Recombinant snake venom metalloproteinase inhibitor BJ46A inhibits invasion and metastasis of B16F10 and MHCC97H cells through reductions of matrix metalloproteinases 2 and 9 activities
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Introduction
Tumor invasion and metastasis are usually major causes of cancer death. Although many advances have been made in cancer therapy, most cancer deaths still result from metastatic disease [1]. The process of cancer cell metastasis can be summarized as a sequence of events that include the formation of tumor embolus in the blood capillary, cell penetration of the extracellular matrix (ECM), trapping at a secondary site, and growth at the secondary site [1]. Degradation of the ECM mediated by major proteases, including serine proteases, cysteine proteases, and matrix metalloproteinases (MMPs), is one of the key steps during the invasion and metastasis of cancer cells, and the MMP family plays an important role in the process. MMPs are therefore amenable to therapeutic intervention by synthetic and natural inhibitors. Multiple therapeutic agents, called matrix metalloproteinase inhibitors (MMPIs), have been developed to target MMPs in an attempt to control their enzymatic activity and to inhibit the invasion and metastasis of cancer cells. However, even though many clinical trials with these MMPIs have been conducted in cancer patients and have proved unsuccessful in most cases, research into MMPIs is ongoing [2,3].

Snake venoms contain several proteolytic enzymes and proteins with different toxicological functions and special pharmacological effects [4,5]. BJ46a, a 46 kDa protein isolated from the serum of Bothrops jararaca, is an inhibitor of snake venom metalloproteinases (SVMPs). A member of the fetuin family and cystatin superfamily, BJ46a interacts with metalloproteinases through the formation of a noncovalent complex, specifically inhibiting atrolysin C (class P-I) and jararhagin (class P-III) proteolytic activities [6]. Because SVMPs share homology with the zinc-binding environment of MMPs, it is possible that BJ46a may be able to modulate anticancer invasion and metastasis through the formation of a complex with MMP. Previous studies in our laboratory have shown that the BJ46a gene can be synthesized by assembly PCR, and pretreatment of the recombinant BJ46a (rBJ46a) protein produced using a Bac-to-Bac system to an anticancer agent. The Enzcheck gelatinase/collagenase assay showed that rBJ46a protein can reduce matrix metalloproteinase (MMP) activities and inhibit invasion and metastasis of melanoma cells. Here, we optimized the Pichia pastoris system to evaluate rBJ46a protein as an anticancer agent. The kinetic analyses using a series of double reciprocal Lineweaver–Burk plots showed that rBJ46a inhibited MMP activities (IC\textsubscript{50} = 0.119 mg/ml). Kinetic analyses using a series of double reciprocal Lineweaver–Burk plots (1/\textit{V} vs. 1/S) showed a competitive mode of inhibition with rBJ46a with inhibitory efficiency against MMPs ($K_i$ = 13.6 nmol/l). Matrigel invasion assays showed significant activity of rBJ46a on tumor cells. For lung colonization assays, C57BL/6 mice were inoculated in the lateral tail vein with B16F10 cells and were treated with three i.v. injections of rBJ46a (1, 2, and 4 mg/kg) 24 h before cell inoculation, and 2 and 24 h after cell inoculation. Administration of rBJ46a suppressed lung tumor colony formation significantly. For spontaneous metastasis assays, MHCC97H cells were inoculated subcutaneously into nude mice. After 24 h, rBJ46a was administered by i.p. injections: 1, 2, and 4 mg/kg once daily for 6 days. rBJ46a decreased lung tumor colony formation significantly. Gelatin zymography showed that MMP2/MMP9 enzymatic activities in tumor cells were suppressed by rBJ46a in a dose-dependent manner, and the $K_m$ values of rBJ46a against MMP2 and MMP9 activities that were expressed in both B16F10 and MHCC97H cells were 3.6 and 1.4 $\mu$mol/l, respectively. Thus, rBJ46a can inhibit the invasion and metastasis of tumor cells by reducing MMP2/MMP9 activities, indicating that rBJ46a may be a novel therapeutic agent for antimetastasis of tumor cells.

**Anti-Cancer Drugs**

**Keywords:** antimetastasis, matrix metalloproteinase, recombinant snake venom metalloproteinase inhibitor BJ46A, tumor

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Baculovirus Expression System could reduce MMP activities and inhibit invasion and metastasis of melanoma cells (B16) in vitro and in vivo [7–9]. However, the Bac-to-Bac Baculovirus Expression System has the disadvantages of low yields, a requirement for costly equipment, and it is tedious, which limits the large-scale production of recombinant protein. To advance research on the biological features of BJ46a and broaden the possibilities of its applications in the pharmaceutical industry, we described the high-level expression, purification, characterization, and structural prediction of rBJ46a in Pichia pastoris [10]. To evaluate further the pharmaceutical potential of rBJ46a protein as a cancer therapeutic agent, in this study, we first optimized the P. pastoris system for recombinant protein synthesis, and then investigated the effects of rBJ46a on the invasion and metastasis of B16F10 cells and MHCC97H cells in vitro by Matrigel invasion assays. We also examined the actions of rBJ46a in vivo by two experimental animal models: human MHCC97H tumors grafted into nude mice and the dissemination of B16F10 melanoma cells to the lungs of syngeneic mice. The results showed that rBJ46a produced by the P. pastoris expression system inhibited the invasion and metastasis of B16F10 cells and MHCC97H cells in vitro and in vivo, possibly through the reduction of MMP2/MMP9 activities.

**Materials and methods**

**Cell culture and animals**

MHCC97H cells were purchased from the Liver Cancer Institute at Fudan University (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (Gibco BRL, Gaithersburg, Maryland, USA) supplemented with 10% fetal bovine serum (Gibco BRL). MHCC97H cells were cultivated by means of alternating cell culture in vitro and growth in nude mice, as described by Tian et al. [11]. MHCC97H cells are a human hepatocellular carcinoma cell line with a high metastatic potential, which were established from a subcutaneous xenograft of a spontaneous lung metastasis model of human hepatocellular carcinoma. B16F10 cells were purchased from the China Center for Type Culture Collection (Wuhan University, Hubei, China) and were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum. B16F10 cells are derived from a spontaneous melanoma in C57BL/6 mice and undergo pulmonary metastasis after injection through the tail vein [12,13]. This is a classic model of experimental lung metastasis. Pathogen-free 5–6-week-old C57BL/6 mice and BALB/c nude mice were obtained from the Chinese Academy of Sciences (Shanghai, China); both the C57BL/6 and the BALB/c nude mice included equal numbers of males and females.

**Production and purification of recombinant BJ46a**

rBJ46a protein was produced according to the manufacturer’s instructions [10,14]. Briefly, recombinant GS115-

BJ46a was amplified to an OD_{600} of 4.0 in log-phase growth in 25 ml of BMGY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) yeast nitrogen base, 0.1 mol/l potassium phosphate buffer pH 6.0, 4 × 10^{-5} (w/v) biotin, 1% (w/v) glycerol, 4 × 10^{-3} (w/v) histidine] at 30°C using a shaking incubator (300 rpm). Cells from the medium were collected by centrifugation (1500g) for 8 min and the pellet was resuspended to an OD_{600} of 1.0 in BMGY [similar to BMGY medium but containing 0.5% (w/v) methanol instead of 1% glycerol] medium for induction and expression in a 11 baffled flask covered with six layers of sterile gauze. The production of protein was carried out in a flask at a final concentration of 1% methanol, with replacement every 24 h. The expression culture supernatants were collected at 24, 48, 72, and 96 h to determine the optimal time for induction. The expression levels were analyzed using 11% Tris-tricine polyacrylamide gels. The collected supernatants were concentrated using an Amicon ultrafiltration device (GE Healthcare, Piscataway, New Jersey, USA).

Purification of polyhistidine-containing recombinant proteins was completed using immobilized metal affinity chromatography (IMAC) using Ni Sepharose 6 Fast Flow (Code Number 17-0921-07; GE Healthcare, Piscataway, New Jersey, USA) according to the manufacturer’s protocol. Briefly, a concentrated expression solution after dialysis was loaded on an IMAC column equilibrated with binding buffer [20 mmol/l sodium phosphate (pH 8.0), 500 mmol/l NaCl, and 20 mmol/l imidazole]. The specifically bound proteins were eluted with elution buffer [20 mmol/l sodium phosphate (pH 8.0), 500 mmol/l NaCl, and 500 mmol/l imidazole]. The eluted fractions that showed inhibitory activity and high purity (98.06%) were pooled, and the resulting mixture was subjected to ultrafiltration and desalting.

**Deglycosylation**

Purified recombinant proteins were enzymatically deglycosylated using Endoglycosidase H (Endo H) using the protocol recommended by the manufacturer [15]. Briefly, 20 μg of recombinant protein was denatured with 1 μl of 10× glycoprotein denaturing buffer, heated at 95°C for 10 min, deglycosylated by adding 2 μl 10× G5 buffer, 1 μl of 500 000 U/ml of Endo H, and 6 μl H2O, and incubated at 37°C for 1 h. The reaction was stopped by boiling and analyzed by SDS polyacrylamide gel electrophoresis (PAGE) and western blotting.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblot analysis**

The purified proteins were electrophoresed in 11% Tris-tricine polyacrylamide gels to determine purities and molecular weights. The separated proteins were used for immunoblot analysis by a Western Breeze chemiluminescent immunodetection kit (catalogue number: WB7106; Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.
Invitrogen, Shanghai, China) with a rabbit anti-BJ46a polyclonal antibody as the primary antibody (BioAsia, Shanghai, China). The purified proteins were sent to the Biology Medical Institute of Fudan University to determine the molecular weight of rBJ46a by MALDI-TOF/TOF mass spectrometry (MS) analysis.

**Assay for the inhibitory activity of recombinant BJ46a**

The ability of the recombinant protein to inhibit the activities of MMPs was determined using an Enzchek Gelatinase/Collagenase Assay Kit (Invitrogen), which provides speed, high sensitivity, and convenience required for measuring gelatinase or collagenase activity for screening inhibitors in a high-throughput format [16,17]. The assay works on the principle that as MMPs (gelatinase) degrade the substrate DQ gelatin, the digestion products can be detected by measuring their absorption maxima at ~495 nm and their fluorescence emission maxima at ~515 nm.

First, 80 μl of 1 × reaction buffer was mixed with 20 μl of DQ gelatin solution in the wells. Then, 100 μl of the dilute enzyme or 100 μl of 1 × reaction buffer as a negative control was added to start the reactions. All the samples were incubated at room temperature and protected from light for 2 h. The digestion products were detected by measuring their absorption maxima at ~495 nm and their fluorescence emission maxima at ~515 nm using an RF-5310PC spectrofluorophotometer (Ying Si Tè, Hangzhou, China).

rBJ46a was diluted with 1 × reaction buffer to a final volume of 80 μl at a series of concentrations and preloaded into each assay well. Next, 20 μl of DQ gelatin substrate solution was added, followed by 100 μl of the dilute enzyme or 100 μl of 1 × reaction buffer as a blank. All the samples were incubated at room temperature and protected from light for 2 h. The digestion products were detected by measuring their absorption maxima at ~495 nm and their fluorescence emission maxima at ~515 nm using an RF-5310PC spectrofluorophotometer (Ying Si Tè).

**Cell growth assays**

Cells (4 × 10^5 cells/well) were separately seeded onto 96-well microtiter plates. After 24 h of incubation, increasing doses of rBJ46a (25, 50, 100, 200, or 400 μg/ml) were added to the cells. Cells were cultured for an additional 72 h and then 0.5 mg/ml MTT was added. After a 4-h incubation, medium was aspirated and dimethyl sulfoxide was added to dissolve the MTT-formazan crystals completely. The light absorbance at 570 nm wavelength (A570), which correlates to the number of cells, was measured on a microplate reader (Model Elx800; Bio-Tek, Shanghai, China).

**Migration and Matrigel invasion assays**

The invasive behavior of the cells in vitro was determined using 24-well transwell inserts fitted with 8 mm pore size polyester membranes. Each bottom chamber was coated with 10 μl of fibronectin (0.5 mg/ml). A cell suspension (500 μl; 1 × 10^5 cells) in serum-free medium after incubation with or without rBJ46a (25, 50, 100, 200, or 400 μg/ml) was seeded on top of each membrane. After 24 h, the migrated cells were counted. For Matrigel invasion assays, the bottom chamber was precoated with 20 μg of Matrigel (BD, San Jose, California, USA). All other conditions were as described for the migration assays. Antitumor effects were evaluated using the following equation: ratio of inhibition (%) = (C – T)/C × 100, where T and C are the relative mean numbers of invading cells in the treatment and the control groups, respectively.

**Experimental lung colonization assay**

C57BL/6 mice (12 mice/group) were inoculated in the lateral tail vein with B16F10 cells (2 × 10^5 cells/animal). Treatment with rBJ46a (1, 2, or 4 mg/kg) was carried out 24 h before cell inoculation, and 2 and 24 h after cell inoculation. Control mice received 200 μl of 0.9% (w/v) NaCl. The mice were killed on day 21. The lungs were removed following tumor colonization, weighed, and processed for histology examination [18,19]. The tumor colonization was enumerated. Antitumor effects were evaluated using the following equation: ratio of growth inhibition (%) = (C – T)/C × 100, where T and C are the relative mean tumor weights in the treatment and the control groups, respectively; ratio of colonization inhibition (%) = (C – T)/C × 100, where T and C are the relative mean numbers of lesions in the treatment and the control groups, respectively.

**Spontaneous metasis assay in nude mice**

MHCC97H cells (1 × 10^7/animal) in 0.2 ml of 0.9% NaCl were inoculated (s.c.) into the right flank of BALB/c nude mice (8 mice/group). After 24 h, rBJ46a was administered by i.p. injections at 1, 2, or 4 mg/kg once daily for 6 days. The mice were killed on day 42, and tumor weights were measured. The tumor volume was calculated using the following equation: V = 4/3πL/2(W/2)^2, where V is the volume (mm^3), L is the largest diameter (mm), and W is the smallest diameter (mm) [19,20]. The tumor and lung tissues were removed for histological examination. The number of metastases were counted. The ratios of growth and metastasis inhibition were calculated as described above.

**Gelatin zymographic assay for matrix metalloproteinases**

MMP2 and MMP9 enzymatic activities were assayed by gelatin zymography [21]. Tumor cells were plated on six-well dishes and grown to 90% confluence in 2 ml of growth medium. The cells were maintained in serum-free media and treated with various concentrations of rBJ46a (the final concentrations of rBJ46a were 25, 50, 100, 200, or 400 μg/ml) for 24 h. Conditioned medium was collected
and concentrated at 10 000 g for 30 min in a SpeedVac concentrator (Savant; E-C Instruments, Niantic, Connecticut, USA). The protein concentration was measured using BCA protein assay reagents. Equal amounts of conditioned media were mixed with no reducing sample buffer, incubated for 15 min at room temperature, and then electrophoresed on 10% SDS-PAGE gels containing 1 mg/ml gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 twice for 30 min, rinsed three times for 30 min with a 50 mmol/l Tris-HCl buffer (pH 7.6) containing 5 mmol/l CaCl₂, 0.02% Brij-35, and 0.2% sodium azide, and then incubated at 37°C overnight. The gels were stained with 0.5% Coomassie brilliant blue R-250 solution containing 10% acetic acid and 20% methanol for 30 min, and then destained with 7.5% acetic acid solution containing 10% methanol. Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background and the gels were scanned with Image Scanner II and analyzed with ImageQuant TLv 2003.03 Software (GE Healthcare, Pittsburgh, Pennsylvania, USA).

Statistical analyses

All the experiments were repeated at least three times. Data are represented as mean±SD. One-way analysis of variance, followed by Fisher’s least significant difference post-hoc test or Tamhane’s t-test (if equal variances were not assumed) was used to compare the data from different groups. *P* less than 0.05 was considered statistically significant.

Results

Production, purification, and identification of recombinant BJ46a

The expression of the rBJ46a supernatant was the highest at 48 h after induction, and the maximum yield was 26 mg/l (Fig. 1a). The culture supernatant was eluted by IMAC, and seven peaks were evident (Fig. 1b). The eluates of peaks III–V showed inhibitory activity. Thereafter, the eluates of peaks III–V were pooled for ultrafiltration and desalting. Approximately 10.15 mg/l of bioactive BJ46a was obtained with a purity of 98.06%. As BJ46a contains four putative N-glycosylation sites,
matched peptides are shown in **bold** type

1. MNSLVALVLL GQIIGSTLSS QVRGDELCDE KDAKEWTDTG VRYINEHKLSS
2. GKYKALNVIK NIVVVPWDGD WVAFLKNL LETECHVLDP TPVKNCTVRP
3. OHHXAVEMDC DVKIMFNVDT FKEDVFAKCH STPDVVENR RNCPCPIILL
4. PSNNPQVVDVS VEYLNKNHEH KLSHYYEVL EISRGQHKYE PEAYYVEFAI
5. VEVNTCAQEL HDDDHHCHPEN TAGEDHIGFC RATVFRSHAS LEKPKDEQFE
6. SDCVILHVKE GHAHSHLIQQ HVEKDSISPE HNNTALNFHV PHNDTSTSHF
7. SHEHLAEVPV AFVKKELPKVD ISDRHTTPVK GCPGKVHHFEL

Alignment of the amino acid sequence of *BJ46a*. The alignment indicated that the amino acid sequence by mass spectrometry analysis matched that expected for *BJ46a* (gi|48428861); matched peptides are shown in **bold** type. The score is 91 and the expected value is 0.0032, ion score is $> 10 \times \log (P)$, where $P$ is the probability that the observed match is a random event. Protein scores greater than 81 are significant ($P < 0.05$).

Located at amino acids 56–59, 165–168, 243–246, and 254–257; the anomalous higher value obtained by SDS-PAGE is probably an artifact as a result of the glycosylated state of *BJ46a*. Fortunately, Endo H can cleave various high mannose glycans. After treatment with Endo H, the deglycosylated recombinant protein migrated as a single band of 38 kDa, which confirmed the occurrence of glycosylation on the recombinant protein (Fig. 1c). Western blot analyses showed that the bioactive *rBJ46a* had a molecular weight of 57.9 kDa (Fig. 1d).

The identity of the purified protein was determined by MS. As shown in Fig. 2, the recombinant protein produced by *P. pastoris* shared 100% identity with the deduced amino acid sequence of *BJ46a*. The actual molecular mass was 38 745 Da by MS and consistent with the literature, in which *BJ46a* in the native state has a mass of 38 166 a.m.u. by MALDI-TOF MS following chemical deglycosylation.

**Recombinant BJ46a shows high inhibitory activity against matrix metalloproteinases**

The inhibitory profile of *rBJ46a* against MMPs was analyzed over 2 h in the presence of increasing concentrations of the inhibitor using the substrate gelatin and assessment of the residual activity was carried out using fluorescence detection. MMP activities decreased with increasing concentrations of the recombinant protein. At 0.5 mg/ml, *rBJ46a* effectively inhibited the activity of the MMPs with an IC50 of 0.119 mg/ml (Fig. 3a and b). Kinetic analyses by a series double reciprocal Lineweaver–Burk plots (1/V vs. 1/S) method indicated a competitive mode of inhibition, with *rBJ46a* showing highly effective inhibitory efficiency against MMPs ($K_i = 13.6$ nmol/l, Fig. 3c). These results suggest that *rBJ46a* is an active MMPI.

**Inhibitory effect of recombinant BJ46a on the invasion of tumor cells in vitro**

To determine the effects of *rBJ46a* on the invasion of the tumor cells *in vitro*, B16F10 and MHCC97H cells were treated with five doses of *rBJ46a* at 50, 100, 200, or 400 μg/ml for 24 h and Matrigel invasion assays were performed. As shown in Fig. 4, the numbers of invading B16F10 cells after treatment with *rBJ46a* were significantly lower than those of the untreated control ($P < 0.01$) (Fig. 4A and C), and the inhibition values were 18.6, 29.2, 33.3, 38.3, and 49.7%, respectively. The numbers of invading MHCC97H cells treated with *rBJ46a* were significantly reduced ($P < 0.01$) (Fig. 4B and C) and the inhibition values were 24.2, 28.3, 33.5, and 35.3%, respectively. These data show that *rBJ46a* can inhibit tumor cell invasion *in vitro*. However, no effect was observed on the proliferation, apoptosis, and migration of tumor cells (data not shown).

**Recombinant BJ46a inhibits the invasion, metastasis, and growth of tumor cells in vivo**

To further evaluate the antitumor activity of *rBJ46a*, experimental lung colonization assays in C57BL/6 mice and spontaneous lung metastases assays in nude mice were carried out. The lung colonization in C57BL/6 mice were 18.6, 29.2, 33.3, 38.3, and 49.7%, respectively. These data show that *rBJ46a* can inhibit tumor cell invasion *in vivo*. However, no effect was observed on the proliferation, apoptosis, and migration of tumor cells (data not shown).
In terms of the tumor growth in vivo, the lung weights in C57BL/6 mice after rBJ46a treatment at 1, 2, and 4 mg/kg increased more slowly than those in untreated mice, and the inhibition values were 21.9, 25.2, and 27.6%, respectively (P < 0.05) (Table 1). In nude mice, the weights of the primary tumors after rBJ46a treatment (4 mg/kg) were lower than those in control mice and the inhibition value was 26.7% (P < 0.05) (Table 1). These data show that rBJ46a can inhibit the growth of B16F10 cells and MHCC97H cells in vivo.

Recombinant BJ46a suppresses matrix metalloproteinase 2 and matrix metalloproteinase 9 enzymatic activities

It has been shown that MMP2 and MMP9 are the prominent MMPs responsible for ECM degradation. Therefore, to test whether the enzymatic activity of these two MMPs is regulated by rBJ46a, the tumor cells (B16F10/MHCC97H) were treated with various concentrations of rBJ46a, the enzymolysis strip volumes of proMMP-2, actMMP-2, proMMP-9, and actMMP-9 were reduced in a dose-dependent manner (Fig. 7). In the MHCC97H cells treated without rBJ46a, the enzymolysis strip volumes of proMMP-2, actMMP-2, proMMP-9, and actMMP-9 were 169±9, 83±15, 710±33, and 210±11 mm³, respectively, and in the MHCC97H cells treated with rBJ46a, the enzymolysis strip volumes of proMMP-2, actMMP-2, proMMP-9, and actMMP-9 were reduced in a dose-dependent manner (Fig. 7b) and the Kₘ values of rBJ46a against MMP2 and MMP9 enzymatic activities that may be expressed in both B16F10 and MHCC97H cells were 3.6 and 1.4 μmol/l, respectively. These results indicate that rBJ46a can suppress MMP9 and MMP2 activities by tumor cells.

Discussion

To further evaluate the anticancer effects of BJ46a, in this study, we first optimized the P. pastoris system for the production of rBJ46a on the basis of previous reports [10,22–24]. The conditions were optimized as follows: culture volume of 30% of the total flask volume; flasks covered with sterile gauze to provide adequate ventilation; and the addition of yeast extract and peptone in the expression medium to improve the yield and reduce the degradation of secretory proteins [25,26].
The maximum yield was 26 mg/l by shaking-flask fermentation. Approximately 10.15 mg/l of bioactive material was obtained with a purity of 98.06%. Enzchek gelatinase/collagenase assay showed that rBJ46a effectively inhibited the activity of the MMPs with an IC_{50} of 0.119 mg/ml. The inhibitory activity was comparable with those of other MMPIs, such as batimastat (IC_{50} values 10 ng/ml) and marimastat (IC_{50} values 3 ng/ml) of peptidomimetic MMPIs [27,28], BAY12-9566 (IC_{50} values below 0.13 μg/ml) and AG3340 (IC_{50} values below 0.13 ng/ml) of nonpeptidic MMPIs [29,30], and Col-3 (IC_{50} values 12 μg/ml) of tetracycline derivatives. Overall, the inhibitory spectrum of rBJ46a was similar to those of synthesized MMPIs [31]. Kinetic analyses showed a competitive mode of inhibition with rBJ46a showing highly effective inhibitory efficiency against MMPs (K_i = 13.6 nmol/l). This specific activity was comparable with those of other MMPIs, such as recombinant TIMP-4 (K_i = 14.5 nmol/l) from P. pastoris expression [32], recombinant TIMP-1 (K_i = 11.8 nmol/l) from CHO-K-1 cells, and recombinant TIMP-2 (K_i = 14.9 nmol/l) from 293-EBNA cells [33]. These data show that the optimized P. pastoris system for recombinant protein production, as reported here, allowed for high recovery, high purity, and high activity, which provided the preconditions for future evaluation of its applications in the pharmaceutical industry.

Subsequently, we showed that the rBJ46a significantly blocked the invasion of B16F10 cells and MHCC97H cells in vitro in a dose-dependent manner by Matrigel invasion assays, indicating that rBJ46a has the potential to degrade...

![Figure 4](image-url)
ECM and basement membranes. In addition, the administration of rBJ46a by multiple i.p. injections prevented spontaneous pulmonary metastases of MHCC97H cells in nude mice, and the administration of rBJ46a by three i.v. injections was sufficient to inhibit pulmonary colonization of B16F10 cells in C57BL/6 mice. The inhibition rate of tumor cell invasion and metastasis is similar to many MMPIs, including synthetic and natural inhibitors [29–34]. However, the inhibitory effect of rBJ46a on tumor metastasis in vivo is relatively modest, which might be associated with the rBJ46a concentration entering the tumor. In addition, the molecular complexity of cancer progression suggests that the appropriate combination of rBJ46a and other MMPIs or molecular targeted agents may increase drug efficacy. In this respect, we recently determined the effects of combining rBJ46a and snake venom cystatin (sv-cystatin) on invasion and metastasis of MHCC97H cells. The results showed that the common expression of BJ46a and sv-cystatin could synergistically inhibit invasion and metastasis of MHCC97H cells in vivo (data not shown). Taken together, these data further show that rBJ46a played an inhibitory role in the invasion and metastasis of tumor cells in vitro and in vivo. It seems that rBJ46a not only inhibits hematogenous invasion of tumor cells but also prevents tumor cells from escaping from the primary tumor and establishing colonies at local and distant sites. These findings provide novel evidence that the use of rBJ46a may be an effective approach to inhibit the invasion and metastasis of tumor cells through multiple targets. However, the molecular mechanism underlying the inhibitory action of rBJ46a on tumor invasion and metastasis remains unclear.

It has been shown that the proteolytic activity of MMPs is required for the degradation of ECM and basement membranes during invasion and metastasis [2], and this study showed that rBJ46a shows high inhibitory activity against MMPs. Therefore, it is reasonable to conclude
that rBJ46a inhibits tumor invasion and metastasis by blocking MMP activities. However, the MMP family consists of a variety of proteolytic enzymes that mediate the degradation of many different components of the ECM [35,36]. In particular, MMP2 and MMP9 are gelatinases that play key roles in the invasion and metastasis of cancer cells through the destruction of the basal membrane and ECM [37]. To further identify the
molecular targets for the inhibition of invasion and metastasis of tumor cells by rBJ46a, a gelatin zymography experiment was performed. We showed that MMP9 and MMP2 enzymatic activities in tumor cells were suppressed by rBJ46a in a dose-dependent manner and the $K_m$ value of rBJ46a against MMP2 and MMP9 enzymatic activities expressed in both B16F10 and MHCC97H cells was 3.6 and 1.4 $\mu$mol/l, respectively, which was comparable with those of other MMPIs, such as TIMP-1, TIMP-2, and TIMP-4 [32,33]. These findings show that rBJ46a may function as a target inhibitor of MMPs or TIMPs to reduce the activities of MMP2/MMP9 in tumor cells, which then decreases the degradation of the ECM and basement membrane to inhibit tumor cell metastasis.

Until now, there has been no direct evidence showing the link of MMP with BJ46a. BJ46a, a 46 kDa protein isolated from the serum of B. jararaca, is an inhibitor of SVMPs. BJ46a is a member of the fetuin family and cystatin superfamily, which interacts with metalloproteinases.
through the formation of a noncovalent complex, specifically inhibiting atrolysin C (class P-I) and jararhagin (class P-III) proteolytic activities [6]. Because SVMP shares homology with the zinc-binding environment of MMPs, it is possible that BJ46a may be able to modulate anticancer invasion and metastasis through the formation of a complex with MMP. Recently, we predicted the 3D structure of rBJ46a and proposed that BJ46a does not interact as a dimer with the metalloproteinase, although it is a dimer protein in its native state. Instead, BJ46a may inhibit MMPs because of its two cystatin-like domains in one monomer, which interact with the catalytic zinc, leading to enzymatic inhibition [10]. Further studies using immunoprecipitation are required to prove the binding of BJ46a and MMPs.

Previous studies in our laboratory have shown that transfection of the BJ46a gene into B16 cells resulted in the inhibition of invasion potential in vitro and in vivo [8]. Currently, we have also shown that transfection of the BJ46a gene into MHCC97H cells results in the inhibition of invasion in vitro and in vivo (data not shown). In this study, we have further shown that rBJ46a can inhibit the invasion and metastasis of B16F10 and MHCC97H cells in vitro and in vivo by blocking MMP2 and MMP9 activities. The role of MMPs is regulated at multiple levels, such as by gene expression, protein amounts, and proteolytic activity. Many studies have shown that the expression of MMP2 and MMP9 by tumor cells is associated with tumor invasion and metastasis [2,3]. However, further studies are required to examine the effects of BJ46a on the expression of MMP2 and MMP9 to gain further insights into its inhibitory mechanism of action on tumor invasion and metastasis.

It has been shown that the expression and activities of MMP2 and MMP9 play a key role in tumor angiogenesis because they activate several proangiogenic factors, such as vascular endothelial growth factor, basic fibroblast growth factor, and degrade the extracellular components, such as collagen type IV, and perlecan, respectively, facilitating tumor angiogenesis [2,3]. This led us to speculate that BJ46a may inhibit tumor angiogenesis by reducing the activities of MMP2 and MMP9. Further studies are required to determine the effects of BJ46a on downstream signaling events associated with each MMP activity.

More recently, we showed that the transfection of MHCC97H cells with sv-cystatin repressed tumor invasion and metastasis, mainly by inhibiting cathepsin B, MMP2, and MMP9 activities, as well as epithelial–mesenchymal transition, suggesting that sv-cystatin may function as a target inhibitor of cysteine protease and cathepsin B, and as an off-target inhibitor of MMPs to inhibit tumor invasion and metastasis. In addition, overexpression of MMP2 and MMP9 has been associated with epithelial to mesenchymal transition. Although classified as members of the cystatin superfamily, BJ46a is unlikely to be a viable cysteine protease inhibitor because the consensus sequence for the cystatin active site, QXVXG, has been extensively altered in this molecule. Corroborating this view, BJ46a failed to inhibit cathepsin B activity [6]. These data indicate that BJ46a may function only as a target inhibitor of MMP2 and MMP9, but not as an off-target inhibitor of cysteine protease and cathepsin B, to inhibit epithelial–mesenchymal transition, and tumor invasion and metastasis [38–40]. It will be necessary to test this hypothesis in the future.

In conclusion, in this study, we showed for the first time that rBJ46a in an optimized P. pastoris expression system can inhibit the invasion and metastasis of tumor cells by reducing MMP2/MMP9 activities. These results indicate that rBJ46a may be a promising candidate therapeutic agent for antimetastasis of tumor cells, and may have potential for pharmaceutical applications. For future studies, we hope to focus on evaluating the safety and efficacy of this agent (including pharmacokinetics, pharmacodynamics, toxicology, tolerability, and immunogenicity) to facilitate studies into pharmaceutical applications.

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Conflicts of interest
There are no conflicts of interest.

References


