0026-895X/02/6204-864–872$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2002 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 62:864–872, 2002

Vol. 62, No. 4
1613/1010927
Printed in U.S.A.

Translocation of Liposomes into Cancer Cells by Cell-Penetrating Peptides Penetratin and Tat: A Kinetic and Efficacy Study

YUN-LONG TSENG, JUN-JEN LIU, and RUEY-LONG HONG

Department of Oncology, National Taiwan University Hospital (Y.-L.T, R.-L.H.); and Institute of Biochemistry, College of Medicine, National Taiwan University (J.-J.L.), Taipei, Taiwan

Received January 5, 2002; accepted June 28, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Unlike conventional liposomes, sterically stabilized liposomes, with their smaller volume of distribution and reduced clearance, preferentially convey encapsulated drugs into tumor sites. Despite these improvements, intracellular delivery is hampered by the stable drug retention of the liposomes, which diminishes the efficacy of the liposomal drug. To facilitate uptake of liposomal drugs into cells, two cell-penetrating peptides, penetratin (PEN) and TAT, derived from the HIV-1 TAT protein, were studied. In contrast to control peptides, both TAT and PEN enhanced the translocation efficiency of liposomes in proportion to the number of peptides attached to the liposomal surface. A peptide number of as few as five could enhance the intracellular delivery of liposomes. The kinetics of uptake was peptide- and cell-type dependent. Intracellular accumulation of TAT-liposomes increased with incubation time, but PEN-liposomes peaked at 1 h and then declined gradually. After treatment with 1 μg/ml doxorubicin equivalents of liposome for 2 h, TAT increased the doxorubicin uptake of A431 cells by 12-fold. However, the improvement of uptake of liposomal doxorubicin was not reflected by cytotoxicity in vitro or tumor control in vivo. Our results demonstrated that merely adding CPP to a liposome encapsulating anticancer drug was inadequate in improving its antitumor activity. An additional approach to enhance the intracellular release of the encapsulated drug is obviously necessary.

Liposomes of conventional formulations are rapidly removed from blood circulation by the reticuloendothelial system, thus preventing them from reaching their target sites. Recently, smaller liposomes with a more stable lipid composition and protection from the reticuloendothelial system by surface coating with polyethylene glycol (PEG) have been developed. They have a smaller volume of distribution and a reduced clearance rate (Allen and Hansen, 1991; Papahadjopoulos et al., 1991; Hong et al., 1999). These sterically stabilized liposomes (second-generation liposomes) circulate for prolonged periods with stable retention of their contents, leading to passive preferential localization into the tumors (Gabizon and Papahadjopoulos, 1988; Papahadjopoulos et al., 1991). Clinical trials of formulations of PEG-coated liposomal doxorubicin also demonstrated improved pharmacokinetic properties and reduced systemic toxicity (Uziely et al., 1995; Hong and Tseng, 2001).

Despite this progress, sterically stabilized liposomal doxorubicin still shows little or no activity against many common cancers, including sarcoma, non–small-cell lung cancer, and hepatoma (Koukourakis et al., 1999; Chidiac et al., 2000; Halm et al., 2000). Although sterically stabilized liposomes are able to traverse the endothelium and to extravasate into the extracellular spaces, after reaching tumor sites, the toughness of the lipid bilayer or presence of surface PEG seems to retard fusion or uptake of the liposomes (Parr et al., 1997; Hong et al., 1999; Ng et al., 2000). A sterically stabilized liposome composed of lipid with a higher transition temperature has an even smaller volume of distribution and a longer β half-life; paradoxically, however, it has lower clinical activity (Hong and Tseng, 2001). To enhance the antitumor effect, further modification to facilitate uptake of liposomes into the target cells must be made.

Recently, several peptides, including penetratin (PEN; DNA binding domain of the *Drosophila melanogaster* transcription factor antennapedia) and the HIV *trans*-activating

The research was supported in part by a grant from the National Science Council, Taiwan (NSC 90-2316-B-002-004).

ABBREVIATIONS: PEG, poly(ethylene glycol); PEN, penetratin; TAT, Tat peptide; CPP, cell-penetrating peptide; HPTS, 8-hydroxypyrenetrisulfonic acid trisodium salt; PBS, phosphate-buffered saline; DSPE, distearoylphosphatidylethanolamine; LD, liposomal doxorubicin; FITC, fluorescein isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TAT-F, fluorescein-conjugated TAT; PEN-F, fluorescein-conjugated PEN; TAT-L-HPTS, TAT-conjugated liposome encapsulating HPTS; PEN-L-HPTS, PEN-conjugated liposome encapsulating HPTS; TAT-LD, TAT-conjugated liposomal doxorubicin.

transcriptional activator (TAT) protein have been shown to translocate across the plasma membrane of eukaryocytes by a seemingly energy-independent pathway (Derossi et al., 1994; Vives et al., 1997). These cell-penetrating peptides (CPPs) have been successfully used for the intracellular delivery of macromolecules with molecular weights several times greater than their own and other large particles in the submicron range (Fawell et al., 1994; Lewin et al., 2000; Torchilin et al., 2001). CPPs, by interacting with charged phospholipids on the outer side of the cell membrane and destabilizing the bilayer, are capable of carrying hydrophilic compounds across the plasma membrane. This property may be useful in facilitating the intracellular delivery of liposomal drugs.

According to the currently accepted mechanism, cellular internalization of rigid and stable sterically stabilized liposomes can be greatly enhanced through the ligand-directed endocytosis pathway (Huang et al., 1983; Connor and Huang, 1985). In this study, we examined a different path for direct translocation of liposomes into the cellular cytoplasm, bypassing the endocytotic pathway. We also explored the benefits of efficacy.

Materials and Methods

Chemicals. Fluorescein-5-maleimide and 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS) were purchased from Molecular Probes (Eugene, OR). Doxorubicin was obtained from Farmitalia Carlo Erba (Milano, Italy). Egg phosphatidylcholine, distearoyl phosphatidylcholine, cholesterol, and polyethylene glycol (average molecular weight, 2000)-derived distearoylphosphatidylethanolamine (*N*-[ω-methoxypoly(oxyethylene)-(*R*)-carbonyl]-distearoylphosphatidylethanolamine) were purchased from Avanti Polar Lipids (Birmingham, AL). The lipids were dissolved in chloroform, sealed in ampoules under argon and stored at −20°C before use. Cell culture materials were obtained from Invitrogen (Carlsbad, CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cells and Cell Culture. Cells of HTB-9 (human bladder carcinoma) and C26 (murine colon carcinoma) were cultured as exponentially growing subconfluent monolayers on 100-mm plates (Corning Glassworks, Corning, NY) or 75-cm^2 tissue culture flasks (TPP, Trasadingen, Switzerland) in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 2 mM glutamine at 37°C, 5% CO_2 in a humidified incubator. A431 cells (human epidermoid carcinoma), SK-BR-3 cells, MCF7/WT, MCF7/ADR (human breast cancer line) and MBT2 cells (murine bladder cancer) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 2 mM glutamine.

Design and Synthesis of Peptides. Peptides were prepared with a peptide synthesizer (model 431; Applied Biosystems, Foster City, CA) and their sequences are: TAT (47–60), *N*-Cys-Tyr^{47}-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln^{60}-COOH; PEN (43–58 of the homeodomain of *D. melanogaster* antennapedia transcription factor, ANTP), *N*-Cys-Arg^{43}-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys^{58}-COOH

The control peptides of PP and PA representing the large hepatitis delta antigen from amino acid residues 198 to 210 and the peptide with Pro205 replaced by Ala as follows, were kindly provided by Dr. Chia-Huei Lee (Lee et al., 2001). Both peptides possessed an additional cysteine residue at the NH_2 termini for conjugation: PP, *N*-Cys^{198}-Ile-Leu-Phe-Ala-Asp-Pro-Pro-Phe-Ser-Pro-Gln-Ser^{210}-COOH; PA, *N*-Cys^{198}-Ile-Leu-Phe-Ala-Asp-Pro-Ala-Phe-Ser-Pro-Gln-Ser^{210}-COOH.

Labeling Peptides with Fluorescent Dyes. Aliquots of the purified peptides were first reacted with Ellman's reagents (Pierce, Rockford, IL) for sulfhydryl quantification to assess the availability of the sulfhydryl group. Briefly, 200 μl of diluted Ellman's reagent solution (0.32 mg/ml of 0.1 M sodium phosphate, pH 8.0) for each well was added to a set of test wells, each containing 20 μl of each standard or unknown, in 96-well microtiter plates. The microtiter plates were mixed and incubated at room temperature for 5 min, and then absorbance was measured at 405 nm with an MRX microplate reader (Dynex Technologies, Inc., Chantilly, VA). The values obtained from the standards were plotted to derive the standard curve and the experimental sample concentrations were determined. One milligram of TAT, PEN, PP, or PA peptides dissolved in phosphate-buffered saline (PBS) was reacted for 2 h in the dark at room temperature with two equivalents of fluorescein maleimide dissolved in dimethylformamide per sulfhydryl group of the peptide. Fluorescein-labeled peptides were purified by a G-10 column (Pharmacia, Uppsala, Sweden). These modified peptides were stored in small volume packages at −80°C in the dark until further use.

Conjugation of Peptides to PEG3400-DSPE. The synthesis of maleimido-PEG3400-DSPE has been described previously (Tseng et al., 1999). Fluorescein-labeled PEN, TAT, or control peptides (PP and PA) were coupled to maleimido-PEG3400-DSPE at a 1:3 molar ratio via the unique free thiol group of cysteine residual in the N terminus of the purified peptide. Dried lipid film containing maleimido-PEG3400-DSPE was hydrated in HEPES-buffered saline (20 mM HEPES-Na, 144 mM NaCl, pH 7.2) and added immediately to the designated peptide with gentle agitation at room temperature. After 2 h of incubation, the reaction was completed and confirmed by quantitation of the remaining sulfhydryl group with Ellman's reagent. The unreacted maleimide group was blocked through incubation with cysteine (3 times the molar ratio to maleimide residues) for 10 min.

Preparation of Liposomes. Small unilamellar vesicles (size <100 nm) were prepared by a combination of standard methods of thin-film hydration and repeated extrusion as described previously (Tseng et al., 1999). Briefly, liposomes composed of distearoyl phosphatidylcholine, cholesterol, and *N*-[ω-methoxypoly(oxyethylene)-(*R*)-carbonyl]-distearoylphosphatidylethanolamine (molar ratio 3:2:0.06) were hydrated at 55°C in ammonium sulfate solution [250 mM $(NH_4)_2SO_4$, pH 5.0, 530 mOsM) and extruded through polycarbonate membrane filters (Costar, Cambridge, MA) of 0.1- and 0.05-μm pore sizes using high-pressure extrusion equipment (Lipex Biomembranes, Vancouver, BC, Canada) at 60°C. Doxorubicin was encapsulated by a remote loading method at a concentration of 1 mg of doxorubicin per 10 μmol of phospholipid. The final concentration of liposomes was estimated with a phosphate assay. After adding 1 ml of acidic isopropanol (81 mM HCl) to 0.2 ml of diluted drug-loaded liposomes, the amount of doxorubicin trapped inside the liposomes was determined with a spectrofluorometer (Hitachi F-4500; Hitachi, Ltd., Tokyo, Japan) using 470 nm as the excitation wavelength and 582 nm as the emission wavelength. Vesicle sizes were measured by dynamic laser scattering with a submicron particle analyzer (model N4+; Beckman Coulter, Fullerton, CA). The prepared liposomes contained 110 to 130 μg of doxorubicin per micromole of phospholipid and the particle sizes ranged from 65 to 75 nm in diameter. For encapsulation of HPTS, small unilamellar vesicles were prepared by reverse-phase evaporation. Egg phosphatidylcholine and cholesterol in a molar ratio of 2:1 were extruded repeatedly through polycarbonate membrane filters of pore sizes of 0.1 and 0.05 μm sequentially. A solution of liposomes encapsulating 30 mM HPTS was prepared in distilled water (pH 6.0, adjusted to about 300 mOsM with 1 M NaCl).

Preparation of Peptidyl Liposomes Containing HPTS or Doxorubicin. Peptidyl-PEG3400-DSPE could be transferred to preformed liposomes after coincubation at temperatures above the transition temperature of the lipid bilayer (Zalipsky et al., 1997; Ishida et al., 1999; Iden and Allen, 2001). To prepare liposomes conjugated with various numbers of peptides, the initial ratio of peptide-PEG3400-DSPE to liposome was varied. Based on a liposome size of 65 nm and an average area of 75 Å^2 per phospholipid molecule, there were 35,000 phospholipid molecules per vesicle (Kirpotin et al., 1997). Aliquots of the peptide-PEG3400-DSPE micelles were incubated for 1 h with preformed liposomes at 37°C for liposome encap-

TABLE 1

Uptake of fluorescein-conjugated peptides and peptidyl liposomes encapsulating HPTS in various cancer cell lines

Values of fluorescence Intensity were obtained from histogram statistics of cell quest software. The data are representative of three similar experiments.

Treatment	Human								Mouse			
	A431		HTB9		MCF7/ADR		SK-BR-3		C26		MBT2	
Groups	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Control	3.0	3.0	4.3	6.2	4.6	7.3	4.0	5.5	3.3	2.1	3.3	4.1
TAT-F	260.0	191.3	100.7	77.6	125.6	106.2	45.3	55.9	45.1	36.2	49.8	64.1
PEN-F	62.0	141.7	30.2	51.2	N.D.		33.2	82.5	16.7	15.5	42.2	125.0
TAT-L-HPTS	567.1	317.5	328.8	363.7	1758.8	1517.1	N.D.		594.7	487.1	N.D.	
PEN-L-HPTS	21.1	19.4	27.7	48.2	N.D.		N.D.		N.D.		N.D.	

sulating HPTS (L-HPTS) and at 60°C for liposomal doxorubicin (LD) followed by chromatography on Sepharose CL-4B columns (Pharmacia) and elution with 0.9% sodium chloride. Fractions of liposome and free doxorubicin (FD) or HPTS were collected. The amount of doxorubicin or HPTS still trapped inside or leaked outside the liposomes was determined with a spectrofluorometer as described above. This insertion method did not appreciably influence the integrality of the liposomes and there was no detectable leakage of entrapped HPTS and less than 5% leakage of doxorubicin. For determination of insertion efficiency, FITC-labeled peptide-PEG3400-DSPE was used in some preparations. The ratio of engrafted FITC-peptide-PEG3400-DSPE in the liposome fractions was assayed by gel electrophoresis. The same amounts of liposomes present before and after passing Sepharose CL-4B columns were suspended in a sample buffer (0.12 M Tris, pH 6.8, 2% SDS, 20% glycerol, and 10% β-mercaptoethanol) and then separated with 17.5% SDS-PAGE and viewed with UV light. The efficiency of insertion was found to be greater than 90% in each preparation.

Fluorescence Microscopy. Exponentially growing cells were dispersed with nonenzymatic cell dissociation medium (5 mM EDTA in PBS) or trypsin (Invitrogen). 5×10^5 cells were plated on 60-mm plates (Corning Glassworks) and cultured overnight. Cells were washed with PBS, pH 7.4, and then treated with fluorescein-labeled peptide or a peptide-conjugated liposome at concentrations indicated in Fig. 6 and incubation time in a culture medium. Treatment was terminated by washing with ice-cold PBS three times followed by

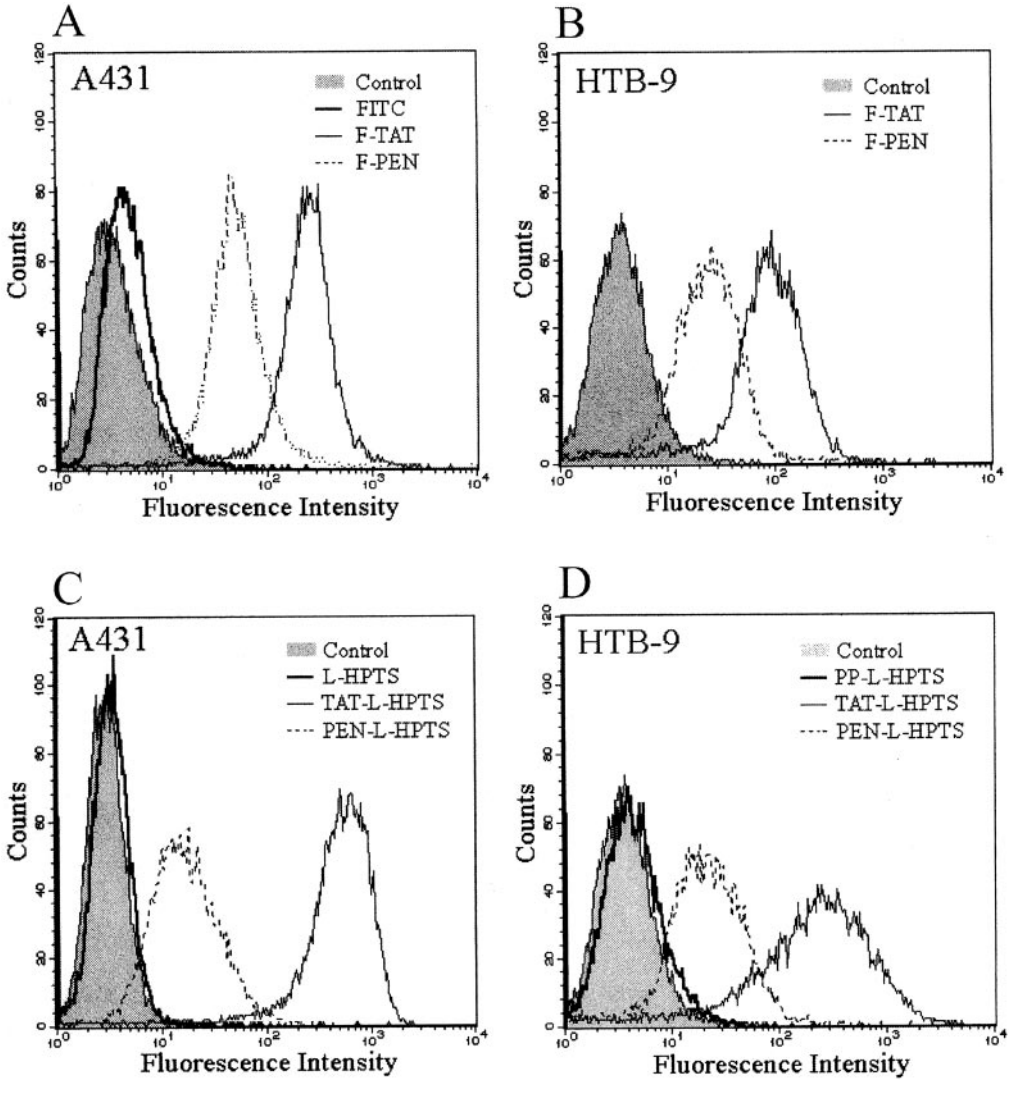


Fig. 1. Uptake of fluorescein labeled cell-penetrating peptides and peptide-conjugated liposomes in various cancer cells. Uptake of TAT-F, PEN-F, TAT-L-HPTS, and PEN-L-HPTS into A431 cells (A, C) and HTB-9 cells (B, D) as determined by flow cytometry. Cells (5×10^5) were incubated with 2 μg of peptide or 20 nmol of phospholipid of liposome in a 1-ml medium at 37°C for 3 h. The background control included cells treated with FITC, L-HPTS, PP-L-HPTS, or PA-L-HPTS. Each peptide-conjugated liposome contained approximately 100 peptides per vesicle. Histograms were obtained from a single experiment and were representative of three similar experiments.

fixation in 4% paraformaldehyde in PBS. After extensive washing, plates were examined under a Leica DM IRB inverted microscope with a 40× objective lens.

Flow Cytometry. To analyze fluorescein-5-maleimide labeled peptides or peptide-conjugated liposome uptake by flow cytometry, cells were washed four times with PBS, trypsinized, again washed with PBS, and analyzed with a flow cytometer (Becton Dickinson, San Jose, CA). A total of 10,000 events per sample were analyzed.

Assays of Cellular Liposome Uptake. Approximately 1×10^6 cells were incubated with FD, plain LD, or peptide-conjugated LD in 1 ml of growth medium containing 1 or 10 μg doxorubicin for the indicated periods. Extraction of doxorubicin from PBS-washed cells was quantified by fluorometry as described above. The amounts of protein were determined by Bio-Rad protein assay.

Cytotoxicity of TAT-Conjugated LD. Cytotoxicity of TAT peptide-conjugated LD was determined with MTT assay. Briefly, 5×10^3 cells were seeded into each well of 96-well microtiter plates. After treatment, 20 μl of MTT stock solution (5 mg/ml) per 200 μl of medium was added into each well. After further incubation at 37°C for 4 h, the medium was carefully removed and any remaining formazan was dissolved in 200 μl of DMSO. Then, 25 μl of Sorensen's glycine buffer was added before measuring the absorbance at 570 nm by enzyme-linked immunosorbent assay.

Animal Tumor Model and Therapeutic Studies. Male BALB/c mice (17–20 g, 6–8 weeks old) were purchased from the Animal Center at the College of Medicine, National Taiwan University (Taipei, Taiwan). C26 cells (2×10^5) were inoculated subcutaneously in the back near the right hind limb. Therapeutic experiments started 7 days after tumor implantation, when the tumor was established as a palpable mass (about 100-mm^3). Animals (groups of 10) were treated with lipid control, LD, or TAT-LD at a dose of 6 mg/kg of doxorubicin through the tail vein on days 7 and 14 after tumor implantation. The tumor size was measured and the survival time of each mouse was recorded. Tumor volume was determined by measuring orthogonal diameters of the tumor and calculated as $0.5 \times (a^2 \times b)$, where a was the smaller of two perpendicular diameters. Animal weights were also recorded at the time of tumor volume measurements as an indicator of drug toxicity. Efficacy of the treatment was assessed by the delay in tumor growth compared with the control group. The animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council). The mice were sacrificed when the diameter of the tumor was greater than 2.5 cm, before the mice became too weak.

Results

Translocation Ability of PEN and TAT Peptide in Various Cancer Cell Lines. First, we explored the ability of PEN-F and TAT to transduce across the plasma membrane of a panel of cancer cells. Various cell lines, including bladder cancer (HTB-9, MBT2), breast cancer (SK-BR-3, MCF7/WT, and MCF7/ADR), squamous carcinoma (A431), and colon cancer (C26) were incubated with 2 μg/ml fluorescein-conjugated peptides for 3 h. Intracellular uptake of TAT and PEN could be identified easily in all tested cell lines under fluo-

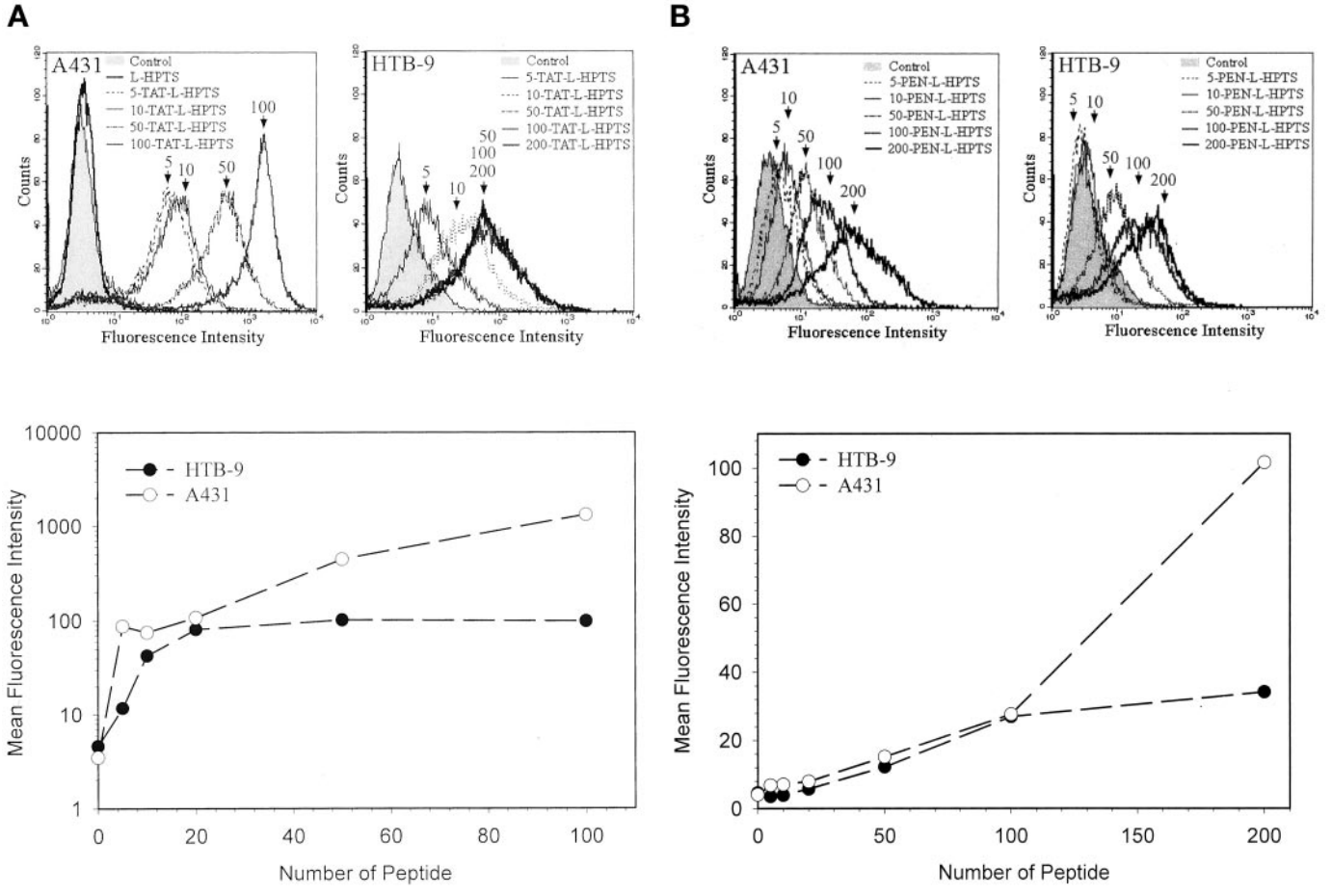
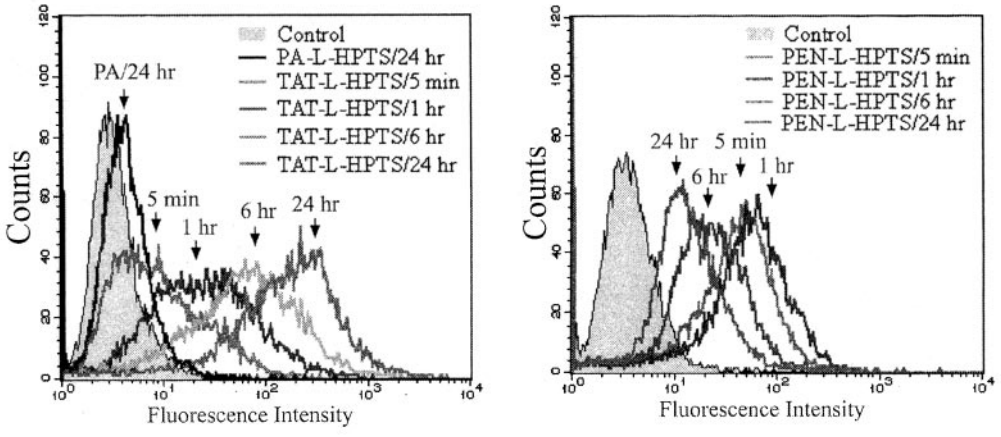


Fig. 2. Effect of peptide number on uptake efficiency of liposome. A431 and HTB-9 cells (5×10^5) were incubated with 20 nmol of phospholipid of L-HPTS conjugated with various numbers of TAT (A) or PEN (B) for 2 h at 37°C before being analyzed with flow cytometry.

rescence microscopy, but the uptake of PA and PP was indiscernible (data not shown). The uptake of peptides was quantitated with flow cytometry (Table 1 and Fig. 1, A and B). Under the same concentration, more TAT accumulated within the cells than PEN, although there was some degree of variation in this preference among the cell lines studied.

Liposomes encapsulating HPTS attached to around 100 peptides were used to evaluate the ability of peptides to translocate liposomes across the bilayer. Compared with the untreated control, the fluorescence intensity of cells was not increased with the use of PA- or PP-liposomes at a concentration of 20 nmol of phospholipid of liposome and incubation of 3 h (Table 1 and Fig. 1, C and D). In contrast, both PEN and TAT markedly increased the uptake of liposomes into



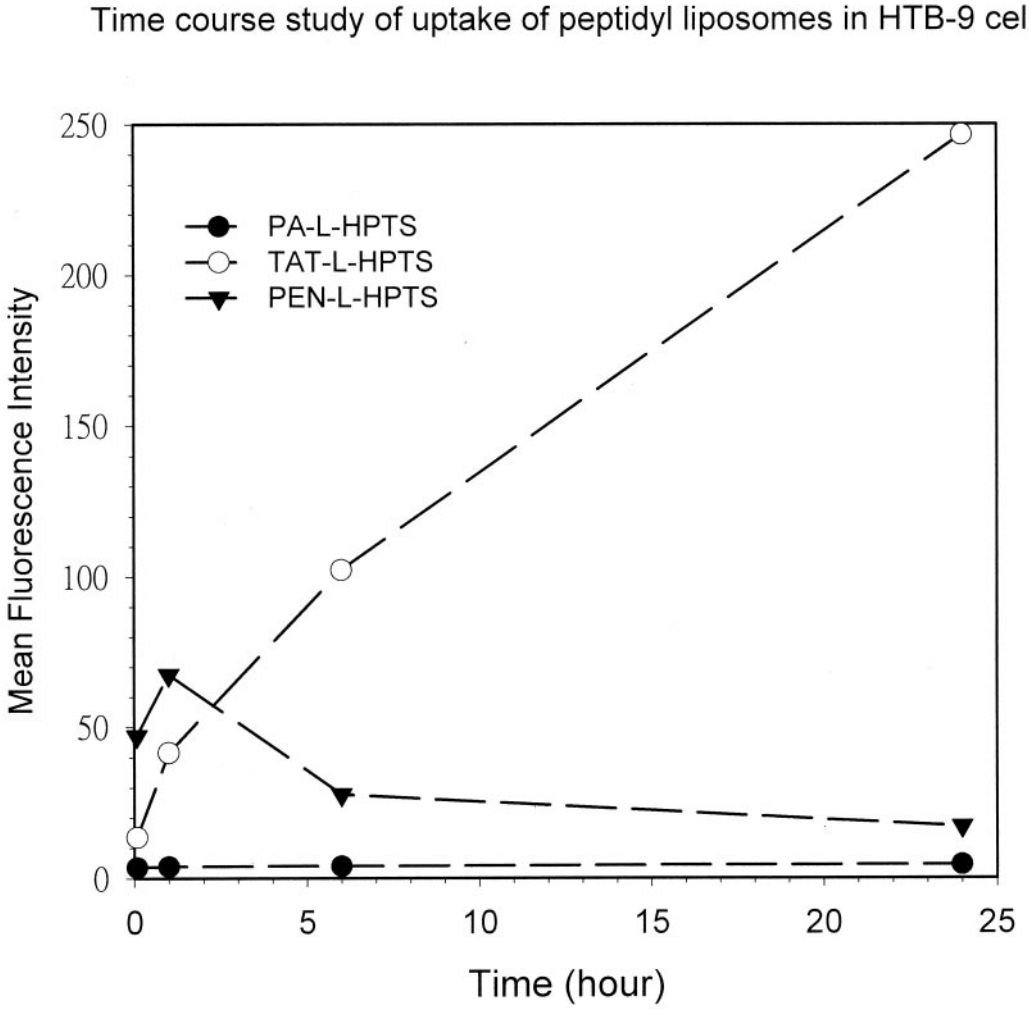


Fig. 3. The relationship between incubation time and uptake efficiency. A431 and HTB-9 cells (5×10^5) were incubated with 20 nmol of phospholipid of L-HPTS conjugated with around 100 TAT or PEN peptides per vesicle for the indicated periods at 37°C before being analyzed with flow cytometry.

TABLE 2
Uptake of FD, LD, and peptidyl-LD in C26, MCF7/ADR, and A431 cells
Values of fluorescence Intensity were obtained from histogram statistics of cell quest software. The data shown are representative of three similar experiments.

Treatment	A431		MCF7/ADR		C26	
Groups	Mean	S.D.	Mean	S.D.	Mean	S.D.
Control	3.5	14.6	3.9	2.8	3.4	3.0
FD	72.6	57.7	23.7	24.4	62.4	33.9
LD	20.7	14.4	7.4	17.1	12.8	6.8
TAT-LD	40.3	22.5	36.8	20.4	36.9	15.5
PEN-LD	45.4	26.8	36.4	20.6	35.1	17.1

PEN-LD, PEN-conjugated liposomal doxorubicin.

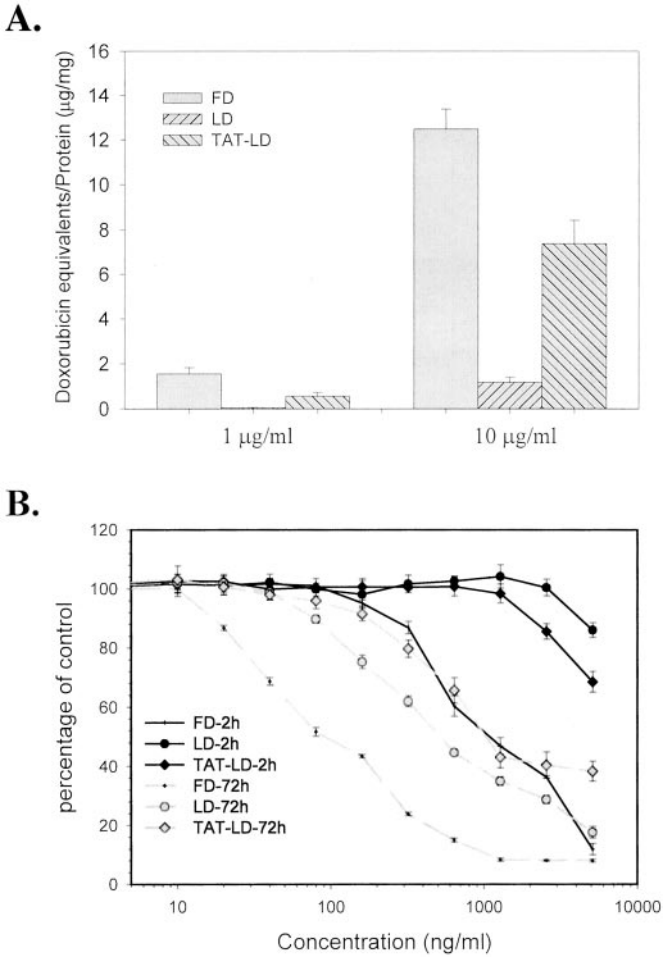


Fig. 4. The relationship between doxorubicin uptake and cytotoxicity. A, TAT-mediated uptake of liposomal doxorubicin. A431 cells were treated with 1 or 10 μg of FD, plain LD, or TAT-LD at 37°C for 2 h. The amount of cellular associated doxorubicin was then extracted and quantitated with a spectrofluorometer using 470 nm as the excitation wavelength and 582 nm as the emission wavelength. B, comparison of antiproliferative effect. A431 cells (5×10^3) in 96-well microtiter plates were treated with FD, plain LD, or TAT-LD for 2 or 72 h. The relative cell growth was determined by MTT assay after 3 days of incubation. Each value is the mean ± S.D. of five determinations.

A431 and HTB-9 cells. Similar to the findings in the studies of labeled peptides alone, TAT was more effective than PEN in transducing ability.

The Relationship between Peptide Number on the Liposomal Surface and Uptake Efficiency. To explore the relationship between peptide density and the efficiency of cellular uptake, a series of PEN- or TAT-coupled liposomes was prepared with the insertion method described above. A431 or HTB-9 cells were incubated with HPTS-loaded liposomes grafted with 5 to 200 TAT or PEN peptides per liposome for 2 h. Increasing the peptide number inserted on the liposome surface enhanced the cellular uptake of liposomes (Fig. 2). A431 cells were more susceptible to intracellular delivery of peptide liposomes. For TAT, a number of as few as five was sufficient to induce significant translocation of liposomes into the cells (Fig. 2A) and the efficiency seemed to be much higher in A431 than in HTB-9 cells. For HTB-9 cells, the plateau was reached at around 50 peptides, but for A431 cells, plateau might not have been reached at 100 peptides. In contrast to the TAT liposome, there was no noteworthy difference between A431 and HTB-9 in the uptake of PEN-coupled liposomes (Fig. 2B). One hundred peptide molecules per vesicle could achieve efficient delivery of liposomes into various cancer cells and this number was chosen for subsequent studies.

Cellular Transport Kinetics of TAT- and PEN-Liposomes. Kinetics of uptake of TAT-L-HPTS and PEN-L-HPTS was studied in HTB-9 cells. TAT-L-HPTS accumulated within cells in a time-dependent manner and the plateau of uptake was not reached after several hours. In contrast, accumulation of PEN-L-HPTS peaked at 5 min to 1 h and then the fluorescence intensity fell gradually after 1 h (Fig. 3). Cell viability after 24 h of incubation was still good as assessed by phase-contrast microscopy and trypan-blue dye assay. There was no increase in PA-L-HPTS uptake up to 24 h of incubation (Fig. 3).

Drug Accumulation and Cytotoxicity of Plain and TAT-Liposomal Doxorubicin in A431 Cells. After 2 h of incubation of 1 μg/ml doxorubicin equivalents, the doxorubicin uptake of cells treated with plain LD was only one thirty-fourth (2.94%) of that treated with free drug (0.05 and 1.56 μg/mg protein, respectively). TAT increased the amount of cellular associated doxorubicin by 12-fold (0.56 μg/mg protein; Fig. 4A). With a dosage of 10 μg/ml doxorubicin equivalents and after being incubated for 2 h, the difference became smaller but the trend was the same (Fig. 4A). Similar results were obtained in C26 and MCF7/ADR cells by measuring doxorubicin uptake with flow cytometry (Table 2).

The effect of CPP on cytotoxicity was also assessed. A431 cells were exposed to doxorubicin preparations for 2, 24, or 72 h and the cell numbers were assessed with MTT assay after an incubation of 3 days (Fig. 4B). Plain LD was minimally cytotoxic with a 2-hour exposure. Although TAT markedly increased the uptake of LD and the amount of doxorubicin delivered was near that of FD, the cytotoxicity was still similar to plain LD. After an exposure of 72 h, the IC_{50} of FD was around 100 ng/ml and the IC_{50} of plain or TAT-coupled LD was around 1 log higher than that of FD.

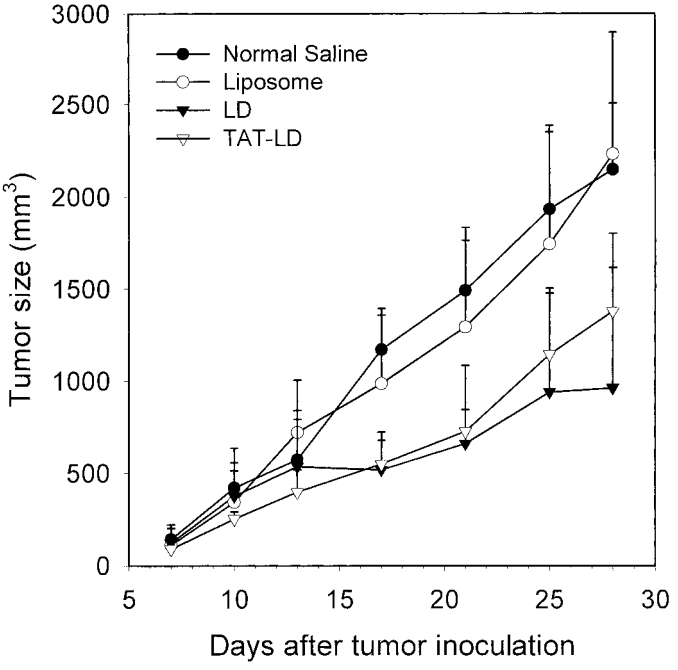


Fig. 5. The C26 tumor growth curves in BALB/c mice after treatment. Cells (2×10^5) were inoculated subcutaneously in the back near the right hind limb. Mice (groups of 10) were treated with saline control, liposome, LD, and TAT-LD at a dose of 6 mg/kg of doxorubicin or an equivalent amount of liposome through the tail vein on days 7 and 14 after tumor implantation.

In vivo study with a C26 syngeneic BALB/c tumor model was also performed. Groups treated with either LD or TAT-LD did not show differences in body weight change (data not shown) or tumor size (Fig. 5).

Localization of TAT-Coupled Liposomal Doxorubicin. Cellular internalization and localization of FD, LD, and TAT-LD in A431 cells was compared directly under fluorescence microscopy (Fig. 6). Free drug readily entered the cell and concentrated in the nucleus (Fig. 6B). In contrast, almost no plain LD could be detected in the cells (Fig. 6D). TAT-LD, compared with plain LD, had increased intracellular doxorubicin, but it was localized in the cytoplasm, preferentially in the perinuclear region (Fig. 6F). There was no discernible nuclear stain of doxorubicin as was observed in the experiment of FD.

Discussion

The leaky microvasculature of tumors combined with the pharmacokinetic properties of a small distribution volume and a long circulation half-life lead to preferential accumulation and retention of liposomal drugs in tumor tissue compared with those in normal tissue (i.e., the beneficial enhanced permeability and retention effect). To have adequate circulation time, liposomal vesicles should be inert and hold the drug tightly.

Once in the tumors, liposomes are localized in the interstitium surrounding the tumor cells (Huang et al., 1992; Yuan et al., 1994) and not seen within tumor cells. To have a better therapeutic effect, the release of the drug from the liposomes should be as complete as possible. However, the accumulated liposomal drug at the tumor sites seems to release slowly because of the stability of the second-generation liposomal system. Cytotoxicity assays in vitro indicate that the IC_{50} decreases by 1 order of magnitude and just counteracts the benefit of drug accumulation. In addition, an extracellularly released drug still cannot bypass the pumping-out activity of multiple drug-resistant transporters. This may partly explain why sterically stabilized liposomal doxorubicin shows little activity in solid tumors known to be refractory to most free drugs. Obviously, active translocation of liposome vesicles into cancer cells is needed to improve the efficacy of a stable liposome delivery system. Ligand-directed endocytosis, conferring some degree of specificity, is the usual process of liposome particle uptake by cells (Ahmad and Allen, 1992; Sarti et al., 1996; Gabizon et al., 1999; Maruyama et al., 1999; Tseng et al., 1999; Cerletti et al., 2000; Eliaz and Szoka, Jr., 2001). However, the ligand-receptor approach depends on the differential expression of receptors between normal and tumor cells to confer selectivity and usually has a limited spectrum. In addition, commonly existing circulating receptors may compete with cell surface receptors for binding to the ligands.

CPPs may enhance the intracellular delivery of second-generation liposomal drugs, given that passive tumor selectivity is maintained. In this study, although we could not subtract the surface-binding portion because of the inherent methodological limitations of flow cytometry, we still demonstrated that two CPPs, TAT and PEN, could translocate liposomes into cells efficiently. The penetrating abilities of CPPs and CPP-coupled liposomes varied and were cell-type dependent. The mechanism of penetration of each CPP, although not thoroughly understood, is probably different. Based on nuclear magnetic resonance studies, penetratin

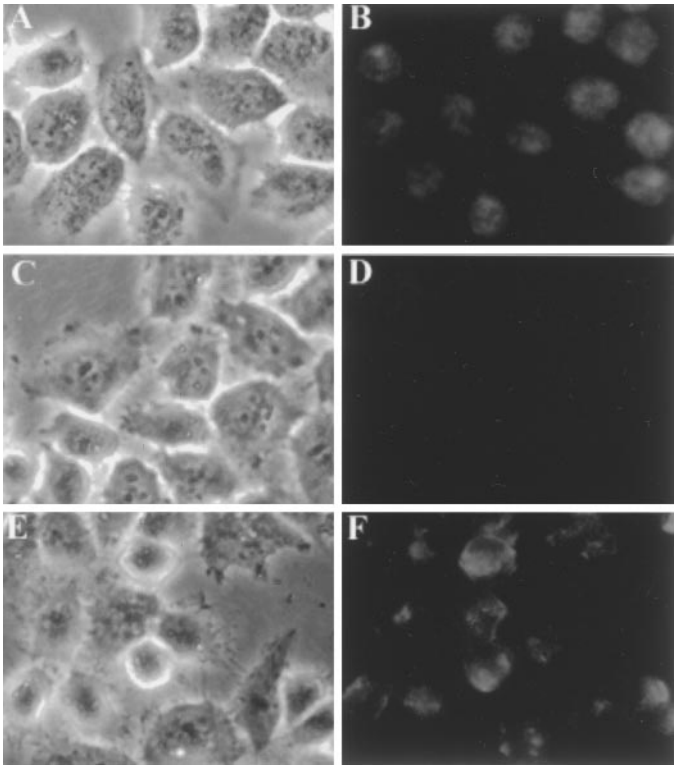


Fig. 6. Bright field (left) and fluorescence (right) microscopy of A431 cells treated with 10 μg/ml doxorubicin of free (A, B), plain liposomal (C, D), or TAT-liposomal (E, F) doxorubicin at 37°C for 3 h. Doxorubicin fluorescence was observed with a rhodamine cube filter.

binds negatively charged phospholipids or glycolipids and induces the formation of an inverted micelle, which should accommodate the peptide and its cargo in its hydrophilic cavity and release its contents into the cytoplasm (Derossi et al., 1996). This cell-penetration model has been suggested to apply to TAT peptides, although without strong experimental evidence (Vives et al., 1997). However, TAT uptake can be partially inhibited by lowering the temperature. Moreover, chloroquine, a lysosomotropic agent, protects TAT from degradation and in some cells stimulates its uptake, suggesting that internalization of TAT can occur by endocytosis (Frankel and Pabo, 1988). By contrast, some cells show very little temperature dependence for TAT uptake (Mann and Frankel, 1991), which suggests the existence of different and, perhaps, competing uptake mechanisms.

With good cell viability after treatment with PEN-L, the possibility of selection bias to make the difference in the uptake kinetics between PEN- and TAT-L-HPTS was excluded. The rapid decline after the initial peak of PEN-L-HPTS might be explained by a difference in the characteristics of peptides. In addition to the penetration pathway, TAT-F and TAT-L might be kept within cells through an endocytotic pathway or by other intracellular interactions after penetration. In contrast to TAT, PEN-F and PEN-L bound to the intercellular matrix extensively and displayed intense background staining under the fluorescence microscope. Without retaining mechanisms to keep the translocated liposomes, the equilibrium might favor extracellular relocation and the distribution would alter gradually. Although the in vitro data suggested that TAT-L might have advantages over PEN-L in intracellular drug delivery, this has to be validated, because the cellular microenvironment is quite different in vivo.

Although CPPs, especially TAT, markedly improved the intracellular delivery of liposomal drugs, the results of in vitro cytotoxicity assays (Fig. 4B), and animal tumor model studies (Fig. 5) could not demonstrate the superiority of CPP-coupled liposomes over plain liposomes. The image study with fluorescence microscopy (Fig. 6) revealed that this approach was hampered by the very limited release of the free drug into the cytoplasm and nucleus. Besides, in vivo, CPPs might increase nonspecific interaction with cells of nontumor tissues. The advantageous enhanced permeability and retention effect of the long-circulating liposomal drugs probably would be decreased by CPPs.

The molecular mechanisms of the binding and internalization of liposomes are not fully understood. However, it is generally accepted that the majority of liposomes enter cells through endocytotic pathway. The possibility of endocytic uptake of liposome is supported by direct electron microscopic analysis (Straubinger et al., 1983; Park et al., 1995; Sarti et al., 1996; Ishida et al., 2001). A pH-sensitive dye, HPTS, has been used to confirm and characterize liposomes encountering a low-pH environment in the lysosome (Daleke et al., 1990; Yoshimura et al., 1995; Tseng et al., 1999). Doxorubicin, which is commonly loaded into liposomes in its membrane-impermeable (cationic) form using an acidic buffer, displays endocytosis-triggered unloading. When the extraliposomal [H^+] increases 250-fold from pH 7.4 outside the cell to pH 5 inside the endosome, the ratio of doxorubicin inside to outside the liposome must decrease by a factor of 250. The collapse of the transliposomal pH gradient indirectly drives an efflux of the drug molecule from the liposome (Lee et al., 1998).

Ligand-mediated liposomal targeting systems use receptor-mediated endocytosis and achieve higher therapeutic activity than plain liposomal drugs or even free drugs in some systems (Ahmad et al., 1993; Park et al., 1997; Tseng et al., 1999). In addition to enhanced endocytosis, ligand-mediated targeting may improve the drug distribution in tumors. Histologic studies using colloidal-gold labeled HER2 immunoliposomes demonstrated efficient intracellular delivery in tumor cells, whereas nontargeted liposomes accumulated within stroma. In the MCF7 xenograft model lacking HER2-overexpression, no difference in tumor cell uptake was seen, with both immunoliposomes and nontargeted liposomes accumulating within stroma (Park et al., 2001). Besides, ligand-mediated endocytic pathways may have the advantage of bypassing multiple drug resistance mechanisms. Folate receptor-mediated cell uptake of targeted liposomal doxorubicin into a multidrug-resistant subline is unaffected by P-glycoprotein-mediated drug efflux, in sharp contrast to uptake of the FD (Goren et al., 2000).

For liposomal drugs, intracellular translocation is only the first step of intracellular molecular targeting. Because the endocytotic pathway is bypassed, CPP-coupled liposomes have low unloading efficiency after internalization into cells. Strategies that can enable rapid release of contents from tissue-accumulated or cellular-internalized liposomes for molecular targeting must be developed. In combination with fusogenic peptide (Bongartz et al., 1994) or releasing agents to trigger intracellular drug release, CPP may still be able to improve the antitumor activity of liposomal drugs. In addition, many antibodies and their derivative immunoliposomes cannot be internalized (Goren et al., 1996). CPPs, possibly in combination with fusogenic peptides (Cho et al., 2001; Hu et al., 2001), might be able to increase the therapeutic value of these immunoliposomes (Maier et al., 1991; Di Lazzaro et al., 1994). Although not successful for drug delivery in terms of cytotoxicity, CPPs could be helpful in liposomal delivery of oligonucleotides or DNA (Bongartz et al., 1994), in which intracellular release may not be a key issue.

References

Ahmad I and Allen TM (1992) Antibody-mediated specific binding and cytotoxicity of liposome- entrapped doxorubicin to lung cancer cells in vitro. *Cancer Res* **52:**4817–4820.

Ahmad I, Longenecker M, Samuel J, and Allen TM (1993) Antibody-targeted delivery of doxorubicin entrapped in sterically stabilized liposomes can eradicate lung cancer in mice. *Cancer Res* **53:**1484–1488.

Allen TM and Hansen C (1991) Pharmacokinetics of stealth versus conventional liposomes: effect of dose. *Biochim Biophys Acta* **1068:**133–141.

Bongartz JP, Aubertin AM, Milhaud PG, and Lebleu B (1994) Improved biological activity of antisense oligonucleotides conjugated to a fusogenic peptide. *Nucleic Acids Res* **22:**4681–4688.

Cerletti A, Drewe J, Fricker G, Eberle AN, and Huwyler J (2000) Endocytosis and transcytosis of an immunoliposome-based brain drug delivery system. *J Drug Target* **8:**435–446.

Chidiac T, Budd GT, Pelley R, Sandstrom K, McLain D, Elson P, Crownover R, Marks K, Muschler G, Joyce M, et al. (2000) Phase II trial of liposomal doxorubicin (Doxil) in advanced soft tissue sarcomas. *Investig New Drugs* **18:**253–259.

Cho JE, Kim HS, Ahn WS, and Park YS (2001) Enhanced cytotoxicity of doxorubicin encapsulated in liposomes with reconstituted sendai F-proteins. *J Microencapsul* **18:**421–431.

Connor J and Huang L (1985) Efficient cytoplasmic delivery of a fluorescent dye by pH-sensitive immunoliposomes. *J Cell Biol* **101:**582–589.

Daleke DL, Hong K, and Papahadjopoulos D (1990) Endocytosis of liposomes by macrophages: binding, acidification and leakage of liposomes monitored by a new fluorescence assay. *Biochim Biophys Acta* **1024:**352–366.

Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G, and Prochiantz A (1996) Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J Biol Chem* **271:**18188–18193.

Derossi D, Joliot AH, Chassaing G, and Prochiantz A (1994) The third helix of the

Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* **269:**10444–10450.
Di Lazzaro C, Digiesi G, Tecce R, Lotti LV, Torrisi MR, and Natali PG (1994) Immunotoxins to the HER-2 oncogene product: functional and ultrastructural analysis of their cytotoxic activity. *Cancer Immunol Immunother* **39:**318–324.
Eliaz RE and Szoka FC Jr (2001) Liposome-encapsulated doxorubicin targeted to CD44: a strategy to kill CD44-overexpressing tumor cells. *Cancer Res* **61:**2592–2601.
Fawell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B, and Barsoum J (1994) Tat-mediated delivery of heterologous proteins into cells. *Proc Natl Acad Sci USA* **91:**664–668.
Frankel AD and Pabo CO (1988) Cellular uptake of the Tat protein from human immunodeficiency virus. *Cell* **55:**1189–1193.
Gabizon A, Horowitz AT, Goren D, Tzemach D, Mandelbaum-Shavit F, Qazen MM, and Zalipsky S (1999) Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: in vitro studies. *Bioconjug Chem* **10:**289–298.
Gabizon A and Papahadjopoulos D (1988) Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc Natl Acad Sci USA* **85:**6949–6953.
Goren D, Horowitz AT, Tzemach D, Tarshish M, Zalipsky S, and Gabizon A (2000) Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump. *Clin Cancer Res* **6:**1949–1957.
Goren D, Horowitz AT, Zalipsky S, Woodle MC, Yarden Y, and Gabizon A (1996) Targeting of stealth liposomes to ErbB-2 (Her/2) receptor: in vitro and in vivo studies. *Br J Cancer* **74:**1749–1756.
Halm U, Etzrodt G, Schiefke I, Schmidt F, Witzigmann H, Mossner J, and Berr F (2000) A Phase II study of pegylated liposomal doxorubicin for treatment of advanced hepatocellular carcinoma. *Ann Oncol* **11:**pp 113–114.
Hong RL, Huang CJ, Tseng YL, Pang VF, Chen ST, Liu JJ, and Chang FH (1999) Direct comparison of liposomal doxorubicin with or without polyethylene glycol coating in C-26 tumor-bearing mice: is surface coating with polyethylene glycol beneficial? *Clin Cancer Res* **5:**3645–3652.
Hong RL and Tseng YL (2001) Phase I and pharmacokinetic study of a stable, polyethylene-glycolated liposomal doxorubicin in patients with solid tumors: the relation between pharmacokinetic property and toxicity. *Cancer* **91:**1826–1833.
Hu Q, Shew CR, Bally MB, and Madden TD (2001) Programmable fusogenic vesicles for intracellular delivery of antisense oligodeoxynucleotides: enhanced cellular uptake and biological effects. *Biochim Biophys Acta* **1514:**1–13.
Huang A, Kennel SJ, and Huang L (1983) Interactions of immunoliposomes with target cells. *J Biol Chem* **258:**14034–14040.
Huang SK, Lee KD, Hong K, Friend DS, and Papahadjopoulos D (1992) Microscopic localization of sterically stabilized liposomes in colon carcinoma-bearing mice. *Cancer Res* **52:**5135–5143.
Iden DL and Allen TM (2001) In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach. *Biochim Biophys Acta* **1513:**207–216.
Ishida O, Maruyama K, Tanahashi H, Iwatsuru M, Sasaki K, Eriguchi M, and Yanagie H (2001) Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors in vivo. *Pharm Res (NY)* **18:**1042–1048.
Ishida T, Iden DL, and Allen TM (1999) A combinatorial approach to producing sterically stabilized (stealth) immunoliposomal drugs. *FEBS Lett* **460:**129–133.
Kirpotin D, Park JW, Hong K, Zalipsky S, Li WL, Carter P, Benz CC, and Papahadjopoulos D (1997) Sterically stabilized anti-Her2 immunoliposomes: design and targeting to human breast cancer cells in vitro. *Biochemistry* **36:**66–75.
Koukourakis MI, Koukouraki S, Giatromanolaki A, Archimandritis SC, Skarlatos J, Beroukas K, Bizakis JG, Retalis G, Karkavitsas N, and Helidonis ES (1999) Liposomal doxorubicin and conventionally fractionated radiotherapy in the treatment of locally advanced non-small-cell lung cancer and head and neck cancer. *J Clin Oncol* **17:**512–3521.
Lee CH, Chang SC, Wu CH, and Chang MF (2001) A novel chromosome region maintenance 1-independent nuclear export signal of the large form of hepatitis delta antigen that is required for the viral assembly. *J Biol Chem* **276:**8142–8148.
Lee RJ, Wang S, Turk MJ, and Low PS (1998) The effects of pH and intraliposomal buffer strength on the rate of liposome content release and intracellular drug delivery. *Biosci Rep* **18:**69–78.
Lewin M, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT, and Weissleder R (2000) Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. *Nat Biotechnol* **18:**410–414.
Maier LA, Xu FJ, Hester S, Boyer CM, McKenzie S, Bruskin AM, Argon Y, and Bast RC Jr (1991) Requirements for the internalization of a murine monoclonal antibody directed against the HER-2/Neu gene product C-ErbB-2. *Cancer Res* **51:**5361–5369.
Mann DA and Frankel AD (1991) Endocytosis and targeting of exogenous HIV-1 Tat protein. *EMBO (Eur Mol Biol Organ) J* **10:**1733–1739.
Maruyama K, Ishida O, Takizawa T, and Moribe K (1999) Possibility of active targeting to tumor tissues with liposomes. *Adv Drug Delivery Rev* **40:**89–102.
Ng K, Zhao L, Liu Y, and Mahapatro M (2000) The effects of polyethyleneglycol (PEG)-derived lipid on the activity of target-sensitive immunoliposome. *Int J Pharm (Amst)* **193:**157–166.
Papahadjopoulos D, Allen TM, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee KD, Woodle MC, Lasic DD, and Redemann C (1991) Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc Natl Acad Sci USA* **88:**11460–11464.
Park JW, Hong K, Carter P, Asgari H, Guo LY, Keller GA, Wirth C, Shalaby R, Kotts C, and Wood WI (1995) Development of anti-P185HER2 immunoliposomes for cancer therapy. *Proc Natl Acad Sci USA* **92:**1327–1331.
Park JW, Hong K, Kirpotin DB, Meyer O, Papahadjopoulos D, and Benz CC (1997) Anti-HER2 immunoliposomes for targeted therapy of human tumors. *Cancer Lett* **118:**153–160.
Park JW, Kirpotin DB, Hong K, Shalaby R, Shao Y, Nielsen UB, Marks JD, Papahadjopoulos D, and Benz CC (2001) Tumor targeting using Anti-Her2 immunoliposomes. *J Control Release* **74:**95–113.
Parr MJ, Masin D, Cullis PR, and Bally MB (1997) Accumulation of liposomal lipid and encapsulated doxorubicin in murine Lewis lung carcinoma: the lack of beneficial effects by coating liposomes with poly(ethylene glycol). *J Pharmacol Exp Ther* **280:**1319–1327.
Sarti P, Ginobbi P, D'Agostino I, Arancia G, Lendaro E, Molinari A, Ippoliti R, and Citro G (1996) Liposomal targeting of leukaemia HL60 cells induced by transferrin- receptor endocytosis. *Biotechnol Appl Biochem* **24:**269–276.
Straubinger RM, Hong K, Friend DS, and Papahadjopoulos D (1983) Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low ph compartment after internalization in coated vesicles. *Cell* **32:**1069–1079.
Torchilin VP, Rammohan R, Weissig V, and Levchenko TS (2001) TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *Proc Natl Acad Sci USA* **98:**8786–8791.
Tseng YL, Hong RL, Tao MH, and Chang FH (1999) Sterically stabilized anti-idiotype immunoliposomes improve the therapeutic efficacy of doxorubicin in a murine B-cell lymphoma model. *Int J Cancer* **80:**723–730.
Uziely B, Jeffers S, Isacson R, Kutsch K, Wei-Tsao D, Yehoshua Z, Libson E, Muggia FM, and Gabizon A (1995) Liposomal doxorubicin: antitumor activity and unique toxicities during two complementary phase I studies. *J Clin Oncol* **13:**1777–1785.
Vives E, Brodin P, and Lebleu B (1997) A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* **272:**16010–16017.
Yoshimura T, Shono M, Imai K, and Hong K (1995) Kinetic analysis of endocytosis and intracellular fate of liposomes in single macrophages. *J Biochem (Tokyo)* **117:**34–41.
Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D, and Jain RK (1994) Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res* **54:**3352–3356.
Zalipsky S, Mullah N, Harding JA, Gittelman J, Guo L, and DeFrees SA (1997) Poly(ethylene glycol)-grafted liposomes with oligopeptide or oligosaccharide ligands appended to the termini of the polymer chains. *Bioconjug Chem* **8:**111–118.

Address correspondence to: Ruey-Long Hong, M.D., Ph.D., Department of Oncology, National Taiwan University Hospital, National Taiwan University, 7 Chung-Shan South Road, Taipei, 10016, Taiwan. E-mail rlhong@ha.mc.ntu.edu.tw