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A potential peptide vector that allows targeted delivery of a desired fusion protein into the human breast cancer cell line MDA-MB-231

WEI QING LIU[1], JUN YANG[2], MIN HONG[1], CHANG E. GAO[1] and JIAN DONG[1,3]

Departments of [1]Internal Medicine Oncology and [2]Oncology, First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan 650032; [3]Department of Internal Medicine Oncology, Third Affiliated Hospital of Kunming Medical University, Kunming, Yunnan 650106, P.R. China

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Abstract. Effective control of breast cancer has been primarily hampered by a lack of tumor specificity in treatments. One potential way to improve targeting specificity is to develop novel vectors that specifically bind to and are internalized by tumor cells. Through a phage display library, an 11-L-amino acid peptide, PI (sequence, CASPSGALRSC), was selected. PI was labeled with fluorescein isothiocyanate (FITC) and named PI-FITC. Subsequently, the specific affinity of PI-FITC to MDA-MB-231 human breast cancer cells and other cancer cell lines was observed by confocal microscopy. Our previous study established that PI-FITC also shows affinity to Calu-1 human lung carcinoma cells and major histocompatibility complex class I antigen molecules; therefore, the cytomembrane proteins of the cell lines were analyzed to determine those that were common to the two cell lines and may be associated with transmembrane transduction. To further test the delivery ability of PI to MDA-MB-231 cells, PI-glutathione-S-transferase (GST) was constructed and the internalization of this fusion protein was visualized by immunofluorescence microscopy. The results revealed that PI exhibited specific affinity to MDA-MB-231 cells. Use of membrane transport inhibitors indicated that macropinocytosis and caveolin-mediated endocytosis may be involved in the endocytosis of PI. In addition, 11 membrane proteins common to MDA-MB-231 and Calu-1 may be associated with transmembrane transduction. In summary, PI was able to deliver PI-GST into MDA-MB-231 cells. Thus, PI could be modified to be a potential vector, and may contribute to the development of targeted therapeutic strategies for breast cancer.

Correspondence to: Dr Jian Dong, Department of Internal Medicine Oncology, Third Affiliated Hospital of Kunming Medical University, 519 Kunzhou Road, Kunming, Yunnan 650106, P.R. China
E-mail: youngjean1982@aliyun.com

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Introduction

Human breast cancer is a malignant breast tumor that primarily affects females. Effective control of breast cancer has been hampered by a lack of specific tumor targets. One potential way to improve the specificity of targeting is to develop novel vectors that specifically bind to and are internalized by tumor cells.

In recent years, receptor-mediated endocytosis proteins, including vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2) and the Arg-Gly-Asp (RGD) motif (1-4), and protein transduction domains (including human immunodeficiency virus trans-activator of transcription protein and VP22) (5-7), have attracted considerable interest in the drug delivery field due to their ability to translocate across biological membranes. Evidence suggests that certain short peptides have promising intracellular delivery activity, and a number of these proteins have been modified and used as drug carriers in preclinical antitumor trials (8,9). However, the low cell specificity of this approach limits its application in tumor-targeted therapy (10,11). Dysregulation of oncogenes and tumor suppressor genes in tumor cells results in abnormal transcription processes, which in turn result in the expression of novel or uncovered ligands on the tumor cell surface.

Recently, Ivanenkov *et al* (12) used phage display to identify a novel peptide that showed a high affinity to HEp-2 human epithelial cells, but no affinity to other types of cells. Subsequently, peptide sequences with unique cell-type specificities have been reported. However, little attention has been focused on the potential uses of these peptides (13-15).

We hypothesized that tumor-targeting efficiency may be greatly improved with the availability of a cell membrane transduction peptides that are able to bind to tumor-specific receptors and provide a higher tumor cell internalization rate. This strategy may provide a novel addition to current

antitumor approaches. In the present study, using phage display technology (16,17), we attempted to select a tumor-targeting peptide with high cell specificity and delivery capacity. In order to study the transmembrane transduction mechanism of the peptide, the peptide was synthesized and labeled with fluorescein isothiocyanate (FITC) green fluorescence at its N-terminus. The association of the specific internalization of the peptide into MDA-MB-231 cells with macropinocytosis and caveolin- and clathrin-mediated endocytosis was investigated. Our previous study found that a lung cancer cell line, Calu-1, also demonstrated an affinity for the peptide, similarly to the human breast cancer cell line MDA-MB-231 (18); thus, the present study investigated the hypothesis that major histocompatibility complex class I (MHC-I) antigen molecules and the cytomembrane proteins of these two cell lines may also be candidate proteins that are involved in the process of transmembrane transduction of PI. MHC-I antigen-mediated transmembrane transduction was investigated, and the membrane proteins of MDA-MB-231 and Calu-1 were extracted and compared by two-dimensional (2-D) electrophoresis (19) to identify those that were common to both cell lines and may be involved in the process of transmembrane transduction of PI. Further, to investigate the delivery efficiency of PI to specific cancer cells, PI-glutathione-S-transferase (GST) was constructed and the internalization of the fusion protein was visualized by immunofluorescence microscopy.

Materials and methods

Chemicals and reagents. The pC89 phage display library of random peptides was provided by Dr Alessandra Luzzago (Integrated Research Biotech Model, Rome, Italy). The RGD-integrin was supplied by Dr Peter J. Stambrook (Department of Cell Biology, Neurobiology, and Anatomy, Vontz Center for Molecular Studies, College of Medicine, University of Cincinnati, Cincinnati, OH, USA). *Escherichia coli* BL21 (DE3) were provided by Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). The plasmid pGEX-2T was maintained in the Key Laboratory of Translational Medicine of Cell Therapy Technology of Yunnan Province (Department of Internal Medicine Oncology, First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, China). The GST agarose affinity chromatography column was purchased from GE Healthcare Life Sciences (Tokyo, Japan). The mouse anti-*Schistosoma japonicum* GST monoclonal antibody was provided by Thermo Fisher Scientific (catalog no., MA4-004-1MG; dilution, 1:500). Horseradish peroxidase (HRP)-labeled rabbit anti-mouse IgG polyclonal antibody was also supplied by Thermo Fisher Scientific (catalog no., 61-6520; dilution, 1:1,000). ProteoPrep® Membrane Extraction Kit and sodium dodecyl sulfate (SDS) were products of Sigma-Aldrich (St. Louis, MO, USA). Polyclonal mouse anti-human MHC-I antibody (catalog no., ab76795; dilution, 1:500) was purchased from Abcam (Cambridge, MA, USA). RPMI-1640 medium, Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Gibco (Thermo Fisher Scientific). The human breast cancer cell lines MDA-MB-231, MDA-MB-435 and MCF-7, and all other tumor cell lines (HeLa, A431, SCC-29, Calu-1, Calu-3, GLC and U251) were purchased from the American Type Culture Collection.

Peptide synthesis. Using the manual solid-phase Fmoc method, PI (sequence, CASPSGALRSC) (18) was synthesized to determine whether the phage-coating protein was required for internalization into target cells. For cellular localization, the synthesized peptide was labeled with FITC at the N-terminus of PI (designated PI-FITC). Purification of the crude product was applied by reverse phase high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA, USA), and identification of chemical structures was conducted by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (JMS-S3000; Bruker Daltonic, Inc., Billerica, MA, USA).

Cell culture. MDA-MB-231 cells were cultured in Leibovitz's L-15 Medium (Gibco; Thermo Fisher Scientific) containing 100 ml/l fetal bovine serum and 50 ml/l CO_2 at 37˚C. Calu-3 cells were cultured in Eagle's Minimum Essential Medium (American Type Culture Collection, Manassas, VA, USA) containing 150 ml/l fetal bovine serum at 37˚C in 50 ml/l CO_2. MDA-MB-435, HeLa, U251, MCF-7, SCC-29 and GLC cells were cultured and maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific) supplemented with 100 ml/l fetal bovine serum at 37˚C in 50 ml/l CO_2. A431 cells were cultured in DMEM-H (Gibco; Thermo Fisher Scientific) containing 150 ml/l fetal bovine serum and 50 ml/l CO_2 at 37˚C. Calu-1 cells were cultured in McCoy's 5A (modified) medium (Gibco; Thermo Fisher Scientific) containing 100 ml/l fetal bovine serum and 50ml/l CO_2 at 37˚C.

Cell-type affinity assay of PI-FITC. MDA-MB-231 cells in the logarithmic phase were grown in a 96-well tissue culture plate for 12 h, and cells were treated with PI-FITC at concentrations of 200, 500 and 1,000 ng/ml. RGD-integrin labeled with FITC was used as a control. The ability of PI-FITC to internalize into other breast cancer cells (MDA-MB-435 and MCF-7) and other solid tumor cells (HeLa, A431, SCC-29, Calu-3, GLC and U251) in the logarithmic phase was also tested by laser scanning confocal microscopy. For microscopy, the MDA-MB-231, MCF-7, HeLa, A431, SCC-29, Calu-3, GLC and U251 cells were treated with PI-FITC and RGD-integrin separately at concentrations of 200, 500 or 1,000 ng/ml. The duration of incubation with PI-FITC and RGD-integrin was 12 h. In this experiment, RGD-integrin was also labeled with FITC. Green fluorescence signals of FITC were observed by scanning confocal microscopy (Zeiss LSM 800; Carl Zeiss AG, Oberkochen, Germany).

Concentration-, temperature- and time-dependence of PI internalization to MDA-MB-231 cells. As it is possible that the affinity of PI-FITC for MDA-MB-231 may be influenced by varying PI-FITC concentrations, incubation times and temperatures, experiments were conducted to investigate the effects of these parameters. For the concentration-dependence experiment, $5x10^4$ MDA-MB-231 cells in the logarithmic phase were grown in 24-well tissue culture plates and divided into four subgroups; 0, 2, 5 or 10 μmol/l of PI-FITC was added to each subgroup, respectively. Following 1 h of incubation, cells were harvested, washed in phosphate-buffered saline (PBS; Beijing Jimei Biotechnology Co., Ltd., Beijing, China), fixed in formaldehyde (Beijing Jimei Biotechnology Co., Ltd.)

and permeabilized with Triton X-100 (Sigma-Aldrich). The PI-FITC distribution of each subgroup was observed by flow cytometry (FACSCanto II; catalog no., 338960; BD Biosciences, Franklin Lakes, NJ, USA).

For the temperature-dependence experiment, $5x10^4$ MDA-MB-231 cells in the logarithmic phase were grown in three 24-well tissue culture plates, 2 μmol/l of PI-FITC was added to each plate, and the plates were incubated for 1 h at temperatures of 4, 25 or 37˚C, respectively. Following incubation, flow cytometry was used to observe the results. MDA-MB-231 cells without PI-FITC were used as a control.

In the time-dependence experiment, $5x10^4$ MDA-MB-231 cells in the logarithmic phase were grown in three 24-well tissue culture plates, 2 μmol/l of PI-FITC was added to each plate, and the plates were incubated for 1, 6 or 12 h. A plate containing MDA-MB-231 cells without PI-FITC was used as a control, and flow cytometry was applied to analyze the results.

Transmembrane transduction inhibition analysis. To investigate whether the transmembrane transduction mechanism of PI was associated with macropinocytosis or caveolin- or clathrin-mediated endocytosis, three inhibitors of cytomembrane transport, consisting of amiloride (Sanofi China, Hangzhou, China) (19), methyl-β-cyclodextrin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) (20) and chlorpromazine (Amresco, Cleveland, OH, USA) (21) were incubated with MDA-MB-231 cells. An MTT assay (Sigma-Aldrich) was used to determine the toxicity of these membrane channel inhibitors to MDA-MB-231 cells.

For each experiment, $5x10^4$ MDA-MB-231 cells in the logarithmic growth phase were grown in 24-well tissue culture plates and equally divided into three subgroups, designated A, B and C; cells in each subgroup were treated with PI-FITC, PI-FITC plus an inhibitor (amiloride, methyl-β-cyclodextrin or chlorpromazine), or normal saline, respectively. The distribution of PI-FITC in MDA-MB-231 cells in each experimental group was subsequently investigated by flow cytometry.

MHC-I antigen analysis. There are 250,000 molecules of each type of human leukocyte antigen (HLA) on the surface of each human cell (22). Our preliminary studies indicated that the human lung cancer cell line Calu-1 had high affinity to PI, similarly to MDA-MB-231 cells. Therefore, the present study compared the MHC-I antigen molecules of these two cell lines and further analyzed whether the MHC-I antigen molecules may be involved in transmembrane transduction. PI-FITC was added to the experimental cultural system of MDA-MB-231 cells and positive cultural system of Calu-1 cells, and both were incubated with an anti-MHC-I antibody. After the MHC-I molecules on the cell surfaces were blocked by the anti-MHC-I antibody, the distributions of PI-FITC in these two cell types were detected under fluorescence microscopy (DM-IL; Leica, Wetzlar, Germany). DNA from MDA-MB-231 and Calu-1 cells were extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. HLA-A and -B DNA fragments were amplified and sequence-specific primer (SSP)-polymerase chain reaction (PCR) was applied to analyze the MHC-I antigen molecules. The MHC-I antigen molecules were analyzed using the AB/DR/DQ SSP UniTray-96 kit from Texas BioGene, Inc. (Richardson, TX, USA). PCR was performed for 21 cycles as follows: Denaturation at 96˚C for 25 sec; annealing at 65˚C for 50 sec; and extension at 72˚C for 45 sec. Extension was then performed at 72˚C for another 10 min. Separation and determination of PCR products were conducted by electrophoresis. The ProFlex PCR machine was provided by Applied Biosystems (Thermo Fisher Scientific).

Membrane protein analysis. To investigate whether the transmembrane transduction mechanism of PI is associated with the same membrane proteins in the MDA-MB-231 and Calu-1 cell lines, the membrane proteins were extracted from the two types of cell using the ProteoPrep Membrane Extraction Kit, according to the instructions, and were compared by 2-D electrophoresis (23). The protein profiles of the two cell lines were analyzed by PDQuest Software for 2-D Gel Analysis, version 7.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), with the help of the Institute of Medical Biology, Chinese Academy of Medical Sciences (Beijing, China).

Construction and expression of the recombinant GST fusion protein PI-GST. The encoding region (5'-TGCGCATCCCCA TCTGGCGCCCTTCGTTGTTGC-3') of PI was synthesized with the addition of a *Bam*HI site upstream and an *Eco*RI site downstream by Takara Bio Inc. (Otsu, Japan). The synthesized product was ligated into the pGEX-2T plasmid at the *Bam*HI and *Eco*RI sites. The insert, cut with the restriction enzymes (Thermo Fisher Scientific), was subject to SDS-polyacrylamide gel electrophoresis (PAGE) and was identified by DNA sequence analysis. The *Escherichia coli* strain BL21 (DE3) was transformed with the recombinant plasmid pGEX-2T-PI or vector alone using the $CaCl_2$ transformation method (24) and then grown in a lysogeny broth solution (Sigma-Aldrich) containing 100 mg/ml of ampicillin (Amresco) at 37˚C. Isopropyl-β-D-thiogalactopyranoside (IPTG; Amresco), an inducer of β-galactosidase activity in bacteria, was added to a final concentration of 1 mM, and the solution was incubated at 37˚C for 1-6 h. Following IPTG induction, bacterial pellets were obtained by centrifugation at 2,000 x g at 4˚C.

Western blot analysis of PI-GST. Fusion protein products expressed in bacterial pellets were released by ultrasonic membrane rupture and centrifuged at 5,000 x g at 4˚C. The supernatant was collected and purified by GST affinity column. One-dimensional SDS-PAGE was performed using 12.5% (wt/vol) polyacrylamide gels. For immunoblotting, proteins were transferred from polyacrylamide gels to Immobilon™ polyvinylidene difluoride membranes (Sigma-Aldrich) using Tris-glycine electroblotting buffer (Sigma-Aldrich) at 15-20 mA overnight. The membranes were blocked with 5% evaporated skimmed milk for 30 min at 37˚C to prevent non-specific binding. The membranes were then incubated with the primary mouse anti-GST monoclonal antibody (dilution, 1:500) overnight at 4˚C and with the secondary HRP-conjugated goat anti-mouse IgG (dilution, 1:1,000) for 30 min at 37˚C. The expressed product was identified by treating with dextran sulfate and 4-methylbenzamide for ~5 min.

Purification of PI-GST. The fused protein products in the supernatant fraction were purified on GST-sepharose columns. Purification was conducted as described by the manufacturer. Briefly, 100 ml of sample was centrifuged to remove any

undissolved membranes and cellular debris before being added to the column. Triton X-100 was then added to the collected supernatants. The column was washed with 5-10 bed volumes of PBS to remove azide. The gel bed was equilibrated with 3-5 bed volumes of PBS containing 1% Triton X-100. Subsequently, the sample was added to the prepared column. The flow-through was collected as a control. The column was washed with 10 bed volumes of PBS until no protein could be detected in the elution. The setup included sufficient Eppendorf tubes for a standard curve with 0, 5, 10, 15, 20 and 25 g of bovine serum albumin (BSA) and the samples to be tested. The harvest was subject to western blotting for identification, as described.

Transduction activity of fusion protein. MDA-MB-231 cells were grown in 24-well tissue culture plates at 37˚C for 12 h. Cells were incubated with PI-GST at 20, 50, 100, 200 or 500 ng/ml for 8-12 h at 37˚C. The cultured cells were fixed in 10% Triton X-100 for 10 min and treated with buffer (1% BSA, 0.025% NaN_3, 0.1% saponin) for 15 min. The GST monoclonal antibody (dilution, 1:800) was added to the cells for 30 min at 4˚C. The cells were then incubated with HRP-labeled rabbit anti-mouse IgG antibody (dilution, 1:1,000) for 30 min at 37˚C, subsequent to washing the cells 3 times. Internalization was visualized by immunofluorescence microscopy (DM-IL; Leica). MDA-MB-231 cells incubated with GST were used as a blank control.

Results

Internalization assay of synthesized peptides in MDA-MB-231 and other cell lines. The green fluorescence signal of PI-FITC was easily detectable in each MDA-MB-231 cell after 12-48 h of incubation, indicating that PI-FITC was efficiently taken up by MDA-MB-231 cells; the internalized PI was predominantly located in the cytoplasm or around the nuclear membrane. No green fluorescence signal could be observed in the MDA-MB-231 cells without PI-FITC (Fig. 1). Compared with RGD-integrin labeled with FITC, the internalization activity of PI in MDA-MB-231 cells was similar (Fig. 2). In contrast to MDA-MB-231 cells incubated with PI-FITC, no fluorescence signal was observed in the other breast cancer cell lines, MDA-MB-435 and MCF-7 (Fig. 3), and the synthesized peptides exhibited no affinity to the other types of tumor cell, HeLa, A431, SCC-29, Calu-3, GLC and U251 (Table I).

Concentration-, temperature- and time-dependence of PI internalization to MDA-MB-231. Flow cytometry results revealed an increase in cell-associated fluorescence with increasing concentration of PI, indicating that the concentration of PI is associated with its internalization (Fig. 4A); both the number of MDA-MB-231 cells and the concentration of PI-FITC were influencing factors on the internalization of PI-FITC. However, the incubation temperature and time had little influence on the internalization of PI (Fig. 4B and C)

Membrane channel inhibition experiments. Following the addition of amiloride, methyl-β-cyclodextrin and chlorpromazine to the MDA-MB-231 cells, MTT results indicated that these three channel inhibitors had no significant toxicity to the growth of MDA-MB-231 cells (Fig. 5). In the amiloride and methyl-β-cyclodextrin experimental groups, flow cytometry results suggested that the distribution of PI-FITC in MDA-MB-231 cells of each group had decreased (Fig. 6A and B). However, in the chlorpromazine group, no significant change in intracellular distribution was observed (Fig. 6C).

Table I. Results of an internalization assay of the synthesized peptide, PI, into various cell lines.

Cell line	Fluorescence signal of PI-FITC
MDA-MB-231	+
MDA-MB-435	-
MCF-7	-
Hela	-
A431	-
SCC-29	-
Calu3	-
GLC	-
U251	-

PI, peptide (CASPSGALRSC); FITC, fluorescein isothiocyanate.

Table II. Analysis of the alleles of HLA-I in the MDA-MB-231 and Calu-1 cell lines.

MDA-MB-231		Calu-1	
HLA-A	HLA-B	HLA-A	HLA-B
A*02:01	B*41:01	A*26:01	B*44:03
A*02:02	B*41:03	A*26:02	B*44:04
A*02:03	B*41:05	A*26:08	B*44:05
A*24:02	B*40:02	A*29:01	B*15:01
A*24:03	B*40:03	A*29:02	B*15:04
A*24:04	B*40:04	A*29:03	B*15:27

Six alleles of HLA-A site were found in MDA-MB-231 and Calu-1 cells, and six alleles of the HLA-B site were also found in MDA-MB-231 and Calu-1 cells. Among all these alleles, no similar allele was found. HLA, human leukocyte antigen.

MHC-I antigen analysis. After the MHC-I antigen had been blocked in the MDA-MB-231+PI-FITC and Calu-1+PI-FITC culture systems, the distributions of PI-FITC in the cells of the two culture systems were consistent with the control groups without MHC-I antibody. DNA samples of MDA-MB-231 cells and Calu-1 cells were detected by PCR-SSP. The results revealed that none of the same MHC-I antigen molecules could be detected. This indicated that the MHC-I antigen molecules of the two cell lines may not be involved in the process of transmembrane transduction of PI (Table II).

Membrane protein comparison by 2-D electrophoresis map. Following the extraction of membrane proteins from the two cell lines, 2-D electrophoresis of each sample was repeated four times, and the protein profiles of the two cell types were

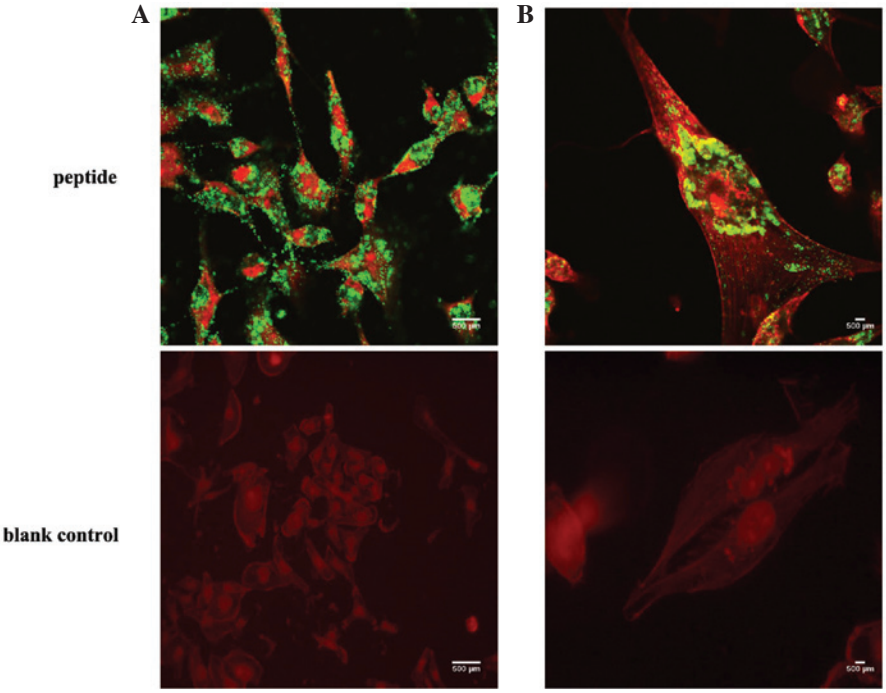

Figure 1. Internalization of PI-FITC in MDA-MB-231 cells observed by laser scanning confocal microscopy. The upper panels show MDA-MB-231 cells treated with PI-FITC. The lower panels show MDA-MB-231 cells without PI-FITC. MDA-MB-231 cells were observed as red fluorescence, and the green fluorescence represents the signal from PI-FITC. (A) x200 and (B) x600 magnification. PI, synthesized peptide (CASPSGALRSC); FITC, fluorescein isothiocyanate.

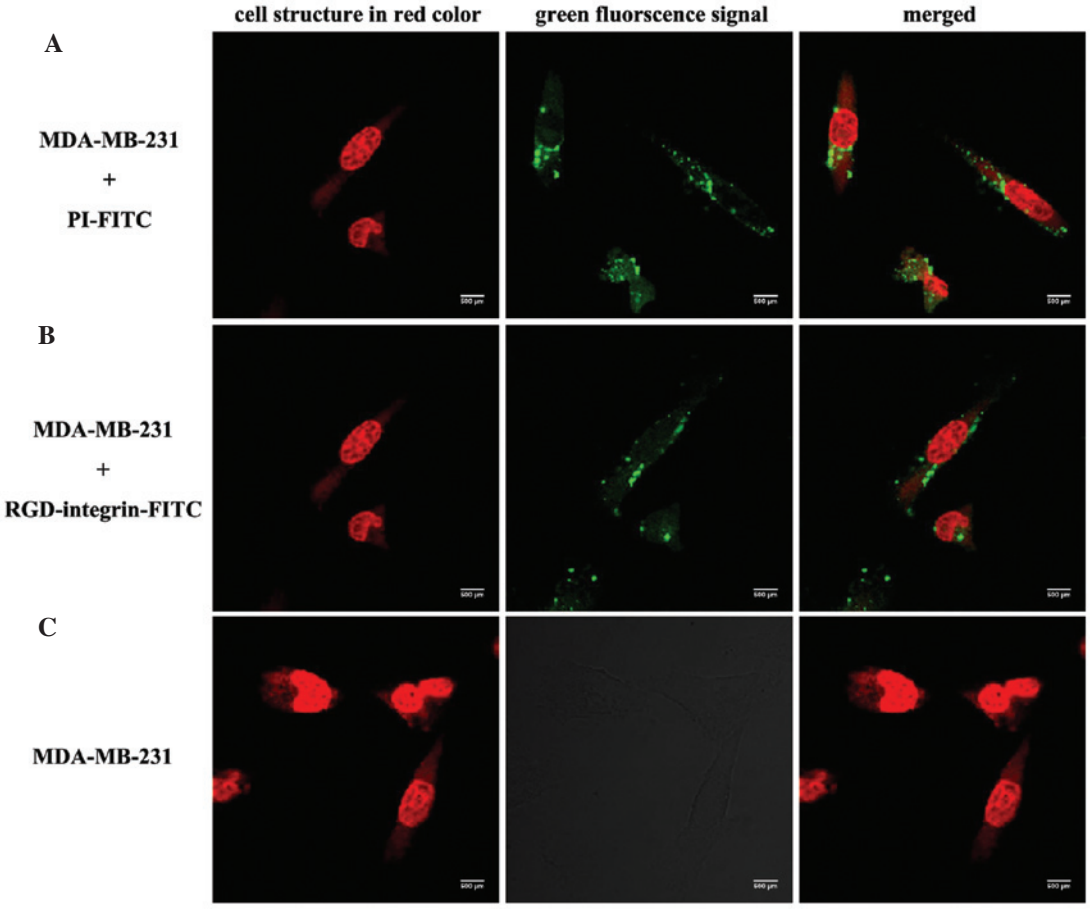

Figure 2. Cell-type affinity assay of PI-FITC observed by laser scanning confocal microscopy (magnification, x200). Left images show representative MDA-MB-231 cells in red. Middle images show green fluorescence signal of FITC. Right images show the merged picture of the cells and the green fluorescence. (A) MDA-MB-231 cells incubated with PI-FITC; (B) MDA-MB-231 cells incubated with RGD-integrin-FITC; (C) MDA-MB-231 cells only (no green fluorescence signal was detected). PI, synthesized peptide (CASPSGALRSC); FITC, fluorescein isothiocyanate; RGD, Arg-Gly-Asp motif.

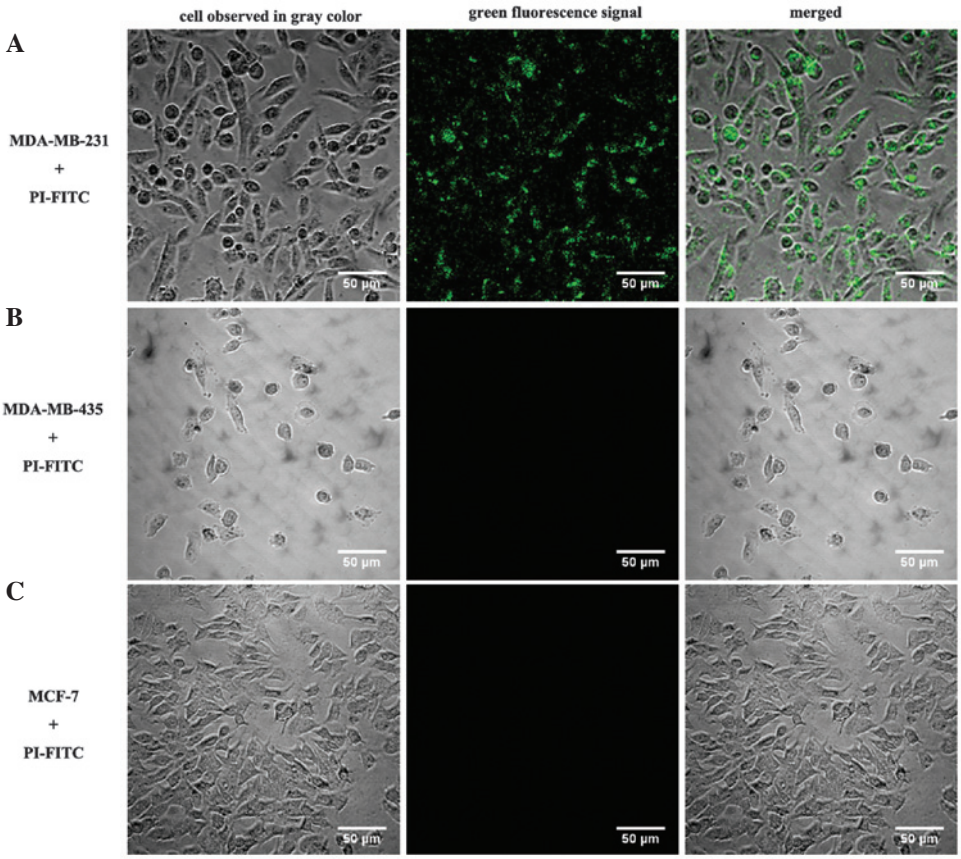

Figure 3. Cell-specific affinity analysis of PI-FITC with various human breast cancer lines under laser scanning confocal microscopy (magnification, x40). Left images show representative areas of MDA-MB-231 cells in gray; middle images show representative areas of FITC green fluorescence; right panels show the merged images of the cells and the green fluorescence. (A) MDA-MB-231 cells incubated with PI-FITC; (B) MDA-MB-435 and (C) MCF-7 cells incubated with PI-FITC (no green fluorescence was observed). PI, synthesized peptide (CASPSGALRSC); FITC, fluorescein isothiocyanate.

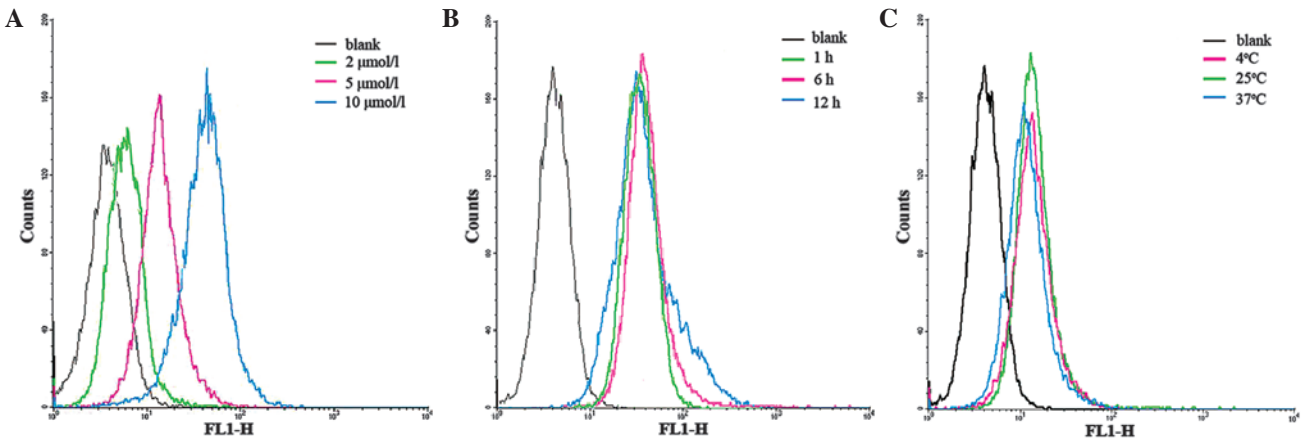

Figure 4. Concentration-, temperature- and time-dependence of PI internalization to MDA-MB-231 cells. (A) Distribution of PI-FITC in MDA-MB-231 cells following incubation with various concentrations of PI-FITC; results indicated an increase in cell-associated fluorescence suggesting that the concentration of PI is associated with its internalization. (B) Distribution of PI-FITC in MDA-MB-231 cells following incubation with PI-FITC for various durations; the internalization of PI-FITC in MDA-MB-231 cells was similar following 1, 6 and 12 h of incubation. (C) Distribution of PI-FITC in MDA-MB-231 cells after different incubation temperatures; the internalization of PI-FITC in MDA-MB-231 cells was similar after incubating at 4, 25 and 37˚C. PI, synthesized peptide (CASPSGALRSC); FITC, fluorescein isothiocyanate.

analyzed by PDQuest Software for 2-D Gel Analysis. Around 260 protein spots of each sample were detected, and a total of 11 common protein spots were identified (Fig. 7).

Expression of PI-GST. Expression of PI-GST and GST was induced by IPTG, and crude bacterial extracts were analyzed by SDS-PAGE. Two constructs produced protein of the expected molecular mass, 26-27 kDa (Fig. 8). Western blot analysis of the crude extract indicated that the fusion protein reacted with an antibody against GST. All data demonstrated that the GST-fused protein PI-GST was present.

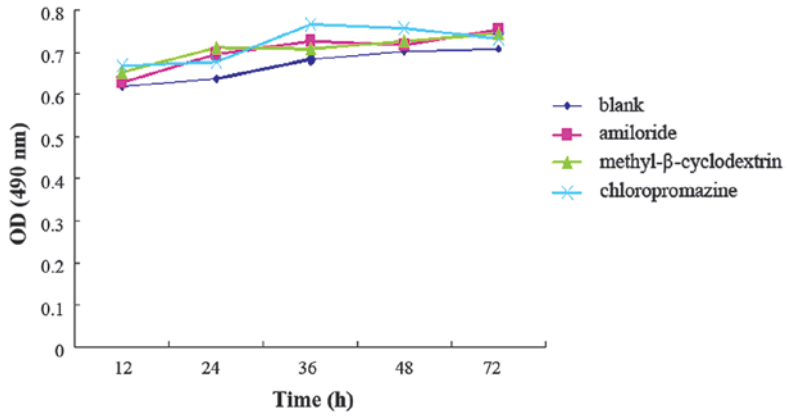

Figure 5. Growth curve of MDA-MB-231 cells treated with amiloride, methyl-β-cyclodextrin and chlorpromazine. The growth states of all the cells of the four groups were consistent, indicating that they were largely unaffected by the three substances. OD, optical density.

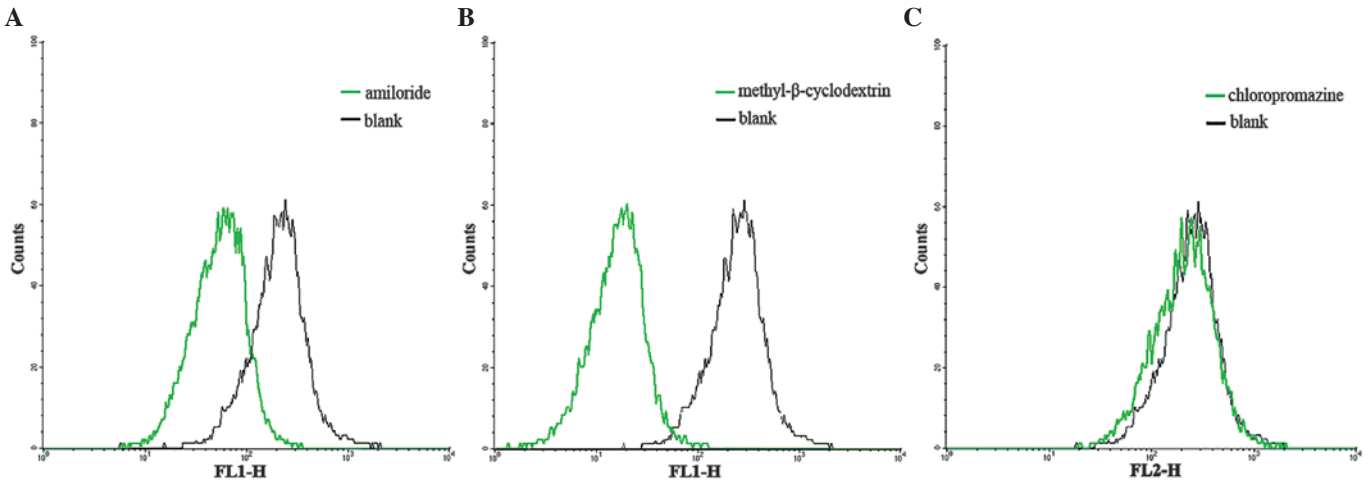

Figure 6. Membrane channel inhibition experiments. (A) Distribution of PI-FITC in MDA-MB-231 cells following addition of amiloride; compared with the MDA-MB-231+PI-FITC culture system (blank), amiloride led to a decrease in the distribution of PI-FITC in MDA-MB-231 cells. (B) Distribution of PI-FITC in MDA-MB-231 cells following addition of methyl-β-cyclodextrin; compared with the blank culture system, methyl-β-cyclodextrin led to a decrease in the distribution of PI-FITC in MDA-MB-231 cells. (C) Distribution of PI-FITC in MDA-MB-231 cells following the addition of chlorpromazine; the distribution of PI-FITC in MDA-MB-231 cells was similar in the chlorpromazine and blank groups. PI, synthesized peptide (CASPSGALRSC); FITC, fluorescein isothiocyanate.

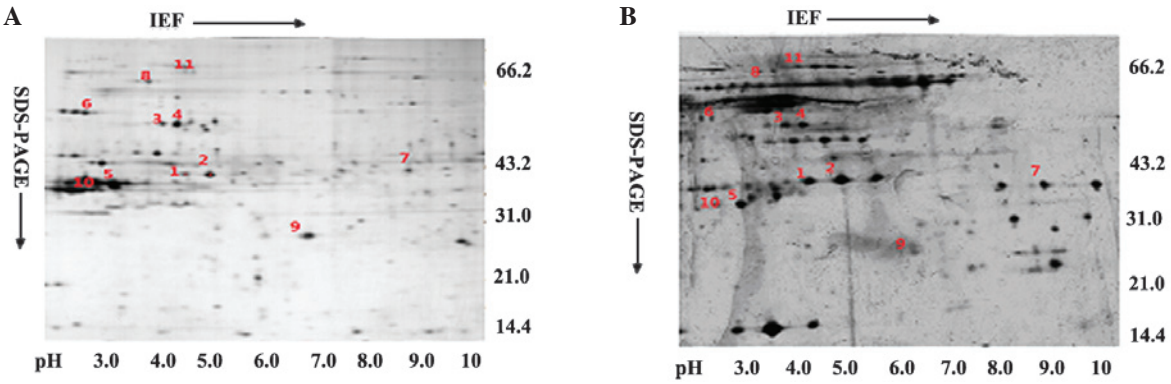

Figure 7. Membrane protein comparison by two-dimensional electrophoresis map: (A) MDA-MB-231 cells and (B) Calu-1 cells. A total of 11 common protein spots were observed. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

Delivery efficiency of fusion protein into target cells. In the presence of increasing concentrations (20-500 ng/ml) of the PI-GST fusion protein for 72 h at 37˚C, cellular viability was 95%. Cellular toxicity was not observed in the experimental cells. At 200 ng/ml, the optimal concentration of the peptide PI-GST was taken up by MDA-MB-231 cells efficiently. In the initial 12-18 h culturing period, the fluorescence intensity of PI-GST in MDA-MB-231 cells was

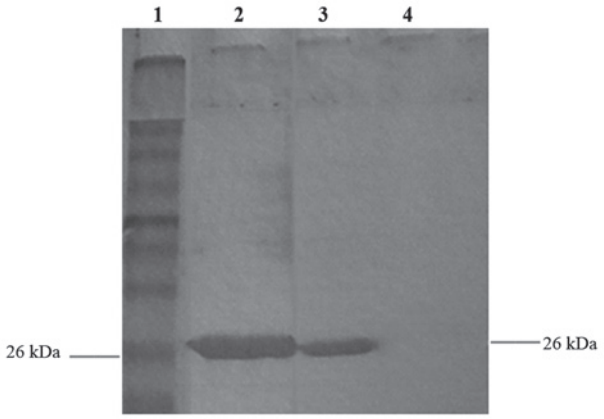

Figure 8. Western blotting of the fusion protein expressed in *E. coli* BL21 (DE3). Lane 1, marker; lane 2, GST; lane 3, PI-GST; lane 4, blank control. GST, glutathione-S-transferase; PI, synthesized peptide (CASPSGALRSC).

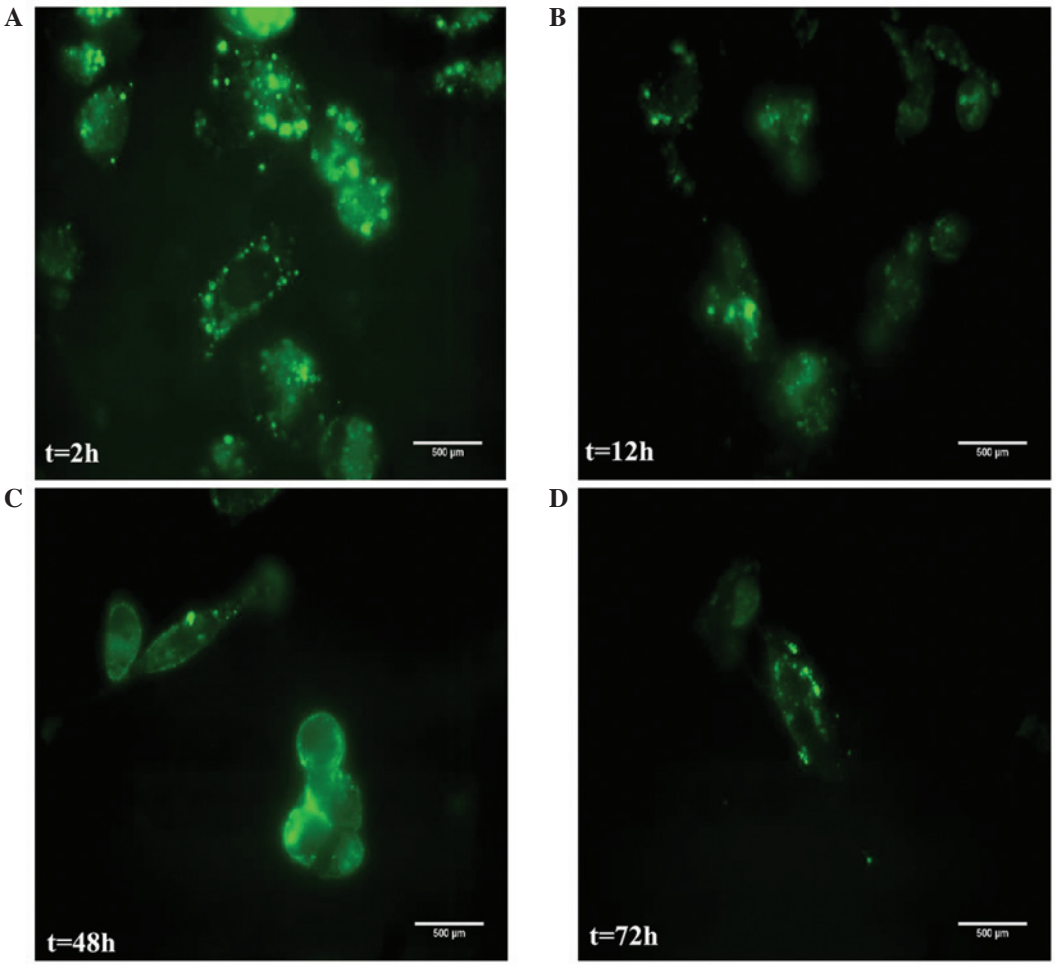

Figure 9. Dynamic imaging of the delivery efficiency of the peptide observed by fluorescence microscopy (magnification, x200). (A) MDA-MB-231 cells after 2 h of incubation with PI-GST; fluorescence microscopy indicated that PI is able to deliver GST protein into MDA-MB-231; (B) after 12 h, the fusion protein was detected in the cytoplasm; (C) 48 h and (D) 72 h later, the signal of the fusion protein in the majority of cells became weak and eventually disappeared. PI, synthesized peptide (CASPSGALRSC); GST, glutathione-S-transferase.

as strong as that observed for PI itself (Fig. 9A); a strong intracellular PI-GST signal was observed in the cytoplasm after 12 h (Fig. 9B). After 48-72 h, the signal of the fusion protein in the majority of the cells became weak and disappeared (Fig. 9C and D).

Discussion

Breast cancer is the most commonly diagnosed cancer in women and is the second leading cause of cancer-associated mortality (25). The risks of local recurrence, metastasis and

drug resistance of tumors remain difficult to overcome and control (26).

Recent targeted and biological therapies reflect novel and promising improvements in the treatment of breast cancer; for example, drugs including trastuzumab, bevacizumab, gefitinib and celecoxib have been designed to focus on identified tumor-associated targets and have demonstrated promising results in clinical practice (27-32). However, these therapeutic targets are expressed in numerous tissues, suggesting that the cellular specificity is extremely low and may cause potential side effects (33,34). Therefore, the development of agents with cancer cell selectivity is urgently required to improve targeted therapy.

It has been reported that use of the phage display system allows selection of peptide sequences with unique cell-type specificity (35,36). We hypothesized that a breast cancer-specific peptide may also be screened through the phage display system. The strategy adopted for the current study was to screen a breast cancer-specific peptide, evaluate whether it has potential as a specific delivery system for tumor-targeting therapy, and investigate the delivery mechanism.

The synthesized PI peptide (CASPSGALRSC) was incubated with MDA-MB-231 cells in order to investigate it as a peptide with tumor cell specificity. To improve comparability, RGD-integrin, which is known to be recognized and internalized by various types of human cells (37,38), was set as a control. The synthesized PI exhibited no affinity to other cancer cell lines MDA-MB-435, MCF-7, HeLa, A431, SCC-29, Calu-3, GLC and U251. These affinity experiments suggested that PI was a novel type of transmembrane peptide with MDA-MB-231 cell specificity. Moreover, PI has no sequence similarity to RGD-integrin or to any other protein sequence available in various protein databases.

To further analyze the transduction mechanism of PI, three inhibitors of cytomembrane transport (amiloride, methyl-β-cyclodextrin and chlorpromazine) were used to investigate whether the specific internalization of the peptide into MDA-MB-231 cells is associated with macropinocytosis, caveolin-mediated endocytosis or clathrin-mediated endocytosis, using flow cytometry. The results revealed that the transduction of the peptide into the cell is partially mediated by macropinocytosis and caveolin-mediated endocytosis. Our initial study established that the lung cancer cell line Calu-1 also demonstrated high affinity to the peptide, similarly to MDA-MB-231 (39). Thus, we hypothesized that MHC-I antigen molecules may mediate transmembrane transduction, and the cytomembrane proteins of the two cell lines may be involved in the process of transmembrane transduction of PI. In the present study, MHC-I antigen molecules and the cytomembrane proteins of the two cell lines were investigated. The results revealed that MHC-I antigen molecules of MDA-MB-231 and Calu-1 share no common alleles, which indicated that these MHC-I antigen molecules have no association with the transmembrane transduction of PI. The results of 2-D electrophoresis identified 11 proteins spots that were common to MDA-MB-231 and Calu-1 cells, which may also be candidates for the transmembrane transduction of PI.

Given the notable cellular specificity of PI, its use as a vector for a therapeutic protein delivery may be a practical way to improve targeting efficiency in tumor therapy. To investigate the ability of PI to deliver a potential therapeutic protein, GST was introduced to represent an exogenous protein. GST was fused with PI and was used as a marker to assess the protein transduction ability of PI *in vitro*. PI was observed to be capable of successfully delivering GST into the cytoplasm of MDA-MB-231 cells, and the exogenous protein did not degrade for ≥72 h. Significant immunofluorescence signals for PI-GST were observed in the MDA-MB-231 cell cytoplasm, while no GST signal was observed in MDA-MB-231 cells. Thus, it may be concluded that PI is able to deliver an exogenous protein of at least 26 kDa into MDA-MB-231 cells. This transduction procedure is also cell-specific, which was confirmed by treating different human breast cancer cell lines with PI-GST. Additionally, PI and PI-GST did not lead to any cytotoxic effects when the cells were maintained in culture medium for 72 h. Thus, the current research shows that PI has the necessary features to be an efficient drug delivery vector for targeted cancer therapy.

Taken together, our studies may lead to the discovery of a novel tumor cell-specific receptor and provide a new therapeutic target for cancer treatment and diagnosis (40). Such a peptide may serve as a specific vehicle for tumor-targeting therapy and may complement other molecular therapeutic approaches for localized and systemic application. Further research is required in this area.

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