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Antitumor Efficacy of Liposomal N-(Phosphonacetyl)-L-Aspartic Acid in C-26 Tumor Bearing Balb/c Mice

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리포좀 포집 PALA의 C-26암 유발 마우스에 대한 항암 효과

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Abstract–We have investigated the efficacy of liposome encapsulated N-(phosphonacetyl)-L-aspartic acid (PALA) for the treatment of the C-26 murine colon tumor in Balb/c mice, and have compared it in this regard to free PALA. Healthy female Balb/c mice and C-26 tumor inoculated mice were randomized for the maximum tolerated dose (MTD) study and the *in vivo* therapy study, and the survival was measured after a single intraperitoneal injection of the drug. The maximum tolerated dose for intraperitoneally administered drug was found to be 750 mg/Kg for free PALA, and was greater than the maximum dose possible (150 mg/Kg) for PALA encapsulated in both DSPC and DSPG liposomes. When drug was administered one day after tumor implantation, 150 mg/Kg of PALA in DSPG liposomes increased the percentage of tumor bearing mice surviving at day 36 from 8% (buffer control) to 88%. In contrast, 150 mg/Kg free PALA increased the day 36 surviving percentage to only 25%. A 150 mg/Kg dose of PALA in DSPC liposomes increased the surviving percentage to 50%, while a 75 mg/Kg dose of PALA in sterically stabilized liposomes increased the surviving percentage to 78%. These results show that PALA in negatively charged or sterically stabilized liposomes can exhibit considerably greater potency than free PALA in C-26 tumor bearing mice.

Keywords–Liposomes, C-26 Colon Carcinoma, *In vivo* Therapy, PALA

Liposomes encapsulating cancer chemotherapeutic agents for experimental *in vivo* and *in vitro* studies have widely been used.[1] Examples include doxorubicin,[2-7] vincristine,[3,8] cytosine arabinoside,[9] cis-platinum,[10] and camptothecin.[11] The me- chanism by which liposomes improve the efficacy of these drugs is not fully elucidated. However, it is clear that the controlled release of the drugs from the liposome is an important factor in many cases. This is perhaps best demonstrated by the fact that encapsulated drug efficacy is greatest when liposomes with prolonged circulating half-life or sterically stabilized liposomes are used.[3, 6-8] In addition to controlled release, there is another mechanism by which liposomes may improve drug efficacy. This mechanism involves the direct delivery of drug to the intracellular target. In order for direct delivery to be relevant, the drug under consideration must have the property of showing improved efficacy when delivered in this way. Such compounds have been termed liposome dependent drugs,[12] and a number have been characterized as such including methotrexate-γ-aspartate,[13] fluoroorotic acid,[14] clodronate,[15] gallium,[16] hygromycin B,[17] and N-phosphonacetyl-L-aspartic acid.[18,19]

Most investigations of liposome dependent drugs have involved *in vitro* test systems. The *in vivo* use of liposome dependent drugs requires that the liposomes used should be capable of direct interaction with the cells, to which the drug must be delivered. *In vivo* studies have been reported for clodronate, which has been shown to eliminate successfully macrophages in animals.[20,21] This clearly showed that direct interaction with the target cell is possible when the target cell is macrophages. Doubts have been raised as to whether tumor cells will be sufficiently accessible to liposomes injected systemically.[22,23] Despite such doubts, sterically stabilized liposomes have proved capable of localizing in tumor.[24]

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In the present study we have explored the use of the li - posome dependent drug, PALA, in a tumor bearing mouse model. We found that encapsulated PALA can be considerably more effective for increasing the surviving percentage of tumor-bearing mice than free PALA, and we discuss the possible role of liposome-mediated intracellular delivery and controlled release in the manifestation of its improved effects.

Experimental

Materials

N-(phosphonacetyl)-L-aspartic acid was a generous gift of Dr. V.L. Narayanan of the Chemical Synthesis Branch, NCI (Bethesda, MD). Polyethylene glycol 1900 derivatized DSPE (PEG-DSPE) was kindly provided by Dr. F. Martin (Liposome Technology Inc., Menlo Park, CA). Calcein was purchased from Molecular Probes (Eugene, OR). Distearoylphosphatidylcholine (DSPC), and distearoylphosphatidylglycerol (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), DSPG:Chol (2:1), or DSPC:Chol:PEG-DSPE (20:10:1) using the method of reverse phase evaporation.[25] 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[26] on triplicate samples, which were first extracted by the method of Bligh and Dyer[27] 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.

Particle Size Analysis

The liposome size was determined using a quasi-elastic laser light scattering instrument (QELS; Model 370, Nicomp, Santa Barbara, CA).

Maximum Tolerated Dose (MTD)

Female Balb/c mice were randomized into 20 groups of 6, which were either untreated (1 group), injected intraperitoneally with 1.4 ml of MES/HEPES (1 group), injected intraperitoneally with 1.4 ml PALA in the free form (6 groups), or injected intraperitoneally with PALA encapsulated in either DSPC (6 groups) or DSPG (6 groups) liposomes. Mouse weight and survival were recorded daily for three weeks. The highest dose that did not cause 15% or more weight loss was taken as the MTD. Animals showing more than 20% weight loss were humanely sacrificed.

C-26 Tumor Bearing Mice

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[28]. 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, 10 mice per group. Treatment was started one day 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

Characteristics of Liposomes

Table I summarizes the physical characteristics of the liposome preparations used for *in vivo* study. 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[29-31].

Determination of the MTD

The results of studies to determine the MTD for free PALA are shown in Figure 1. Free PALA exhibited an MTD of 750 mg/Kg. At this dose, the maximum weight loss observed was 12%, which occurred 5 days after injection. As none of the DSPC-PALA and DSPG-PALA doses injected caused more than a 3% weight loss in this study, the MTD for these two formulations was greater than 150 mg/Kg (data not shown). Based on drug concentration and injection volume, this was the highest 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. The doses of PALA in sterically stabilized liposomes used were 100 mg/Kg, 50 mg/Kg, and 30 mg/Kg doses. No MTD study was done on the latter, and the doses were chosen to be comparable to those of the other liposome preparations, but were lower owing to the limited availability of the material.

Immediate (One-Day) *In Vivo* Therapy

The results of tumor therapy initiated one day after tumor implantation are shown in Figures 2-5 and Table II. The median survival time (MST) of the control (buffer) group was 20 days, and by day 36, 92% had died. In contrast, the MST of the group receiving 750 mg/Kg free PALA was 29 days, and all had died by day 34. At day 36, 25% of the 375 mg/Kg or 150 mg/Kg free PALA groups were alive. Free PALA caused a marked weight loss in many animals. We conclude that the therapeutic effects of free PALA were minimal, and were limited by the toxicity of the drug. Of the mice receiving PALA in DSPC liposomes, 50% of those receiving 150 mg/Kg survived to day 36, and 25% of those receiving either 75 mg/Kg or 50 mg/Kg survived to day 36. All doses of PALA in DSPC liposomes caused an increase in the percent surviving at day 36, with no evidence of toxicity. The increase in the percent surviving was commensurate with dose. Moreover, the percent surviving at day 36 was greater in the group receiving 150 mg/Kg of PALA in DSPC liposomes than it was in the group receiving the same dose of free PALA. All doses of

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] Determined by quasi-elastic laser light scattering.
[d] Stealth liposome contains 5% PEG-DSPE additionally in DSPC liposome formulation.

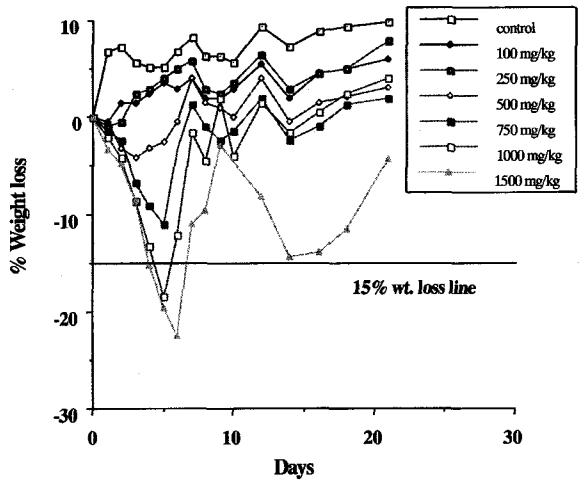

Figure 1–Determination of the maximum tolerated dose (MTD) for free PALA after a single intraperitoneal injection in Balb/c mice.

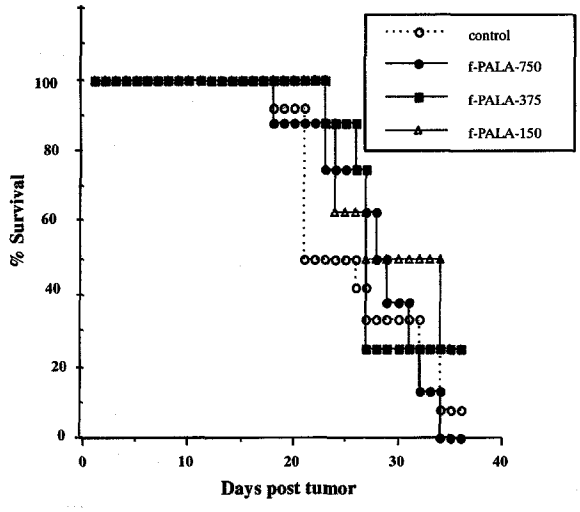

Figure 2–Survival of mice bearing the C-26 tumor following intraperitoneal injection of free PALA 1 day after tumor implantation. Doses given were 750 mg/kg (closed circles, solid line), 375 mg/kg (closed squares, solid line), and 150 mg/kg (open triangles, solid line), and buffer (control: open circles, broken line).

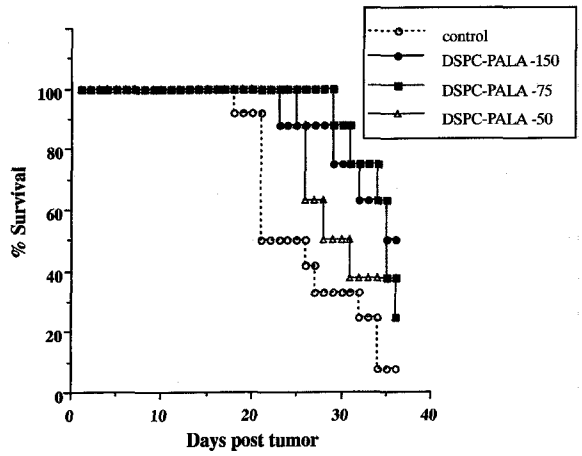

Figure 3–Survival of mice bearing the C-26 tumor following intraperitoneal injection of PALA encapsulated in DSPC-Chol (2:1) liposomes 1 day after tumor implantation. Doses given were 150 mg/kg (closed circles, solid line), 75 mg/kg (closed squares, solid line), and 50 mg/kg (open triangles, solid line), and buffer (control: open circles, broken line).

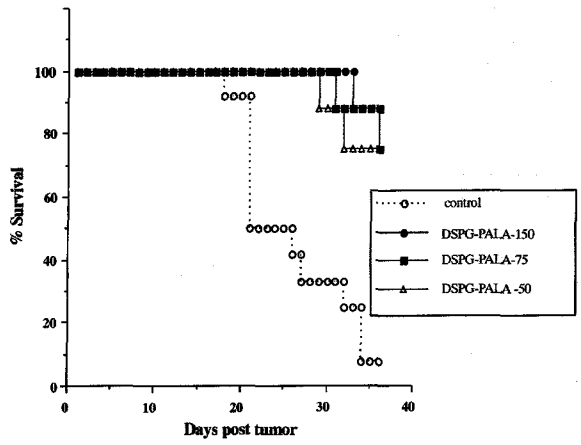

Figure 4–Survival of mice bearing the C-26 tumor following intraperitoneal injection of PALA encapsulated in DSPG:Chol (2:1) liposomes 1 day after tumor implantation. Doses given were 150 mg/kg (closed circles, solid line), 75 mg/kg (closed squares, solid line), and 50 mg/kg (open triangles, solid line), and buffer (control: open circles, broken line).

PALA encapsulated in DSPG liposomes caused a significant increase in the percent surviving at day 34. Only one mouse of the group receiving 150 mg/Kg had died (88% surviving) by day 36. Two mice had died by day 36 (75% surviving) in each of the two groups, one receiving 75 and one receiving 50 mg/Kg, respectively. Owing to the limited number of deaths it is not possible to assign a value to the MST of the groups receiving PALA in DSPG liposomes. However, based on a comparison of the death curve for the groups receiving PALA in DSPG liposomes to that of the control group (MST for control is 22.5 days), we estimate an MST of 43 days for the 75 and 50 mg/Kg doses, and 45 days for the 150 mg/Kg dose, and hence increases in life span of 95% and 100%, respectively.

A significant increase in the percent surviving among the groups that had received PALA in PEG-DSPE liposomes was observed at day 36. The percent surviving at day 36 from the respective groups of mice was 38% for the group receiving 100 mg/Kg, 78% for the group receiving 75 mg/Kg and 56% for the group receiving 50 mg/Kg. The increase in survival at

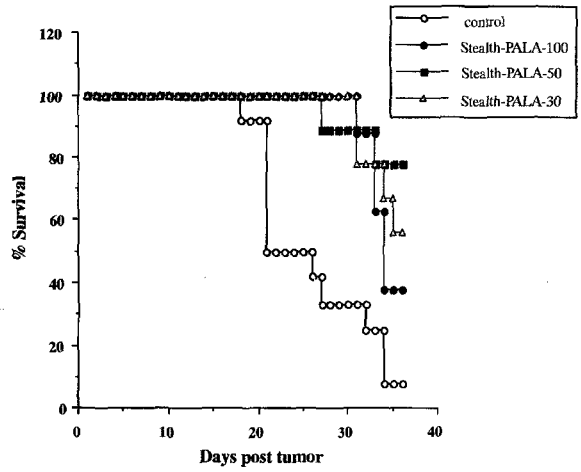

Figure 5–Survival of mice bearing the C-26 tumor following intraperitoneal injection of PALA encapsulated in DSPC:Chol : PEG-DSPE (20: 10:1) liposomes 1 day after tumor implantation. Doses given were 100 mg/kg (closed circles, solid line), 50 mg/kg (closed squares, solid line), and 30 mg/kg (open triangles, solid line), and buffer (control: open circles, broken line).

Table II–*Percent of Mice Surviving at Day 36 Following a Single Intraperitoneal Injection of PALA One Day after Tumor Implantation*

Lipid Composition	# of Mice	PALA Dose (mg/kg)`	% Survival at Day 36
Saline Control	12	0	8
Free PALA	8	750	8
	8	375	25
	8	150	25
DSPG:Chol (2:1)	8	150	88
	8	75	75
	8	50	75
DSPC:Chol (2:1)	8	150	50
	8	75	25
	8	50	25
DSPC:Chol:PEG-DSPE (20 : 10 : 1)	8	100	38
	9	50	78
	9	30	56

day 36 for the highest dose of PALA in PEG-DSPE-liposomes (38%) is not as great as was seen for the two lower doses (56% and 78%). Based on the percent surviving at the time of termination, the MST is estimated to be 38 days for 30 mg/Kg, and 40 days for 50 mg/kg, which are increases of 67% and 78%, respectively. Hence, the PEG-DSPE liposome formulation is significantly more effective than the DSPC liposome formulation, though not as effective as the DSPG liposome formulation. In addition, PALA in PEG-DSPE liposomes caused significant toxicity at the highest dose, as indicated by the observed reduction in the percent surviving. When the experiments were terminated at day 36, all of the surviving mice were examined for any sign of intraperitoneal or subcutaneous tumors, which were found in all of the mice, except for two mice that had received 75 mg/Kg of PALA in DSPG liposomes. These two mice showed no visible tumor at all, suggesting that a single intraperitoneal injection of PALA in DSPG liposomes may eradicate the tumor in some of the tumor bearing mice.

Discussion

The above results demonstrate that the potency of PALA in the treatment of the C-26 colon carcinoma bearing mice can be markedly improved by encapsulation in liposomes, and it is useful to consider the possible mechanism, by which this improvement takes place. The *in vitro* growth inhibitory potency of PALA is increased by encapsulation only if the liposomes are DSPG, showing that, of the three formulations studied, only DSPG liposomes can deliver PALA directly to C-26 cells (data not shown). 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[18] . In fact, DSPG liposomes have shown the most improved *in vivo* therapeutic efficacy (88%) in C-26 tumor model. It is probable that liposome-mediated direct intracellular delivery makes this anionic liposome more effective than any others. Furthermore, PALA in sterically stabilized liposomes and in DSPC liposomes also show considerable improvement in their potency as compared to free drug. These two liposome formulations must improve the efficacy of PA LA *in vivo* by mechanisms other than liposome-mediated intracellular delivery, because our *in vitro* studies, though not shown here, do not show them to be active in this way. Therefore, controlled release is the most likely mechanism, by which the efficacy of PALA is improved by DSPC and sterically stabilized liposomes. The conclusion that controlled release is the mechanism of action of these two liposome compositions is also consistent with the fact that sterically stabilized liposomes are the better of the two. DSPC liposomes will exhibit a shorter *in vivo* half life than the sterically stabilized liposomes, and, therefore, will be present as a release depot for a shorter period

of time.

It is also important to note the toxic effects, which may arise through administration of PALA in liposomes. While the effects of PALA in DSPG liposomes occur strictly in accordance with dose, the effect of PALA in sterically stabilized liposomes is markedly reduced when the dose is increased from 50 to 100 mg/Kg. This suggests that this preparation is toxic at the highest dose, and that its use would be limited by such toxicity. Therefore, improvement of PALA potency seems to be accompanied by an increase in its toxicity if the mechanism of improvement is controlled release, but not if the improvement is caused by direct delivery to the tumor cells.

In conclusion, 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. 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 sterically 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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References

1) J. H. Senior, Fate and behavior of liposomes *in vivo*: a review of controlling factors, *Crit. Rev. Ther. Drug Carrier System*, **3**, 123-193 (1987).
2) E. Mayhew, M. Cimino, J. Klemperer, R. Lazo, J. Wiemikowski and S. Arbuck, Free and liposomal doxorubicin treatment of intraperitoneal colon 26 tumor: Therapeutic and pharmacologic studies, *Selective Cancer Therapeutics*, **6**, 193-209 (1990).
3) J. Vaage, D. Donovan, E. Mayhew, P. Uster and M. Woodle, Therapy of mouse mammary carcinomas with vincristine and doxorubicin encapsulated in sterically stabilized liposomes, *Int. J. Cancer*, **54**, 959-964 (1993).
4) S. S. Williams, T. R. Alosco, E. Mayhew, D. D. Lasic, F. J. Martin and R. B. Bankert, Arrest of human lung tumor xenograft growth in severe combined immunodeficient mice using doxorubicin encapsulated in sterically stabilized liposomes, *Cancer Res.*, **53**, 3964-3967 (1993).
5) K. Maruyama, S. Unezaki, S. Yuda, O. Ishida, T. N. A. Suginaka, L. Huang and M. Iwatsuru, Enhanced delivery and antitumor effect of doxorubicin encapsulated in long-circulating liposomes, *J. Liposome Res.*, **4**, 143-165 (1994).
6) D. Papahadjopoulos, T. M. Allen, A. Gabizon, E. Mayhew, K. K. Matthay, S. K. Huang, K-D. Lee, M. C. Woodle, D. D. Lasic, C. Redemann and F. J. Martin, Sterically stabilized liposomes: Improvements in pharmacokinetics and antitumor therapeutic efficacy, *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11460-11464 (1991).
7) J. Vaage, D. Donovan, E. Mayhew, R. Abra and A. Huang, Therapy of human ovarian carcinoma xenografts using doxorubicin encapsulated in sterically stabilized liposomes, *Cancer*, **72**, 3671-3675 (1993).
8) N. L. Boman, D. Masin, L.D. Mayer, P. R. Cullis and M. B. Bally, Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing p388 tumors, *Cancer Res.*, **54**, 2830-2833 (1994).
9) Y. Rustum, C. Dave, E. Mayhew and D. Papahadjopoulos, Role of Liposome type and route of administration in the antitumor activity of liposome-entrapped 1-beta-D-arabinofuranosylcytosine against mouse L 1210 leukemia, *Cancer Res.*, **39**, 1390-1395 (1979).
10) B. Sur, R. R. Ray, P. Sur and D. K. Roy, Effect of liposomal encapsulation of cis-platinum diamminedichloride in the treatment of Ehrlich ascites carcinoma, *Oncology*, **40**, 372-376 (1983).
11) S. S. Daoud, M. I. Fetouh and B. C. Giovanella, Antitumor effect of liposome-incorporated camptothecin in human malignant xenografts, *Anti-Cancer Drugs*, **6**, 83-93 (1995).
12) T. D. Heath, N. G. Lopez and D. Papahadjopoulos, The effects of liposome size and surface charge on liposome-mediated delivery of methotrexate-γ-aspartate to cells *in vitro*, *Biochim. Biophys. Acta*, **820**, 74-84 (1985).
13) T. D. Heath, J. A. Montgomery, J. R. Piper and D. Papahadjopoulos, Antibody-targeted liposomes: Increase in specific toxicity of methotrexate-γ-aspartate, *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 1377-1381 (1983).
14) T. D. Heath, N. G. Lopez, W. H. Stem and D. Papahadjopoulos, 5-Fluoroorotate: a new liposome-dependent cytotoxic agent, *FEBS Lett.*, **187**, 73-75 (1985).
15) J. Monkkonen and T. D. Heath, The effects of liposome-encapsulated and free clondronate on the growth of macrophage-like cells *in vitro*: The role of calcium and iron, *Calcif. Tissue Int.*, **53**, 139-146 (1993).
16) J. Monkkonen, C. S. Brown, T. T. Thompson and T. D. Heath, Liposome-mediated delivery of gallium to macrophage-like cells *in vitro*: Demonstration of a transferrin-independent route for intracellular delivery of metal ions, *Pharm. Res.*, **10**, 1130-1135 (1993).

17) O. Sechoy, M. Vidal, J. R. Philippot and A. Bienvenue, Interactions of human lymphoblasts with targeted vesicles containing Sendai virus envelope proteins, *Exp. Cell Res.*, **185**, 122-131 (1989).
18) T. D. Heath and C. S. Brown, Liposome dependent delivery of N-(phosphonacetyl)-L-aspartic acid to cells *in vitro*, *J. Liposome Res.*, **1**, 303-317 (1989).
19) A. Sharma, N. L. Straubinger and R. M. Straubinger, Modulation of human ovarian tumor cell sensitivity to N-(phosphonacetyl)-L-aspartate by liposome drug carriers, *Pharm. Res.*, **10**, 1434-1441 (1993).
20) N. Van Rooijen, N. Kors, N. Van der Ende and C. D. Dijkstra, Depletion and repopulation of macrophages in spleen and liver of rat after intravenous treatment with liposome-encapsulated dichloromethylene diphosphonate, *Cell Tissue Res.*, **260**, 215-222 (1990).
21) N. VanRooien, The liposome-mediated macrophage 'suicide' technique, *J. Immunol. Methods*, **124**, 1-6 (1989).
22) S. Matzku, H. Krempel, H. P. Weckenmann, V. Schirr-rnacher, H. Sinn and H. Stricker, Tumor targeting with antibody-coupled liposomes: failure to achieve accumulation in xenografts and spontaneous Ever metastases, *Cancer Immunol. Immunother.*, **31**, 285-291 (1990).
23) G. Poste, R. Kirsh and T. Koestler, The challenge of liposome targeting *in vivo*: In *Liposome Technology*, G. Gregoriadis (Eds.), CRC Press, pp. 188-195 (1984).
24) S. K. Huang, K-D. Lee, D. S. Friend and D. Papahadjopoulos, Microscopic localization of sterically stabilized liposomes in colon carcinoma-bearing mice, *Cancer Res.*, **52**, 5135-5143 (1992).
25) F. Szoka and D. Papahadjopoulos, Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 4194-4198 (1978).
26) G. R. Bartlett. Phosphorus assay in column chromatography, *J. Biol. Chem.*, **234**, 466-468 (1958).
27) E. G. Bligh and W. J. Dyer, A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.*, **37**, 911-917 (1959).
28) T. H. Corbett and D. P. Grisword, A mouse colon-tumor model for experimental therapy, *Cancer Chemotherapy Rep.*, **5**, 169-186 (1975).
29) F. Olson, C. A. Hunt, F. C. Szoka, W. J. Vail and D. Papahadjopoulos, Preparation of liposomes of defined size distribution by extrusion through polycarbonate membrane, *Biochim. Biophys. Acta*, **557**, 9-23 (1979).
30) F. Szoka, F. Olson, T. Heath, W. Vail, E. Mayhew and D. Papahadjopoulos, Preparation of unilamellar liposomes of intermediate size (0. 1-0.2 μm) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes, *Biochim. Biophys. Acta*, **601**, 559-571 (1980).
31) L. D. Mayer, M. J. Hope and P. R. Cullis, Vesicles of variable sizes produced by a rapid extrusion procedure, *Biochim. Biophys. Acta*, **858**, 161-168 (1986).