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Design of a Tumor-Homing Cell-Penetrating Peptide

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Chemotherapy is often limited by toxicity to normal cells. Therefore, an ideal anticancer drug should discriminate between normal tissue and tumors. This would require a target receptor molecule mostly present in tumors. The cyclic peptide cCPGPEGAGC (PEGA) is a homing peptide that has previously been shown to accumulate in breast tumor tissue in mice. PEGA peptide does not cross the plasma membrane *per se*; however, when attached to the cell-penetrating peptide *p*VEC, the conjugate is taken up by different breast cancer cells *in vitro*. Additionally, the homing capacity of the PEGA-*p*VEC is conserved *in vivo,* where the conjugate mainly accumulates in blood vessels in breast tumor tissue and, consequently is taken up. Furthermore, we show that the efficacy of the anticancer drug, chlorambucil, is increased more than 4 times when the drug is conjugated to the PEGA-*p*VEC chimeric peptide. These data demonstrate that combining a homing sequence with a cell-penetrating sequence yields a peptide that combines the desirable properties of the parent peptides. Such peptides may be useful in diagnostics and delivery of therapeutic agents to an intracellular location in a specific tumor target tissue.

INTRODUCTION

Breast cancer is one of the major causes of death in developed countries, and there is a 1 in 7 risk of developing cancer during a woman's lifetime. Today, there are several choices available for treating breast cancer, including hormone therapy, aromatase inhibitors, and cytotoxins (for review, see ref (*1*)). Still, there is a need for improvement, i.e., to target the cytotoxic agents to the cancer cells in order to reduce side effects and toxicity in noncancer cells.

Blood vessels in normal individual tissues and different tumor tissues express different molecular markers (*2, 3*). In tumors, both blood and lymphatic vessels differ from their normal counterparts (*4*). Furthermore, lymphatic and blood vessels are distinguishable in different tumor types and various stages of tumor development (*5*). Numerous peptides that detect such differences have been isolated by combining *ex vivo* and *in vivo* screening of phage display libraries for tumor homing (*6–9*). One of these vascular homing peptides is a cyclic nonapeptide, cCPGPEGAGC, henceforth referred to as PEGA peptide, with a disulfide bridge between the two cysteines in the sequence (*8*). The PEGA peptide accumulates in breast vasculature as well as in premalignant breast tissue and primary breast tumors, and is thought to bind to the membrane-bound aminopeptidase P which is expressed at high levels in mouse breast tissue (*8, 10*).

Cell-penetrating peptides (CPPs)[1] are peptides which translocate the cell membrane and have the ability to transport different cargos such as oligonucleotides (*11*), plasmids (*12*), and proteins (*13*) across the cell membrane. CPPs can be divided into different classes depending on their origin (for review, see ref (*14*)); a common trait is that the CPPs are rich in basic amino acid residues. The translocation mechanism for CPPs is still not clear and might vary between the different classes of CPPs, as well as depending on cargo, and so forth; however, there is increasing evidence that endocytosis plays a significant role in the CPP uptake at least in cases where cargo molecules are attached (*15, 16*). A major drawback with most CPPs is that they are not cell-type specific.

The nitrogen mustard, chlorambucil (Cbl), is a well-known cytotoxic agent, which acts by alkylating and cross-linking the DNA, concomitantly inhibiting further cell proliferation. The effect is mediated through alkylation of guanine N7 in the major groove of DNA and subsequent interstrand cross-linking (*17, 18*). Moreover, this interstrand cross-link may induce apoptosis (*18, 19*). Cancer chemotherapy is often limited by toxicity to normal cells and tissues (for review, see ref (*20*)). Whether a drug such as chlorambucil can be administered at a dose that eliminates a tumor generally depends on the toxicity of the same dose to normal cells. An ideal anticancer drug is targeted toward cancer cells that are subsequently killed, but it has little or no effect on normal cells.

In the present study, we have designed and synthesized a breast tumor homing cell-penetrating peptide in order to provide a more effective and specific vector for anticancer drug delivery. We combined a tumor-specific vascular homing peptide cCPGPEGAGC (PEGA) sequence (*8*) with a well-studied CPP, *p*VEC (*21*), and demonstrated that the resultant PEGA-*p*VEC peptide colocalizes with the tumor vasculature marker and furthermore accumulates in tumor cells *in vivo*. We also showed *in vitro* that the efficacy of chlorambucil toward breast cancer cells is several fold higher when conjugated to PEGA-*p*VEC as compared to treatment with chlorambucil alone.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. Peptides (Table 1) were synthesized on an Applied Biosystems model 433A by solid-phase method using a small-scale (0.1 mmol) *t*-Boc strategy on *p*-methylbenzhydrylamine resin (Neosystem) generating C-terminally ami-

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[1]Abbreviations: c, cyclic; CPP, cell-penetrating peptide; DAPI, 4′,6-diamidino-2-phenylindole; Cbl, chlorambucil; FBS, fetal bovine serum; Fluo, fluoresceinyl-; PBS, phosphate buffered saline; PEGA, cCPGPEGAGC; RT, room temperature.

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Table 1. Sequences of the Studied Peptides

name	sequence
PEGA	cCPGPEGAGC-NH_2[a,b]
PEGA-*p*VEC	cCPGPEGAGC-LLIILRRRIRKQAHAHSK-NH_2[a,b,c]
*p*VEC	LLIILRRRIRKQAHAHSK-NH_2[b,c]
TP10	AGYLLGKINLKALAALAKKIL-NH_2

[a] PEGA, PEGA-*p*VEC are cyclic peptides, cyclized between the two cysteins. [b] For uptake studies, PEGA, PEGA-*p*VEC, and *p*VEC were fluoresceinyl-labeled at the N-terminus of the peptide. [c] For toxicity studies, PEGA-*p*VEC and *p*VEC were conjugated to chlorambucil at the N-terminus of the peptide.

dated peptides as reported previously (*22*). Stepwise coupling reactions were performed with *t*-Boc-protected amino acids (Neosystem), 1-hydroxybenzotriazole, *N,N′*-dicyclohexylcarbodiimide (4:4:4 equiv, 35 min, RT), followed by N-terminal deprotection of the *t*-Boc group with TFA/dichloromethane (1:1) (14 min, RT). For fluorophore labeling, the peptides were coupled N-terminally with 5(6)-carboxyfluorescein succinimidyl ester (Molecular Probes) (2 equiv) and diisopropylethylamine (2 equiv) in dimethyl sulfoxide/dimethylformamide (1:1) in the dark overnight. For chlorambucil coupling, chlorambucil (4 equiv) was coupled to the N-terminal amino group of the peptide with activation by 1-hydroxybenzotriazole and diisopropylethylamine (4:4 equiv, 30 min, RT) in dimethyl sulfoxide/dimethylformamide (1:1). After deprotection and cleavage of peptides, the disulfide bond formation between the two cysteines in the PEGA and PEGA-*p*VEC sequences was carried out by oxidation in excess of dimethyl sulfoxide overnight (*23*). Deprotection of the dinitrophenyl group was performed by treating the peptide (*p*VEC and PEGA-*p*VEC) with thiophenol:dimethylformamide (1:5) (1 h, RT), and the formyl group was removed by treating the peptide (TP10) with piperidin/dimethylformamide (1:5) (30 min, RT). Final cleavage of the peptides from the resin was performed in liquid hydrofluoric acid (1 h, 0 °C) in the presence of *p*-cresol and *p*-thiocresol. All peptides were purified by RP-HPLC (Discovery C-18 HPLC column, 25 cm, 21.2 mm, 5 μM) using a gradient of acetonitrile/water containing 0.1% TFA (50 min, 8 mL/min). The identity of the purified products was verified by matrix-assisted laser desorption–ionization time-of-flight mass spectrometer (Voyager-DE STR, Applied Biosystems). The mass spectra were acquired in positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix (Sigma-Aldrich) (10 mg/ml, 7:3 acetonitrile/water, 0.1% TFA).

Cell Culture. The human breast cancer cell lines MDA-MB-231 and MCF-7 (ATCC) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), sodium pyruvate (1 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and 1% nonessential amino acids (further denoted as complete medium) (Invitrogen). The human breast cancer cell line MDA-MB-435 was maintained in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). All cells were incubated at 37 °C in 5% CO_2.

Quantitative Cellular Uptake Studies. 150 000 cells/well were seeded two days before the experiment on a 12 well plate to get cells at 80% confluence. On the day of the experiment, the cells were washed with phosphate buffered saline (PBS) (1000 μL) and incubated with fluoresceinyl peptides (500 μL, 5 μM) in complete medium for 30 min at 37 °C. Cells were washed with PBS (2 × 1000 μL), whereafter trypsin (200 μL) was added and incubated for 5 min at 37 °C. PBS was added (1000 μL), and cells were transferred to Eppendorf tubes and centrifuged (10 min, 1000 *g*). Cells were lysed in sodium hydroxide solution (0.1 M, 300 μL) for 1 h and centrifuged (10 min, 10 000 *g*). The fluorescence (494/518 nm) was measured with SPECTRAmax GEMINI XS spectrofluorometric plate reader (Molecular Devices). The amount of internalized peptide was normalized against the amount of protein (Lowry, BioRad).

Microscopic Analysis of Cellular Peptide Uptake. MCF-7, MDA-MB-231, and MDA-MB-435 cells (20 000 cells/well) were seeded two days before the experiment on a Laboratory-Tek 8-well chamber slide (NUNC). The cells were washed with PBS (400 μL) and exposed to fluoresceinyl peptides (400 μL, 5 μM) in complete medium for 30 min at 37 °C. The cells were washed with PBS (3 × 400 μL) and visualized with an UltraView ERS Confocal Live Cell Imager microscope (Perkin-Elmer).

Toxicity Studies. Cell viability was measured using the Promega CellTiter-Glo assay according to the manufacturer's instructions. MCF-7 and MDA-MB-231 cells (20 000 cells/well) were seeded in a 48 well plate two days before the experiment. The cells were exposed to chlorambucil-conjugated peptide solution or free chlorambucil solution at different concentrations in complete media containing 1% FBS. After 3 h, complete media containing 10% FBS was added to achieve 5% FBS and then incubated for another 45 h at 37 °C. After reaching RT, CellTiter-Glo Reagent was added, the plate was incubated for 10 min at RT, and then the luminescence was measured. In the CellTiter-Glo assay, luciferin and luciferase are used to measure ATP content in the cells after incubation with peptides in different concentrations for 48 h. The ATP content is proportional to the amount of viable, metabolically active cells.

***In Vivo* Tumor Homing Analysis of Chimeric Peptides.** To produce tumors, nude BALB/c mice were orthotopically injected with 1 × 10^6 MDA-MB-435 tumor cells into the mammary fat pad. The tumor-bearing mice were used for homing analysis of peptides when the tumor diameter reached about 10 mm. Tissue distribution of fluoresceinyl-labeled peptides was studied by intravenously injecting the peptides (100 μM in 200 μL PBS) into tumor-bearing mice. The injected peptides were allowed to circulate 2 h, and the mice were perfused with 4% paraformaldehyde through the left ventricle of the heart. Tumors and various control tissues were dissected and frozen in OCT embedding medium (Tissue-Tek, Elkhart, IN). Frozen sections were prepared for immunohistological analysis and were observed under a Radiance MP confocal microscope to determine the internalization of peptides into cells *in vivo*. The immunohistochemical staining of tumor blood vessels was performed as previously described (*5, 9*). Briefly, frozen tissue sections were air-dried, fixed with cold acetone for 1 min, and washed with PBS three times. The slides were incubated with blocking buffers (PBS with 5% normal goat serum and 1% BSA) for 30 min at RT and then incubated with rat antimouse MECA-32 antibody (BD PharMingen) (1:50) overnight at 4 °C. After washing, the corresponding secondary antibody AlexaFluor-594 goat antirat or rabbit IgG (1:1000; Molecular Probes) was added and incubated for 1 h at RT. The slides were washed three times with PBS and mounted in Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

RESULTS

Selection of CPP. We tested the toxicity of two well-known CPPs, *p*VEC (*21*) and TP10 (*24*), in MCF-7 cells using a luciferase-based assay (Figure 1). TP10 shows considerable toxicity already at a concentration of 10 μM resulting in 48% of viable cells. *p*VEC exhibited toxicity first at 50 μM, where the amount of viable cells was reduced to 60%. Since *p*VEC was less toxic among the two studied CPPs, it was selected for coupling to a homing peptide sequence.

Cellular Uptake of Homing CPP *in Vitro*. The uptake of fluoresceinyl-labeled PEGA, PEGA-*p*VEC, and *p*VEC was studied by fluorescence microscopy in MDA-MB-231, MCF-7, and MDA-MB-435 cells. As expected, fluoresceinyl-*p*VEC was internalized into the tested cells. The homing peptide,

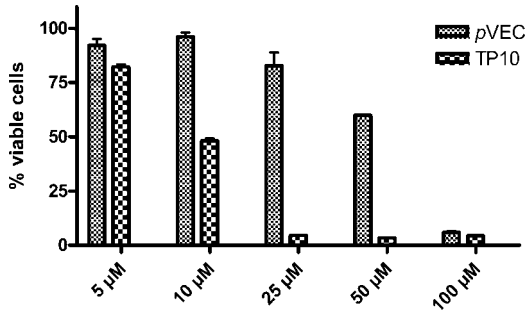

Figure 1. Effects of CPPs *p*VEC and TP10 on MCF-7 cell viability. The cells were exposed to different concentrations of peptides in media for 48 h followed by measurement of the amount of ATP (i.e., viable cells). Cells in untreated cultures were set to 100% viable. Mean ± SEM is presented.

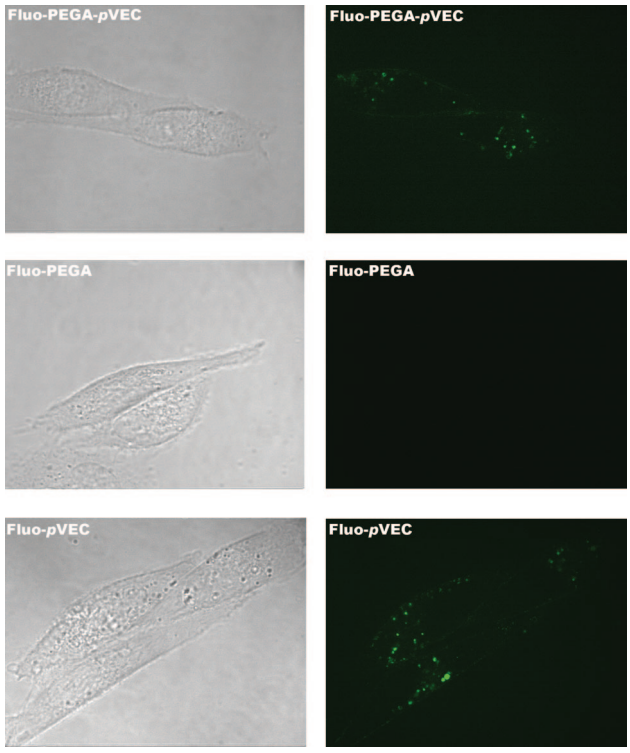

Figure 2. Detection of fluoresceinyl peptides in MDA-MB-231 cells by confocal laser scanning microscopy. Cells were incubated with 5 μM fluoresceinyl peptide, as indicated in the figure, for 30 min followed by imaging in bright field (left column) and corresponding image in fluorescence mode (green; right column).

fluoresceinyl-PEGA, alone did not enter the cells, while the conjugate, fluoresceinyl-PEGA-*p*VEC, was internalized in MDA-MB-231 (Figure 2), MCF-7, and MDA-MB-435 cells (data not shown).

Cellular uptake of the peptides was quantified by the detection of the intracellular amount of fluoresceinyl-labeled PEGA, PEGA-*p*VEC, and *p*VEC in MCF-7 and MDA-MB-231 cells. The results are presented in Figure 3 where the amount of the internalized peptide is normalized against the amount of total cellular protein in the culture well. The pattern of uptake was similar in both MDA-MB-231 cells and MCF-7 cells but slightly higher in MDA-MB-231 cells. As shown in Figure 3 for MDA-MB-231 cells, we confirmed that the homing peptide fluoresceinyl-PEGA alone did not display cell-penetrating properties; its uptake was 1 pmol/mg protein, whereas when conjugated to *p*VEC, the uptake increased to 630 pmol/mg protein. Fluoresceinyl-*p*VEC showed the highest uptake at 2400 pmol/mg protein.

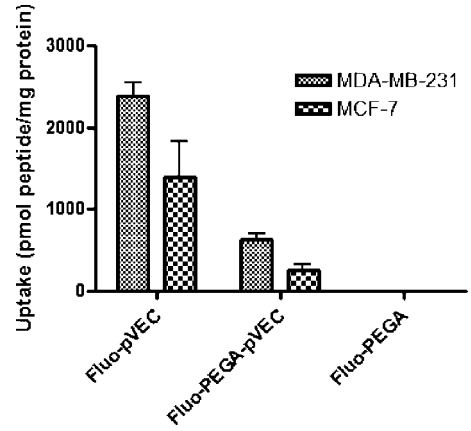

Figure 3. Uptake of fluoresceinyl peptides into MDA-MB-231 and MCF-7 cells. Cells were exposed to 5 μM fluoresceinyl peptide solution for 30 min, followed by removal of extracellular peptide by washing and trypsination of the cells. The cells were lysed in 0.1 M sodium hydroxide, and fluorescence at 494/518 nm was measured. The amount of internalized peptide was calculated on the basis of linear relation between fluorescence and concentration and normalized to the amount of protein. Mean ± SEM is presented.

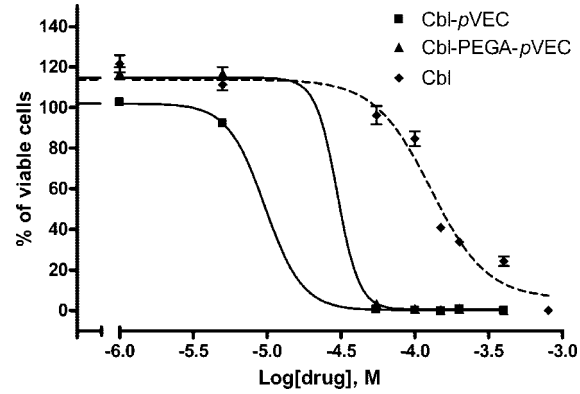

Figure 4. Cytotoxicity of chlorambucil (Cbl), Cbl-*p*VEC, and Cbl-PEGA-*p*VEC to MCF-7 cells. Cells were exposed to different concentrations of the chlorambucil-conjugated peptide solution or free chlorambucil in media for 48 h. Cells in untreated cultures were set to 100% viable. The results are presented as mean ± SEM. Data are analyzed using nonlinear regressions, *GraphPad Prism* software.

***In Vitro* Drug Activity.** In order to evaluate whether the activity of chlorambucil is improved when conjugated to PEGA-*p*VEC, we exposed chlorambucil or chlorambucil-conjugated peptides at different concentrations to MCF-7 and MDA-MB-231 breast cancer cells for 48 h. After incubation, the ATP content was measured. The IC_{50} was similar in both MCF-7 (Figure 4) and MDA-MB-231 cells (data not shown). The calculated IC_{50} for chlorambucil alone in MCF-7 cells was 128 μM, while the IC_{50} for chlorambucil conjugated to *p*VEC was 9.6 μM. When chlorambucil was conjugated to the PEGA-*p*VEC, the IC_{50} value was 30 μM. The toxicity appears to be due to the chlorambucil moiety of the conjugate, since *p*VEC alone displayed little toxicity at concentrations below 50 μM (Figure 1).

Homing Specificity of PEGA-pVEC Peptide *in Vivo*. To evaluate the homing specificity of the PEGA-*p*VEC, MDA-MB-435 tumor-bearing mice were intravenously injected with 100 μM of fluoresceinyl-PEGA-*p*VEC chimeric peptide or fluoresceinyl-PEGA or fluoresceinyl-*p*VEC as controls in 200 μL PBS. Each of the peptides was detected in tumor tissue (Figure 5), where they partially colocalized with a blood vessel specific marker MECA-32 that stained with anti-MECA-32 antibody; the colocalization was particularly prominent with fluoresceinyl-

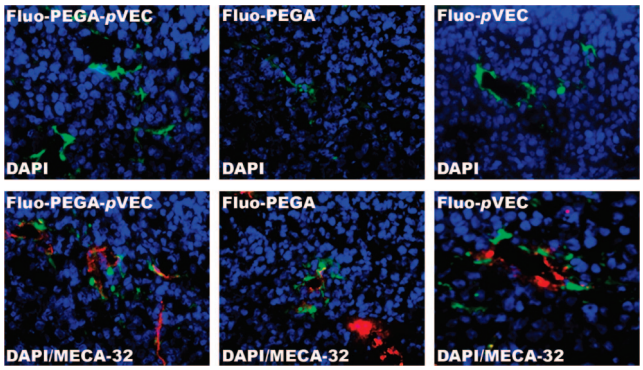

Figure 5. Localization of fluoresceinyl peptides in MDA-MB-435 tumors. The mice were injected i.v. with fluoresceinyl peptides as indicated in the figure, and tissue sections were examined for fluorescence. Fluoresceinyl peptides (green) and blood vessels stained with anti-MECA-32 antibody (red; bottom row) in tumor tissue. Anti-MECA-32 antibody is specific for vascular endothelial cells. Nuclei were detected by DAPI staining (blue).

PEGA-*p*VEC (Figure 5). At some parts, fluoresceinyl-PEGA-*p*VEC and fluoresceinyl-*p*VEC appeared to be in nuclei in the tumor cells. Apparently, the amount of fluoresceinyl-PEGA-*p*VEC in nontumor tissues was much lower than that of fluoresceinyl-*p*VEC (Figure 6). These data show that the peptide chimera combines the main desirable properties of the component peptides, specific homing to tumors and internalization into target cells.

DISCUSSION

In this report, we show, for the first time, that by combining homing and cell-penetrating peptide sequences a new effective tumor-specific peptide with the capability to translocate cargo inside target cells is obtained. Moreover, this new peptide shows considerable improvement in anticancer drug cytotoxicity.

Here, we used a cyclic homing peptide cCPGPEGAGC (PEGA) that has been previously isolated by *in vivo* phage display library screening (*8*). The PEGA peptide is not cell-penetrating *per se*; however, by conjugation it to the well-studied CPP, *p*VEC (*21*), both the cell-penetrating property of *p*VEC and the homing property of PEGA are achieved in a chimeric peptide. The quantitative and qualitative *in vitro* uptake studies in MCF-7, MDA-MB-231, and MDA-MB-435 cells (data not shown) confirmed that PEGA-*p*VEC is a CPP. There are studies of homing peptides with cell-penetrating abilities (*7, 9, 25*), but they are not extensively characterized so far.

There are different classes of CPPs depending on their origin (for review, see ref (*14*)); common for all classes is that CPPs are rich in basic amino acids. The PEGA peptide withholds no basic amino acids but a negatively charged glutamic acid, and as expected, the PEGA peptide has no CPP properties (Figures 2 and 3). However, when conjugated to the well-characterized CPP, *p*VEC, the lack of basic amino acids in the PEGA sequence is compensated with the basic residues in *p*VEC. The reduced uptake of the PEGA-*p*VEC peptide compared to *p*VEC might be a result of the reduced net positive charge of the PEGA-*p*VEC peptide in relation to *p*VEC. These results are in accordance to a CPP prediction method outlined by Hällbrink et al. (*26*) suggesting differential cell-penetrating properties of these two peptides.

The PEGA-peptide has previously been shown to accumulate in breast vasculature as well as in premalignant breast tissue and to primary breast tumors *in vivo* (*8*). We confirmed with our study that the fluoresceinyl-PEGA-*p*VEC translocates breast cancer cells and also human aortic endothelial cells *in vitro* (unpublished data).

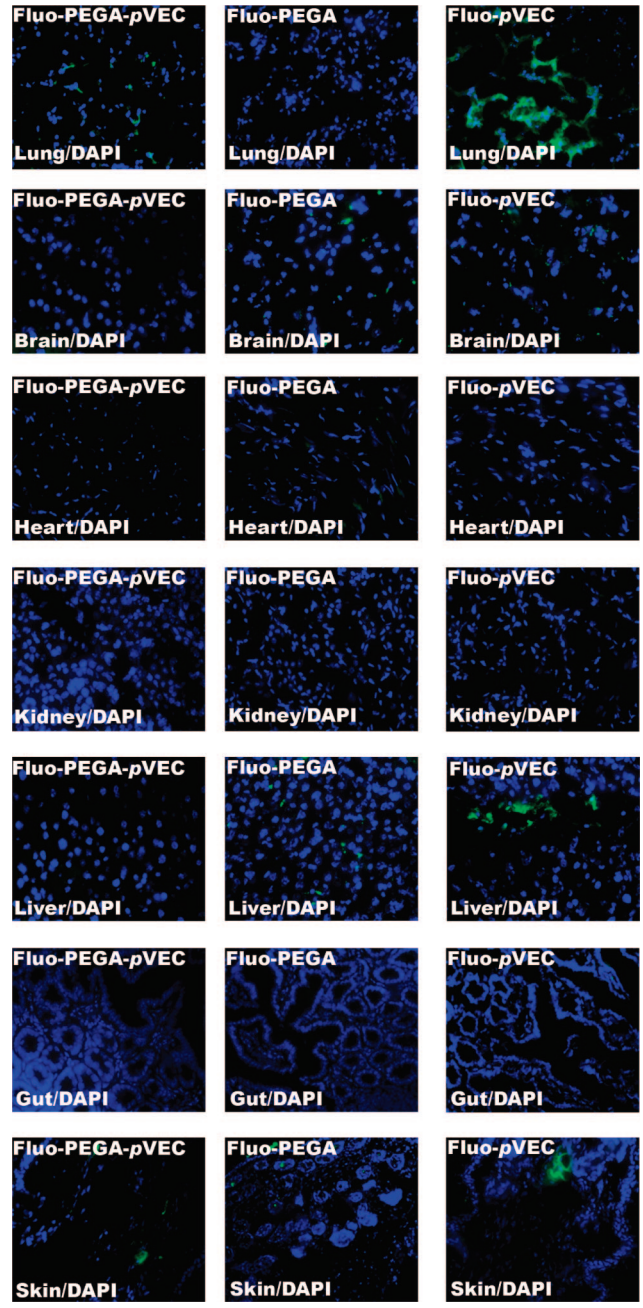

Figure 6. Distribution of fluoresceinyl peptides (green) in nontumor tissues of mice bearing MDA-MB-435 tumors. The mice were injected i.v. with fluoresceinyl-PEGA-*p*VEC (left column), fluoresceinyl-PEGA (middle column), and fluoresceinyl-*p*VEC (right column).The tissue sections, as indicated in the figure, were examined for fluorescence. Nuclei were detected by DAPI staining (blue).

The major obstacle in cancer treatment is the lack of specificity of therapeutic agents to cancer cells. It has been shown that drug therapy can be improved if the drug is targeted to tumor cells (*27, 28*). *In vivo* accumulation of fluoresceinyl-labeled PEGA-*p*VEC into the MDA-MB-435 tumor vasculature as well as tumor cells and not into control tissues suggested that our chimeric peptide could also be used for this purpose. In order to test this idea, we conjugated the DNA alkylating anticancer drug chlorambucil (*17, 18*) to PEGA-*p*VEC and studied the cytotoxicity of the conjugate in breast cancer cells. The efficacy of cytotoxicity measured as IC_{50} value of chlorambucil is lowered by more than 4-fold when the drug is conjugated to PEGA-*p*VEC (Figure 4). Chlorambucil is believed to be taken up by passive diffusion (*29*); however, that mechanism is less

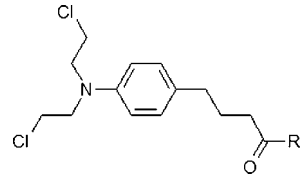

Figure 7. Chlorambucil coupled to the N-terminus of the *p*VEC and PEGA-*p*VEC (R), via an amide bond.

efficient as compared to conjugation to a CPP. *p*VEC contributes to transport of chlorambucil over the cell membrane, enhancing the efficiency of the drug. Chlorambucil is conjugated to the N-terminus of the peptides via an amide bond (Figure 7) leaving the alkylating moiety of chlorambucil available for the reaction to be able to exert its effect.

The uptake mechanism of CPPs is not yet clear and might vary among different CPPs; endocytosis seems to be one route for cellular import of CPPs (for review, see ref (*30*)). The uptake mechanism for PEGA-*p*VEC will be a subject for further studies. Preliminary experiments show that the fluoresceinyl-PEGA-*p*VEC is not colocalized with lysosomal marker (LysoTracker Red DND-99, Molecular Probes) (data not shown). In the *in vivo* studies, the fluoresceinyl peptides are injected intravenously in the mice; already after 2 h, it is possible to detect the accumulation of the peptides in the tumor cells (Figure 5). The colocalization of fluoresceinyl-PEGA-*p*VEC with the blood vessel marker, MECA-32, and the translocation of the fluoresceinyl-PEGA-*p*VEC in the breast cancer cells in the tumor, indicates that chlorambucil-PEGA-*p*VEC could be used for *in vivo* targeting of breast cancer cells as well as tumor microvascular endothelial cells.

Another possible approach for fluoresceinyl-labeled PEGA-*p*VEC is to use the peptide for imaging of tumors. This application could be useful in detection of tumors in early as well as progressed stages and for studying the response to therapy, i.e., imaging of tumor growth. Imaging of tumors and cancer cells has successfully been carried out by other homing peptides, i.e., fluoresceinyl-labeled Lyp-1 peptide *in vivo* in mice (*31*).

In summary, we have characterized a breast tumor-homing peptide, PEGA-*p*VEC, which translocates the cell membrane *in vitro* and homes to breast tumor tissue *in vivo*. We have also shown that by conjugating chlorambucil to PEGA-*p*VEC the efficacy of chlorambucil to tumor cells was increased more than 4-fold *in vitro*.

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