Published in final edited form as:

Nanomedicine. 2009 March ; 5(1): 73–82. doi:10.1016/j.nano.2008.07.007.

Targeting of albumin-embedded paclitaxel nanoparticles to tumors

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Abstract

We have used tumor-homing peptides to target abraxane, a clinically approved paclitaxel-albumin nanoparticle, to tumors in mice. The targeting was accomplished with two peptides, CREKA, and LyP-1 (CGQKRTRGC). Fluorescein (FAM)-labeled CREKA-abraxane, when injected intravenously into mice bearing MDA-MB-435 human cancer xenografts, accumulated in tumor blood vessels, forming aggregates that contained red blood cells and fibrin. FAM-LyP-1-abraxane co-localized with extravascular islands expressing its receptor, p32. Self-assembled mixed micelles carrying the homing peptide and the label on different subunits accumulated in the same areas of tumors as LyP-1-abraxane, showing that LyP-1 can deliver intact nanoparticles into extravascular sites. Untargeted, FAM-abraxane was detected in the form of a faint meshwork in tumor interstitium. LyP-1-abraxane produced a statistically highly significant inhibition of tumor growth compared to untargeted abraxane. These results show that nanoparticles can be effectively targeted into extravascular tumor tissue and that targeting can enhance the activity of a therapeutic nanoparticle.

Keywords

peptides; tumor targeting; tumor vessels; drug delivery; tumor markers

Background

The idea of an antibody type “magic bullet” for targeted cancer treatment has been around for 100 years, but the application of this concept to the treatment of solid tumors has proven challenging. The main reason for the limited success is that tumor tissue is not readily penetrable to compounds introduced through the blood stream¹. Targeting tumor vasculature obviates this limitation, as tumor blood vessels are fully accessible to agents in the blood and

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express numerous marker molecules that are not present, or are expressed only at low levels in normal resting blood vessels. Many of these molecules are linked to angiogenesis, the growth of new blood vessels from existing ones, a process that is needed for tumor growth²⁻⁴.

Markers that are functionally important in angiogenesis can be targeted with agents that inhibit the angiogenesis related function. Another way of utilizing vascular markers is to have a probe such as an antibody or peptide bind to a specific vascular marker and carry a diagnostic or therapeutic payload with it, so that the payload becomes concentrated at the target site⁵. We have introduced the term “synaphic” (affinity-based) targeting to distinguish this approach from activity-based targeting that is based on the specificity of drug action.

We have assembled a battery of tumor-homing peptides that selectively recognize tumor blood vessels or tumor lymphatics^{4, 6-12}. Two peptides from this collection are relevant to the present report: CREKA (cysteine-arginine-glutamic acid-lysine-alanine) is a pentapeptide that binds to clotted plasma proteins and homes to tumors because interstitial tissue of tumors¹³ and the vessels wall¹¹ contain clotted plasma proteins, while the vessels in normal tissues do not. LyP-1 is a cyclic 9-amino-acid peptide (Cys-Gly-Gln-Lys-Arg-Thr-Arg-Gly-Cys) that provided the first demonstration that lymphatic vessels in tumors can differ molecularly from normal lymphatics⁸. A protein known as p32 or gC1qR receptor¹⁴ is the target molecule for the LyP-1 peptide and, in addition to overexpression in tumors, it also exhibits aberrant cell surface expression in tumor lymphatics, tumor cells, and, a subset of myeloid cells which contributes to the tumor specificity of LyP-1 homing¹⁵.

Iron oxide nanoparticles coated with the CREKA peptide bind to clotted plasma proteins in tumor vessels and induce additional clotting thus amplifying their own homing¹¹. Quantum dots coated with the LyP-1 peptide specifically target tumor cells and lymphatic vessels upon intravenous injection in mice bearing MDA-MB-435 tumors¹⁶. LyP-1 coated Iron oxide nanoparticles extravasate into the tumor and specifically bind p32 expressing tumor cells¹⁷. We have used these peptides in tumor targeting of an anti-tumor nanoparticle drug.

Abraxane is a 130-nm, albumin-based nanoparticle formulation of paclitaxel that is clinically used for the treatment of metastatic breast cancer¹⁸. Abraxane obviates cremaphor, the emulsifier used to solubilize paclitaxel, eliminating the need for pre-medication with steroids and anti-histamines and a long infusion. Moreover, a higher dose of paclitaxel (260 mg/m²) can be administered as abraxane than in cremaphor (175mg/m²). We reasoned that coating the surface of abraxane nanoparticles with tumor homing peptides could further increase the accumulation of albumin bound paclitaxel in tumors. This, in turn, should improve the therapeutic efficacy of the drug.

We show here that the biodistribution of abraxane nanoparticles can be changed by conjugating the particles with tumor-homing peptides, and that the localization of the conjugates within tumor tissue is dictated by the specificity of the peptide. Using a modular nanoparticle system, self-assembled mixed micelles, we show that a homing peptide can effectively deliver intact nanoparticles into the extravascular compartment in tumors. Finally, we show that a targeted abraxane can be more effective in inhibiting tumor growth than unmodified abraxane.

Methods

Reagents, cell lines and tumors

Abraxane (Abraxis Biosciences, Los Angeles, CA, USA) was obtained from the Cancer Center Pharmacy at University of California, San Diego. Sulfo-SMCC (Sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate) and NHS-Fluorescein 5-(and 6-) carboxyfluorescein succinimidyl ester were from Pierce, Rockford, IL, USA. All the lipids

were purchased from Avanti Polar Lipids, Alabaster, AL, USA. MDA-MB-435 cells were maintained in DMEM supplemented with 10% FBS and 1% Glutamine Pen-Strep at 37°C/5% CO₂. To induce MDA-MB-435 tumors, Balb/c nude mice were injected with 2×10^6 MDA-MB-435 cells in PBS into the mammary fat pad region.

Peptides

Peptides were synthesized with an automatic Microwave assisted peptide synthesizer (Liberty; CEM Corporation, USA) using standard solid-phase Fmoc/t-Bu chemistry. During synthesis, the peptides were labeled with 5(6)-Carboxyfluorescein (FAM) with a 6-aminohexanoic acid spacer separating the dye from sequence. The synthesis of LyP-1 peptide used for the chemoselective ligation described with an extra *N*-terminus cysteine will be described elsewhere (Kotamraju et al., in preparation).

Peptide-nanoparticle conjugates

Peptide-abraxane conjugates were prepared by coupling peptides to abraxane through their cysteine sulfhydryl group using a sulfo-SMCC cross-linker. Abraxane was suspended in nitrogen-purged sterile PBS at a concentration of 5 mg per ml and 0.3 mg of sulfo-SMCC was added. After incubation at room temperature for 30 minutes, the excess crosslinker was removed by filtration through a Nap-10 column (GE Healthcare, UK). Peptide labeled with FAM was dissolved in sterile nitrogen-purged water and added to the abraxane-sulfo-SMCC conjugate in small aliquots over a period of 1 hour. The peptide was used at 3-fold molar excess relative to albumin. Excess peptide was removed by repeated washing in ultracentrifugal devices (Amicon; MWCO 10K). Fluorescence measurements revealed approximately one peptide per albumin molecule in the final conjugate. FAM-abraxane was prepared by directly coupling of NHS-Fluorescein (Pierce) to abraxane in PBS for 1 hour at room temperature. Particle integrity was confirmed by hydrodynamic diameter measurements using dynamic light scattering (Brookhaven Instruments). The measurements were performed in deionized water at an angle of 90°. To further confirm the presence of paclitaxel in these modified particles, they were digested with proteinase K (Qiagen, Valencia, CA, USA) for 1 hour at 60°C. The released paclitaxel was then extracted in chloroform and analyzed by mass spectrometry. Separately, chloroform was removed by evaporation, the residue was dissolved in methanol and absorbance measured at 227nm. The concentration of paclitaxel was calculated from the slope of standard curve generated with pure paclitaxel (Sigma, St. Louis, MO, USA) in methanol.

Lipopeptides were prepared by coupling unlabeled or FAM-labeled peptides to 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)₂₀₀₀; DSPEPEG₂₀₀₀-maleimide] (Avanti Polar Lipids) at a 1:1 molar ratio by making use of a free cysteine sulfhydryl group on the peptide. The reaction was performed in aqueous solution at room temperature for 4 hours. The resulting DSPE-PEG₂₀₀₀ lipopeptides were lyophilized and stored at -20°C until used. DSPE-PEG₂₀₀₀-FAM was either purchased (Avanti polar lipids) or prepared by coupling 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Amino(Polyethylene Glycol)₂₀₀₀; (DSPEPEG₂₀₀₀-amine) with NHS-Fluorescein (Pierce) at a 1:1 molar ratio in PBS for 1h at room temperature. Excess NHS-Fluorescein was removed by overnight dialysis against water using slide-a-lyzer dialysis cassettes (3.5K MWCO, Pierce). The dialyzed product was then lyophilized and stored at -20°C until used.

To prepare micelles, lyophilized DSPE-PEG₂₀₀₀-peptide and DSPE-PEG₂₀₀₀-FAM were dissolved in methanol and transferred to a glass tube at appropriate molar ratios. Methanol was evaporated under nitrogen flow. Residual solvent was removed by storing the film under vacuum for 12 hours. The film was then hydrated for 15 minutes in MilliQ water (18 MΩ-cm) heated to 80°C. After the solution cooled to room temperature, it was filtered through a

polyvinylidene fluoride centrifuge filter (100 nm nominal pore size, Amicon Bioseparations). and washed with PBS using the ultracentrifugal devices (Amicon; MWCO 10K). Dynamic light scattering system (Brookhaven Instruments Corporation, Holtsville, New York, USA) was used to confirm spherical shape and to measure the hydrodynamic diameter of the micelles. The first cumulant (Γ) of the first order autocorrelation function was measured as a function of scattering vector, q in the range of 0.013 to 0.024 nm⁻¹. The quantity, Γ/q^2 , which corresponds to the translational diffusion coefficient was found to be independent of q , confirming the presence of isotropic spherical particles. Thus, the Stokes-Einstein relation was used to calculate a hydrodynamic diameter of 12.5 ± 0.5 nm for the micelles.

Nanoparticle injections

To analyze nanoparticle biodistribution, tumor-bearing mice were anesthetized with intraperitoneal Avertin, and abraxane nanoparticles (20mg of paclitaxel per kg body weight) or micelles (100 µl of a 1 mM solution) were injected into the tail vein. The mice were sacrificed 3 hours post-injection by cardiac perfusion with PBS under anesthesia, and organs were dissected and analyzed for particle homing. In tumor treatment experiments, nude mice bearing MDA-MB-435 tumors of approximately 100 mm³ were treated with LyP-1-conjugated abraxane, unmodified abraxane, free LyP-1 peptide, or saline four times a week for 3 weeks (at a paclitaxel equivalent of 3 mg/kg/day for LyP-1-abraxane and unmodified abraxane). The total cumulative dose was 30 mg/kg. LyP-1 peptide was used at a dose equivalent to what was injected on the particles. There were 10 mice per group. Tumor volume was calculated using the following formula: volume (mm³) = (d²×D)/2 where d and D are the smallest and biggest tumor diameters respectively. Similar treatment studies were conducted with CREKA-abraxane. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of UCSB and animal research committee of Burnham Institute for Medical Research. For humane reasons, mice were sacrificed when the tumor volumes became more than 2000mm³.

Immunohistochemistry

Tissues from mice injected with nanoparticles were fixed in 4% Paraformaldehyde (PFA) overnight at 4°C, cryo-protected in 30% sucrose for 10 hours and frozen in OCT embedding medium. For histological analysis, 5 µm sections were cut. For immunostaining, tissue sections were first incubated with 10% serum from the species in which secondary antibody is generated for 1 hour at room temperature followed by incubation with the primary antibody overnight at 4°C. The following antibodies were used: rat monoclonal anti-mouse CD31 (10 µg/ml; BD Pharmingen, San Jose, CA, USA), hamster monoclonal anti-mouse podoplanin (10 µg/ml; Abcam, Cambridge, MA, USA) and rabbit polyclonal anti-p32 (10 µg/ml). The p32 antibody was produced against a peptide from the p32 sequence near the N-terminus and extensively characterized by immunoblotting and tissue staining. The details will be published elsewhere (Fogal et al., 2008, submitted). T7 phage was detected by immunostaining with a polyclonal rabbit anti-T7 antibody (8). The primary antibodies were detected with Alexa 594 goat anti-rat, Alexa 594 goat anti-rabbit and Alexa 594 goat anti-hamster secondary antibodies (1:1000; Molecular Probes, Eugene, OR, USA). Each staining experiment included sections stained with secondary antibodies only as negative controls. Nuclei were counterstained with DAPI (5 µg/mL; Molecular Probes, Eugene, OR, USA). The sections were mounted in gel/mount mounting medium (Biomedex, Foster city, CA) and visualized under an inverted fluorescent (Leica Microsystems, Wetzlar, Germany) or confocal microscope (Olympus, Melville, NY).

Statistical analysis

Differences between the various treatments were statistically tested using two tailed Student's unpaired t-test. *P* values of less than 0.05 were considered statistically significant.

Results

Peptide-abraxane conjugates

We coupled the CREKA and LyP-1 peptides to the surface of abraxane particles through a cysteine sulfhydryl group. The CREKA peptide contains a cysteine residue, which can be used for conjugation to nanoparticles without loss of homing activity¹¹. LyP-1 is a cyclic peptide with a disulfide bond. We added a free cysteine to this peptide using chemistries that will be described elsewhere (Kotamraju, et al., in preparation). The integrity of the cyclic structure was ascertained by mass spectrometry and activity was confirmed by *in vitro* cell-binding and internalization assays¹⁹. Both peptides had a fluorescein label on their N-terminus and the C-terminus was blocked as an amide. The peptides were coupled to abraxane particles through a sulfo-SMCC crosslinker. Fluorescence measurements revealed one peptide moiety per molecule of albumin in the final conjugate. Fluorescein-labeled abraxane particles were also prepared and used as a control. Abraxane particles conjugated with fluorescein-labeled peptides or with fluorescein exhibited only a small increase in hydrodynamic diameter (~130 to ~150nm) as measured by dynamic light scattering. To further confirm the presence of paclitaxel in these modified particles, they were digested with proteinase K and the released paclitaxel was extracted in chloroform. Mass spectrometric analysis of the chloroform extract demonstrated the presence of paclitaxel (see Methods).

CREKA-abraxane and LyP-1-abraxane target different structures in MDA-MB-435 tumors

We next tested the peptide-coated abraxane preparations for *in vivo* homing to MDA-MB-435 xenograft tumors grown in nude mice. We injected CREKA-abraxane, LyP-1-abraxane and FAM-abraxane at a dose of 20 mg/kg of paclitaxel equivalent and allowed them to circulate for 3 hours. CREKA-abraxane mostly accumulated in the tumor blood vessels as evident from its co-localization with CD31 staining (Figure 1A). Some of the blood vessels appeared to have their lumens filled with a fluorescent mass that also contained trapped erythrocytes. This fluorescent material was positive for anti-fibrin(ogen) staining, indicating the presence of clotted plasma proteins (Figure 1B). These results suggest that CREKA-abraxane induces a clotting event similar to what has been previously shown with CREKA-coated iron oxide nanoparticles¹¹, and that the nascent clot may then trap circulating erythrocytes. In addition to its vascular homing, CREKA-abraxane was also present in a faint meshwork pattern throughout the tumor. This meshwork in tumors was also present in tumors of mice injected with FAM-abraxane (Figure 1C). Among the tissues most likely to trap nanoparticles, there was modest uptake of CREKA-abraxane in the liver and in the spleen, while the lungs and kidneys showed no fluorescence. The heart, pancreas and brain also contained no detectable CREKA-abraxane fluorescence; FAM-abraxane was not detected in any normal organ. In agreement with what has been shown for CREKA-coated iron oxide nanoparticles¹¹, no occluded vessels were seen in any of the non-tumor tissues examined (Supplementary Figure 1).

LyP-1-abraxane showed greatly enhanced accumulation in extravascular tumor tissue compared with CREKA-abraxane or FAM-abraxane. This conjugate homed to tumor cell clusters that were positive for p32 (Figure 2A), the receptor for the LyP-1 peptide¹⁵. These cell clusters were typically located in regions that had interspersed podoplanin-positive structures, presumably lymphatic vessels (Figure 2B), but few blood vessels (Figure 2C). Similar distribution has been previously noted for the LyP-1 peptide^{8, 19}. LyP-1-abraxane was also detected in tumor stroma in a meshwork pattern similar to, but more strongly fluorescent than that seen with FAM-abraxane. The presence of faint fluorescent meshwork in tumor tissue with untargeted FAM abraxane and CREKA abraxane could possibly be due to an albumin dependent transport mechanism²⁰. However, the enhanced accumulation observed with LyP-1-abraxane shows that the LyP-1 peptide causes increased extravasation of abraxane compared to untargeted or CREKA targeted abraxane. LyP-1-abraxane was not detectable in the control

tissues examined, with the exception of the liver and spleen, which like CREKA-abraxane, showed some uptake (Supplementary Figure 1). The liver uptake could be due to the presence of basic amino acids in the peptide sequences. Quantification of fluorescence intensities showed that FAM-LyP-1-abraxane delivered 4-fold more fluorescence into the tumors than FAM-abraxane (Figure 2F, $p < 0.01$), whereas FAM-CREKA-abraxane was not significantly different from FAM-abraxane. LyP-1 abraxane delivered 4-fold more fluorescence into tumors than to the liver (Figure 2F, $p < 0.01$).

LyP-1 phage and micelles extravasate into tumor tissue

The strong accumulation of fluorescence from LyP-1-abraxane in locations away from blood vessels suggested that LyP-1 can carry a nanoparticle payload into extravascular tumor tissue. However, since the fluorescent label was attached to the peptide, the formal possibility remained that the abraxane particle had disintegrated, and that we were actually observing the labeled peptide without its payload. As there was no practical way of designing an abraxane particle where the label and the peptide were uncoupled, we used phage (a 50 nm particle) and micelles^{21, 22} to study the ability of LyP-1 to deliver an intact particle outside the confines of blood vessels.

Immunohistochemical staining tumor sections with antibodies against T7 phage coat protein showed extensive staining for the LyP-1 phage as early as 10–15 minutes after an intravenous injection (Figure 3 A and B). Co-staining with anti-CD31 showed no co-localization of the phage coat protein with tumor blood vessels, indicating extravascular localization of the phage. A control phage displaying the sequence Cys-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Cys was not detected in the tumor tissue (Figure 3C). As the T7 phage is highly resistant to disassembly, for example, it remains infective after treatment with denaturing agents such as 1 % SDS, 4M urea, and 2M guanidine-HCl²³, this result suggests that the intact LyP-1 phage particle had extravasated and penetrated the tumor interior.

To further test the presumed peptide-guided extravasation of LyP-1-coated nanoparticles, we constructed micelles displaying the targeting peptides on their surface. We separately conjugated the peptide or fluorophore to 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)₂₀₀₀] (DSPE-PEG), and prepared mixed micelles composed of unlabeled lipopeptide and labeled lipid. Upon intravenous injection into mice bearing MDA-MB-435 tumors, the mixed micelles showed the same localization in tumors as the corresponding abraxane conjugates; LyP-1 micelles accumulated extravascularly, whereas CREKA based micelles accumulated in tumor blood vessels (Figure 4). We also tested micelles labeled on the peptide and found the same homing pattern as with the lipid-labeled compounds. Micelles prepared from a FAM-labeled lipid did not show any tumor homing. These results strongly support the notion that LyP-1 peptide can deliver intact nanoparticles into extravascular locations, and that this process only takes minutes.

LyP-1 coating increases the anti-tumor activity of abraxane

Encouraged by the effective tumor homing of the peptide-abraxane conjugates, we decided to treat MDA-MB-435 tumors with these conjugates. We performed initial experiments to find the highest dose level at which unmodified abraxane had no effect on the growth of the tumors. We then compared the peptide-abraxane conjugates to unmodified abraxane at this dose level. CREKA-abraxane treatment resulted in a modest inhibition in tumor growth compared to unmodified abraxane, but the difference was not statistically significant (data not shown). In contrast, treatment with LyP-1-abraxane resulted in a significant ($p=0.013$) inhibition of tumor growth (Figure 5).

Discussion

We show here that the clinically used nanoparticle drug, abraxane, can be modified with homing peptides, and that peptides with different tumor-targeting specificities deliver abraxane to their target sites. We describe a peptide-targeted abraxane that is more effective than untargeted abraxane in tumor treatment.

The two homing peptides used in this work both selectively target tumors, but with a very different specificity. The CREKA peptide recognizes clotted plasma proteins in tumor vessels and tumor stroma. Iron oxide nanoparticles delivered to tumors bind to the walls of tumor vessels and cause clotting in them. As this additional clotting creates new binding sites for the CREKA peptide, the homing of the nanoparticles becomes self-amplifying¹¹. The CREKA-coated abraxane nanoparticles and micelles studied also accumulated in tumor vessels and caused clotting in them as shown by co-localization of the particles with fibrin staining and trapping of red blood cells inside the clots.

Iron oxide particles, even when coated with PEG, tend to accumulate in the liver (although they do not cause any detectable clotting in the liver^{11, 24}. CREKA-abraxane or CREKA micelles showed only modest uptake into the liver. Abraxane that was modified only by FAM without a peptide was not detectable in the liver, indicating that adding the CREKA peptide causes liver binding. However, the increase obtained with CREKA coating in tumor accumulation of abraxane seemed to outweigh the slight diversion of some of the CREKA-abraxane into the liver. The LyP-1 peptide also caused some liver uptake of abraxane and micelles. The presence of basic amino acids in both the peptides might favor some liver uptake but the liver accumulation of the peptide-coated abraxane particles may not be entirely charge-based, as the net charge is +1 for CREKA and +3 for LyP-1, and the liver uptake of abraxane coated with these peptides appeared to be equal.

The distribution of LyP-1 abraxane and LyP-1 micelles within tumors was very different from that of the CREKA-coated particles. The LyP-1 particles were not detectable in blood vessels, and instead accumulated outside the blood vessels in islands of cells that were positive for the known receptor for this peptide, p3215. The vessels in these areas are primarily lymphatics, and there are few blood vessels, which may be the reason why these areas are hypoxic¹⁹. Tumor cells in these hypoxic (and apparently also nutrient-deficient) areas tend to be resistant to chemotherapeutic agents, which generally target dividing cells. There is also growing evidence indicating that these cells are genetically unstable, and a significant source of metastasis^{25–27}. Thus, the ability of LyP-1 to deliver payloads to these otherwise difficult-to-access sites opens up new possibilities in tumor therapy.

LyP-1 is a cell-penetrating peptide with activity similar to that of widely used cell-penetrating peptides such as Tat and penetratin^{28, 29}, except that LyP-1 is cell type-specific. The ability of LyP-1 to penetrate into tumor tissue and take payloads with it may be related to the cell type specificity of its binding and internalization. Non-selective cell-penetrating peptides are taken up by all cells in all tissues³⁰ and will concentrate in the cells that are in or close to blood vessels, whereas LyP-1 is not consumed by these cells and can accumulate in the specific target cells. We are also investigating the possibility that there may be an active tissue-penetrating element to the tumor homing of LyP-1.

Nanoparticles do not readily extravasate, and their penetration into tumor tissue beyond the vicinity of blood vessels is particularly problematic because of the high interstitial pressure in tumors^{1, 31}. Importantly, our results show that synthetic particles coated with LyP-1 extravasate and spread into tumor tissue. As the abraxane nanoparticles did not lend themselves to determining whether the particles were still intact when they reached the p32-rich extravascular sites, we studied this question by using mixed micelles that have the label on one amphiphile

and the peptide on another. The presence of the label at the sites the peptide homes to indicates that LyP-1 can deliver intact micelles and other nanoparticles to extravascular sites. Synaptic targeting may make it possible to change the pharmacokinetics of a drug such that more of the drug is delivered into the tumor, rendering it more effective and thereby reducing the toxicity. We did not perform toxicity studies, but our targeted abraxane nanoparticles seem to fulfill two of these expectations: non-targeted, FAM-labeled abraxane was detectable as a fluorescent network in tumor tissue, but this network was stronger when FAM-LyP-1-abraxane was injected, and p32-rich islands were intensely positive.

Our tumor treatment results with the 3 abraxane compounds were in good agreement with the bio-distribution results. The CREKA compound was not significantly different from non-targeted abraxane. In contrast, LyP-1-abraxane improved the efficacy of abraxane in a statistically highly significant matter ($p = 0.001$ and 0.013 in two independent experiments; combined $p = 0.007$). The reason for the difference may be that, although the accumulation of CREKA-abraxane in tumor blood vessels was impressive, the diffuse spread into the tumor was similar to unmodified abraxane. The accumulation in the blood vessels alone may not be sufficient to exert an enhanced anti-tumor effect compared to untargeted abraxane. However, there was an indication that CREKA-abraxane could have been slightly more active than non-targeted abraxane. Future studies will explore the possibility LyP-1 abraxane and CREKA-abraxane might synergize, as the fact that the two peptides deliver abraxane to largely non-overlapping sites in tumor tissue suggests that combining them should result in a broader distribution of the drug than either one alone. At this time, our results indicate that LyP-1-abraxane is a promising compound for tumor treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Steven Howell for discussions and comments on the manuscript. We also thank Drs. Kazuki Sugahara, Tambet Teesalu for their help and discussions on various occasions and Dr. Valentina Fogal for p32 antibody.

This work was supported by National Cancer Institute grants CA119335, CA124427, CA115410, CA104898 and, in part, National Heart, Lung and Blood Institute grant HL080718 and MRSEC Program of the National Science Foundation under Award DMR05-20415.

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Figure 1. Localization of CREKA-abraxane in tumor tissue

Balb/c nude mice bearing MDA-MB-435 tumors ($\sim 0.5 \text{ cm}^3$) were intravenously injected with abraxane conjugated to labeled CREKA peptide (CREKA-abraxane) or to fluorescein (FAM-abraxane), and adjusted to 20 mg/kg of paclitaxel equivalent. The mice were sacrificed by perfusion through the heart 3 hours later and tissues sections were examined for fluorescence (green). CREKA-abraxane accumulates in tumor blood vessels (A; anti-CD31, red) and co-localizes with anti-fibrin(ogen) (red) staining (B). FAM-abraxane showed some accumulation in tumor interstitium (C). Nuclei were counterstained with DAPI (blue). The results are representative of three independent experiments. Magnification: 200x.

Figure 2. Localization of LyP-1-abraxane in tumor tissue

LyP-1-abraxane was injected into nude mice bearing MDA-MB-435 tumors as described in the legend of Figure 1. LyP-1-abraxane (green) co-localizes with p32 (red), the receptor for LyP-1 (A). Cell clusters positive for LyP-1-abraxane are interspersed with podoplanin-positive structures (red), presumed to be lymphatic vessels (B), but these areas are mostly devoid of blood vessels (C). LyP-1-abraxane exhibits increased accumulation in extravascular tissue compared to FAM-abraxane (D, E, F). Blood vessels were stained with anti-CD31 and lymphatic vessels with anti-podoplanin. Nuclei were counterstained with DAPI. The results are representative of three independent experiments. Magnification: 600x (A & B), 200x (C, D & E). Quantification of fluorescence (F) in tumor micrographs (FAM-abraxane, CREKA-abraxane and LyP-1-abraxane) and liver micrographs (LyP-1-abraxane) was performed using Image J (NIH, USA). Five random fields were quantified per tumor and liver for LyP-1-abraxane (n=3), four random fields per tumor for CREKA-abraxane (n=3) and five random fields per tumor for FAM-abraxane (n=3). Error bars represent S.E.M.

Figure 3. Rapid extravasation of LyP-1 phage

Nude mice with MDA-MB-435 tumors were intravenously injected with 100 ml of 2×10^{10} plaque forming units (p.f.u.) of T7 phage displaying 415 copies of LyP-1 peptide (Cys-Gly-Gln-Lys-Arg-Thr-Arg-Gly-Cys) (A and B) or CG7C peptide (Cys-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Cys) (C) on the phage capsid protein. The mice were sacrificed 15 minutes post-injection by perfusion with PBS and tissues were collected for histology. The T7 phage was detected by immunostaining with polyclonal rabbit anti-T7. Blood vessels were stained with anti-CD31 and nuclei were counterstained with DAPI. The images are representative of 2 independent experiments. Scale bar: $50 \text{ }\mu\text{m}$

Figure 4. Peptide- targeted micelles are delivered intact to tumor tissue

Nude mice bearing MDA-MB-435 tumors ($\sim 0.5 \text{ cm}^3$ in diameter) were intravenously injected with $100 \mu\text{l}$ of 1 mM solution of DSPE-PEG₂₀₀₀-FAM-CREKA micelles (A), DSPE-PEG₂₀₀₀-FAM-LyP-1 micelles (B), DSPE-PEG₂₀₀₀-FAM micelles (C), mixed CREKA micelles, (D) or mixed LyP-1 micelles (E). (The mixed micelles were prepared from DSPE-PEG₂₀₀₀-CREKA (unlabeled) and DSPEPEG₂₀₀₀-FAM or DSPE-PEG₂₀₀₀-LyP-1 (unlabeled) and DSPE-PEG₂₀₀₀-FAM). The mice were sacrificed 3 hours post-injection by perfusion through heart with PBS and tissues were collected for histology. Blood vessels were visualized by staining with anti-CD31 (red). Nuclei were counterstained with DAPI (blue). The images are representative of 3 experiments. Magnification: 200x (A, B, C, E). Scale bar: $50 \mu\text{m}$ (D).

Figure 5. Effect on tumor growth by LyP-1-abraxane conjugate

Nude mice bearing MDA-MB-435 xenograft tumors were treated with LyP-1-conjugated abraxane, and unmodified abraxane, free LyP-1 peptide, or saline four times a week for 3 weeks (at a paclitaxel equivalent of 3 mg/kg/day for LyP-1-abraxane and unmodified abraxane). The total cumulative dose was 30 mg/kg. LyP-1 peptide was used at a dose equivalent to what was injected on the particles. There were 10 mice per group and the treatment started when the mean tumor volume for each group was about 100 mm³. Two independent experiments were performed and gave similar results; one is shown here. LyP-1-abraxane was significantly more effective in inhibiting tumor growth than unmodified abraxane ($p = 0.013$), LyP-1 alone ($p < 0.01$) and saline ($p < 0.01$). Error bars represent S.E.M.