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Therapeutic Potency of N-(Phosphonacetyl)-L-Aspartic Acid in Liposome in Established Tumor Bearing Mice

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진행된 암 동물모델에서의 리포좀 포집 PALA의 항암 치료 효과

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ABSTRACT–Previously, we have reported an antitumor efficacy of liposomal N-(phosphon-acetyl) -L-aspartic acid (or PALA) in C-26 tumor bearing Balb/c mice, where PALA in liposome was administered one day after tumor inoculation.[1] In this report, we have investigated the therapeutic potency of liposomal formulation of PALA, which was administered eight days after tumor inoculation in the same C-26 tumor bearing mice. The C-26 murine colon tumor inoculated mice were randomized for the *in vivo* therapy and the survival was measured after a single intraperitoneal injection of the drug. When the therapy was initiated eight days after tumor inoculation, DSPC-PALA at 150 mg/kg resulted in a significant increase in median survival time (MST) of 56% over the control group which received MES/HEPES buffer alone. However, none of the free PALA and DSPG-PALA liposome doses caused a statistically significant increase in MST over control group at the 95% confidence level. At 750 mg/kg dose, free PALA caused a marginally significant improvement in MST by 34%, but both 375 mg/kg and 150 mg/kg doses of free PALA caused only a 2% and a 4% increase in MST, respectively. These results show that PALA in neutrally charged liposome can exhibit considerably greater potency than free PALA in established C-26 tumor bearing mice.

Keywords–Liposomes, C-26 Murine Colon Carcinoma, PALA

Since the first discovery in 1964,[2] liposomes have extensively been used not only as tools for the study of model biological membranes but as drug carriers.[3] Among the many favorable properties of liposomes as drug carriers include that the phospholipids and cholesterols used to form liposomal structure are usually derived from natural sources such as egg yolks or soybean oil. Phospholipids have long been known to be safe when administered intravenously as emulsifying agents for total parenteral nutrition (TPN) as in Intralipid™. Doxil™, a doxorubicin HCl containing Stealth™ liposome formulation, was the first liposomal medicine approved by US FDA, which was originally indicated for AIDS-related Kaposi's sarcoma (AIDS-KS) and recently for the treatment of metastatic carcinoma of the ovary in patients with disease that is refractory to both paclitaxel- and platinum-based chemotherapy regimens.

In order for the liposomal drug delivery to be successful, it is required that the drugs to be tested have the characteristic of selective action on the cells, to which it is delivered by liposomes. We have previously referred to this kinds of compounds as liposome dependent drugs, and have defined how this property may be evaluated.[4,5] Despite the potential utility of liposome dependent drugs for clinical protocols, many of the compounds that have previously been described with this property are not clinically relevant for cancer chemotherapy. However, N-(phosphonacetyl)-L-aspartic acid (PALA) has recently been shown to be a liposome dependent drug when encapsulated in negatively charged liposomes,[6] and to be effective in ovarian cancer cells in culture.[7] Moreover, PALA in this form has shown effects against some human ovarian tumor cell lines.[8] However, its potency has generally proved limited despite its potential utility. This is at least in part caused by its limited ability to enter cells, as demonstrated

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by its low apparent volume of distribution.[9] This limited capacity for cell entry, which has been shown to occur only through non-adsorptive endocytosis,[10] is also the reason why this drug is liposome dependent. Therefore, it seems likely that its potency for treatment of tumors might be improved by encapsulation.

In this report, we have explored the use of liposome dependent drug, PALA, in a C-26 tumor bearing mouse model, where drug was administered intraperitoneally eight days after tumor inoculation and compared the anticancer potency of different liposomal PALA formulations with its free form.

Experimental

Materials

N-(phosphonacetyl)-L-aspartic acid was a generous gift of Dr. V.L. Narayanan of the Chemical Synthesis Branch, NCI (Bethesda, MD). Calcein was purchased from Molecular Probes (Eugene, OR). Distearoylphosphatidylcholine (DSPC), and distearoylphosphatidyl-glycerol (DSPG) were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol (Chol), protease (Type IX), collagenase (Type IV), and DNase were obtained from Sigma (St Louis, MO). DSPC, DSPG and cholesterol were stored ampouled in chloroform under argon at -20°C until use. Female Balb/c mice weighing 16-21 g were purchased from Harlan Sprague-Dawley (Indianapolis, IN). All other materials were reagent grade or better.

Preparation of Liposomes

Liposomes were prepared from either DSPC:Chol (2:1) or DSPG:Chol (2:1) using the method of reverse phase evaporation.[11] Typically, 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 mmol/Kg, and the pH was 7.2. Liposomes were extruded 5 times using a thermostatted stainless steel filtration cell (Mico Instruments, Middleton, WI) 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 NJ). The column was eluted with 50 mM MES, 50 mM HEPES, and 80 mM NaCl, 290 mmol/Kg pH 7.2 (MES/HEPES). The concentration of phospholipid in the liposome fractions was determined by a phosphorus assay[12] on triplicate samples, which were first extracted by the method of Bligh and Dyer[13] 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 detergent. 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.

C-26 Tumor Mouse Model

C-26 cells grown *in vitro* were harvested and resuspended at 1×10^6 cells/ml, and were inoculated subcutaneously on each flank of a female Balb/c mouse. After two to three weeks, the tumors, which were about 1000 mm^3 in diameter, were removed, and used to prepare a tumor inoculum by protease-collagenase treatment.[14] The viability of the cell suspension was tested by trypan blue exclusion and was over 95%. Experimental mice were then injected intraperitoneally with 1×10^6 tumor cells per mouse, and were randomly assigned to different groups, 5-9 mice per group. Treatment was started eight days after tumor inoculation. Control mice were injected with 1.4 ml of MES/HEPES. After initiation of drug treatment, the mice were weighed three times a week and were inspected daily during the experimental period. Deaths were recorded daily, and mice whose weight was less than 80% of the starting weight, or mice that were obviously in discomfort were humanely sacrificed. In cases where mice were humanely sacrificed, the day of sacrifice was recorded as the day of death. All animal experiments, including the MTD study, adhered to the "Principles of Laboratory Animal Care (NIH publication #85-23, revised 1985).

Results and Discussion

Characteristics of PALA-Liposome Formulations

Many of the basic physico-chemical characteristics of liposomal PALA formulations and *in vitro* cytotoxicity in cultured cells were as reported elsewhere.[1,15] Briefly, the mean diameter of PALA-liposome formulations was about 200 nm and the drug encapsulation ratio was about 15%. Also, the IC_{50} of DSPG-PALA and DSPC-PALA liposome formulations against C-26 cells *in vitro* were 0.09 M and 11.8 M, respec-

tively. As none of the DSPC-PALA and DSPG-PALA doses injected caused more than a 3% weight loss in this study, the maximum tolerated dose (MTD) for these two formulations was greater than 150 mg/kg (data not shown). Based on drug concentration and injection volume, this was the largest possible dose for encapsulated PALA. Based on these determinations, the doses of free and encapsulated PALA were chosen for antitumor therapy studies. The doses of free PALA were 750 mg/kg (the MTD), 375 mg/kg (one-half the MTD), and 150 mg/kg (one-fifth the MTD). The doses of PALA encapsulated in DSPC and DSPG liposomes were 150 mg/kg, 75 mg/kg, and 50 mg/kg.

Eight-Day Therapy of C-26 Tumor Bearing Mice with PALA

Eight days after C-26 tumor cells were inoculated, intraperitoneal (i.p.) therapy was initiated using free PALA or DSPC- or DSPG-PALA liposome formulations. As shown in Figure 1-3 and Table 1, treatment with DSPC-PALA at 150 mg/kg resulted in a significant increase in median survival time (MST) of 56% over the control group which received MES/HEPES buffer alone. However, none of the free PALA and DSPG-PALA liposome doses caused a statistically significant increase in MST over control group at the 95% confidence level. At 750 mg/kg dose, free PALA caused a marginally significant improvement in MST by 34%, but both 375 mg/kg and 150 mg/kg doses of free PALA caused only a 2% and a 4% increase in MST, respectively. The DSPG-PALA liposomes did not cause a significant increase in MST either.

It seems likely that the C-26 tumor may have advanced too much to respond to a single i.p. therapy of PALA in any of the

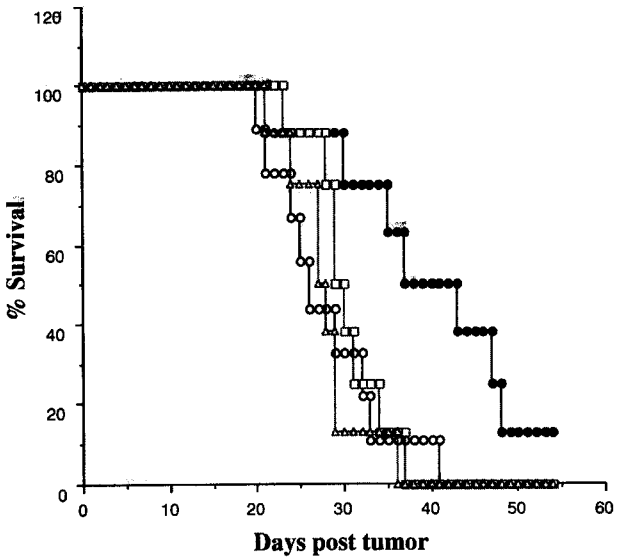

Figure 2–Survival of C-26 tumor bearing mice by a single i.p. injection of DSPC-PALA liposomes eight days after tumor implantation. [○: control (buffer), ●: DSPC-PALA liposome, 150 mg/kg, □: DSPC-PALA liposome, 75 mg/kg, △: DSPC-PALA liposome, 50 mg/kg].

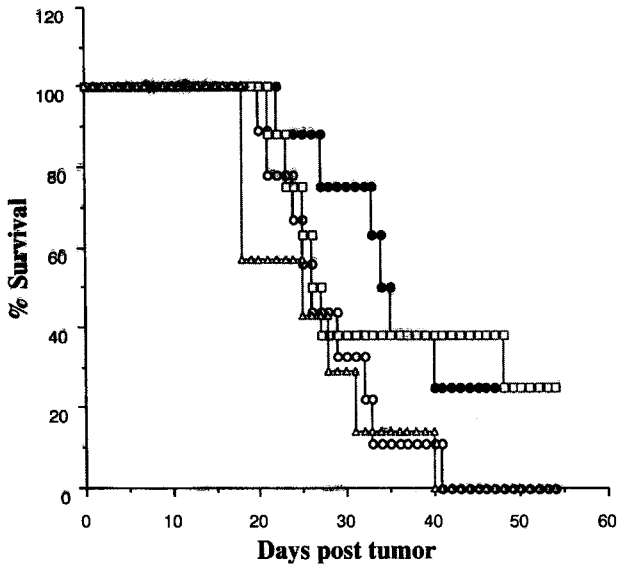

Figure 1–Survival of C-26 tumor bearing mice by a single i.p. injection of free PALA eight days after C-26 tumor implantation. [○ : control (buffer), ●: free PALA, 750 mg/kg, □: free PALA, 375 mg/kg, △: free PALA, 150 mg/kg].

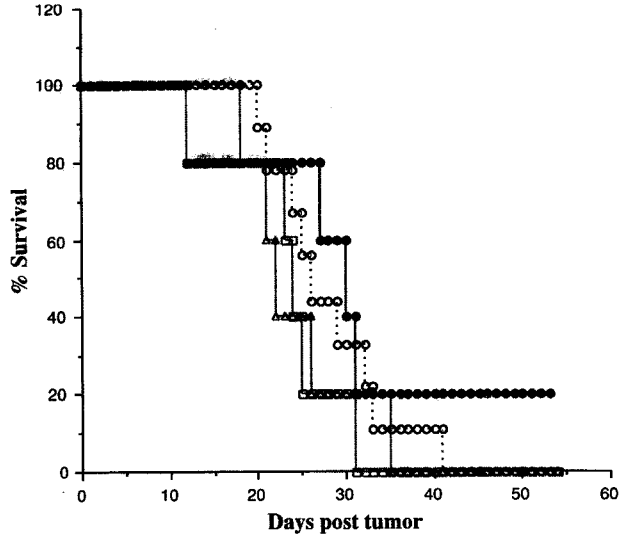

Figure 3–Survival of C-26 tumor bearing mice by a single i.p. injection of DSPG-PALA liposomes eight days after tumor implantation. [○: control (buffer), ●: DSPG-PALA liposome, 150 mg/kg, □: DSPG-PALA liposome, 75 mg/kg, △: DSPG-PALA liposome, 50 mg/kg].

Table I–*Summary of The Survival of C-26 Tumor-Bearing Mice with The Treatment of PALA in Various Formulations 8 Days After Tumor Implantation*

Treatment	# mice/group	PALA (mg/kg)	Median Survival Time (days)	Increase in life span (ILS) (%)[a]
control	9	none	25 ± 6.6 [b]	
free PALA	8	750	33.5 ± 12	+34
	8	375	25.5 ± 4.1	+2
	7	150	24 ± 8.3	-4
DSPG -PALA Lipo.	5	150	29 ± 15.4	+16
	5	75	23 ± 6.9	-8
	5	50	21 ± 6.5	-16
DSPC -PALA Lipo.	8	150	39 ± 11	+56
	8	75	28.5 ± 4.2	+14
	8	50	26.5 ± 4.4	+6

[a] % increase in life span (ILS) = [(median survival time of treated ÷ median survival time of control) × 100] - 100
[b] median ± s.d.

formulations used, though DSPC-PALA liposomes at a 150 mg/kg dose caused a slight increase in MST over control group. It is probable that the DSPC-PALA liposomes are taken up by tumor cells much more efficiently than any other PALA formulations, because they remain at the tumor sites longer. They may also deliver drugs in a controlled release fashion, because the *in vitro* cytotoxicity of DSPC-PALA liposomes on C-26 cells was even less than free PALA.[15] It is not clearly known currently why DSPG-PALA liposomes did not show the same effect as DSPC-PALA liposomes but it is postulated that the lower physical stability of DSPG-PALA liposomes in body fluid and the shorter residence time in body cavity might have affected the uptake of PALA by tumor cells.

As we have previously reported the therapeutic efficacy of PALA-liposome formulations in C-26 tumor bearing mice one day after tumor inoculation,[1] the issue was raised whether this increased therapeutic effect will remain if they are injected eight days after tumor inoculation or not. Here, we have exploited the use of liposomal formulations of PALA in established C-26 tumor mouse model. Almost 90% of the mice from control groups died by Day 36, though the majority of the deaths occurred by Day 21. DSPC-PALA and DSPG-PALA liposomes increased the median life span (ILS) by 56% and 16%, respectively, when the therapy was initiated eight days after tumor inoculation. Free PALA at 750 mg/kg caused a significant increase in life span (34% ILS), though this dose caused severe toxicity. A Stutent's t-test revealed that only the 34% ILS caused by 750 mg/kg free PALA and the 56% ILS caused by 150 mg/kg DSPC-PALA liposomes were statistically significant over the control group at the 95% confidence level. When liposomes are injected intraperitoneally, they will be removed from the intraperitoneal cavity via the abdominal lymphatics with subsequent drainage into the venous system for the circulation. It is conceivable that DSPC-PALA liposomes remain longer in the peritoneal cavity due to the higher stability than the DSPG-PALA liposomes, and free PALA is released from liposomes in a controlled-release fashion, and exhibit a better therapeutic efficacy than the DSPG-PALA liposomes. As the drug (PALA) itself has a negative charge at the experimental condition, less charge repulsion from the encapsulated drug is expected in DSPC-PALA liposome than DSPG-PALA liposome, which might result in higher stability of DSPC-PALA liposome.

The above results demonstrate that the potency of PALA in the treatment of the established C-26 colon carcinoma bearing mice can be improved by encapsulation in liposomes, especially in neutrally-charged liposome. This result seems to be in contrary to the previous report, where the negatively charged liposomal formulation was the most effective when the therapy was initiated one day after tumor inoculation. Possible reason for this includes, but not limited to, that the C-26 tumor has been established too much to be easily accessed to the free or liposomal PALA, and longer residence in and/or around the tumor site is important in getting the desired antitumor effect.

Together with the previous one-day therapy result, it is concluded that the potency of PALA for therapy in the C-26 tumor bearing mice can be considerably improved by encapsulation in liposomes. This result may have considerable benefit for the use of PALA as an antitumor agent. However, it is important to start the therapy as early as possible for the

best result regardless of the type of liposomal formulations. The increase in the potency of PALA appears to occur by direct delivery to the tumor cells when the liposomes are anionic, and by controlled release when the liposome are neutral or stoically stabilized. While controlled release is potentially useful when liposomes cannot deliver drug directly to the tumor cells, it appears to be limited in its effects by toxicity. We hope to explore these factors in more detail in future studies.

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