyclohexaneglutamatoplatinum(II), [Pt(dach)(Glu)], which was formulated in long-circulating stealth liposomes, and evaluated the usefulness of [Pt(dach)(Glu)] encapsulated with stealth liposomes to increase its biological activity and overcome cisplatin resistance.

Results and Discussion

Evaluation of the liposomal [Pt(dach)(Glu)]

We have prepared the liposomal [Pt(dach)(Glu)] using the basic formulation of PC/PEG2000-PE/CH, varying the long acyl chain of glycerol backbone of each phospholipid, PC and PE. Those acyl groups employed in this study are DM- (1,2-dimyristoyl), DO- (1,2-dioleoyl), and DS- (1,2-distearoyl). The average size of liposomes prepared in this study was found to be in the range of 150–300 nm. The percentage entrapment efficiencies (%EE) of the liposomal [Pt(dach)(Glu)] depending on the liposomal composition and the drug to lipids ratio are shown in Table 1. The entrapment efficiencies for the formulations including DO, DS, and DM groups were similar (38.7, 42.8 and 42.4, respectively) at the molar ratio of PC/PEG2000-PE/CH = 50/5/45. The formulations including DM and DO groups were found to form stable and uniform suspensions whereas the liposomal suspension with DS formulation was somewhat aggregated at room temperature. The DO formulation was found to form a nice liposome, which, however, was a little unstable for long storage probably due to the unsaturated double bond in the acyl chain of DO. After all, the DM formulation turned out to be most suitable for the preparation of the liposomal platinum complexes. The entrapment efficiency of the DM formulation increased appreciably (42–52%) as the portion of PEG2000-DMPE increased four times (5–20%) in the liposome, but as shown in Table 2, the platinum complex encapsulated with the liposomes containing the larger portions of the hydrophilic PEG2000-DMPE exhibited lower cytotoxicity. Furthermore, the content of PEG2000-DMPE does not influence very much on the entrapment efficiency of the stealth liposome. However, the entrapment efficiency decreased sharply from 42.4 to 33.5 and 11.9% as the drug to lipid ratio increased from 1/20 to 1/10 and 1/5, respectively. In other words, the lower drug to lipid ratio resulted in the better encapsulation efficiency with this hydrophilic platinum drug. Therefore, we have decided to use the DM formulation of DMPC/PEG2000-DMPE/CH = 50/5/45 at the weight ratio of drug/lipid = 1/20. This optimal formulation product to be represented as L-[Pt(dach)(Glu)] was used for further animal study.

Antitumor activity

All the in vitro cytotoxicity data are summarized in Table 2. The stealth liposome alone did not show any significant cytotoxicity in both sensitive and resistant cells. Among the formulations in the table, the liposo-

Table 1. Percentage of entrapment efficiency (%EE) of liposomal [Pt(dach)(Glu)]

Lipid type	Drug/lipid ratio (w/w)	Lipid mole ratio	%EE[a]	Drug leakage (%$EE_{6\ h}$-%$EE_{0\ h}$)
DOPC/PEG2000-DOPE/Chol	01:20	50:5:45	38.7	3.2
DSPC/PEG2000-DSPE/Chol	01:20	50:5:45	42.8	2.7
DMPC/PEG2000-DMPE/Chol	01:20	50:5:45	42.4	5.5
	01:20	50:10:40	40.1	3.5
	01:20	50:20:30	52.5	3.5
	01:10	50:5:45	33.5	4.7
	01:05	50:5:45	11.9	2.3

[a]All the %EE values are means of three separate experiments with SD $< \pm 3.0\%$.

Table 2. Cytotoxicity of liposomal [Pt(dach)(Glu)] in sensitive and cisplatin-resistant A2780 and ME180 cell lines[a]

Drug type	Drug/lipid ratio(w/w)	Lipid mole ratio[b]	A2780 ID_{50} (μM)	A2780/PDD ID_{50} (μM)	RI[c]	ME180 ID_{50} (μM)	ME180/PDD ID_{50} (μM)	RI[c]
Liposome only			200[d]	<500[d]		200[d]	<500[d]	2.5
[Pt(dach)(Glu)] only			20	100	5	45	200	4.4
Liposomal [Pt(dach)(Glu)]	01:20	50:05:45	10	25	2.5	19	25	1.3
	01:20	50:10:40	15	20	1.3	20	30	1.5
	01:20	50:20:30	30	50	1.7	39	70	1.8
	01:10	50:05:45	10	40	4	20	40	2
	01:05	50:05:45	10	40	4	24	50	2.1
Cisplatin			20	120	6	30	150	5

[a]All ID_{50} values calculated based on the Pt-content are means ± SD $< \pm 3.0 \sim 10$ from at least three separate experiments.
[b]Liposomes are comprised of DMPC/PEG2000-DMPE/Chol with the mole ratio.
[c]Resistance index = the ratio of ID_{50} in resistant cells to ID_{50} in sensitive cells.
[d]ID_{80} values.

Table 3. In vivo antitumor activity of the liposomal [Pt(dach)(Glu)] in mouse L1210 leukemia

Drug type	Dose (mg/kg)	T/C (%)[a]	60-day survivors
[Pt(dach)(Glu)] only	40	>182	1/8
	20	>459	4/8
L-[Pt(dach)(Glu)][b]	400	Toxic	
	200	>442	3/8
Cisplatin	4	184.4	

[a]Liposome is composed of DMPC/PEG2000-DMPE/Chol = 50:5:45 (mole ratio) and the drug to lipid ratio is 1:20 (w/w).
[b]T/C (%) = Median survival of treated animals/median survival of untreated animals×100.

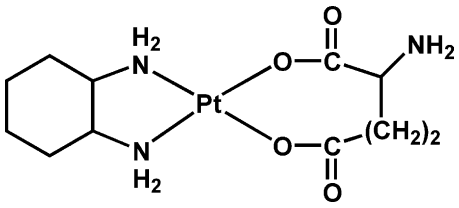

Figure 1. The molecular structure of *trans*(±)-1,2-diaminocyclohexaneglutamato-Platinum(II).

mal [Pt(dach)(Glu)] with the lipid formulation of PC/PEG2000-PE/CH = 50/5/45 and at the drug to lipid ratio of 1/20, that is, L-[Pt(dach)(Glu)] exhibited 2∼3 times more cytotoxic than the free complex [Pt(dach)(Glu)] and cisplatin in sensitive cells, and 4–8 times more cytotoxic in resistant cells. Furthermore, the drug resistance index (RI) of L-[Pt(dach)(Glu)] was found to be 1.3–2.5 in the A2780 cells and 1.3–1.8 in the ME180 cells, which implies that L-(dach)Pt(Glu) could overcome the cisplatin resistance. However, at the drug to lipid ratios of 1/10 and 1/5, the RI values were found to be larger than 2.0 in both cell lines.

The in vivo antitumor activity of L-[Pt(dach)(Glu)] against the leukemia L1210 cell line is shown in Table 3. The optimal doses of the free and liposomal platinum complex were 20 and 200 mg/kg, respectively. It is seen from the table that the activity of the free and the liposomal platinum complex are comparable at this optimal dose, and therefore, considering the amount of the platinum compound in the liposomal [Pt(dach)(Glu)] (1/20,w/w), L-[Pt(dach)(Glu)] displayed about 2-fold higher drug potency than the free platinum compound. Therefore, we have found that the stealth liposomal formulations not only significantly increased the drug potency of the platinum complex but also greatly improved the drug resistance index.

Biodistribution study

The optimal liposomal platinum complex, L-[Pt(dach)-(Glu)] and carboplatin as a reference were injected iv into LE52 tumor-bearing mice. At 2 and 24 hr after injection, the mice were sacrificed to analyze the concentration of platinum distributed in tumor, blood, kidney, muscle, liver, and lung by means of ICP, and the results are listed in Table 4.

Surprisingly, L-[Pt(dach)(Glu)] showed a remarkable organ targeting properties to lung. The reason for such organ selectivity of L-[Pt(dach)(Glu)] is not clear, but it may be conjectured that the liposomal platinum complex injected as lyophilic particles of nano sizes(150–300 nm) may preferentially accumulate in the lung organ by endocytosis mechanism. Another aspect to notice is that the tumor/tissue(muscle) ratio of L-[Pt(dach)(Glu)] at 2 h after injection is 1.28/1.09 = 1.17 but the ratio increased to 1.59/0.55 = 2.89 at 24 h after injection probably due to the EPR(enhanced permeability and retention) effect of the nano particles whereas the tumor/tissue ratios of carboplatin did not change. Carboplatin instead of the free [Pt(dach)(Glu)] may be used as a reference, since the dicarboxylatoplatinum(II) complexes shows very similar profiles of organ distribution.

Conclusion

The antitumor platinum(II) compound, [Pt(dach)(Glu)] (dach = *trans*(±)-1,2-diaminocyclohexane, Glu = glutamate) was formulated with a stealth liposome to improve its biological activity. Liposomes were composed of PC/PEG2000-PE/CH (PC = 1,2-diacyl-glycero-3-phosphocholine; PEG2000-PE = poly(ethylene glycol)2000-1,2-diacyl-glycero-3-phosphoethanolamine; CH = cholesterol) involving different acyl moieties of phospholipids such as DO (dioleoyl), DM (dimyristoyl) or DS (distearoyl) group. Among the different acyl formulations in the stealth liposomes, the DM formulation was optimal for the preparation of the liposomal [Pt(dach)(Glu)] at the mole ratio of DMPC/PEG2000-DMPE/CH = 50/5/45 and at the weight ratio of drug/lipid = 1/20. This optimal formulation product, L-[Pt(dach)(Glu)], meets standard physico-chemical properties, and shows excellent antitumor activity and drug

Table 4. Biodistribution of the liposomal [Pt(dach)(glu)] and carboplatin

Organs	L-[Pt(dach)(Glu)][a], Pt-concentration (mg/kg)		Carboplatin, Pt-concentration (mg/kg)	
	2 h	24 h	2 h	24 h
Blood	7.10	1.21	2.89	0.83
Tumor	1.28	1.59	3.87	0.67
Muscle	1.09	0.55	2.98	0.58
Kidney	16.8	7.94	8.31	2.13
Liver	12.6	14.7	6.71	1.44
Lung	46.7	25.9	2.84	0.73

[a]Liposome is composed of DMPC/PEG2000-DMPE/Chol = 50:5:45 (mole ratio) and the drug to lipid ratio is 1:20 (w/w).

potency. Furthermore, L-[Pt(dach)(Glu)] has enough capability to overcome the drug resistance to cisplatin and shows excellent lung targeting prperties. Further studies to understand and improve the lung targeting properties of L-[Pt(dach)(Glu)] are underway.

Experimental

Platinum complexes and chemicals

The parent complex, [Pt(dach)(Glu)], in Figure 1 was prepared by the reaction of (dach)$PtSO_4$ (0.81 g, 2.0 mmol) with the equimolar barium salt of glutamic acid (0.60 g, 2.0 mmol) in 30 mL of water. After $BaSO_4$ was filtered off, the filtrate was freeze-dried. This complex is soluble in water and methanol. Lipids of DMPC, DOPC, DSPC, PEG2000-DMPE, PEG2000-DOPE, PEG2000-DSPE and cholesterol purchased from Avanti Polar Lipids (Alabaster, AL, USA) were used without further purification.

Preparation of the liposomal platinum complex

Stealth liposomes containing the platinum complex, [Pt(dach)(Glu)] were prepared by lyophilization–hydration method.[4] Briefly, lipids in chloroform were mixed at the desired mole ratio of each formulation (PC/PEG2000-PE/CH), and the chloroform was removed in a rotary evaporator. To the dried lipid film, drug dissolved in methanol was added at different weight ratios of drug to lipid (1:5, 10, or 20), and the methanol was removed in a rotary evaporator. Then, *tert*-butanol was added and the whole solutions were shaked at 50 °C for 10 min to obtain the homogeneous solutions. Aliquoted samples in vials were frozen in dry ice/acetone bath, and *tert*-butanol was removed by lyophilization overnight to give the lyophilized preliposomal powders. To reconstitute the preliposomes, saline was added at the concentration of 50 mg/mL, and the resulting suspension was shaked at 40 °C for 1 h with vigorous vortexing and sonicated for 5 min twice (Laboratory Supplies Co., NY, USA).

Entrapment efficiency

The entrapment efficiency was determined by the protamine aggregation method.[15] Briefly, the liposome suspension (20 mg/mL) was mixed with an equal volume of the protamine solution (10 mg/mL) and allowed to stand for 5 min. After adding 3 volumes of saline to the mixture, it was centrifuged at 3,000 rpm for 20 min at room temperature. The supernatant was taken and the pellet was resuspended in 10% Triton-X 100. The amounts of platinum drug in the supernatant (S) and pellet (P) were analyzed by ICP (inductive coupled plasma) and percentage of entrapment efficiency (%EE) was calculated as:

$$\%EE = [\text{Total Pt}(S + P) - \text{Pt}(S)]/\text{Total Pt}(S + P) \times 100$$

ICP-atomic emission spectrometric measurements were performed using JY38Plus (Jobin Yvon, France) for checking the platinum amounts in solutions.

Cytotoxicity measurement

Human ovarian A2780 and cervical ME180 cancer cell lines derived from a patient prior to chemotherapy and their cisplatin-resistant cells A2780/PDD and ME180/PDD were maintained at 5% CO_2, 37 °C in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, 100 μg/mL of streptomycin, 100 U/mL of penicillin, 0.3 mg/mL of glutamine, and 0.3 U/mL of insulin (bovine, GibcoBRL). Cell cytotoxicity was determined by methylthiazoletetrazolium (MTT) dye reduction assay.[16] Cells were seeded, allowed to attach overnight, and then exposed to various concentrations of drugs for 48 h. After washing the cells with PBS solution twice, MTT solution was added. After incubation for 4 h at 37 °C, the cells were lyzed by dimethyl sulfoxide and incubated for another 2 h. The cell survival fractions were determined by reading the absorbance at 570 nm in a microplate reader (Model MCC/340, Titertek multiscan). All the ID_{50} (50% inhibitory dose) values of liposomal platinum complexes were normalized against those of the corresponding empty liposomes. The reported values are the averages of at least triplicate experiments. The resistance indexes were calculated as the ratio of ID_{50} in resistant cells to ID_{50} in sensitive cells.

In vivo antitumor activity

In vivo activity was assayed using the ascites cells of L1210 lymphoid leukemia, which was obtained from BDA/2 donor mice bearing 3–5-day tumor growth. L1210 leukemia cells (1×10^6 cells in 0.2 mL saline) were inoculated ip in BDF mice (6–8 weeks old, 20–25 g; eight mice per group) on day 0. Drug was administered ip on days 1, 5 and 9. The results were expressed as:

$$\text{T/C}\ (\%) = \text{Median survival of treated animals/Median survival of untreated animals} \times 100$$

Biodistribution study

Male C57 BL/6N mice (8–9 weeks old, 25–27 g) were adapted for 4 days to a 12 h/12 h light/dark cycle, and then inoculated subcutaneously with B16F10 melanoma cells (1×10^6 cells) in the back region. After 2 weeks when the tumor had grown to 10 mm in diameter, the liposomal platinum complex was injected in a tail vein. The animals were sacrificed at 2 and 24 h after drug administration. Blood samples were collected by heart puncture with a syringe. Tumor, muscle, liver, lung and kidney were removed from animals, and stored at −80 °C for analysis. Analysis of the drugs in the biological samples was based on measurement of platinum as reported in the literature.[17] After the samples were treated with c-H_2SO_4, c-HNO_3 and finally aqua regia, platinum content was measured by ICP-MS (Model ELAN5000, Perkin-Elmer, Norwalk, CT, USA). Mean concentration ± S.E.M. was calculated for each time point.

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