Antiangiogenic Targeting Liposomes Increase Therapeutic Efficacy for Solid Tumors

It is known that solid tumors recruit new blood vessels to support tumor growth, but the molecular diversity of receptors in tumor angiogenic vessels might also be used clinically to develop better targeted therapy. *In vivo* phage display was used to identify peptides that specifically target tumor blood vessels. Several novel peptides were identified as being able to recognize tumor vasculature but not normal blood vessels in severe combined immunodeficiency (SCID) mice bearing human tumors. These tumor-homing peptides also bound to blood vessels in surgical specimens of various human cancers. The peptide-linked liposomes containing fluorescent substance were capable of translocating across the plasma membrane through endocytosis. With the conjugation of peptides and liposomal doxorubicin, the targeted drug delivery systems enhanced the therapeutic efficacy of the chemotherapeutic agent against human cancer xenografts by decreasing tumor angiogenesis and increasing cancer cell apoptosis. Furthermore, the peptide-mediated targeting liposomes improved the pharmacokinetics and pharmacodynamics of the drug they delivered compared with nontargeting liposomes or free drugs. Our results indicate that the tumor-homing peptides can be used specifically target tumor vasculature and have the potential to improve the systemic treatment of patients with solid tumors.

One of the primary goals of a cancer treatment regimen is to deliver sufficient amounts of a drug to targeted tumors while minimizing damage to normal tissues. Most chemotherapeutic but cytotoxic agents enter the normal tissues in the body indiscriminately without much preference for tumor sites. The dose reaching the tumor may be as little as 5–10% of the dose accumulating in normal organs (1). One reason is that interstitial fluid pressure in solid tumors is higher than in normal tissues, which leads to decreased transcapillary transport of chemotherapy or anticancer antibodies into tumor tissues (2–4). Cancer cells are therefore exposed to a less than effective concentration of the drug than normal cells, whereas the rest of the body must be subjected to increased toxicity and decreased effectiveness. This phenomenon often limits the dose of anti-cancer drugs that can be given to a patient without severe harm, resulting in incomplete tumor response, early disease relapse, and drug resistance.

The development of drug delivery systems represents the ongoing effort to improve the selectivity and efficacy of antineoplastic drugs. Compared with conventional administration methods for chemotherapeutic agents, lipid- or polymer-based nanomedicines have the advantage of improving the pharmacological and therapeutic properties of cytotoxic drugs (5, 6). Most small molecule chemotherapeutic agents have a large volume of distribution upon intravenous administration (7) and a narrow therapeutic window because of severe toxicity to normal tissues. By encapsulating drugs in drug delivery particles, such as liposomes, the volume of distribution is significantly reduced, and the concentration of drug within the tumor is increased (8).

The coupling of polyethylene glycol (PEG) to liposomes (PEGylated liposomes), which have a longer half-life in the blood (9–11), is regarded as having great potential in a drug delivery system. For example, PEGylated liposome-encapsulated doxorubicin has been reported to significantly improve the therapeutic index of doxorubicin in preclinical (10, 12, 13) and clinical studies (14–16). Many of these drug delivery systems have entered the clinic and have been shown to improve the pharmacokinetics and pharmacodynamics of the drugs they deliver (6).

The growth of solid tumors is dependent on their capacity to induce the growth of blood vessels to supply them with oxygen and nutrients. However, the blood vessels of tumors present specific characteristics not observed in normal tissues, including extensive angiogenesis, leaky vascular architecture, impaired lymphatic drainage, and increased expression of permeability mediators on the cell surface (17, 18). These characteristics might be used to develop antiangiogenic target therapy for cancer. The hyperpermeability of tumor vasculature, for example, is a key factor for the success of liposome-delivered chemotherapy agents. The angiogenic tumor vasculature is estimated to have an average pore size of 100–600 nm (19). These pores are significantly larger than the gaps found in normal endothelium, which are typically <6 nm wide (8). After
intravenous administration, liposomes with diameters of ~65–75 nm (20–22) are small enough to passively infiltrate tumor endothelium but large enough to be excluded from normal endothelium. In solid tumors, the permeability of the tumor vasculature increases to the point that particulate liposomes can extravasate and localize in the tissue interstitial space (19). In addition, tumor tissues frequently lack effective lymphatic drainage (3), which promotes liposome retention. The combination of these factors leads to an accumulation of the drug delivering liposome within the tumor. This passive targeting phenomenon has been called the “enhanced permeability and retention effect” (23, 24).

The use of liposomes for passive targeting has some disadvantages. Normal organ uptake of liposomes leads to accumulation of the encapsulated drug in mononuclear phagocytic system cells in the liver, spleen, and bone marrow, which may be toxic to these tissues. With the increased circulation time and confinement of the particulate liposomes, hematological toxicities, such as neutropenia, thrombocytopenia, and leucopenia, have also appeared (25, 26). Ongoing research aims to enhance the tumor site-specific action of the liposomes by attaching them to ligands that target tumor cell (21, 27) and tumor vasculature (20, 28) surface molecules. These liposomes are called active or ligand-mediated targeting liposomes.

Combinatorial libraries displayed on phage have been used successfully to discover cell surface-binding peptides and have thus become an excellent means of identifying tumor specific targeting ligands. Phage-displayed peptide libraries have been used to identify B-cell epitopes (29–31). They can also be used to search for disease-specific antigen mimics (32, 33) and identify tumor cells (21, 34) and tumor vasculature-specific peptides (35). Screening phage display libraries against specific target tissues is therefore a fast, direct method for identifying peptide sequences that might be used for drug targeting or gene delivery. By combining a drug delivery system with tumor-specific peptides, it is possible that targeting liposome can deliver as many as several thousand anticancer drug molecules to tumor cells via only a few targeting ligand molecules.

In this in vivo study, we developed a method capable of selecting peptides that home to tumor tissues. We identified several targeting peptides able to bind specifically to tumor vasculature in surgical specimens of human cancer and xenografts. Coupling these peptides with a liposome containing the anticancer drug doxorubicin (Lipo-Dox; LD) enhanced the efficacy of the drug against several types of human cancer xenografts in SCID mice. Our results indicate that these targeting peptides can potentially play an important role in the development of more effective drug delivery systems.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture**—SAS (oral cancer), HCT116 (colon cancer), BT483 (breast cancer), Mahlavu (liver cancer), and PaCa-2 (pancreatic cancer) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 3.7 g of bicarbonate and 40 mg of kanamycin per liter, 2 mM l-glutamine, 5% fetal bovine serum (Invitrogen) in a 10% CO₂ incubator. H460 (lung cancer) and PC3 (prostate cancer) were grown in RPMI 1640 supplemented with 2 g of bicarbonate and 40 mg of kanamycin per liter, 2 mM l-glutamine, and 10% fetal bovine serum at 37 °C under a humidified atmosphere of 95% air and 5% CO₂ (v/v). Preparation of HUVECs from umbilical vein was described in a previous report (20).

**In Vivo Phage Display Biopanning Procedures**—SAS cells were injected subcutaneously into the dorsolateral flank of 4 – 6-week-old SCID mice to produce oral cancer xenografts. A phage-displayed peptide library (New England Biolabs, Inc.) was injected intravenously into the tail vein of SCID mice bearing size-matched SAS-derived tumors (~500 mm³). After 8 min of phage circulation, the mice were perfused with 50 ml of PBS to wash unbound phage. The organs (such as lungs, heart, and brain) and tumor masses were removed, weighed, and washed with cold PBS. The organs and tumor samples were homogenized, and the phage particles were rescued by ER2738 bacteria (New England BioLabs). The phages were titered on agar plates in the presence of 1 mg/liter isopropyl 1-thio-β- d-galactopyranoside/X-gal. The bound phages were amplified and titered in ER2738 culture. Recovered phages were subjected to four consecutive rounds of biopanning using oral cancer xenografts prepared as described above. The phages eluted from the fifth round were titered on LB/isopropyl 1-thio-β- d-galactopyranoside/X-gal plates. The candidate tumor-homing phage clones were randomly selected and identified by in vivo homing experiments. DNA sequences of the selected phages and targeting peptide synthesis were described in our previous reports (20, 22).

**In Vivo Homing Experiment and Phage Binding in Xenograft Tumor Vessels**—The phage clones or control phage (insertless phage) were injected into the tail vein of tumor xenograft mice. The inputs for all of the individual phage-homing experiments were 1 × 10⁹ plaque-forming units except for the IVO-8 in the BT483 animal model, which was 6 × 10⁹ plaque-forming units. After perfusion, xenograft tumors and organs were removed and titered. In peptide competitive inhibition experiments, phage clones were co-injected with 100 μg of synthetic peptide. After injection of targeting or control phage clones, the organs and tumors were removed and divided into two parts. One part was titered by ER2738, and the other was embedded in optimal cutting temperature (Tissue-Tek). The optimal cutting temperature-embedded frozen tissues were sectioned at 5 μm and transferred to cold PBS buffer. The sections were fixed with acetone-methanol (1:1), washed with PBS, and immersed in blocking buffer (1% bovine serum albumin in PBS) for 1 h. Then the sections were incubated with rat anti-mouse CD31 (BD Pharmingen) and rabbit anti-rat antibody (Ab) (Stressgen) and immersed in rhodamine-labeled goat anti-rabbit Ab (Jackson ImmunoResearch). The slides were further incubated with mouse anti-M13 phage monoclonal antibody (mAb) (American Biosciences), followed by fluorescein isothiocyanate-labeled goat anti-mouse Ab (Jackson ImmunoResearch), and immersed in rhodamine-labeled goat anti-rabbit Ab (Jackson ImmunoResearch). The slides were washed and mounted with mounting medium (Vector). Slides were examined under a Leica confocal microscope (TCS-SP5-AOBS). Images were merged by Leica application suite advanced fluorescence software.

**Peptide Synthesis**—Targeting peptides PIVO-8 (SNPF-SKPYGLTV) and PIVO-24 (YPHYSLPGSSTL) and control
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Peptide (TDSLRSYDGGG) (36) were synthesized and purified by reverse phase high performance liquid chromatography to >95% purity by Academia Sinica (Taipei, Taiwan).

Detection of VEGF-stimulated HUVECs and Human Cancer Surgical Specimens by Tumor-homing Phages—HUVECs were plated and grown to ~80% confluence on coverslips. The cells were pretreated with 20 ng/ml VEGF (B&D Systems) and 2 ng/ml basic fibroblast growth factor (PEPROTECH, London, UK) for 48 h. The VEGF-stimulated HUVECs were washed with serum-free M199 plus 3% bovine serum albumin and incubated in blocking buffer for 30 min at 4 °C and then with phages at 4 °C for 1 h. They were washed and fixed with 3% formaldehyde for 10 min, followed by incubation with mouse anti-M13 mAb (Amersham Biosciences) for 1 h and then fluorescein isothiocyanate-labeled anti-mouse Ab (Jackson ImmunoResearch), followed by staining in Hoechst 33258 (Molecular Probes, Inc., Eugene, OR). The coverslips were finally washed and mounted. Images were merged by SimplePCI software (C-IMAGING). Tumor blood vessels on human cancer frozen sections were stained with ulex europaeus agglutinin I (UEA-1) (37). Sections were incubated in blocking buffer for 30 min and then treated with phages and biotinylated UEA-1 (Vector). Slides were washed and followed by incubation with mouse anti-M13 mAb plus fluorescein isothiocyanate-conjugated streptavidin (Pierce) for 1 h and then treated with phycocyanin-conjugated goat anti-mouse Ab (Jackson ImmunoResearch). Slides were examined under a Zeiss Axiovert 200M inverted microscope. Images were merged by MetaMorph software (Molecular Devices).

Preparation of Peptide-conjugated Liposomal Doxorubicin—Peptide-conjugated liposomes containing doxorubicin were prepared as described in previous studies (20, 22, 36). Briefly, the peptide was coupled to N-hydroxysuccinimido-carboxylpolyethylene glycol (M, 3400)-derived distearoylphosphatidyl ethanolamine (NOF Corp.) in a 1:1.5 molar ratio. The reaction was completed and confirmed by quantitation of the remaining amino groups using trinitrobenzenesulfonate reagent (Sigma). Doxorubicin and vinorelbine were encapsulated in liposomes using a remote loading method at a concentration of 1 mg of drug/10 μmol of phospholipids. Peptidy-PEG-derived distearoylphosphatidyl ethanolamine was transferred to preformed liposomes after co-incubation at a transition temperature of the lipid bilayer. There were 500 peptide molecules/liposome, as described previously (38).

Endocytosis of Liposome Conjugates with PIVO by HUVECs—Liposomes containing sulforhodamine B (SRB), a fluorescent dye, were prepared as described previously (21). HUVECs were incubated at 37 or 4 °C, with different formulation of liposomal SRB (LS), including PIVO-8-LS, PIVO-24-LS, control peptide-Lipo-SRS (CP-LS), and LS. After 5 min of incubation, the cells were washed with PBS, stained with DAPI, and then detected with confocal microscopy.

Animal Models for Study of Ligand-targeted Therapy—Human cancer xenografts were established in SCID mice. Human cancer cells were injected subcutaneously into the dorsolateral flank in mice 4–6 weeks of age. Mice with size-matched tumors (tumor sizes of ~100 mm³) were randomly assigned to different treatment groups and treated with PIVO-8-conjugated liposome/doxorubicin (PIVO-8-LD), doxorubicin-free drug (FD), or equivalent volumes of saline through the tail vein. The dosage of doxorubicin was 1 mg/kg injected twice a week for 4 weeks (total doxorubicin dose of 8 mg/kg). Mouse body weight and tumor size were measured twice a week using calipers. The tumor volumes were calculated using the equation, length × (width)² × 0.52. Animal care was carried out in accordance with the guidelines of Academia Sinica (Taipei, Taiwan).

Pharmacokinetic and Biodistribution Studies—SCID mice bearing H460-derived tumor xenografts (~300 mm³) were injected through the tail vein with various formulations of liposomal doxorubicin (PIVO-2-LD, PIVO-8-LD, PIVO-24-LD, and LD) and FD at a dose of 2 mg/kg. At selected time points, three mice in each group were anesthetized and sacrificed. Blood samples were collected through submaxillary punctures, and plasma samples were prepared. After perfusion, xenograft tumors and mouse organs were removed and homogenized. Procedures for isolating tumor cell nuclei and extracting nuclear doxorubicin were carried out as previously reported (39, 40). Total doxorubicin concentration was measured using a method described by Mayer et al. (40). Total doxorubicin was quantified using spectrofluorometry at λ ex 485/20 nm and λ em 645/40 nm (Synergy HT multidetection microplate reader; BioTek Instruments, Winooski, VT).

To determine the presence of the drug localized in tumor tissues, doxorubicin autofluorescence was detected using a Zeiss Axiovert 200M inverted microscope with a 100-watt HBO mercury light source equipped with a 546/12-nm excitation and a 590-nm emission filter set. Tissue sections were imaged with a FLUAR ×10/0.50 numerical aperture lens and captured with a Roper Scientific CoolSnap HQ CCD camera.

Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) Staining—The frozen tumor tissue sections were incubated with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling reaction mixture (Roche Applied Science) at 37 °C for 1 h. The slides were counterstained with mounting medium with DAPI. The slides were then visualized under a fluorescent microscope and analyzed with MetaMorph software.

Vessel Staining—Tissues were removed from mice treated with anticancer drugs, fixed with 4% paraformaldehyde, and embedded with paraffin. Blood vessels were detected by staining of Lycopersicon esculentum (tomato) lectin conjugated to biotin (Vector). The biotinylated lectin was visualized with streptavidin-conjugated rhodamine (Pierce).

Statistical Analyses—We analyzed the data of phage titer, tumor volume, body weight, and doxorubicin concentration using a two-sided unpaired Student’s t test. A p value below 0.05 was considered significant for all analyses. All values are represented as mean ± S.D.

RESULTS
Isolation of Tumor-homing Peptides by in Vivo Phage Display—To isolate tumor-homing phages from tumor tissues, we used a phage-displayed peptide library to treat the oral cancer (SAS)-bearing mice for five rounds of in vivo affinity selection (bio-panning). The number of phages recovered from tumor tissues...
markedly increased in fourth and fifth rounds of biopanning, whereas the number of phages recovered from the lungs, which was used as a control organ, remained largely unaffected (Fig. 1A). Enriched phages from the fifth biopanning rounds were randomly selected and sequenced. Using GCG software, the sequences of 23 clones had displayed the same, related or non-related peptide sequence (Table 1). Searching the peptides displayed in our phage-homing system, we identified the IVO-2 phage displaying the same sequence as was found in one of our previous papers (20). The tumor homing potential of these selected phages was further characterized using in vivo homing assay by injecting them individually. Finally, we identified six novel phages (IVO-5, -8, -12, -24, -25, and -29) but not control phages that had higher homing ability in the tumor mass, exhibiting a concentration from 4.0- to 481-fold higher in the tumor mass than in normal organs, including the brain, lungs, and heart (Fig. 1B). Two phages, IVO-8 and -24, particularly showed higher tumor homing ability (Fig. 1B).

Specificity of Tumor-homing Phages in Solid Tumors—To test whether the target of tumor-homing phages may be universally expressed in the solid tumors, we examined the homing ability of IVO-8 and IVO-24 in six other types of human cancers, including human lung (H460), colon (HCT116), breast (BT483), prostate (PC3), pancreatic (PaCa-2), and liver (Mahlavu) cancer xenografts. We measured the titers of the phage in tumor masses and normal control organs (brain, heart, and lungs) (20, 22). Interestingly, in all of these human cancer xenografts, these phages targeted tumor tissues but not normal organs like brain, lungs, or heart (Fig. 2A). Control phages without these targeting ligands had no such homing ability (Fig. 2A).

To investigate the tumor targeting ability and specificity of the IVO phages in vivo, we injected phages into the tail vein of mice bearing H460-derived tumors. They were recovered after perfusion. IVO-8 was found to home specifically to tumor masses, and the tumor homing ability of IVO-8 was completely inhibited by cognate synthetic peptide PIVO-8 but not by PIVO-24 (Fig. 2B). IVO-24 was found to home specifically to tumor masses, and the tumor homing ability of IVO-24 could be inhibited by PIVO-24. The PIVO-8 did not inhibit IVO-24 phage homing to tumor tissue (Fig. 2C). These data indicated

FIGURE 1. Isolation of tumor-homing peptides by in vivo phage display. A, selection of oral cancer targeting phages by in vivo phage display. A phage-displayed peptide library was injected through the tail vein of tumor-bearing mice. Eight minutes after injection, the mice were perfused through the heart. Phage recovered from the tumor was amplified and reinjected in four consecutive rounds. The number of phages recovered from tumor tissues but not control lungs had markedly increased by the fifth round of biopanning. B, tumor-homing ability of phages. SCID mice bearing human oral cancer xenografts were injected intravenously with IVO phages, and phage was recovered after perfusion. Phage titer in control organs compared with tumor tissues is indicated. Recovery of IVO-8 and -24 phages in the tumor tissues was significantly higher than in the brain, lung, or heart tissues.
TABLE 1
Alignment of phage-displayed peptide sequences selected from lung cancer cell xenographs

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>Phage-displayed peptide sequence*</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVO-2, 21, 26</td>
<td>SVSVGMKPSRPR</td>
<td>3/23*</td>
</tr>
<tr>
<td>IVO-7</td>
<td>ASPMTAIAKLRFA</td>
<td>1/23</td>
</tr>
<tr>
<td>IVO-10</td>
<td>NWWTNRPNTSPP</td>
<td>1/23</td>
</tr>
<tr>
<td>IVO-17</td>
<td>MNNDHVPSVQG</td>
<td>1/23</td>
</tr>
<tr>
<td>IVO-4</td>
<td>AYWSNPSHPXO</td>
<td>1/23</td>
</tr>
<tr>
<td>IVO-8</td>
<td>SNFPKSPGYTL</td>
<td>1/23</td>
</tr>
<tr>
<td>IVO-28</td>
<td>WDNSYTPRPLM</td>
<td>1/23</td>
</tr>
<tr>
<td>IVO-3</td>
<td>ATNPUPHPQLPV</td>
<td>1/23</td>
</tr>
<tr>
<td>IVO-14</td>
<td>NPSLHHPLTISI</td>
<td>1/23</td>
</tr>
<tr>
<td>IVO-20</td>
<td>NPYQHRNWAYVVG</td>
<td>1/23</td>
</tr>
<tr>
<td>IVO-16</td>
<td>HARENSLWLHA</td>
<td>1/23</td>
</tr>
<tr>
<td>IVO-24</td>
<td>YPHYSLPGSSTL</td>
<td>1/23</td>
</tr>
<tr>
<td>IVO-29</td>
<td>LPLALPRINSV</td>
<td>1/23</td>
</tr>
<tr>
<td>IVO-13</td>
<td>SSLKHLHPTLDA</td>
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</tr>
<tr>
<td>IVO-25</td>
<td>SSLERPWRRTSR</td>
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<td>GNFQFESYTVPI</td>
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<td>IVO-6</td>
<td>FHILSWKPRKA</td>
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</tr>
<tr>
<td>IVO-5</td>
<td>NYLHHPPTGTVG</td>
<td>1/23</td>
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</tbody>
</table>

* Phage-displayed consensus amino acids are shown in boldface type.
+ From 30 random selected phage clones, 23 phage-displayed peptide sequences were identified and aligned.

Peptide-mediated Targeting to Tumor Vasculature

that the two tumor-homing phages had different peptide sequences, and each might be capable of recognizing different molecules expressed on tumor tissues.

For verification of the specificity of tumor-homing phages (IVO-8 and -24), tissue sections of tumor and normal organs derived from the homing and competition experiments were immunostained by anti-phage antibody. Only tumor tissues but not control lungs revealed immunoreactivity of IVO phages (Fig. S1). However, when IVO-8 and -24 phages were co-injected with the cognate synthetic peptide PIVO-8 and -24, no immunoreactivity was found in the tumor tissues (Fig. S1).

The Tumor-homing Peptides Target Tumor Vasculature—To investigate the body distribution of IVO phages, we used an immunofluorescent assay to identify the binding site of the phage after perfusion. Results indicated that phage particles reacted with xenograft tumor sections of lung cancer but not with normal lung tissues (Fig. S1 and Fig. 3A). However, the phages did not localize to cancer cells. Using frozen sections from tumor tissues incubated with anti-M13 mAb (green) and anti-CD31 mouse endothelial cell marker (red), IVO phages were co-localized with CD31 in the tumor vasculature of xenograft tumor tissues (Fig. 3A). The phage was not found in blood vessels of normal lungs (Fig. S1).

This study found IVO phages capable of targeting mouse vasculature endothelia of tumor tissues. To identify whether these phages had affinity for human endothelia cells, we applied these phage particles to VEGF-stimulated human vascular endothelial cells (HUVECs), which mimic angiogenic endothelial cells. IVO-8 and IVO-24 bound to VEGF-stimulated HUVECs (Figs. 3B, a and d); the control phages without these peptides showed no binding activity (Fig. 3B, c and f). HUVECs without VEGF stimulation did not display phage binding (Fig. 3B, b and e).

We found that tumor-homing phages could bind to the vessels in surgical specimens of malignant tumors taken from humans through immunofluorescent localization (Fig. 3C and Tables 2 and 3). IVO-8 and -24 phages were found co-localized with tumor vasculature in surgical specimens of human breast cancer (Fig. 3C) but not with the blood vessels of normal counterparts taken from the same specimen (Fig. S2). To verify that these peptides could be used to develop ligand-targeted therapy or imaging agents for human solid tumors, we also used these phages to detect several cancer types. These peptides recognized target molecules from surgical specimens from breast, lung, colon, liver, oral, and pancreatic cancer patients (Table 2). These surgical specimens from six types of cancer patients could be detected by IVO-8 and -24, with positive rates ranging from 50 to 80% (Table 3). These data indicate that these peptides can recognize unidentified molecules expressed on tumor vasculatures in mice and humans.

Enhanced Therapeutic Efficacy of PIVO-conjugated Liposomal Doxorubicin—Doxorubicin (M, 543.54), much like other small molecule chemotherapeutic agents, has a poor pharmacokinetic profile. However, the pharmacokinetic profile of LD was markedly better than that of free doxorubicin (Fig. 6A). We found it to be much more efficacious therapeutically than free doxorubicin at the same concentrations in human lung (H460), breast (BT483), and liver (Mahlavu) cancer xenografts (Fig. 4A).

We treated five types of human cancer xenografts with the PIVO-8-LD and PIVO-24-LD to determine whether they could be improved to use the therapeutic efficacy of LD in solid tumors. Interestingly, PIVO-LD increased therapeutic efficacy to each of the five human cancers: lung (H460), breast (BT483), liver (Mahlavu), pancreatic (PaCa-2), and colon (HCT116) (Fig. 4B). These results indicate that conjugation of LD with the targeting ligands PIVO-2, -8, and -24 enhances the efficacy of the doxorubicin in its inhibition of human solid tumor xenografts in all of the animal models we tested.

Histopathological Examination and Immunofluorescent Detection of Tumor Blood Vessels and Apoptotic Cells in the Study of Ligand-targeted Therapy—The histopathology of H460-derived tumor tissues in each treatment group was examined by hematoxylin/eosin (H&E) staining. Marked disseminated necrotic/apoptotic areas were present throughout the whole section of PIVO-LD-treated tumors, whereas moderate amounts of necrotic/apoptotic areas were found in the LD-treated tumors. The PBS-treated group showed no necrotic/apoptotic area (Fig. 5A). TUNEL was used to detect apoptotic cells. Representative microscopic fields from the tumors show more apoptotic cancer cells in the PIVO-LD groups than in LD group (Fig. 5A). TUNEL-positive areas in tumor tissues were quantified under low power magnification. TUNEL-positive areas were higher in the PIVO-LD groups compared with those in the LD group (n = 6, p < 0.01) (Fig. 5D).

We removed the tumor tissues and analyzed the effect PIVO-LD on tumor blood vessels. Tumor vessels were found to be markedly decreased in PIVO-LD-treated mice. LD-treated mice showed a limited reduction in tumor vasculature (Fig. 5B). Areas of lectin-positive tumor blood vessels were counted.
under low power magnification.

The PIVO-LD groups had significantly fewer tumor blood vessels than the LD group \((n = 6, p < 0.01)\) (Fig. 5C). The PBS group had high tumor vascular density and very few apoptotic cells (Fig. 5). The severe damage to tumor vasculature and increase in apoptosis of tumor cells caused by a low dose of PIVO-conjugated liposomal doxorubicin throughout the tumors suggested significant improvement in chemotherapeutic efficacy over liposomal doxorubicin and free doxorubicin.

Enhanced Tumor Drug Delivery of PIVO-conjugated Liposomes—The uptake of PIVO-Lipo-SRB (PIVO-LS) to HUVECs was studied by immunofluorescent localization. When HUVECs were incubated at 37 °C with PIVO-8-LS or PIVO-24-LS, the fluorescence was distributed in the cytoplasm surrounding the nuclei (Fig. 3D). Internalization of Lipo-SRB fluorescence was lost when HUVECs were incubated at 4 °C with PIVO-8-LS or PIVO-24-LS (Fig. 3D). No specific fluorescence could be detected on HUVECs when cells were incubated with nontargeting LS at 37 or 4 °C (Fig. 3D). Likewise, if control peptide-Lipo-SRB (CP-LS) was used, the results were similar to the application of Lipo-SRB (Fig. 3D). These results suggest that targeting ligands PIVO-8 or PIVO-24 could enhance drug uptake by endothelial cells through receptor-mediated endocytosis.

We wanted to verify the enhancement of therapeutic efficacy by PIVO-LD. To do this, we measured the area under the concentration-time curve \((AUC_{0–48h})\) of doxorubicin in tumor tissues to determine drug delivery into the tumor tissues. The results revealed that \(AUC_{0–48h}\) was 10.2 \(\mu\)g·h/g, 31.3 \(\mu\)g·h/g, 49.6 \(\mu\)g·h/g, and 49.5 \(\mu\)g·h/g in the FD, LD, PIVO-8-LD, and PIVO-24-LD groups, respectively (Fig. 6B and Table 4). The mean intratumor doxorubicin concentration in the PIVO-8-LD group was 4.9- and 1.6-fold higher than in the FD and LD groups. It was 4.8- and 1.6-fold
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FIGURE 2. Verification of tumor homing ability of IVO phages in vivo. A, IVO phages homed to a variety of human cancer xenografts. SCID mice bearing human lung (H460), colon (HCT116), breast (BT483), prostate (PC3), pancreatic (PaCa-2), and liver (Mahalavu) cancer xenografts were injected intravenously with IVO or control phages. After perfusion with PBS buffer, xenograft tumor masses were removed, and phage titers were measured. Phage titer in control organs compared with tumor tissues is indicated. The level of IVO phage titer in the tumor masses was markedly higher than in the tissues of control organs in all of the human cancer xenografts. B and C, SCID mice bearing lung cancer xenografts were injected intravenously with IVO phages or phages plus peptides, and phages were recovered after perfusion. The titer of IVO phages recovered from tumor tissues was higher than that from control lungs. The targeting activity of IVO phages to tumor tissues was competitively inhibited by their cognate peptide PIVO but not by other peptides.

TABLE 2
Detection of human cancer surgical specimens by IVO phages using immunohistochemistry

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cancer</th>
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FIGURE 3. The tumor-homing peptides target tumor vasculature. A, SCID mice bearing lung cancer (H460) xenografts were injected intravenously with tumor-homing phages. Eight minutes after the injection, the mice were perfused with PBS buffer. Tumor and organ tissues were immunostained with anti-M13 phage and anti-CD31 antibodies. Anti-phage immunofluorescence was colocalized with anti-CD31 on tumor neovasculature endothelia after the injections with IVO-8 or -24 phages (bar, 20 μm). B, tumor-homing phages reacted with VEGF-stimulated HUVECs. IVO phages were incubated with VEGF-stimulated HUVECs. Unbound phage particles were washed off, and the HUVECs were immunostained with fluorescein isothiocyanate-anti-M13 antibodies. The specific reactivity of IVO phages on stimulated HUVECs is shown in a and d. IVO phages exhibited no specific reactivity with nonstimulated HUVECs (b and e). The control helper phage did not react with stimulated HUVECs (c and f). Nuclear staining was by H33258 (bar, 10 μm). C, immunofluorescent staining of IVO phages on surgical specimens of human breast infiltrating ductal carcinoma. The IVO phages were incubated with frozen sections of surgical specimens, followed by anti-M13 phage and UEA-1 staining. Anti-phage immunofluorescence was colocated with UEA-1 on tumor neovasculature endothelia after treatment with IVO-8 or -24 phages (bar, 20 μm). D, internalization of PIVO-8-LS, PIVO-24-LS, and non-targeted LS (CL-LS and LS) by HUVECs was studied by fluorescence microscopy after incubation at 37 and 4 °C. Nuclear staining was by DAPI (scale bar, 30 μm).

To assess the bioavailability of the liposomal drugs, we used the accumulation of nuclear doxorubicin as an indicator of drug cytotoxicity (39). The AUCAUC0–48 of bioavailable doxorubicin (i.e. bound to nuclei) for FD, LD, PIVO-8-LD, and PIVO-24-LD was 3.7, 6.7, 14.8, and 13.9 μg/h/g, respectively (Fig. 6C and Table 4). The intratumor nuclear doxorubicin concentration in the PIVO-8-LD group was 4.0- and 2.2-fold higher than in the FD and LD groups. It was 3.8- and 2.1-fold higher in the PIVO-24-LD group than in the FD and LD groups (Fig. 6C and Table 4).

To compare the drug delivery profile of the various doxorubicin formulations, we detected the drug in tumor tissues using a fluorescence microscope. Doxorubicin was visualized clearly in the tumor nuclei 4 h after the administration of PIVO-8-LD and PIVO-24-LD (Fig. 6D). Over time, there was an increase in the number of areas with tumor sections secreting doxorubicin. The areas with detectable doxorubicin were significantly larger in PIVO-8-LD- and PIVO-24-LD-treated tumors than they were in LD-treated tumors at each time point (Fig. 6D). The sections of FD-treated tumors showed no detectable doxorubicin (Fig. 6D). Liposomal doxorubicin appeared to escape from the vasculature and be delivered directly to the tumor interstitial space (Fig. 6D). The nuclei of cancer cells in lung cancer xenografts displayed doxorubicin fluorescence after the injection of targeting liposomes, indicating that once the drug is released from the liposomes, it finds its way to the target site.

DISCUSSION

Most chemotherapy for cancer is accompanied by strong side effects and acquired drug resistance. The development of more selective anticancer drugs with better discrimination between tumor cells and normal cells is the most important
goal of current anticancer research. In this study, we developed an antitumor therapeutic strategy that utilizes selective expression of vascular receptors in tumors combined with the passive targeting property of liposome to create a means of better targeting tumor cells. We discovered that IVO-8 and -24, neovasculature-specific phages, could specifically bind to both tumor vessels of xenografts in animal models and the blood vessels of six types of human solid tumors. By coupling PIVO peptides to the polyethylene glycol terminus of sterically stabilized liposomes, we were able to optimize liposome-based delivery to solid tumors and improve therapeutic efficacy (Fig. 4). We have demonstrated that this targeted delivery of liposomal doxorubicin increased cancer cell apoptosis and decreased tumor angiogenesis in mice, resulting in a marked regression in tumors (Fig. 5). Tumor vessel-specific liposomes may be used effectively to deliver cytotoxic drugs to tumor endothelial cells, but they also may be used to directly reach tumor cells by passive targeting (Fig. 6).

Using in vivo phage display, we have identified IVO-8 and -24, each with a different peptide sequence (Table 1), able to precisely home to tumor tissues (Fig. 1). In this study, we found that phages IVO-8 and IVO-24 displayed two new peptides that were capable of binding specifically to the vasculature of solid tumors. We showed that IVO-8 and -24 peptides bound to molecules expressed in tumor vessels and were not expressed in normal vessels (Figs. 2 and 3). When a peptide competitive inhibition assay was performed, the binding activities of IVO

### TABLE 3
Summary of IVO-positive surgical specimens of human cancer

<table>
<thead>
<tr>
<th>Sample</th>
<th>Breast</th>
<th>Lung</th>
<th>Colon</th>
<th>Liver</th>
<th>Oral</th>
<th>Pancreas</th>
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FIGURE 4. Treatment of SCID mice bearing human cancer xenografts with targeting liposomes. A, SCID mice bearing human lung (H460), breast (BT483), and liver (Mahlavu) cancer xenografts were injected intravenously with LD, FD, or PBS (n = 6; **, p < 0.01; *** , p < 0.005). Median tumor volume over time in mice treated with LD was markedly decreased compared with the FD group. B, SCID mice bearing human lung (H460), breast (BT483), liver (Mahlavu), pancreatic (PaCa-2), and colon (HCT116) cancer xenografts were treated with PIVO-8-LD, PIVO-24-LD, LD, or PBS. PIVO-8-LD and PIVO-24-LD significantly enhanced therapeutic efficacy for all tumor types (n = 6; *, p < 0.05; ***, p < 0.005).
Peptide-mediated Targeting to Tumor Vasculature

IVO-conjugated targeting liposomes increased the therapeutic efficacy through decreased tumor angiogenesis and enhanced cancer cell apoptosis. A, the histopathology of tumor tissues of each treatment group was examined after staining with hematoxylin/eosin (H&E) (bars, 200 μm (upper panels) and 50 μm (lower panels)). The sections were TUNEL-labeled to visualize apoptotic tumor cells (green). The TUNEL-positive tumor cells were distributed more extensively in the PIVO-8-LD- and PIVO-24-LD-treated groups than in the LD groups (bar, 200 μm). B, the tumor sections of each treatment group were stained with tomato lectin to visualize tumor blood vessels (red) and counterstained with DAPI (blue) (bar, 50 μm). C, areas of tumor blood vessels were counted (n = 6) at low power magnification. There were much fewer tumor vessels in the PIVO-8-LD- and PIVO-24-LD-treated groups than in the LD or PBS group (n = 6; **, p < 0.01). D, areas of TUNEL-positive tumor cells were measured (n = 6) at low power magnification. The TUNEL-positive areas are more increased in the PIVO-8-LD- and PIVO-24-LD-treated groups than in either the LD or PBS group (n = 6; **, p < 0.01).

phages with tumor tissues were inhibited only by their cognate synthetic peptides and not other peptides (Fig. 2, B and C). Furthermore, we were able to use IVO-8 and -24 to detect six types of human cancers. Each specimen was shown to have different staining reactivity, depending on the phage we used (Tables 2 and 3), suggesting that these two targeting peptides bind the different target molecules on tumor blood vessels.

IVO phages were found to specifically home to tumor tissues from lung, colon, breast, prostate, pancreatic, liver, and oral cancer xenografts but not to normal tissues from organs such as the brain, lungs, or heart (Figs. 1B and 2A). A closer examination revealed that IVO phages were co-localized with endothelial markers in most xenograft vasculatures but were rarely detected in tumor cells or normal organs (Fig. 3A and Fig. S1), suggesting that IVO phages might specifically target the endothelial cells in the vasculature of tumors but not the vasculature in normal organs. Moreover, IVO-8 and -24 bound specifically to the VEGF-stimulated HUVECs (Fig. 3B) and tumor vessels of six types of human solid tumors (Fig. 3C and Tables 2 and 3), suggesting that the vasculature in solid tumors may express...
unidentified universal receptors not expressed in normal mature vasculature and that these molecules can be recognized by the PIVO peptides.

Although some ligands isolated in mouse models have been used in humans (20, 35), it is unlikely that ligand-targeted delivery would always be achieved in humans using mouse-derived ligands. To evaluate the utility of targeting ligands for human cancer therapy, we investigated whether these peptides, which we selected using the murine angiogenic model, had the same binding activity for the neovasculature endothelia in human tumors. We found that IVO phages specifically bound to VEGF-stimulated HUVECs (for mimic of angiogenic endothelial cells) and human cancer surgical specimens from patients with breast, lung, colon, liver, oral, and pancreatic cancer (Fig. 3C and Tables 2 and 3), suggesting that they can potentially be used as targeting ligands in the development of antiangiogenic therapy for human cancers. The recovery rates for the phages varied among the different tumor types in phage homing experiments (Fig. 2A), which may be due to differences in blood vessel densities of the different tumor types or different expression levels of target molecules in those vessels. In our immunohistochemical staining surgical specimens from cancer patients, we found that the detection rate of the method using one IVO phage alone varied from 50 to 80% depending on tumor type (Tables 2 and 3). When two phages were combined, however, the detection rate increased to

**FIGURE 6.** PIVO-conjugated liposomes enhanced drug delivery to tumor. A, the pharmacokinetic profile of doxorubicin and liposomal doxorubicin formulations in a lung cancer xenograft mouse model. At selected time points (1, 4, 24, 48, and 72 h) after injection, doxorubicin concentration in blood was measured (n = 3 at each time point). B and C, tumor accumulation of doxorubicin in lung cancer-bearing mice treated with different formulations of liposomal and free doxorubicin. At selected time points (1, 4, 24, and 48 h) after injection, doxorubicin concentration in tumor tissues (B) and nuclei (C) was measured (n = 3 at each time point; *, p < 0.05; **, p < 0.01, for black and red asterisk p value versus FD and LD, respectively). D, representative two-color images showing the distribution of doxorubicin (red) in relation to nuclei (blue) in tissue sections. Accumulation of doxorubicin in tumor nuclei was examined at 1, 4, and 24 h postinjection. Bar, 25 μm.

**TABLE 4**

<table>
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<th>Tumor pharmacokinetics of free doxorubicin versus liposomal doxorubicin formulations</th>
<th>Formulation (2 mg/kg)</th>
<th>Tumor AUC&lt;sub&gt;0–48 h&lt;/sub&gt;</th>
<th>Nucleus AUC&lt;sub&gt;0–48 h&lt;/sub&gt;</th>
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<tr>
<td></td>
<td>Free doxorubicin</td>
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<td></td>
<td>Liposomal doxorubicin</td>
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<td></td>
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Peptide-mediated Targeting to Tumor Vasculature

has improved the therapeutic index of cancer patients (46, 47). At present, angiogenesis inhibitors have been shown to prolong progression-free survival but have only been found to have a small effect on overall survival in patients with cancer (47). VEGF has been found to play an important role in hematopoiesis, myelopoiesis, and endothelial cell survival (48, 49). Therefore, antiangiogenic therapy might cause bleeding, disturbed wound healing, thrombosis, hypertension, hypothyroidism and fatigue, proteinuria and edema, skin toxicity, leukopenia, and lymphopenia (18, 47, 50). The inhibition of angiogenesis has given us a straightforward means of treating cancer, although many antiangiogenic agents have side effects. Tumors treated with antiangiogenic targeting liposomes have a marked decrease in tumor vessel density, a higher level of cancer cell death, and more inhibited tumor growth than tumors treated with nontargeting liposomes (Figs. 4 and 5). This tumor site-specific target therapy may overcome the adverse effect caused by the present use of systemic therapy using antiangiogenic drugs and increase the therapeutic index.

In summary, using in vivo phage display to isolate ligands that home to molecules on tumor endothelial cells, we identified two novel peptides capable of targeting the neovasculature in seven human tumor xenografts and the surgical specimens of six types of human cancer. Using the ligands to develop targeting liposomes resulted in a significant improvement of anti-tumor effect and may potentially be used for targeted drug delivery systems. The current study indicates that antiangiogenic ligand-targeted therapy offers significantly therapeutic improvements over conventional anticancer drug therapy.

Acknowledgments—We thank Yun-Long Tseng for preparation of liposome complex and the Core Facility of the Institute of Cellular and Organismic Biology, Academia Sinica (Taipei, Taiwan) for assistance in fluorescence microscopy.

REFERENCES


70–90% (Table 2). This finding suggests that when using this method to treat cancer, combining two targeting peptides may reduce drug resistance.

Our finding that these peptides targeted the neovasculature suggests that they possess great potential in detection of tumors and the delivery of chemotherapeutic drugs. We found that the targeting ligands markedly increased the therapeutic efficacy of liposomal doxorubicin in five types of human cancer xenografts (Fig. 4B). Furthermore, we observed markedly decreased microvessel density and substantially increased cell apoptosis in tumor tissues after treatment with these targeting liposomes (Fig. 5). The severe damage to tumor vasculature caused by PIVO-LD throughout the tumors clearly demonstrates an improvement in chemotherapeutic efficacy in a xenograft animal model. This improvement resulted from the enhanced peptide-mediated targeting liposomes uptake by endothelial cells through endocytosis (Fig. 3D) and increased accumulation of targeting liposomal drugs in the tumor tissues (Fig. 6). Therefore, these ligand-mediated liposomal formulations were found to be much more effective for treatment of solid tumors than conventional anticancer therapy.

As mentioned earlier, the high interstitial fluid pressure of solid tumors presents a barrier to efficient delivery of chemotherapeutic drugs (2, 4), since it contributes to decreased transcapillary transport in tumors and the subsequent decrease in uptake of drugs. These phenomena are accompanied by the development of drug resistance, metastatic disease, and eventual therapeutic failure (41–44). The ligand-targeted therapy described in this study made possible more specific targeting and the delivery of higher dosages of anticancer drug to tumor tissues (Figs. 5 and 6), which may help overcome problems like high tumor interstitial fluid pressure and cancer cell heterogeneity through the affinity of targeting ligand, passive targeting liposome, and bystander effect (Figs. 3D, 5A, and 6D).

Delivery of liposomal doxorubicin to tumors by passive targeting is the main mechanism behind the successful clinical use of liposomal drugs like Doxil/Caelyx (45). The anticancer efficacy of these nontargeting liposomes can be increased by the use of targeting liposomes (Fig. 4). The use of antiangiogenic targeting liposomes may increase the ability of the encapsulated drug to target tumor blood vessels and destroy tumor cells. PIVO-mediated targeting liposomes (PIVO-LD, including PIVO-8-LD and PIVO-24-LD) kill tumor-associated endothelia, resulting in the death of the tumor cells that these vessels support (Fig. 5). The PIVO-LD can also penetrate the interstitial space of the tumor and there, functioning as a sustained release system like Doxil, directly kill cancer cells (Figs. 5 and 6). The dual action of the PIVO-targeted liposomes may contribute to the development of a more efficacious, more durable anticancer approach than the conventional chemotherapeutic approaches (Fig. 4), which are often hindered by tumor cell heterogeneity and high tumor interstitial fluid pressure. The antiangiogenic targeting liposomes may serve as the basis of a new pharmacological approach for the treatment of malignancies, since they can allow for the delivery of cytotoxic drugs to both tumor vasculature and the tumor cells themselves.

Tumor angiogenesis has been considered as an especially useful target for anticancer therapy, and antiangiogenic therapy...
Peptide-mediated Targeting to Tumor Vasculature

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