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Liposome Formulations for Effective Administration of Lipophilic Malonatoplatinum(II) Complexes

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For effective administration of lipophilic *trans*(±)-1,2-diaminocyclohexaneplatinum(II) complexes of malonate derivatives [(dach)PtL, L=allylmalonate (AM), diallylmalonate (DAM), allylbenzylmalonate (ABM), or dibenzylmalonate (DBM)] in aqueous solution, we have applied three different liposome formulations and evaluated their physical and chemical properties, along with their *in vitro* cytotoxicity. The liposome formulations were composed of DMPC/DMPG [DMPC=1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, DMPG=1,2-dimyristoyl-*sn*-glycero-3-(phospho-*rac*-1-glycerol) (sodium salt)] in different molar ratios (7/3 or 3/7) or an equimolar DOTAP/DOPE formulation (DOTAP=1,2-dioleoyl-3-trimethylammonium propane, DOPE=1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine). Preliposomal powders of the platinum complexes were prepared by lyophilization, and reconstituted in aqueous solution to obtain the final liposomal platinum complexes. Due to the lipophilicity of the malonatoplatinum complexes, the entrapment efficiency of drugs within the liposomes was over 90% except for the AM complex, and platinum drug stability was also satisfactory (>90%) in these liposomal systems. *In vitro* cytotoxicity was tested in human ovarian carcinoma cells sensitive (A2780) and resistant to cisplatin (A2780/PDD). In both cell lines, the liposomal DBM complex was much more cytotoxic than the corresponding DAM and ABM complexes, which means that the more hydrophobic benzyl substituent affords higher cytotoxicity than the allyl substituent in the malonato leaving group. Furthermore, the DBM complex in DMPC/DMPG formulations was effective against both sensitive and resistant A2780 cells (resistance indexes (RI)=1.10–1.49), showing lack of cross-resistance to cisplatin. Therefore, the liposomal DBM complex in the DMPC/DMPG formulations is a promising candidate for stable pharmaceutical liposomal platinum complexes.

Key words: Lipophilic platinum(II) complex — Liposome — Cytotoxicity — Drug resistance

Cisplatin [*cis*-diamminedichloroplatinum(II)] is very effective in the treatment of various types of human cancers.[1)] However, its use is limited due to its significant toxic side effects, such as acute nephrotoxicity and chronic neurotoxicity as well as acquired drug resistance. The *trans*(±)-1,2-diaminocyclohexaneplatinum(II) complexes, (dach)Pt(II), have attracted significant attention for many years because they do not show cross-resistance with cisplatin,[2)] probably as a result of inducing Pt-DNA adducts that are poorly repaired in resistant cells, even though they are identical to those induced by cisplatin,[3)] or of inhibiting essential processes such as replication or transcription.[4)] In a series of studies, (dach)Pt(II) complexes of malonate derivatives as a leaving group were designed with a wide range of lipophilicity and hydrophilicity, and the correlation of their antitumor activities to their water-solubility was examined. A good relationship was observed between the *in vitro* toxicity and water-solubility, but no relationship could be established between the *in vivo* antitumor activity and water-solubility of the malonatoplatinum(II) complexes.[5)] This indicates that other pharmacokinetic factors may play an important role in *in vivo* systems.

Liposomes have been explored as delivery tools for antitumor drugs in an attempt to alter the pharmacokinetics and tumor/organ targeting of the drugs, and even to overcome multi-drug resistance.[6)] Liposomes have been successfully used in the clinic as carriers of antitumor agents such as adriamycin,[6–8)] taxol,[9, 10)] and platinum antitumor drugs.[11–18)] In addition to increasing the therapeutic indexes of the drugs, liposomes have made it possible to deliver lipophilic drugs practically in aqueous suspensions. The leading compound among the lipophilic platinum drugs is NDDP [*cis*-bis-neodecanoato-*trans*-*R*,*R*-1,2-diaminocyclohexane platinum(II)] entrapped in multilamellar vesicles composed of DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) and DMPG [1,2-dimyristoyl-*sn*-glycero-3-(phospho-*rac*-1-glycerol) (sodium salt)]. Liposomal NDDP (L-NDDP) did not show cross-resistance with cisplatin in *in vitro* and *in vivo* experiments,[11–15)] and was not nephrotoxic in mice and dogs. In phase I and II clinical studies, L-NDDP was non-nephrotoxic and its limiting toxicity was myelosuppression.[13)] However, L-NDDP exerts its biological activity through intraliposomal degrada-

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tion of the original drug, NDDP, into one or more active platinum species *ex vivo*.[13, 14] This chemical degradation and activation process requires the presence of acidic lipids such as DMPG [1,2-dimyristoyl-*sn*-glycero-3-(phospho-*rac*-1-glycerol) (sodium salt)] or DMPA [1,2-dimyristoyl-*sn*-glycero-3-phosphate (monosodium salt)] in the liposomes.[15] This intraliposomal instability of NDDP makes it difficult to develop L-NDDP as a pharmaceutical product.

Therefore, we need to develop an effective formulation affording antitumor activity while preserving the chemical stability of the platinum complexes within the liposomes before administration to the patient. We have synthesized more stable lipophilic (dach)Pt(II) complexes using chelating dicarboxylic malonate derivatives instead of two monocarboxylate ligands as a leaving group in NDDP. We have investigated the entrapment efficiency and stability of these malonatoplatinum(II) complexes within the liposomes and then examined their *in vitro* cell cytotoxicity and resistance index in human ovarian carcinoma cell lines sensitive (A2780) and resistant (A2780/PDD) to cisplatin.

MATERIALS AND METHODS

Platinum complexes and chemicals A series of (dach)Pt(II) complexes of malonate derivatives was prepared by the reaction of (dach)$PtSO_4$ with barium salts of the corresponding malonic acid derivatives, and the final products, (dach)PtL [L=AM (allylmalonate), DAM (diallylmalonate), ABM (allylbenzylmalonate) and DBM (dibenzylmalonate)] were recovered by solvent extraction using methanol, as previously reported.[5] All these complexes are less soluble in water than carboplatin, but more soluble in polar organic solvents such as methanol (at 50°C), dimethylformamide (DMF) and dimethylsulfoxide (DMSO). Lipids such as DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), DMPG, DOTAP (1,2-dioleoyl-3-trimethylammonium propane) and DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) were purchased from Avanti Polar Lipids (Alabaster, AL).

Preparation of liposomal platinum complexes Multilamellar vesicles containing the platinum complexes were prepared by a lyophilization-rehydration method. Briefly, lipids in chloroform were mixed at the desired molar ratio (DMPC/DMPG=7/3 or 3/7, DOTAP/DOPE=1/1), and the chloroform was removed in a rotary evaporator. To the dried lipid film, the platinum complexes dissolved in methanol were added at the weight ratio of drug to lipid of 1:5, 1:10, and 1:15, and subsequently the methanol solvent was removed in the rotary evaporator. Then, *tert*-butanol was added and the solution was shaken at 40–50°C for 10–30 min to obtain a clear solution. Aliquoted samples in vials were frozen in a dry ice/acetone bath, and *tert*-butanol was removed by lyophilization overnight to give the lyophilized preliposomal powders. To reconstitute the preliposomes, saline or PBS (phosphate-buffered saline) was added at the concentration of 10–50 mg/ml, and the resulting suspension was shaken at 40°C for 60 min with vigorous vortexing. The size distribution of liposomal preparations was determined with a Nicomp submicron particle sizer model 370 (Nicomp Particle Sizing Systems, Santa Barbara, CA).

Entrapment efficiency and intraliposomal drug stability The entrapment efficiency and stability of the platinum complexes incorporated in different liposomes were determined as described previously.[11] Briefly, the final liposome suspension (1 mg/ml) was centrifuged at 20 000*g* for 1 h at 4°C and a sample of the supernatant was taken. The amount of platinum drug was analyzed with an ICP (inductive coupled plasma) device and percent entrapment efficiency (%EE) was calculated as:

$$\%EE = \frac{\text{Total platinum complex } (T) - \text{Platinum complex in supernatant } (S)}{\text{Total platinum complex } (T)} \times 100$$

ICP-atomic emission spectrometric measurements were performed using a JY38Plus (Jobin Yvon, Longjumeau Cedex, France) to determine platinum amounts in solutions.

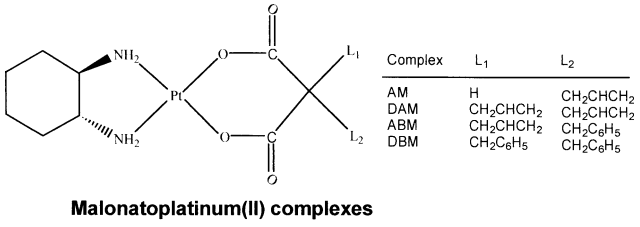
To calculate drug stability, aliquoted samples of the suspension were diluted 5–10 times with methanol to make clear solutions for HPLC at 10 min, 2 h, and 6 h after liposome preparation, and then each sample was monitored by HPLC. The retention times in CH_3OH/H_2O (80/20 volume ratio) eluent were 19.6, 20.4, 20.0, and 20.2±0.5 min for the AM, DAM, ABM, and DBM complexes, respectively. HPLC was performed using a Waters Associates unit: 510 pump, model 80 gradient controller, 712 automatic sampler, 481 LC detector and Youngrin analysis software. The samples were eluted through a C_{18}-μBondapak column using aqueous methanol as the eluent. The flow rate was 1.0 ml/min, and the samples were detected by a UV detector set at 224 nm.

Cell cytotoxicity The *in vitro* cell cytotoxicity was assessed by MTT (methylthiazoletetrazolium) dye reduction assay using mouse leukemia L1210 cells or human ovarian carcinoma cells sensitive (A2780) and resistant (A2780/PDD) to cisplatin. Both cells grown in RPMI medium were seeded in the wells of 96-well plates, allowed to attach overnight, and then exposed to various concentrations of drugs for 48 h and 72 h. The cells were washed with PBS twice, and the cell survival was determined by means of MTT assay. All the cell cytotoxicity data were normalized against the cytotoxicities of the corresponding empty liposomes excluding the platinum complexes. All the ID_{50} (50% inhibitory dose) values were calculated from at least three separate experiments. The resistance indexes were calculated as the ratio of ID_{50} in resistant cells to ID_{50} in sensitive cells. Cytotoxicity to mouse leukemia L1210 cells grown in RPMI medium was also checked by MTT assay as described above.

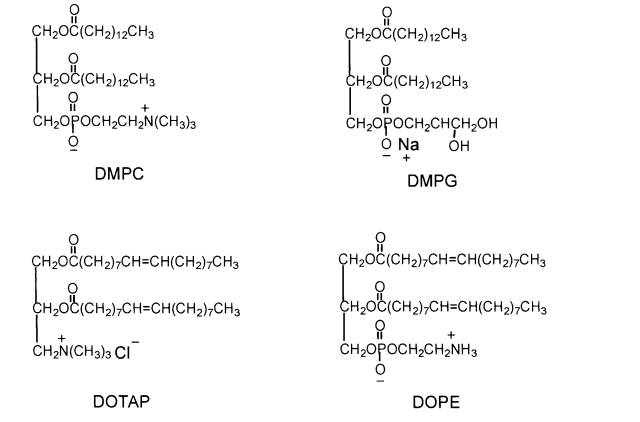
RESULTS AND DISCUSSION

Preparation of liposomal platinum complexes The malonatoplatinum(II) complexes, (dach)PtL (L=AM, DAM, ABM, and DBM), employed in this study are expected to be more stable than the early monocarboxylate complexes such as NDDP,[15] since the malonate derivatives (L) are all chelating dicarboxylates. The present malonatoplatinum compounds are soluble in polar organic solvents such as methanol, DMF, and DMSO, but practically insoluble in water. Therefore, liposomal formulations were introduced for efficient *in vitro/vivo* administration of these lipophilic malonatoplatinum drugs in aqueous solution. The liposomes were composed of DMPC/DMPG with different molar ratios (7/3 or 3/7) or an equimolar cationic DOTAP/DOPE formulation. All the malonatoplatinum(II) complexes and lipids used are shown in Fig. 1.

The drug-to-lipid ratios examined were 1:5, 1:10 and 1:15, but we have found that the 1:10 ratio was optimal in terms of the physical and chemical stability of the liposomes. The average size of the multilamellar vesicles of the liposomes prepared in saline was 500–800 nm. The liposomal malonatoplatinum complexes were found to form stable and uniform suspensions at room temperature.



Malonatoplatinum(II) complexes



Lipids

Fig. 1. Chemical structures of the malonatoplatinum(II) complexes, (dach)PtL (L=AM, DAM, ABM, DBM) and lipids (DMPC, DMPG, DOTAP, DOPE).

The liposomal entrapment efficiency and the stability of the liposomal malonatoplatinum drugs are shown in Table I.

The percent entrapment efficiency of all the liposomal malonatoplatinum complexes was between 90.7% and 95.3%, except for the AM complex (65.5%), indicating good compatibility between these lipophilic malonatoplatinum complexes (dach)PtL (L=DAM, ABM, DBM) and the DMPC/DMPG or DOTAP/DOPE liposomes used. No crystals of the free drug were observed in any of these preparations within 24 h as assessed by optical microscopy. Further, the intraliposomal stability of the platinum complexes except the AM complex was over 90% (91.6–95.0%) even after 6 h in all of the liposomal preparations. These results proved that the (dach)Pt(II) complexes of chelating dicarboxylates such as malonate derivatives are more stable than those involving two monocarboxylate leaving groups, as in NDDP described previously, under these liposomal conditions (pH= 6.0–7.5). Thus, the drug leakage was not significant (3.3–4.7%) due to high compatibility between the lipophilic malonatoplatinum complexes and liposomes, and good stability of the malonatoplatinum complexes within the liposomal suspensions. Therefore, these liposomal malonatoplatinum complexes could be applied without further purification due to the high entrapment efficiency and could be a candidate pharmaceutical due to the excellent stability of the platinum complexes within the liposomes.

Cell cytotoxicity Table II shows the cytotoxicity and resistance index of the liposomal malonatoplatinum complexes compared with those of cisplatin against sensitive A2780 and resistant A2780/PDD cells for continuous drug

Table I. Percent Entrapment Efficiency (%EE) and Drug Stability of the Liposomal Malonatoplatinum(II) Complexes[a]

Liposomal[b] (dach)PtL		$\%EE_{0\,h}$	Drug leakage ($\%EE_{6\,h}-\%EE_{0\,h}$)	Stability		
				0 h	2 h	6 h
L=AM	7/3	65.5	10.5	—	—	—
L=DAM	7/3	90.7	3.3	100	95.0	94.1
	3/7	92.1	3.9	100	94.6	93.1
L=ABM	7/3	95.3	3.5	100	93.5	94.3
	3/7	93.5	4.0	100	92.8	90.9
L=DBM	7/3	94.6	3.5	100	91.6	90.5
	3/7	90.9	4.1	100	92.3	90.9
	1/1	92.2	4.7	100	93.1	91.4

a) All the values are means of at least three separate experiments with SD >±3.0%.
b) 7/3, 3/7, and 1/1 mean the lipid compositions of DMPC/DMPG=7/3 or 3/7 and DOTAP/DOPE=1/1 molar ratio, respectively.

exposure. All the cytotoxicity data were normalized to the cytotoxicities of the corresponding empty liposomes without the platinum complexes.

It can be seen from the table that the DMPC/DMPG formulations of the DBM complex were very cytotoxic (ID_{50}=2.0−10.7) towards A2780 cells in 48 h drug treatment, while the liposomal DAM and ABM complexes exhibited low cytotoxicity (ID_{50}=20.7−52.5) even after 72 h drug exposure. In A2780/PDD cells, the liposomal DBM complexes were still cytotoxic (ID_{50}=11.2−14.8) in 48 h, while the liposomal DAM and ABM complexes gave significantly lower cytotoxicity (ID_{50}=54.0−152.8) in 72 h. From these data, it is concluded that the cytotoxicity of the liposomal malonatoplatinum complexes increased in the order of the DAM, ABM, and DBM complexes: the hydrophobic benzyl substituent in the malonate leaving group resulted in higher cytotoxicity than the allyl substituent. Due to the substantial cytotoxicity of the liposomal DBM complex compared to cisplatin, further studies focused on the liposomal DBM complex. Subsequently, the equimolar cationic DOTAP/DOPE formulation was introduced for the liposomal DBM complex to increase the cell permeation and absorption. The cationic DOTAP/DOPE formulation of the DBM complex (ID_{50}=2.0) was 5-fold more cytotoxic than the neutral/anionic DMPC/DMPG formulations (ID_{50}=9.9−10.7) in sensitive cells, but showed similar cytotoxicity (ID_{50}=11.2) to those of the DMPC/DMPG formulations (ID_{50}=11.8−14.8) in resistant A2780/PDD cells. There was not much difference in cytotoxicity depending on the molar ratio of DMPC/DMPG formulations. Namely, the DBM complex with DMPC/DMPG formulations similarly affected both sensitive and resistant cells, but the cationic DOTAP/DOPE liposomes affected the sensitive cells more than resistant cells.

Based on the ID_{50} values, the calculated resistance indexes (RI) are shown in Table II. The RI values calculated for the DAM and ABM complexes with the DMPC/DMPG formulations were between 2.42 and 2.91, being lower than that of cisplatin (3.90). The RI values of the DBM complex in the formulations of DMPC/DMPG=7/3, 3/7 and DOTAP/DOPE=1/1 were 1.49, 1.10 and 5.60, respectively. Thus, the ID_{50} values of the DBM complex in the DMPC/DMPG formulations indicated no cross-resistance to cisplatin. However, the higher RI value of the DBM complex in the cationic DOTAP/DOPE formulation seems to be due to its high sensitivity and interaction in sensitive cells, which suggests that the cationic formulation may play an important role in cell killing or drug influx in the sensitive cells. Furthermore, in mouse leukemia L1210 cells, the ID_{50} values of the liposomal DBM complex were 14.0 and 52.1 μM with the formulations of DOTAP/DOPE and DMPC/DMPG=7/3, respectively. Again, the DBM complex with the cationic DOTAP/DOPE formulation also showed 4-fold higher toxicity than with the neutral/anionic DMPC/DMPG formulations, as seen with A2780 sensitive cells.

To examine further the effects of liposome composition on overcoming cisplatin-resistance, the time-dependent cytotoxicity was checked. The results (Fig. 2) also indicated that the DOTAP/DOPE formulation was more cytotoxic to the sensitive than the resistant cells, while the DMPC/DMPG formulations were equally toxic to both. The resistance indexes of the DMPC/DMPG formulation were 1.13, 1.49 and 1.58 while those of the DOTAP/DOPE formulation were 2.14, 5.60 and 4.25 for 24, 48 and 72 h drug exposure, respectively. These results also showed that the DBM complex in the DMPC/DMPG formulations showed no cross-resistance to cisplatin regard-

Table II. Cell Cytotoxicity and Resistance Index of Liposomal Malonatoplatinum(II) Complexes in A2780 and A2780/PDD Cells[a)]

Liposomal[b)] (dach)PtL		ID_{50} (A2780)		ID_{50} (A2780/PDD)		Resistance index[c)]	
		48 h	72 h	48 h	72 h	48 h	72 h
L=DAM	7/3		52.5±5.8		152.8±10.7		2.91
	3/7		20.7±2.9		54.0±4.3		2.61
L=ABM	7/3		30.5±2.9		63.5±6.1		2.53
	3/7		25.1±3.0		58.1±5.1		2.42
L=DBM	7/3	9.9±1.8		14.8±1.9		1.49	
	3/7	10.7±2.0		11.8±1.6		1.10	
	1/1	2.0±1.2		11.2±0.7		5.60	
Cisplatin		2.1±1.9		8.3±2.5		3.90	

a) All ID_{50} values are expressed as μM and are mean±SD from at least three separate experiments.
b) 7/3, 3/7, and 1/1 mean the lipid compositions of DMPC/DMPG=7/3 or 3/7 and DOTAP/DOPE=1/1 molar ratio, respectively.
c) Resistance index=ratio of ID_{50} in resistant cells to ID_{50} in sensitive cells.

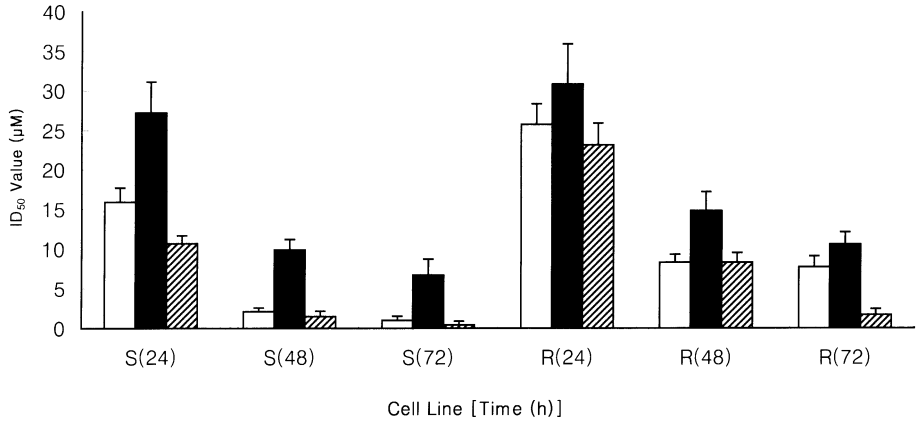


Fig. 2. Time-dependent cell cytotoxicity of liposomal (dach)Pt[DBM] complexes in A2780 cells. A2780 cells sensitive (S) and A2780/PDD cells (R) resistant to cisplatin were treated with a) cisplatin (□) and the liposomal DBM complexes for 24, 48, and 72 h. The lipid compositions of b) DMPC/DMPG (■) and c) DOTAP/DOPE (▨) were DMPC/DMPG=7/3 and DOTAP/DOPE=1/1 molar ratio, respectively.

less of drug exposure time. However, the DBM complex in the cationic DOTAP/DOPE formulation again showed much higher sensitivity in sensitive cells rather than resistant cells with time.

In spite of the encouraging preclinical results with the lead compound, NDDP, of the liposomal lipophilic platinum complex family, broad clinical studies were deferred due to the instability of the monocarboxylate leaving group of NDDP within liposomes containing acidic lipids such as DMPG or DMPA.[13–15] Therefore, the present lipophilic malonatoplatinum complexes with chelating dicarboxylates were developed, since they were expected to be more stable than platinum complexes with two monocarboxylates as a leaving group, such as NDDP. In fact, the experimental results on the entrapment efficiency and drug stability of our liposomal malonatoplatinum complexes confirm satisfactory stability of drugs and liposomes. Neither the substituent effect on the malonato group nor the lipid composition significantly affected %EE or drug stability within the liposomes. In particular, the cell cytotoxicity data showed that, among the malonatoplatinum complexes, the DBM complex with the DMPC/DMPG formulations not only met the required cytotoxicity standard compared with cisplatin, but also showed no cross-resistance to cisplatin. Furthermore, the cationic DOTAP/DOPE formulation in the DBM complex exhibited high sensitivity in sensitive cells, rather than the corresponding resistant cells. Further studies on these cationic liposomal systems are needed to elucidate the difference of cell killing mechanisms between sensitive and resistant cells.

In summary, our findings suggest that the lipophilic DBM complex with the DMPC/DMPG formulations meets the required criteria of physical and chemical stability of the liposomal drugs and cytotoxicity for a pharmaceutical product. Furthermore, this drug, liposomal (dach)Pt[DBM], has some capability to overcome cross-resistance to cisplatin.

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