Arch Pharm Res Vol 23, No 2, 167-171, 2000

In Vitro Cytotoxic Effect of *N*-(Phosphonacetyl)-L-Aspartic Acid in Liposome Against C-26 Murine Colon Carcinoma

Jin-Seok Kim[1] and Timothy D. Heath[2]

[1]College of Pharmacy, Sookmyung Women's University, Chungpa-Dong 2-Ga, Yongsan-Gu, Seoul, Korea 140-742 and [2]School of Pharmacy, University of Wisconsin, 425 N. Charter St., Madison, WI 53706, USA

(Received August 17, 1999)

We have investigated the *in vitro* cytotoxic effect of liposome-encapsulated N-(phosphonacetyl)-L-aspartic acid (PALA) against C-26 murine colon cancer cells, and have compared it in this regard to free PALA. Three different PALA-containing liposomal formulations using distearoylphosphatidylcholine (DSPC), distearoylphosphatidylglycerol (DSPG), and polyethyleneglycol-derivatized distearoylphosphatidylethanolamine (PEG-DSPE) were made and their cytotoxicity was measured. In 72 hr continuous exposure experiment with C-26 cells, the 50% growth inhibitory concentration (IC_{50}) of DSPG-PALA liposome formulation was 0.09 μM, which showed about 65-fold more potent than unencapsulated free PALA (5.1 μM). Similar degree of increase in cytotoxicity was also observed in 1 hr exposure experiment. However, the IC_{50} of PEG-DSPE-PALA liposome and DSPC-PALA liposome were 10.7 μM and 11.8 μM, respectively, which showed slightly less potent than unencapsulated free PALA. Physical characteristics of PALA-liposomes, such as the size and drug:lipid ratio were also determined. In conclusion, negatively-charged DSPG-PALA liposome showed the highest cytotoxic effect among tested on the C-26 cells *in vitro*.

Key words: Liposomes, C-26 Murine Colon Carcinoma, PALA, Cytotoxicity

INTRODUCTION

The use of liposomes as a drug carrier to improve the therapeutic efficacy of antitumor agents has been frequently proposed (Senior, 1987). Among those antitumor agents that have shown improved antitumor efficacy when encapsulated in liposomes are doxorubicin (Mayhew *et al.*, 1990; Vaage *et al.*, 1993; Williams *et al.*, 1993; Maruyama *et al.*, 1994; Papahadjopoulos *et al.*, 1991), vincristine (Boman, *et al.*, 1994; Vaage, *et al.*, 1993), cytosine arabinoside (Rustum *et al.*, 1979), cis-platinum (Sur *et al.*, 1983), and camptothecin (Daoud *et al.*, 1995). Even though the mechanism by which liposomes improve the efficacy of these drugs is not fully elucidated, direct delivery of drugs to the intracellular target site was proposed as one of the possible mechanisms. In order for the direct delivery relevant, the drug under consideration must have the property of showing improved efficacy when delivered in liposomal formulation. Such compounds have been termed as liposome-dependent drugs (Heath *et al.*, 1985a), and a few have been characterized as such including methotrexate-γ-aspartate (Heath *et al.*, 1983), fluoroorotic acid (Heath *et al.*, 1985b), clodronate (Monkkonen *et al.*, 1993a), gallium (Monkkonen *et al.*, 1993b), hygromycin B (Sechoy *et al.*, 1989), and N-phosphonacetyl-L-aspartic acid (Heath *et al.*, 1989; Sharma *et al.*, 1993).

N-phosphonacetyl-L-aspartic acid (PALA) has been extensively explored in clinical trial as a potential chemotherapeutic agent for cancer treatment (Grem *et al.*, 1988). However, its potency has generally proved limited despite of potential utility. This is, at least in part, caused by its limited ability to enter the cells, as demonstrated by its low apparent volume of distribution (Loo *et al.*, 1980). This limited capacity of cell entry, or sorptive endocytosis, is also the reason why this drug is liposome dependent. Therefore, it seems likely that its potency for treatment of tumor might be improved by encapsulation in liposome.

In this paper, we explore whether PALA can be more effective in several liposomal formulations of different lipid compositions. Their cytotoxicity and the effects of exposure time on cytotoxicity were tested in C-26 murine colon cancer cells *in vitro*.

Correspondence to: Jin-Seok Kim, College of Pharmacy Sookmyung Women's University Chungpa-Dong 2-Ga, Yongsan-Gu Seoul, Korea 140-742
E-mail: jskim@sdic.sookmyung.ac.kr

MATERIALS AND METHODS

Materials

N-(phosphonacetyl)-L-aspartic acid was a generous gift of Dr. V.L. Narayanan of the Chemical Synthesis Branch, National Cancer Institute (Bethesda, USA). Polyethylene glycol 1900 derivatized DSPE (PEG-DSPE) was kindly provided by Dr. F. Martin (Liposome Technology Inc., Menlo Park, USA). Calcein was purchased from Molecular Probes (Eugene, USA). Distearoylphosphatidylcholine (DSPC), and distearoylphosphatidylglycerol (DSPG) were purchased from Avanti Polar Lipids (Birmingham, USA). Cholesterol (Chol) was obtained from Sigma (St. Louis, USA). DSPC, DSPG and cholesterol were stored ampouled in chloroform under argon at -20°C until use. All other materials were reagent grade or better.

Cell Culture

A murine colon tumor cell line, C-26, was obtained from Dr. E. Mayhew, Roswell Park Memorial Institute, and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units/ml of penicillin and 100 µg/ml of streptomycin (Gibco, Grand Island, USA). The C-26 cells were grown in 25 cm^2 polystyrene tissue culture flasks (Coming, USA) in a humidified incubator (Napco) at 37°C with a 4% CO_2 atmosphere. Confluent flasks were split by treatment with 0.05% trypsin with 1 mM EDTA for 20 min to release the cell monolayer. Serum was added to stop the action of the trypsin, and the cells were harvested by centrifugation at 300 g for 10 min. The cells were resuspended in fresh medium prior to use.

Preparation of liposomes

Liposomes were prepared from either DSPC:Chol 2:1, DSPG:Chol 2:1, or DSPC:Chol:PEG-DSPE 20:10:1 using the method of reverse-phase evaporation (Szoka *et al.*, 1978). A 60 mM PALA solution for encapsulation was prepared in 50 mM MES, 50 mM HEPES, 2 mM Calcein, and NaCl to give a tonicity of 290 mOsm/Kg, and the pH was 7.2. Liposomes were extruded five times using a thermostatted stainless steel filtration cell (Mico Instruments, Middleton, USA) with a 1.5 ml capacity chamber and a 13 mm diameter, 0.2 µm pore size polycarbonate membrane. Liposomes were separated from the unencapsulated PALA by gel chromatography on a 1 × 15 cm sterile sephadex G-50 column (Pharmacia, Piscataway, USA). The column was eluted with 50 mM MES, 50 mM HEPES, and 80 mM NaCl, 290 mOsm/Kg, pH 7.2 (MES/HEPES). The concentration of phospholipid in the liposome fractions was determined by a phosphorus assay (Bartlett, 1958) on triplicate samples, which were first extracted by the method of Bligh and Dyer (Bligh *et al.*, 1959) to eliminate PALA. The concentration of PALA was determined indirectly from the concentration of co-encapsulated calcein after the liposomes were solubilized using 0.1% Triton X-100. The concentration of calcein was determined spectrophotometrically assuming a molar extinction coefficient at 493 nm of 18,258 L $mole^{-1}$ cm^{-1} in MES/HEPES.

Particle size analysis

The liposome size was determined using a quasi-elastic laser light scattering instrument (QELS; Model 370, Nicomp, Santa Barbara, USA). The wavelength of the light source was 632.8 nm. Typically, data was collected from an appropriate dilution of freshly made liposomes by running the instrument for 10 min at a room temperature with a count rate of 300-326 kHz. Owing to the uniformity of the particles, the diameters were determined using a volume-weighted Gaussian analysis, and are shown in Table I.

In Vitro Growth Inhibition of C-26 Cells

C-26 murine colon carcinoma cells were plated at 2×10^4 cells/ml, 1 ml per well, in 24-well plates, and were incubated overnight prior to treatment. Triplicate wells were then treated with either free PALA or PALA in liposome. Triplicate control wells were treated with MES/HEPES. Three wells were counted at the time of drug treatment to give the original cell count. After 72 h, growth medium was removed, and 1 ml of 0.05% trypsin in PBS, 1 mM EDTA was added, and the plate was incubated for 20 min at 37°C to release the cells from the wells. The released cells were counted using a cell counter (Coulter Model ZM, Hialeah, USA) after 1/50 dilution. The percent growth was calculated and the IC_{50} determined from these values as previously described (Heath *et al.*, 1985b). In one experiment, the drug exposure period was reduced to 1 h by washing the wells twice with 1 ml of warm PBS containing Ca^{++}/Mg^{++} one hour after addition of drug. After washing, the cells were allowed to grow for an additional 71 h in fresh growth medium, and were counted to determine the IC_{50} in the usual way.

Table I. Physical properties of liposomes

Liposome Formulation	PALA Conc. (mg/ml)[a]	PALA Encapsulation (%)	Lipid Conc. (mM)[b]	Drug:Lipid Ratio (mol:mol)	Liposome Diameter (µm)[c]
DSPC-PALA	3.3	16	17.2	0.55	215±112
DSPG-PALA	2.1	11	20.6	0.3	132±62
Stealth[d]-PALA	1.8	7	18.8	0.28	181±57

[a] Determined indirectly from the encapsulation of calcein .
[b] Determined by phosphorus analysis.
[c] Deterinined by quasi-elastic laser light scattering.
[d] Stealth liposome contains 5% PEG-DSPE additionally in DSPC liposome formulation.

RESULTS AND DISCUSSION

Characteristics of liposomes

Table I summarizes the physical characteristics of the liposome preparations used for the *in vitro* study. The highest PALA encapsulation was achieved in DSPC liposome formulation (16%), followed by DSPG (11%) and Stealth (7%) liposome formulation. There seems to be charge repulsion between negatively charged glycerol head group of DSPG and PALA, which might have resulted in a decrease in drug encapsulation ratio in DSPG liposome formulation. Also, the polyethyleneglycol group of PEG-DSPE seems to render steric hindrance of drug encapsulation in PEG-DSPE liposome formulation as shown in lower PALA encapsulation ratio (7%). Lipid concentration doesn't seem to vary much regardless of the lipid composition used. The drug: lipid ratio was greatest for the DSPC liposomes, and least for the sterically stabilized liposomes. The size of the liposomes, determined by QELS, was also greatest for the DSPC liposomes, but was least for the DSPG liposomes. For unilamellar liposomes, the drug:lipid ratio and diameter should be directly related to one another. Deviation from this relationship normally suggests that one or more of the liposome preparations being compared are not truly unilamellar. On this basis, the sterically stabilized liposomes may have been oligolamellar. An alternative explanation would be that the larger size than was expected from the drug : lipid ratio may be caused by the presence of the PEG-DSPE. All diameters were either less than or close to 0.2 μm, which is the pore size of the membranes used for liposome extrusion. This is expected based on the known effects of extrusion (Olson *et al.*, 1979; Szoka *et al.*, 1980; Mayer *et al.*, 1986).

In Vitro Growth Inhibition of C-26 Cells

The growth inhibitory potency of free and encapsulated PALA for C-26 cells in culture is shown and summarized in Fig. 2-3 and Table II, respectively. In 72 h exposure experiment, PALA in DSPG liposomes was 65-fold more potent than free drug (0.09 mM vs. 5.1 mM in IC_{50}). In contrast to this, PALA in both DSPC and PEG-DSPE liposomes was half as potent as free drug. This result is consistent with previous findings from other studies, where negatively charged liposomes delivered PALA to a number of *in vitro* cell lines more efficiently than neutral liposomes (Heath *et al.*, 1989; Sharma *et al.*, 1993). It also suggests that sterically stabilized liposomes are not taken up appreciably by these cells. Parallel studies were carried out with liposomes prepared from the same lipid compositions, but without any encapsulated PALA. There was no inhibition of cell growth at lipid concentrations up to 2 mM (data not shown). This concentration is 10 times higher than the highest lipid concentration used in the

Table II. *In vitro* growth inhibitory potency of PALA encapsulated in liposomes for the C-26 murine colon carcinoma

Lipid Composition	Continuous Exposure (72 h)		Short-Term Exposure[a] (1 h)	
	IC_{50} (mM)	PIF[b]	IC_{50} (μM)	PIF
Free Drug	5.1±0.3[c]	1	823±66	1
DSPG: Chol(2 : 1)	0.09±0.008	57	14.3±1.3	58
DSPC: Chol(2 : 1)	11.8±1.1	0.4	2040±178	0.4
DSPC:Chol:PEG-DSPE (20 : 10 : 1)	10.7±0.8	0.5	1400±123	0.6

[a] Cells were exposed to drug for 1 h, washed three times with buffer, and incubated for a further 71 h in drug-free medium.
[b] PIF is the potency increase factor (IC_{50} of free PALA/IC_{50} of encapsulated PALA), and shows by how much a given preparation is more potent than free PALA.
[c] Data presented as mean±s.d. (n=9)

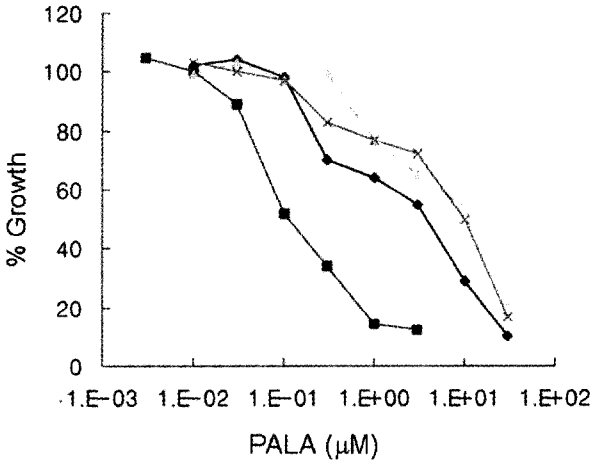

Fig. 1. 72 h growth inhibition of C-26 cells *in vitro* by PALA in different liposomal formulation such as Free PALA (closed diamond), DSPG-PALA (closed rectangle), DSPC-PALA (closed triangle), and PEG-DSPE (or Stealth)-PALA (cross).

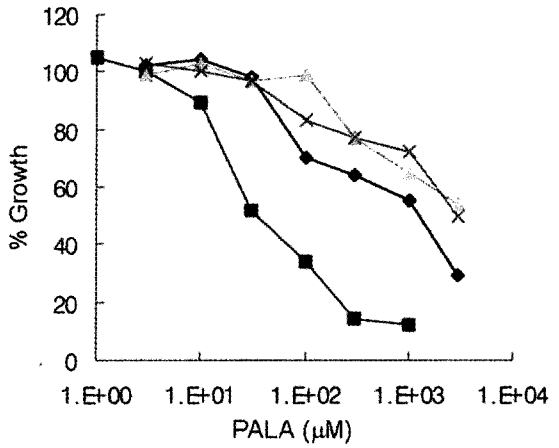

Fig. 2. 1 h growth inhibition of C-26 cells *in vitro* by PALA in different liposomal formulation such as Free PALA (closed diamond), DSPG-PALA (closed rectangle), DSPC-PALA (closed triangle), and PEG-DSPE (or Stealth)-PALA (cross).

growth inhibition experiments with encapsulated PALA. Therefore, PALA, and not the lipid, is the agent causing the observed growth inhibition.

Table II also shows the IC_{50} of free and encapsulated PALA when the exposure length was only 1 hr. All the IC_{50} values were increased by > 130 times, compared to 72 h exposure, but the rank-order of potency remained the same, and the potency increase factor (PIF) for the liposome preparations was similar to that observed in the 72 h exposure experiment. This agrees with previous observations on the short-term exposure of other cell lines to PALA (Heath *et al.*, 1989). As with the 72 h exposure study, no inhibition of cell growth by non-loaded liposomes was observed at lipid concentrations up to 2 mM.

Based on these observations, PALA in DSPG liposomes is selectively delivered to C-26 cells among tested. Therefore, this formulation should be capable of acting *in vivo* by selective delivery to the tumor cells in situations where the exposure of the tumor cells to the liposomes is either transient or prolonged. In contrast, PALA in both DSPC and PEG-DSPE liposomes is not selectively effective for *in vitro* inhibition of C-26 cells.

In conclusion, the *in vitro* growth inhibitory potency of PALA is increased by encapsulation only if the liposomes are negatively-charged DSPG, showing that, of the three formulations studied, only DSPG liposomes can deliver PALA directly to C-26 cells. Therefore, assuming that the liposome uptake properties of C-26 tumor cells are the same *in vivo* and *in vitro*, only DSPG liposomes should improve the *in vivo* therapeutic efficacy of PALA by liposome-mediated intracellular delivery.

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