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Phenotypic Knockout of CXCR4 by a Novel Recombinant Protein TAT/54R/KDEL Inhibits Tumors Metastasis

Wei-Feng Ma,[1] Jun Du,[2] Liu-Peng Fu,[3] Rui Fang,[2] Hong-Yuan Chen,[4] and Shao-Hui Cai[3]

[1]Department of Microbiology, School of Public Health and Tropical Medicine, Southern Medical University, [2]Center of Microbiology, Biochemistry and Pharmacology, Sun Yat-sen University, [3]Department of Clinical Pharmacology, College of Pharmacy, Jinan University, and [4]Department of Immunology and Microbiology, Guangdong Pharmaceutical University, Guangzhou, Guangdong Province, P.R. China

Abstract

The chemokine receptor, CXCR4, and its specific ligand, CXCL12, have been proven to regulate the directional trafficking and invasion of breast cancer cells to sites of metastases, and similar phenomena have also been identified in many malignant tumors that aberrantly overexpress CXCR4. Therefore, blocking the interaction between CXCR4 and CXCL12 is considered a possible approach to efficiently prevent cancer metastasis. Employing a cellular phenotypic knockout strategy based on intrakines, we developed a novel recombinant chimeric protein, TAT/54R/KDEL, which contains three distinct functional domains: CXCL12/54R, a mutant of CXCL12 with CXCR4 antagonism, as well as HIV-derived TAT (47-57) and an endoplasmic reticulum retention four-peptide sequence KDEL that links at its NH_2 and COOH termini, respectively. Using the MOLT-4 cell line, which expressed CXCR4 highly and stably *in vitro*, we determined that TAT/54R/KDEL was able to efficiently transfer into the endoplasmic reticulum of tumor cells, where it specifically binds to the newly synthesized CXCR4 and prevents the latter from reaching the surface. Chemotaxis assays showed that the cells treated with TAT/54R/KDEL failed to migrate toward CXCL12. Furthermore, we observed that the systemic treatment of TAT/54R/KDEL could impair lung metastasis in a highly metastatic mammary cancer cell line, 4T1 cells, with the decrease of CXCR4 on their membrane. Our results suggest that the phenotypic knockout strategy of CXCR4 using a novel recombinant protein TAT/54R/KDEL might be a possible approach for inhibiting relative tumor metastasis mediated by CXCR4/CXCL12 interaction. (Mol Cancer Res 2009;7(10):1613–21)

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Introduction

Evidence shows that the interaction between CXCR4 (CXC chemokine receptor 4) and its specific ligand CXCL12 (stromal cell–derived factor-1) plays an important role in facilitating the growth and metastasis of many malignant tumors (1-3). It has been recognized that most breast cancer cells aberrantly overexpress CXCR4, and the CXCR4-CXCL12 gradient drives CXCR4-expressed breast cancer cells to migrate to specific metastatic sites such as the lung, kidney, and bone where CXCL12 is abundantly expressed (4, 5). Therefore, blocking the interaction between CXCR4 and CXCL12 with CXCR4 antagonists may be a possible strategy to efficiently prevent metastasis. Based on the structure-activity relationship of the interaction of CXCL12 with CXCR4, we previously developed a mutant mouse CXCL12 by deleting the 55th to 67th residues of its COOH terminus (CXCL12/54R). It was confirmed *in vitro* that this novel mutant of CXCL12 was able to quickly eliminate CXCR4 on the cell surface by inducting CXCR4 internalization without activating downstream signals mediated by CXCR4, resulting in the inhibition of tumor cell migration (6). However, this inhibitory effect of CXCL12/54R seemed to be temporary due to the recruitment of internalized CXCR4 on cell membranes. Accordingly, more reasonable approaches are expected which might produce a longer or more permanent inhibition of CXCR4 expression on the cellular surface.

Various studies of cellular phenotypic knockout strategies based on intrabody/intrakine for inhibition of the expression of some functional proteins have shown potential application value in the prevention and cure of serious diseases such as AIDS and tumors (7). An intercellular body/chemokine located at an intercellular compartment such as the endoplasmic reticulum (ER) is used to trap target proteins, consequently resulting in longer or permanent blockage of the transport of newly synthesized target proteins to the cell surface. Recently, CXCR4 phenotype knockout strategies were widely used not only to resist the infection of HIV-1 viruses but also to inhibit tumor metastasis mediated by the CXCR4/CXCL12 axis. For example, lymphocytes with a phenotypic knockout of CCR5 and CXCR4 were found to have a broad resistance to the infection of M-tropic, T-tropic, and dual-tropic HIV-1 virus infection (8). CXCR4 expression and function in CT-26 colon carcinoma cells were inhibited by transfection of CXCL12 extended with a KDEL sequence, and the metastatic phenotype of these cells to the liver and lung was also blocked (9). Based on these findings, we hypothesized that it might be possible to directly deliver recombinant protein CXCL12/54R into tumor cells to abrogate CXCR4 expression

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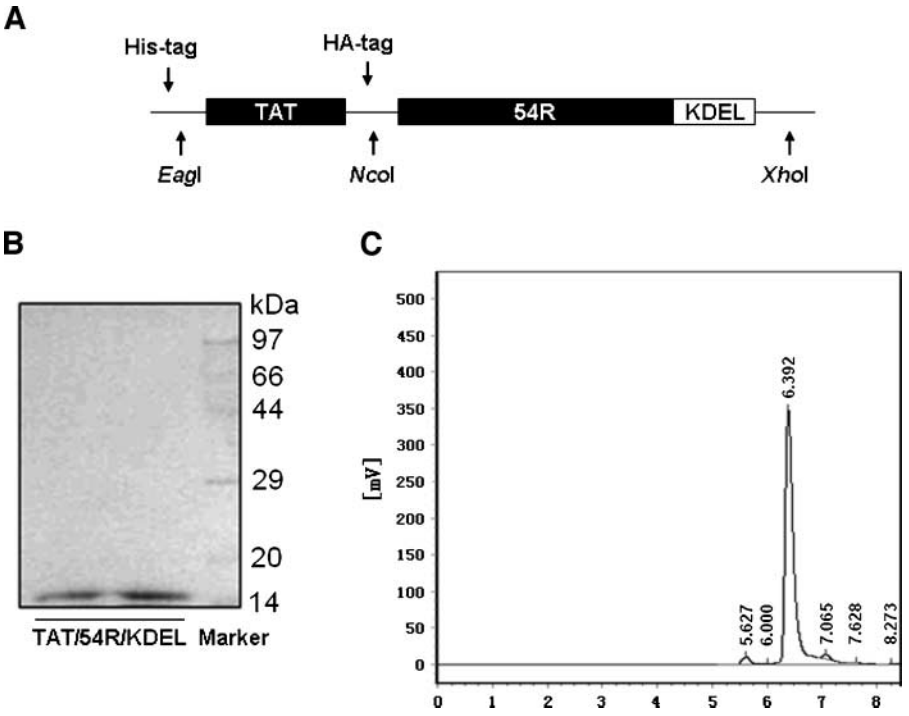

FIGURE 1. Schematic representation of the construction and purification of TAT/54R/KDEL. **A.** Strategy for the construction of TAT/54R/KDEL. **B.** The fusion protein TAT/54R/KDEL was purified by nickel affinity chromatography and identified by SDS-PAGE. **C.** The fusion protein TAT/54R/KDEL was purified by high-performance liquid chromatography with a C-18 column. The purified fusion protein was found to be nearly homogeneous and the purity was >95%.

on the cellular surface and sequentially restrain tumor metastasis. However, the question of how to deliver CXCL12/54R protein to the endochylema and retain the protein at the ER is the primary challenge.

The transmembrane efficiency of HIV-1 TAT (47-57) (YGRKKRRQRRR) has been increasingly recognized. TAT (47-57) is able to permeate the plasma membrane of cells either alone or fused with full-length proteins or peptides (10, 11). It can deliver proteins ranging from 10 to 120 kDa into almost all the tissues and cells, even through the blood-brain barrier, without damage to cells (12-15). Therefore, the TAT fusion protein system is probably considered as an appropriate carrier to deliver CXCL12/54R recombinant protein into cells.

Almost all of the secreted proteins need to be modified and processed in the ER, in which only the proteins with correct steric configuration are permitted to pass to the Golgi apparatus, otherwise, they are detained and then degraded in the ER. Retention of resident proteins in the lumen of the ER in eukaryon cells is achieved by their continual retrieval from the cis-Golgi or a pre-Golgi compartment. Studies have confirmed that four-peptide KDEL or DDEL is a retention signal which prevents the secretion of soluble ER-resident proteins by interacting with KDEL receptors localized in the intermediate compartment and Golgi apparatus (16-20). Thus far, more than 10 endogenous proteins containing KDEL have been discovered (21-23).

In the present study, we developed a novel recombinant chimeric protein, TAT/54R/KDEL, in which TAT (47-57) and KDEL were, respectively, linked to the NH_2-terminal and COOH-terminal of CXCL12/54R. Using the MOLT-4 cell line which expressed CXCR4 highly and stably *in vitro*, we determined that TAT/54R/KDEL was able to efficiently transfer into the ER of tumor cells, in which it specifically binds to the newly synthesized CXCR4 and prevents the latter from reaching the cell surface, resulting in these cells failing to migrate toward CXCL12. Also, we observed the systemic inhibitory effect of TAT/54R/KDEL on the metastasis of CXCR4-positive breast cancer cells to lungs in the transplanted tumor model mice.

Results

Construction and Expression of TAT/54R/KDEL

According to previous reports, TAT derived from HIV-1 at the NH_2 terminus of the fusion protein provides a more intensive delivery, and KDEL is usually designed at the COOH terminus of proteins that is expected to be delivered to the ER. Considering that it is necessary for this new chimeric peptide to contain three distinct functional domains, their genetic linking sequence was therefore framed as TAT/54R/KDEL. It is known that pTAT-HA is a common vector that expresses TAT fusion proteins, which has 6× His-Tag and HA-Tag at promoters downstream. In this study, 54R/KDEL was inserted into multiple clone sites downstream of the TAT coding region to construct the recombinant vector of TAT/54R/KDEL. The design and construction of TAT/54R/KDEL is illustrated in Fig. 1A. The recombinant plasmid TAT/54R/KDEL was confirmed by sequencing and transformed into BL21 (DE3). Following the induction of isopropyl-L-thio-β-D-galactopyranoside, TAT/54R/KDEL proteins were expressed in insoluble inclusion bodies. Then, TAT/54R/KDEL was purified by nickel affinity chromatography and high-performance liquid chromatography with a C-18 column. As shown in Fig. 1B, the molecular weight of TAT/54R/KDEL was ~16 kDa, which is consistent with the predicted size, and the purified fusion protein was found to be nearly homogeneous at a purity of >95%, as determined by high-performance liquid chromatography analysis (Fig. 1C). The

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eluted proteins were refolded by gradually altering the buffer to natural conditions.

CXCR4 Knockout Effect of TAT/54R/KDEL *on the Cell Surface*

In vitro *Assay Results*. One of the most critical steps for this study was undoubtedly to validate the CXCR4 knockout function of TAT/54R/KDEL to achieve the prospective target of this strategy. The reliability of the experimental results was closely dependent on the stability of CXCR4, which were highly expressed on the cell membrane. We previously screened CXCR4 expression on a variety of breast cancer cell lines, such as cell lines derived from human, MCF-7, MDA-MB-231, MDA-MB-435, and mouse-derived 4T1 cell lines. Although the expression of CXCR4 could be detected by flow cytometry in all of the above cell lines, the percentage of positive cells varies, even within the same cell lines in different experiments, which inevitably influences the evaluation of the CXCR4 knockout function of TAT/54R/KDEL. In a previous study, we tested CXCR4 expression in MOLT-4 cells (human acute lymphoblastic leukemia) which showed not only a high level (almost 100% positive cells) but also comparative stability *in vitro*, suggesting that the MOLT-4 cell line may be an appropriate cell model to test the CXCR4 knockout function of TAT/54R/KDEL. Thus, the effect of TAT/54R/KDEL on CXCR4 expression in MOLT-4 cell surfaces was examined by a flow cytometer. As shown in Fig. 2, treatment with 0.5 and 2 μmol/L of TAT/54R/KDEL resulted in a continued decrease of fluorescence density for 2 to 72 hours, and even by 72 hours, the fluorescence density was still <50%. Furthermore, the same concentration of CXCL12/54R also displayed an apparent inhibitory effect on the expression of CXCR4 in MOLT-4 cells. When the cells were treated with 0.5 μmol/L of CXCL12/54R for 4 hours, the amount of CXCR4 on the cell membrane decreased to ~24%. However, this inhibitory effect was apparently temporary, and evidently tended to lower as the reaction time progressed. CXCR4 expression of the cells treated with 0.5 μmol/L of CXCL12/54R for 8 hours had almost reverted to the primary levels. Although 2 μmol/L of CXCL12/54R could produce stronger and longer inhibition of CXCR4 expression, both the intensity and persistence of its inhibitory effect were inferior to TAT/54R/KDEL at the same concentration. We suppose that this advantage of TAT/54R/KDEL in continued downregulation of CXCR4 should be attributed to its unique mechanism of action. Based on its structural characteristics, the inhibitory effect of TAT/54R/KDEL on CXCR4 expression in the cell surface may be achieved by two approaches. In the first, TAT/54R/KDEL could rapidly bind to CXCR4 on the cell membrane and induce CXCR4 internalization; the other approach is to penetrate the cell membrane and locate at the ER, where it specifically binds to newly synthesized CXCR4, and accordingly, efficiently block CXCR4 expression on the cell surface.

Mechanism of Action of TAT/54R/KDEL. To verify this hypothesis and clarify the mechanism of action of TAT/54R/KDEL, we further surveyed the transduction efficiency and ER localization of TAT/54R/KDEL in MOLT-4 cells by fluorescence microscope and confocal microscope. As shown in Fig. 3A, the fluorescence signal was readily detected in MOLT-4 cells that were treated with 1 μmol/L of TAT/54R/KDEL for 5 minutes, and the amount of TAT/54R/KDEL up-take increased along with the time and amount of protein added. However, the transduction efficiency of TAT/54R/KDEL was lower in CXCR4-negative CNE2 cells and A431 cells (data not shown). Therefore, the outstanding transduction efficiency of TAT/54R/KDEL not only benefits from efficient delivery of TAT but also from the targeting capability of CXCR4 on the cell surface. Synchronously, the ER localization of TAT/54R/KDEL in MOLT-4 cells was examined. TAT/54R/KDEL was added to the culture medium of MOLT-4 cells at a concentration of 1 μmol/L for 3 hours and the transduced cells were stained with Cy5-conjugated anti-HA antibody and the ER-specific marker ER-Tracker, and was observed by confocal microscopy. The results (Fig. 3B) showed that the position in which

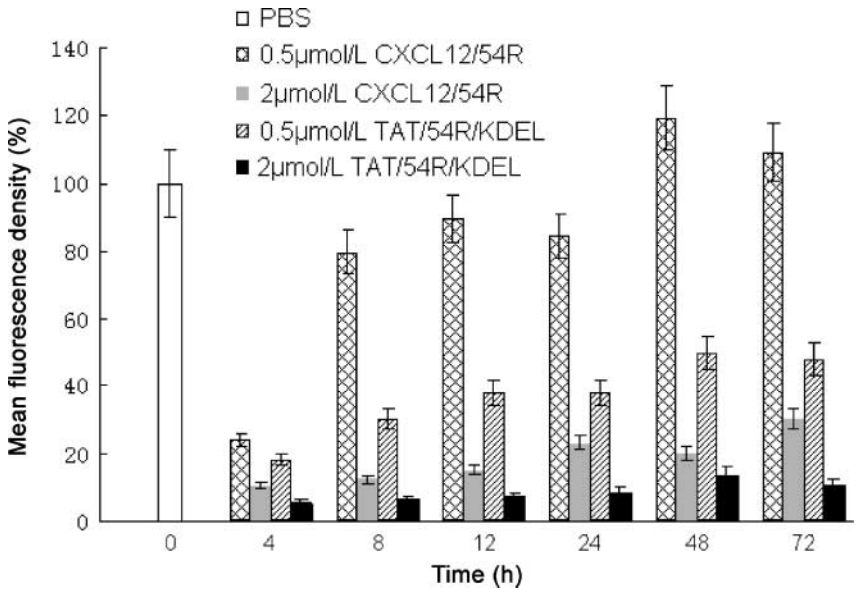

FIGURE 2. Effects of TAT/54R/KDEL on CXCR4 expression on MOLT-4 cell surface by flow cytometry. MOLT-4 cells were treated with TAT/54R/KDEL and CXCL12/54R at the same concentration for the indicated times. The CXCR4 on MOLT-4 cell surface was stained with CXCR4 antibody (12G5) and detected by flow cytometry. CXCR4 levels on MOLT-4 cell surface with mean fluorescence density.

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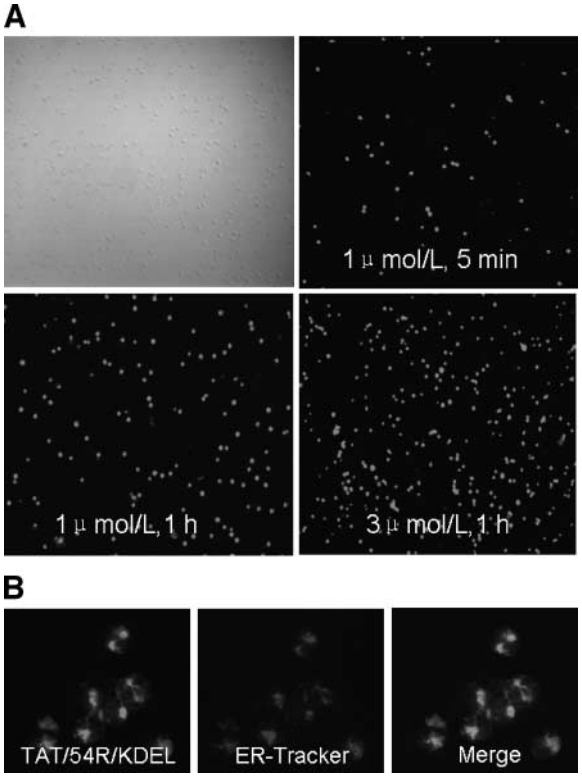

FIGURE 3. The transduction ability and ER localization of TAT/54R/KDEL. **A.** The transduction ability of TAT/54R/KDEL at various concentrations was analyzed by fluorescence microscope (magnification, ×100) in MOLT-4 cells for the indicated times. Fluorescence signal was readily detected in MOLT-4 cells that were treated with 1 μmol/L of TAT/54R/KDEL for 5 min and stained with Cy5-conjugated anti-HA antibody, and the amount of TAT/54R/KDEL up-take increased along with the time and amount of protein added. **B.** ER localization of TAT/54R/KDEL visualized by confocal microscopy (magnification, ×630). MOLT-4 cells were treated with 1 μmol/L of TAT/54R/KDEL for 3 h, and were stained with anti–HA-Tag antibody and ER-Tracker. The position in which TAT/54R/KDEL was located was also marked by ER-Tracker; the merged domain meant that most of the TAT/54R/KDEL could be located at the ER in transduced cells.

TAT/54R/KDEL was located (*red*) was also marked by ER-Tracker (*blue*); the merged domain (*purple*) meant that most TAT/54R/KDEL could be located at the ER of the transduced cells. This property of TAT/54R/KDEL established a necessary foundation to capture CXCR4 at the ER.

In vivo *Assay Results.* Furthermore, it was necessary to detect the CXCR4 knockout effect of TAT/54R/KDEL *in vivo*. Therefore, the influence of TAT/54R/KDEL on CXCR4 expression in transplanted tumors was investigated in mice treated with 5 mg/kg of TAT/54R/KDEL or PBS daily for 35 days. Results of reverse transcription-PCR showed that the primary tumor cells were positive for CXCR4 expression, in which there was no significant difference between the TAT/54R/KDEL-treated group and the PBS control (Fig. 4A). However, the results from flow cytometry did not show positive expression of CXCR4 on the primary tumor cells from mice treated with TAT/54R/KDEL. In contrast, primary tumor cells from the PBS group presented high levels of expression (Fig. 4B). These results indicate that TAT/54R/KDEL could inhibit CXCR4 expression in the cell membrane under systemic treatment, and this effect took place at the protein level, but not at the gene transcription level.

CXCR4 Phenotypic Knockout Mediated by TAT/54R/KDEL *Restrained Tumor Metastasis*

Induction of the migration of T lymphocytes through interaction with CXCR4 is not only an important physiologic function of the native CXCL12, but also a major concern for potential tumor metastasis (24, 25). To investigate the influence of CXCR4 phenotypic knockout mediated by TAT/54R/KDEL on the migration of CXCR4-positive tumor cells, the migration of MOLT-4 cells to CXCL12 was determined by chemotaxis assay. MOLT-4 cells were preincubated with various concentrations of TAT/54R/KDEL or CXCL12/54R for 12 hours, and then were subjected to chemotaxis assay with 0.1 μmol/L of CXCL12 treatment. CXCL12-induced cell migration was significantly suppressed by both TAT/54R/KDEL and CXCL12/54R in a dose-dependent manner (0.5-2 μmol/L; Fig. 5). Noticeably, the migration inhibition of TAT/54R/KDEL was more potent than CXCL12/54 at the same concentration ($P < 0.05$), which may benefit from the CXCR4 phenotype knockout effectiveness of TAT/54R/KDEL.

To evaluate whether TAT/54R/KDEL-induced CXCR4 phenotypic knockout could influence tumor metastasis *in vivo*, we injected 1×10^5 4T1 breast cancer cells overexpressing CXCR4 s.c. proximal to the right abdominal mammary gland of female BALB/c mice (26). Two days after transplantation, mice were injected s.c. with TAT/54R/KDEL (5 mg/kg) solution daily or with the same volume of PBS. The results showed that the primary tumor from PBS-treated mice grew more rapidly than the mice treated with TAT/54R/KDEL, and the difference between these two groups was significant ($P < 0.05$; Fig. 6A), indicating that TAT/54R/KDEL could inhibit 4T1 tumor cell growth. When the lungs were examined 35 days posttransplantation, TAT/54R/KDEL-treated mice had developed fewer metastases than PBS-treated mice (95 versus 168 metastases, $P < 0.05$; Fig. 6B). The weight of the lungs showed the same trend, the lungs derived from TAT/54R/KDEL-treated mice were significantly lighter than PBS-treated lungs ($P < 0.05$; Fig. 6C). In addition, a few sporadic metastases were observed with the naked eye in livers from the PBS-treated group but not in livers from the TAT/54R/KDEL-treated group (data not shown). Moreover, we noticed that at 5 weeks posttransplantation, mice from the control group were dying in succession, and by 7 weeks, all of the mice had died due to severe lung metastasis. Conversely, 60% of the mice treated with TAT/54R/KDEL were still alive at 7 weeks ($P < 0.05$; Fig. 6D). To eliminate the possibility that TAT/54R/KDEL inhibits lung metastases on account of its cytotoxicity to tumor cells, trypan blue stain assays were done. 4T1 tumor cells were treated with 1 to 6 μmol/L of TAT/54R/KDEL or PBS for 48 hours. Cell viability showed no significant difference between the TAT/54R/KDEL group and the PBS group (Fig. 7). These *in vivo* evidences further indicate that TAT/54R/KDEL possessed visible inhibitory activity in tumor growth and metastasis, and taking into account the *in vitro* evidence of the inhibitory effect of TAT/54R/KDEL on CXCR4 expression in 4T1-transplanted tumors, it may be considered that the TAT/54R/KDEL

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antagonism for tumor growth and metastasis contributes to its CXCR4 phenotypic knockout function.

Discussion

Based on intrabody/intrakine mechanism and TAT characteristics, we first developed a novel recombinant chimeric protein, TAT/54R/KDEL, which contains three distinct functional domains: CXCL12/54R, a mutant of CXCL12 with CXCR4 antagonism, and TAT (47-57) and KDEL which link at its NH_2 terminus and COOH terminus, respectively. Here, we present essential evidence indicating that TAT/54R/KDEL is able to efficiently transfer into the ER of tumor cells and downregulate the expression of CXCR4 on the cell surface, and consequently, block the following CXCR4-positive tumor metastasis mediated by CXCR4/CXCL12 interaction. Importantly, compared with the current DNA-based intrabody/intrakine strategy, the protein-based intrabody/intrakine strategy seems more advantageous for future clinical applications due to its relative safety, facility, and manageability (27). Moreover, TAT/54R/KDEL could produce a more efficient CXCR4 antagonism than the antagonists which competitively inhibit CXCR4 just on cell membranes, such as one of the CXCL12 mutants, CXCL12/54R, because the former antagonism was derived from retention at ER before newly synthesized CXCR4 was transited to the cell membrane.

Considering the interaction of CXCR4 and its only specific ligand, CXCL12, native CXCL12 is conceivably eligible as a candidate for the CXCR4 phenotypic knockout strategy. However, CXCL12 may widely cause inflammatory reactions or other side effects resulting from CXCR4 activation induced by CXCL12 in systemic treatment. Therefore, it might be supposed that the mutant CXCL12 with CXCR4 antagonism is more feasible for developing the recombinant protein with CXCR4 phenotypic knockout.

Despite the potential advantages of protein-based intrabody/intrakine compared with gene-based intrabody/intrakine, the lower membrane permeability of proteins is certainly a major obstacle for the use of protein-based intrabody/intrakine. Therefore, improved transduction of target proteins into cells and tissues is considered as a critical step for the application of this strategy. It is known that fragment TAT (47-57) (YGRKKRRQRRR) cut from HIV-1 transcription factor is a relatively ideal transmembrane carrier because of its unique features. TAT is proven to be able to traverse the cell membrane either alone or fused with proteins or peptides without obvious cytotoxicity, and can deliver proteins ranging from 10 to 120 kDa into almost all the tissues and cells, even through the blood-brain barrier, without damage to cells. It has been reported that TAT can deliver a variety of proteins, including ovalbumin, horseradish peroxidase, β-galactosidase, cyclin-dependent kinase inhibitor p27 Kip1, caspase-3, cyclin, DNA modification enzyme, signal protein and antiapoptosis protein, and so on, and most of them have antineoplastic activity (28-30). Furthermore, *in vivo* studies have reported that TAT could deliver some proteins such as TAT-galactosidase into various mouse tissues, including the brain, to exert their bioactivities (31-33). Being consistent with these findings, our data revealed that the fusion protein, TAT/54R/KDEL, could efficiently enter cells with the induction of TAT.

Another important issue for this strategy is in further promoting the accumulation of exogenous intrabodies/kines at ER where endogenous proteins are synthesized, assembled, and secreted. It has been proven that retention of resident proteins in ER is dependent on a COOH-terminal tetrapeptide signal. KDEL (Lys-Asp-Glu-Leu) has been confirmed as a ubiquitous ER-trapping signal peptide. By interacting with its receptors localized in the intermediate compartment and Golgi apparatus, KDEL plays a key role for the localization in the ER of mammal cells of many soluble proteins contained in the cisternal lumen and of type I transmembrane proteins, etc. (16-20). Therefore, KDEL was linked at the COOH-terminal of CXCL12/54R in this recombinant chimeric protein design. Our data indicated that the position where TAT/54R/KDEL is located (*red*) was also marked by ER-Tracker (*blue*), which suggests that most of TAT/54R/KDEL could be located at the ER of the transduced cells. This

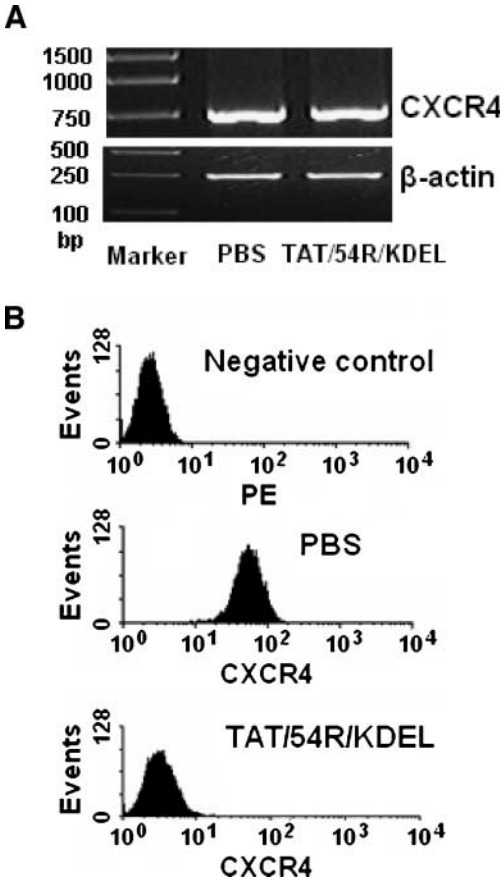

FIGURE 4. Identification of CXCR4 expression of transplant tumor by reverse transcription-PCR and flow cytometry. **A.** Identification of CXCR4 expression of transplant tumor by reverse transcription-PCR. The primary tumor cells appeared to have a positive expression; there was no significant difference between TAT/54R/KDEL and PBS. **B.** CXCR4 expression on the cell surface of transplant tumors was detected by flow cytometry. A positive expression of CXCR4 was not found on the primary tumor cells derived from mice treated with TAT/54R/KDEL, whereas primary tumor cells from the PBS group presented a high level of expression.

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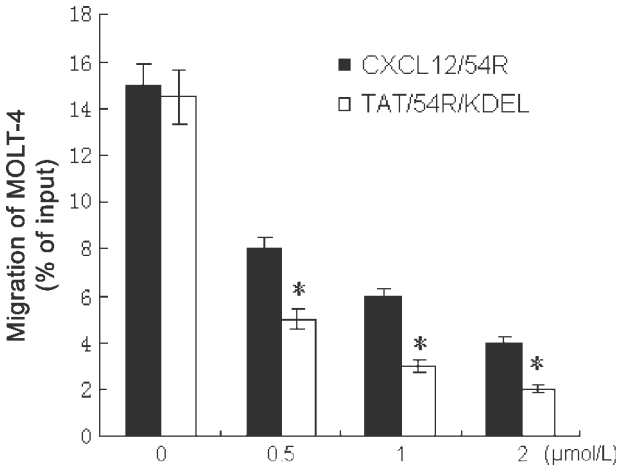

FIGURE 5. Inhibitory effect of TAT/54R/KDEL on the chemotactic ability of CXCL12 in MOLT-4 cells. MOLT-4 cells were preincubated with TAT/54R/KDEL or CXCL12/54R with the indicated concentrations at 37°C for 12 h. The chemotactic activity of CXCL12 (0.1 μmol/L) on the cells was detected. The experiments were repeated thrice. *, $P < 0.05$, significant difference between TAT/54R/KDEL versus CXCL12/54R at a range of 0.5 to 2 μmol/L.

property of TAT/54R/KDEL established a necessary foundation to capture CXCR4 at the ER.

According to the structural characteristics of TAT/54R/KDEL, we supposed that this novel recombinant protein might take three necessary steps to promote CXCL12/54R into tumor cells and produce the phenotypic knockout effect on CXCR4: (*a*) CXCL12/54R specifically identifies and binds to CXCR4 on the cell membrane and rapidly induces CXCR4 internalization; (*b*) TAT helps TAT/54R/KDEL to effectively traverse tumor cell membranes; and (*c*) KDEL leads the localization of TAT/54R/KDEL and captures CXCR4 at the ER to block CXCR4 expression on the cell surface. In this study, we observed that TAT/54R/KDEL revealed higher transduction efficiency in CXCR4-positive cells, MOLT-4, than those in CXCR4-negative cells such as CNE2 and A431 cells (data not shown), which should be ascribed to the CXCL12/54R-mediated targeting capability of CXCR4 on the cell surface. This feature of TAT/54R/KDEL also hints that it might have the potential to block the metastasis of tumor cells with high expression of CXCR4. However, we cannot ignore the possibility that treatment with TAT/54R/KDEL *in vivo* causes some side effects due to TAT-mediated nonspecific cell penetration as well as CXCR4 expression in normal cells (34), although we did not observe obvious toxic effects in the heart, spleen, and kidney of mice injected with TAT/54R/KDEL at primary tumor sites. For this reason, we need to improve the targeting of TAT/54R/KDEL to tumors for future practical applications. Indeed, we are exploring several strategies to promote targeting enrichment of TAT/54R/KDEL in tumors, such as enveloping TAT/54R/KDEL with folate-conjugated liposome, which may increase the distribution of TAT/54R/KDEL in tumors because the cellular membrane of most tumor cells show high specific expression of folate receptor (35).

In summary, we developed a novel recombinant chimeric protein, TAT/54R/KDEL, that has been shown to possess the ability to efficiently transfer into the ER of tumor cells and specifically block the expression of CXCR4 on the cell surface, consequently leading to the inhibition of tumor metastasis mediated by CXCR4/CXCL12 interaction. This attempt explores a possible novel approach in inhibiting relative tumor metastasis mediated by CXCR4/CXCL12 interaction. Undoubtedly, a large number of relative studies are required to optimize this therapeutic approach.

Materials and Methods

Plasmids, Escherichia coli*, and Antibodies*

Plasmid pTAT-HA was donated by Prof. Dowdy (Howard Hughes Medical Institute, and Department of Cellular and Molecular Medicine, University of California San Diego School of Medicine, La Jolla, CA). *Escherichia coli* BL21 was purchased from Novagen, whereas phycoerythrin mouse anti-human CXCR4 monoclonal antibody (12G5) was from R&D Systems. ER-Tracker Blue-White DPX was from Molecular Probes, HA-Tag (6E2) mouse monoclonal antibody (Cy5 conjugated) was from CST, rabbit anti-CXCR4 polyclonal antibody was from Abcam, and phycoerythrin-conjugated goat anti-rabbit IgG was from Zhongshan.

Tumor Cell Lines. Human acute lymphoblastic leukemia (MOLT-4) cells and human nasopharyngeal carcinoma (CNE2) cells were from the China Center for Type Culture Collection, and were cultured in RPMI 1640 with 10% fetal bovine serum. 4T1 cells, a breast cancer cell line derived from a BALB/c mouse, and human epidermoid carcinoma cells (A431) were obtained from the China Center for Type Culture Collection and cultured in DMEM, supplemented with 10% fetal bovine serum (Gemini Bioproducts), 1.5 mg/mL of sodium bicarbonate, 2 mmol/L of L-glutamine, and 100 units/mL of penicillin/streptomycin in 5% CO_2 atmosphere.

Construction and Expression of TAT/54R/KDEL

Primers for cloning 54R/KDEL were synthesized by Shanghai Sangon Biological Engineering Technology according to cDNA sequences of native CXCL12 and encoding sequences of KDEL. Their sequences included sense primers, 5′-CATGCCATGGGCAAGCCCGTCAGCCTGAGCTACAGAT-3′ and antisense primer 5′-CCGCTCGAGTTAGAGCTCGTCCTTCTTCGGGTCAATGCACACTTGTC-3′. The amplified PCR products were linked to pTAT-HA after digestion with *Nco*I and *Xho*I. The recombinant plasmid TAT/54R/KDEL/pTAT-HA was sequenced and transformed into expression bacteria *E. coli* BL21. The positive clone was induced with 0.5 mmol/L of isopropyl-L-thio-β-D-galactopyranoside at an OD_{600} of 0.5. The target proteins were purified by nickel affinity chromatography and high-performance liquid chromatography with a C-18 column. The eluent was diluted to a quadruple volume with a renaturing buffer (20 mmol/L Tris-HCl, 0.5 mmol/L oxidized glutathione, 5 mmol/L reduced glutathione, 0.5 mmol/L L-arginine, 0.5 mmol/L L-histidine, 20 nmol/L $CuSO_4$, 1% glycine, and 5% glycerol) and dialyzed twice against renaturing buffer and PBS at 4°C. Next, the solution was ultrafiltrated and lyophilized for desalination and condensation.

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Transmembrane Ability and ER Localization of TAT/54R/KDEL

MOLT-4 cells (1×10^5) were inoculated in 24-well tissue culture plates. Various concentrations of TAT/54R/KDEL were added to the culture medium and incubated at 37°C for various periods of time. Then the cells were washed with PBS to remove nonspecific extracellular proteins bound to the cell surface. Then the cells were fixed in 1% paraformaldehyde for 30 min at 4°C, permeabilized with 0.1% saponin solution for 15 min at 20°C, and stained with Cy5-conjugated anti-HA antibody for 30 min at 4°C. The cells were washed twice with PBS and transmembrane ability was analyzed with a fluorescence microscope. To detect the stagnation state of TAT/54R/KDEL at the ER, the abovementioned cells labeled by HA-Tag were stained for 15 min with ER-Tracker at 37°C, and then transferred to a chamber on a Zeiss Axiovert S100 microscope and observed with a confocal laser-scanning system (Bio-Rad; MRC-1024ES).

Chemotaxis Assay

The migration of MOLT-4 cells was assessed in disposable Transwell trays (Kurabo) with 6-mm diameter chambers and a membrane pore size of 5 μm. The MOLT-4 cells were preincubated with various concentrations of TAT/54R/KDEL and CXCL12/54R at 37°C for 12 h. Two hundred microliters of preincubated cells were added to the upper wells, and 700 μL of diluted CXCL12 (0.1 μmol/L) was added to the lower wells. After incubating in humidified 5% CO_2 at 37°C for 2 h, the cells that migrated to the lower wells were counted. Migrating cells that were not treated were used as negative controls.

Measurement of CXCR4 on the Cell Surface

The cells treated with TAT/54R/KDEL were stained with mouse anti-human monoclonal antibody CXCR4-PE and analyzed by flow cytometry. Cell suspensions derived from transplanted tumors were done and CXCR4 on the cell surface was measured with rabbit anti-mouse polyclonal antibody (Abcam) using flow cytometry.

Animal Studies

BALB/c female mice were purchased from the Experimental Animal Center of Sun Yat-sen University, P.R. China (certification no. 2004A084) and housed in a specific pathogen–free facility. All animal studies were conducted in accordance with NIH Animal Care and Use Committee guidelines. Viable 4T1 cells (1×10^5) were implanted into the right inguinal mammary gland of 6- to 8-wk-old female BALB/c mice. Tumor diameters were measured with calipers twice weekly (converted to tumor volume by the formula: length × width^2 × 0.5). Two days after

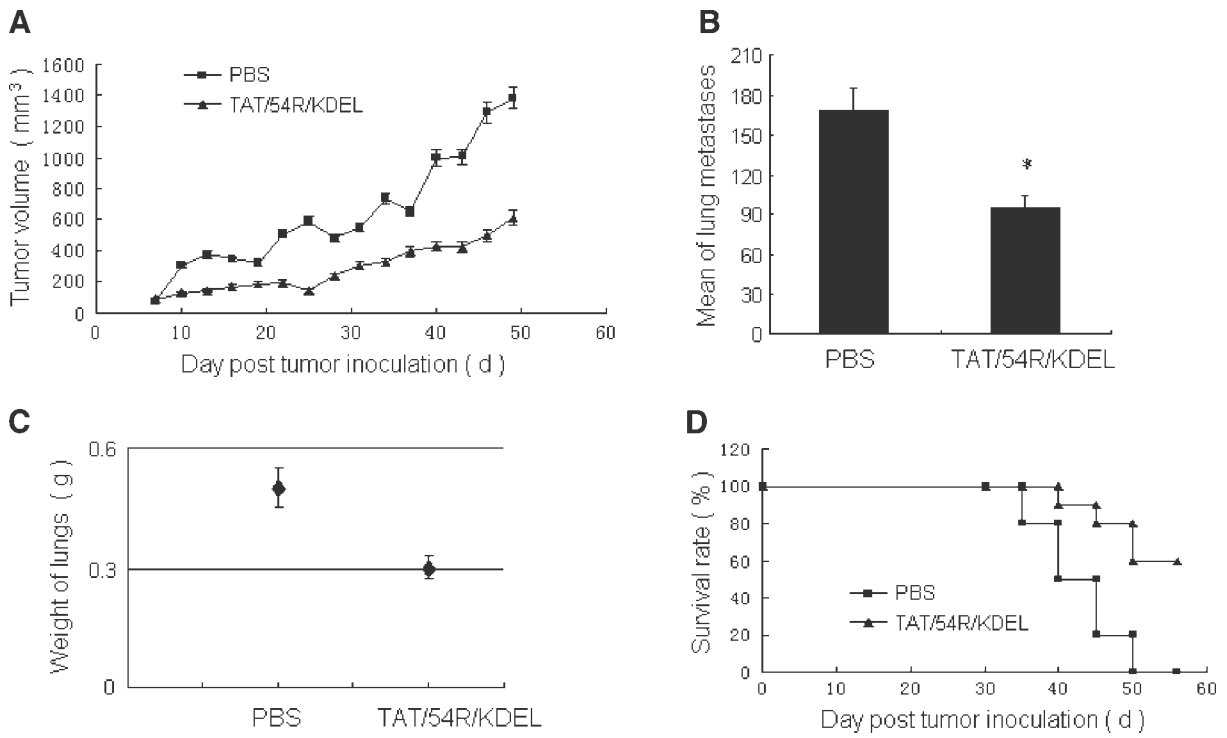

FIGURE 6. Inhibition of TAT/54R/KDEL on the growth and lung metastases of 4T1 cells. **A.** The growth curve of tumors *in situ*. Viable 4T1 tumor cells (1×10^5) were injected s.c. proximal to the right abdominal mammary gland of female BALB/c mice. Two days posttransplantation, mice were injected daily with 5 mg/kg of TAT/54R/KDEL or s.c. with the same volume of PBS. Tumor diameters were measured by caliper twice weekly. The tumor size *in situ* in the PBS group increased more rapidly than the mice treated with TAT/54R/KDEL, and the difference between these two groups of mice was significant ($P < 0.05$). **B.** Inhibition of TAT/54R/KDEL on lung metastases. The lungs were examined 35 d posttransplantation. TAT/54R/KDEL-treated (5 mg/kg) mice had developed fewer metastases than PBS-treated mice ($P < 0.05$). **C.** Comparison of the weight of lungs. The lungs derived from TAT/54R/KDEL-treated mice were significantly lighter than PBS lungs ($P < 0.05$). **D.** Survival curve of tumor-bearing mice. At 5 wk posttransplantation, the mice from the control group were dying in succession, and by 7 wk, all of the mice had died due to severe lung metastasis. On the contrary, 60% of mice treated with TAT/54R/KDEL were still alive at 7 wk ($P < 0.05$).

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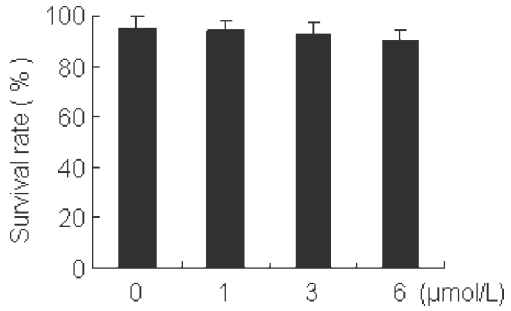

FIGURE 7. The cellular toxicity of TAT/54R/KDEL in trypan blue stain assay. 4T1 tumor cells were treated with 1 to 6 μmol/L of TAT/54R/KDEL or PBS for 48 h. Then the cells were stained with 0.4% trypan blue solution. The living cells were counted with a counting plate for 3 min to calculate the rate of cell survival. The results were from three separate experiments conducted in triplicate for each concentration. There was no significant difference in cell viability between the TAT/54R/KDEL group and the PBS group at a range of 1 to 6 μmol/L.

transplantation, the mice were injected daily with a TAT/54R/KDEL solution (5 mg/kg) at the primary tumor site, and control mice were injected with the same volume of PBS. The experiments were carried out according to two protocols. One was that the mice were euthanized on an individual basis when the subcutaneous tumor measured 18 mm in diameter or earlier if the mice seemed moribund, which focused on observing the effect of TAT/54R/KDEL on the primary tumor. The other protocol was aimed at the inhibitory activity of TAT/54R/KDEL on metastasis. In this experiment, all mice were euthanized 35 d posttransplantation and their lungs were removed and weighed. Then, the surface tumor colonies of the lungs were quantified in a blinded fashion under a dissecting microscope.

Analysis of mRNA Content of CXCR4 in Tumor Cells

Total RNA of transplanted tumor tissue was extracted using TRIzol Reagent (Invitrogen) according to the instructions of the manufacturer. Total RNA (2 μg) was reverse-transcribed to cDNA using SuperScript II RNase H Reverse Transcriptase, and 2 μL of the cDNA product was amplified by PCR. The sequences of the gene-specific primers were as follows: CXCR4 forward 5′-ATAAGAATGCGGCCGCTATGGAGGGGATCAGTATATACACTT-3′, reverse 5′-CGCTCTAGATTAGCTGGAGTGAAAACTTGAAGACT-3′; β-actin forward 5′-GAGACCTTCAACACCCCAGC-3′, reverse 5′-ATGTCACGCACGATTTCCC-3′. The PCR procedure included an initial denaturation at 94°C for 4 min, followed by 30 cycles of three PCR steps of 10 s each at 94°C, 55°C, and 72°C. The reaction was completed at 72°C for 10 min. The PCR amplification products were analyzed on a 1.5% agarose gel.

Statistical Analyses

All data were expressed as mean ± SD. Differences between groups were assessed by one-way ANOVA. If the variances between groups were homogenous (Levene's test), groups were subjected to a multiple comparison Bonferroni's test. $P < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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