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Antitumour activity of the recombination polypeptide GST-NT21MP is mediated by inhibition of CXCR4 pathway in breast cancer

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Background: CXC chemokine receptor 4 (CXCR4) and its ligand stromal cell-derived factor-1 α (SDF-1 α , also known as CXCL12) have important roles in promoting tumour growth and metastasis. Therefore, targeting CXCR4 could be a promising strategy for treatment of human cancer.

Methods: To achieve this goal, we developed a highly purified recombination polypeptide (GST-NT21MP), which is a synthetic 21-mer peptide antagonist of CXCR4 (NT21MP) derived from the viral macrophage inflammatory protein II by fermentation technology, affinity chromatography and fast protein liquid chromatography. In this study, we used multiple methods such as MTT assay, FACS, invasion assay, RT-PCR and western blot to explore the efficacy and mechanism by which GST-NT21MP inhibits cell growth, migration and invasion of breast cancer *in vitro* and *in vivo*.

Results: We found that blockade of CXCR4 pathway by GST-NT21MP decreased SDF-1-induced cell growth, adhesion and migration capacities in breast cancer cells. Moreover, GST-NT21MP significantly retarded pulmonary metastasis *in vivo*. Furthermore, GST-NT21MP-mediated antitumour activity was found to be associated with reduced phosphorylated Src, Akt, FAK and ERK1/2 as well as decreased Bcl-2.

Conclusions: Our results suggest that GST-NT21MP could be a potential anticancer agent for the treatment of breast cancer.

Breast cancer is one of the most frequently diagnosed cancers and the leading cause of cancer-related death in women in the United States (Siegel *et al*, 2013). Approximately 226 870 new breast cancer cases were expected to occur and 39 510 patients were expected to die due to this deadly disease in 2012 (Siegel *et al*, 2013). Although the current therapies including surgery, radiation, chemotherapy, hormonal therapy or combined therapies have improved the 5-year survival rate, the patients with breast cancer still suffer from long-term survival partly due to cancer metastasis

(Scully *et al*, 2012). Metastasis is responsible for survival rate from 90% for localised breast cancer to 20% for metastatic breast cancer (Trape and Gonzalez-Angulo, 2012). Therefore, it is pivotal to explore the underlying mechanism of metastasis and develop novel therapeutic agents for inhibition of metastasis in breast cancer patients.

Recently, it has been reported that stromal cell-derived factor-1/CXC chemokine ligand 12 (SDF-1/CXCL12) and its G-protein-coupled receptor CXC chemokine receptor 4 (CXCR4) have a

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critical role in breast cancer growth and metastasis (Jin *et al*, 2012; Mukherjee and Zhao, 2013). It is known that SDF-1 binds to CXCR4 and subsequently activates their multiple target genes including extracellular regulated kinase 2 (ERK2), protein kinase B (PKB), phosphatidylinositol 3-kinase (PI3K), steroid receptor coactivator (SRC) and mitogen activated protein kinase (MAPK), resulting in the regulation of cell growth, survival and metastasis (Epstein, 2004). Indeed, overexpression of CXCR4 has been observed in breast cancer cells, malignant breast tumours and metastasis sites (Muller *et al*, 2001). Moreover, the interaction between CXCL12 and CXCR4 leads to actin polymerisation and pseudopodia formation, resulting in cell chemotactic migration and metastasis in breast cancer cells (Hinton *et al*, 2010; Hirbe *et al*, 2010). Thus, targeting CXCR4 signalling pathway could be a novel strategy to block tumour metastasis, which could ultimately improve the survival rate in patients diagnosed with breast cancer.

The viral macrophage inflammatory protein-II (vMIP-II) encoded by Kaposi's sarcoma-associated herpesvirus is unique among all known chemokines, in that vMIP-II shows a broad-spectrum interaction with both CC and CXC chemokine receptors including CXCR4 (Kledal *et al*, 1997). A peptide corresponding to amino-acid residues 1–21 of the N-terminal of vMIP-II has been shown to strongly and selectively bind to CXCR4 (Khan *et al*, 2007), suggesting that the N-terminus of vMIP-II is essential for its function via CXCR4. Therefore, in this study, we developed a synthetic peptide derived from the 21-residue N-terminal of vMIP-II (NT21MP) against CXCR4 pathway. Owing to high cost for chemical synthesis of peptides, we subsequently developed a highly purified recombination polypeptide (GST-NT21MP) by fermentation technology, affinity chromatography, fast protein liquid chromatography (FPLC) and ultrafiltration. In this study, we assessed the effect of GST-NT21MP on breast cancer cell growth, apoptosis, migration and invasion, and further explored the mechanism underlying antitumour activity of GST-NT21MP *in vitro* and *in vivo*.

MATERIALS AND METHODS

Reagents. Anti-Bcl-2, anti-Bax, anti-caspase-3, anti-focal adhesion kinase-1 (FAK-1), anti-ERK1/2, anti-phospho-ERK1/2, anti- β -actin, anti-mouse IgG-FITC and polymerised HRP-anti Rb IgG antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-Akt and anti-phospho-Akt antibodies were obtained from Signalway Antibody (College Park, MD, USA). Anti-CXCR4 was purchased from Abcam (Cambridge, MA, USA). Anti-Src and anti-phospho-Src antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Hoechst 33258 was purchased from Sigma-Aldrich (St Louis, MO, USA). *In Situ* Cell Apoptosis Detection Kit II was purchased from Wuhan Boshide Biotechnology (Wuhan, China). Elivision™ plus Polymer HRP (mouse/rabbit) IHC Kit was purchased from Maixin Biology (Fuzhou, China). TdT-mediated dUTP nick end labelling (TUNEL) kit was obtained from Wuhan Boster Biological Technology (Beijing, China). Human-SDF-1 α was purchased from PeproTech Systems (Rocky Hill, NJ, USA). AMD3100, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and fibronectin were obtained from Sigma-Aldrich.

Cell culture. The breast carcinoma cell lines SKBR-3, MCF-7 and 4T-1 were cultured in RPMI-1600 (Invitrogen, Carlsbad, CA, USA) supplemented with 5% or 10% foetal bovine serum, 50 units ml⁻¹ penicillin and 50 μ g ml⁻¹ streptomycin. All cells were maintained in a 5% CO₂-humidified atmosphere at 37 °C.

Development of GST-NT21MP. The N-terminal GST fusion protein derived from viral macrophage inflammatory protein II (GST-NT21MP) was developed. Specifically, the N-terminal of

vMIP-II cDNA was derived by chemical synthesis method. DNA fragment encoding the NT21MP peptide was inserted into pGEX-KG vector and the recombinant plasmid was expressed in *E. coli* BL21 (DE3). NT21MP peptide, which was recombined with a glutathione S-transferase (GST) at the N-terminal, was expressed in *E. coli* cells. SDS-PAGE and western blot analysis showed that the recombinant protein existed mainly in the supernatant of *E. coli* lysates. The supernatant was further purified by affinity chromatography, ultrafiltration and FPLC.

Immunofluorescence staining. Cells were fixed with 4% paraformaldehyde at room temperature for 20 min, then permeabilised with PBS-T solution at 4 °C for 10 min and subsequently blocked with PBS-B solution at 37 °C for 30 min. The cells were then incubated with primary antibody solution at 4 °C overnight. After washing with PBS, cells were incubated with secondary antibody for 1 h at room temperature. After washing, nuclei were stained in 10 μ g ml⁻¹ Hoechst solution. Cell images were observed under a confocal laser scanning microscope.

Cell growth inhibition by MTT assay. The effect of GST-NT21MP on the cell viability was determined by MTT assay. Briefly, MCF-7, SKBR-3 and 4T-1 cells were seeded in 96-well culture plates in serum-free medium at an optimal density. After 24-h incubation, cells were treated with different concentrations of GST-NT21MP (0.1, 1.0, 2.0 μ g ml⁻¹), 2.0 μ g ml⁻¹ GST and 1 μ g ml⁻¹ AMD3100 (CXCR4 antagonist) for 30 min, respectively. Cells were then added to SDF-1 α (100 ng ml⁻¹). MTT assay was performed after 48 and 72 h as described earlier (Xia *et al*, 2012). Each experiment was performed in triplicate.

Soft agar assay for colony formation. In all, 0.7% and 1.2% low melting point agar (Sigma-Aldrich) was prepared and mixed with equal ratio in RPMI-1640 in 60 °C after sterilisation. In total, 0.6 ml 1.2% agar was added in the bottom of the 24-well plate. About 1000 cells and 0.6 ml 0.7% agar were mixed and the mixture was added to the upper portion of 1.2% agar. After routine culture for 2 weeks, colony numbers were counted under a microscope.

The cell adhesive ability assay. Cell adhesion assay was performed in 96-well flat bottom plates coated with fibronectin. Briefly, 50 μ l per well solution of fibronectin (10 μ g ml⁻¹) in PBS were pre-added to 96-well plates and incubated at 37 °C overnight. The plates were then rinsed and blocked with 0.2% BSA for 2 h at room temperature followed by three times washing in PBS. In all, 4 \times 10⁵ cells were added to each well in triplicate and incubated for 1 h at 37 °C. After washing, cells attached to the plates were fixed by 4% paraformaldehyde and stained with a solution containing 0.1% (w/v) crystal violet in PBS. Superfluous crystal violet was washed out with distilled water and airing thoroughly. The staining crystal violet was dissolved by 0.1 mol l⁻¹ sodium citrate (PH4.2)/50% ethanol. Automatic microplate reader (BIO-RAD550, Hercules, CA, USA) was used to measure the absorbance value of optical density (OD) at 550 nm.

Real-time reverse transcription-PCR analysis for gene expression. The total RNA was isolated with Trizol (Invitrogen) according to the manufacturer's protocols. The PCR reactions were described previously (Wu *et al*, 2013). The primers used in PCR reaction are as follows: CXCR4, forward primer (5'-GAAC CCTGTTTCCGTGAAGA-3') and reverse primer (5'-CTTGTC GTCATGCTTCTCA-3'); β -actin, forward primer (5'-TCACCCA CACTGTGCCCATCTACGA-3') and reverse primer (5'-CAGCG GAACCGCTCATTGCCAATGG-3').

Western blotting. Cells were lysed in lysis buffer (50 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40) supplemented with protease inhibitors and phosphatase inhibitors. The protein concentrations of the lysates were measured using the Bio-Rad protein assay reagent using a spectrophotometer. The lysates were then resolved

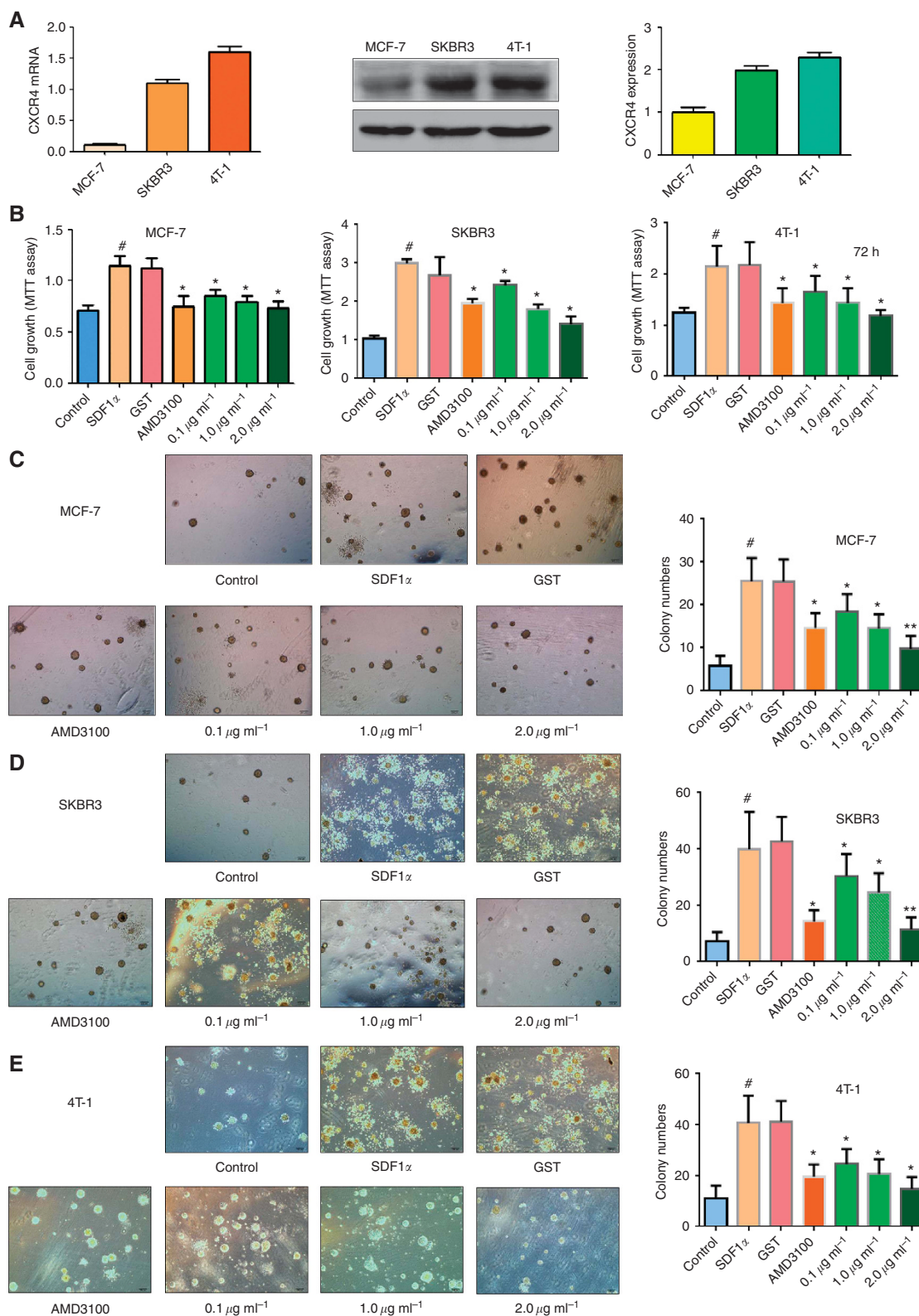


Figure 1. GST-NT21MP inhibited cell proliferation and colony formation in MCF-7, SKBR-3 and 4T-1 breast cancer cells. 0.1 g ml $^{-1}$: GST-NT21MP 0.1 g ml $^{-1}$; 1.0 g ml $^{-1}$: GST-NT21MP 1.0 g ml $^{-1}$; 2.0 g ml $^{-1}$: GST-NT21MP 2.0 g ml $^{-1}$. (A) CXCR4 expression in different human breast cancer cell lines. Left panel: real-time RT-PCR was performed to detect CXCR4 mRNA levels in three breast cancer cell lines. Middle panel: western blotting was conducted to detect CXCR4 protein levels. Right panel: quantitative analysis of CXCR4 expression in middle panel. (B) The effect of GST-NT21MP on cell viability was determined by the MTT assay. # $P < 0.05$, compared with control; * $P < 0.05$, compared with the SDF1 α treatment. (C–E) The effect on cell survival by GST-NT21MP was measured by soft agar colony formation assay in MCF-7 (C), SKBR3 (D) and 4T-1 (E) cells. Left panel: microphotographs of cell survival at indicated treatment. Right panel: quantitative analysis of colony assay. # $P < 0.05$, compared with control; * $P < 0.05$, ** $P < 0.01$, compared with the SDF1 α treatment.

by SDS-PAGE and immunoblotted with indicated antibodies for western blotting as described earlier (Wu *et al*, 2013).

Invasion assay. The invasive capacity of MCF-7, SKBR-3 and 4T-1 cells followed by GST-NT21MP treatment were performed using 8.0 μm pore size Transwell inserts (Costar, Cambridge, MA, USA). Briefly, after incubation with GST-NT21MP or AMD3100 for 30 min at 37 °C, breast cancer cells were then added to the upper chamber of the inserts. DMEM medium with or without SDF-1 α was added to the lower chamber. After incubation for 12 h, the upper surfaces of the transwell chambers were wiped with cotton swabs and the invading cells were fixed and stained with Giemsa solution. The stained invasive cells were photographed and counted in five randomly selected fields under a microscope.

Apoptosis assay by annexin V-FITC and PI staining. Annexin V-FITC and PI staining were performed to detect early stage apoptosis in MCF-7, SKBR3 and 4T-1 cells treated with AMD3100 or GST-NT21MP. Cells were washed with PBS and harvested using a commercially available formulation (Accutase; Innovative Cell Technologies Inc., San Diego, CA, USA). The resultant cell pellets were resuspended in binding buffer (Caltag Laboratories, Burlingame, CA, USA) and stained with annexin V-FITC (Caltag Laboratories) and PI (Sigma-Aldrich). After incubation for 10 min at room temperature in the dark, the samples were immediately analysed by flow cytometry (FACSCalibur system; BD Biosciences, San Jose, CA, USA).

Animal experiments. The BALB/c female mice, aged 6–8 weeks, were purchased from Shanghai SLAC Laboratory Animal Co. Ltd

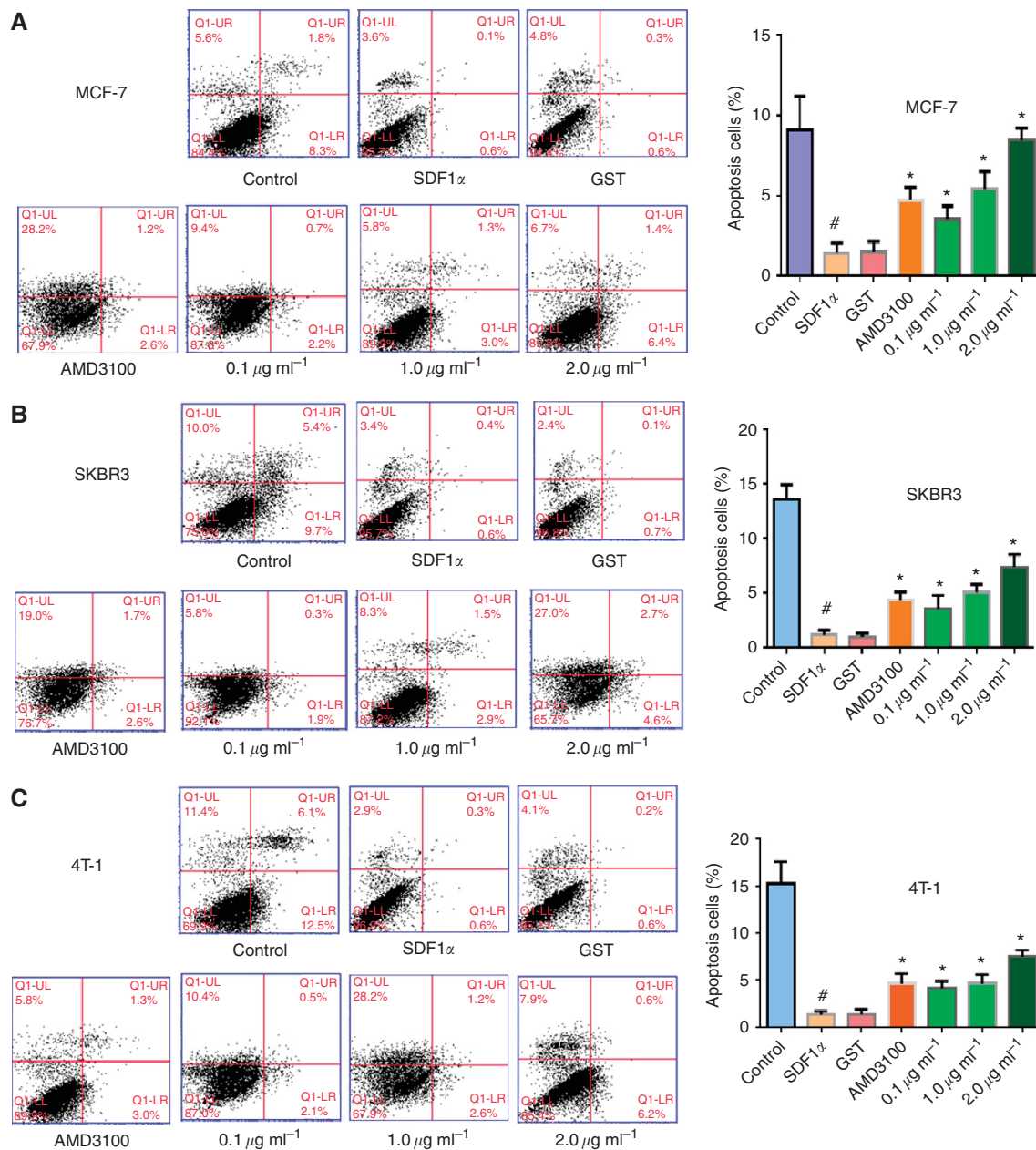


Figure 2. GST-NT21MP induced apoptosis in MCF-7, SKBR-3 and 4T-1 breast cancer cells. 0.1 g ml^{-1} : GST-NT21MP 0.1 g ml^{-1} ; 1.0 g ml^{-1} : GST-NT21MP 1.0 g ml^{-1} ; 2.0 g ml^{-1} : GST-NT21MP 2.0 g ml^{-1} . Effect of GST-NT21MP on apoptosis in MCF-7 (A), SKBR3 (B), 4T-1 (C) cells. Left panel: apoptosis analysis by annexin V FITC flow cytometry analysis at 72 h under indicated treatments in breast cancer cells. Right panel: representative % apoptosis after GST-NT21MP treatment. # $P < 0.05$, compared with control; * $P < 0.05$, compared with the SDF1 α treatment.

(Shanghai, China). The mice were housed and maintained under sterile conditions and used in accordance with Animal Care and Use Guidelines of Bengbu Medical College. The animal experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Bengbu Medical College Institutional Users of Animal Care Committee. Mice were randomly divided into six

groups (12 mice per group). In all, 1×10^6 4T-1 cells were injected in the second right mammary gland. After 24 h, the mice were treated with saline, GST, AMD3100 (5 mg kg^{-1}) and different doses of GST-NT21MP (50, 500 or $5000 \mu\text{g kg}^{-1}$). GST-NT21MP, saline and GST were injected through the tail vein, 5 days per week for 4 weeks, whereas AMD3100 was injected through the tail vein once

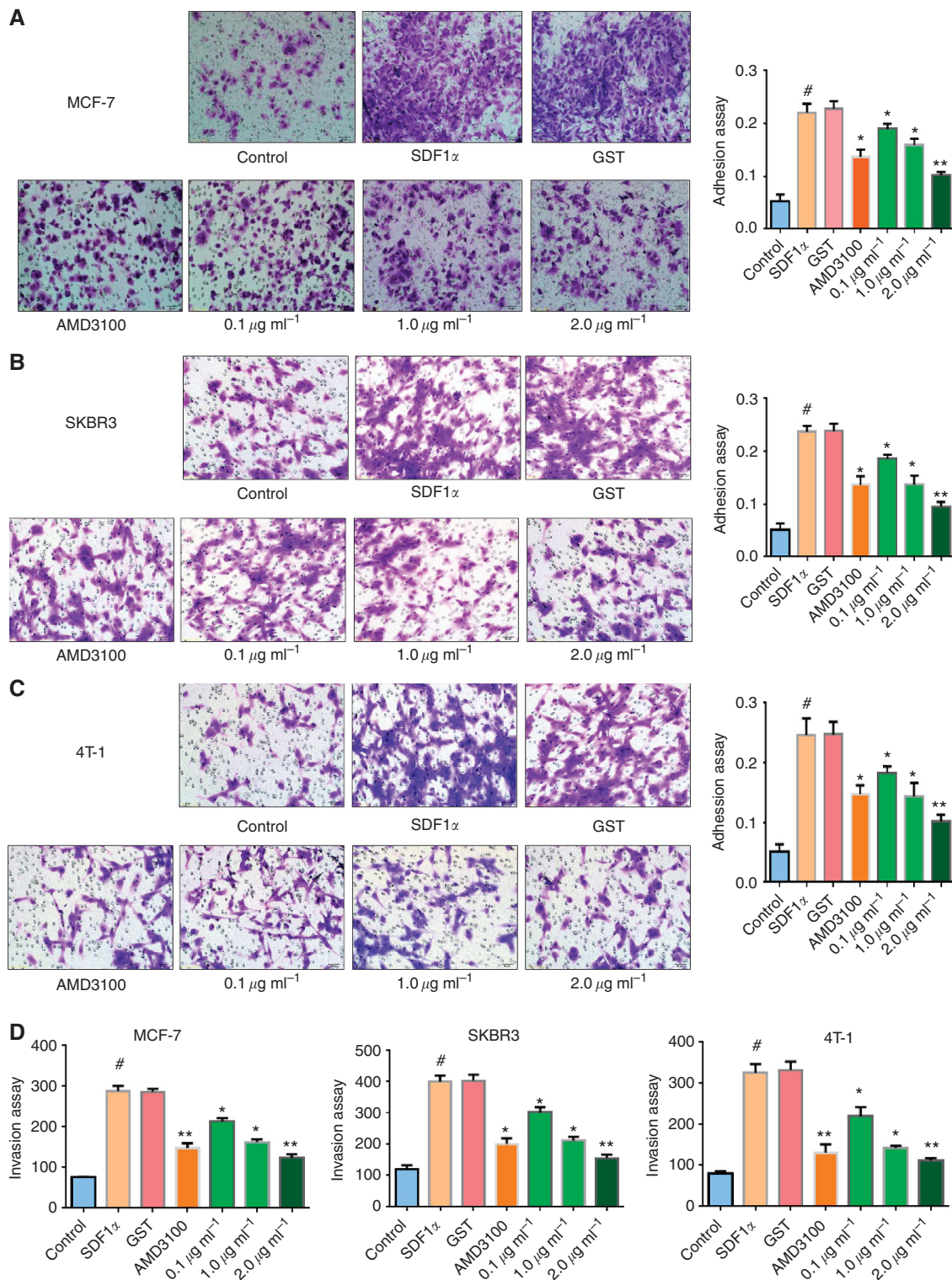


Figure 3. GST-NT21MP inhibited cell adhesion and invasion. 0.1 g ml^{-1} : GST-NT21MP 0.1 g ml^{-1} ; 1.0 g ml^{-1} : GST-NT21MP 1.0 g ml^{-1} ; 2.0 g ml^{-1} : GST-NT21MP 2.0 g ml^{-1} . # $P < 0.05$, compared with control; * $P < 0.05$, ** $P < 0.01$, compared with the SDF1 α treatment. (A–C) Left panel: effect of GST-NT21MP on cell adhesion in MCF-7 (A), SKBR3 (B), 4T-1 (C) cells. Right panel: quantitative analysis of adhesion assay. (D) Effect of GST-NT21MP on breast cancer cell invasion.

per week. After 4-week treatment, mice were killed. Tumours, lungs and blood were harvested and processed for molecular analysis. The tumours were excised neatly, weighed and subsequently processed for H&E, immunohistochemical staining and preparation of protein extracts for western blotting.

Histologic sections and immunohistochemistry. Immunohistochemical studies were performed to determine the expression of p-Akt, p-ERK, p-FAK, Akt, ERK1/2, FAK, Bcl-2, Bax and caspase-3 in tumours as described before (Wang *et al*, 2012b). Tumour proliferation was assessed by PCNA staining and tumour apoptosis was evaluated by TUNEL as described previously (Wang *et al*, 2009).

Statistical analysis. To determine significant differences between individual groups, the data were analysed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test of multiple comparisons. Data presented are mean \pm s.d. of at least three repeats and all differences were considered significant at $P < 0.05$.

RESULTS

CXCR4 is highly expressed in breast cancer cell lines. The baseline expression of CXCR4 was determined using real-time RT–PCR and western blot analysis, respectively. Our results showed that CXCR4 was frequently but differentially overexpressed in different human breast cancer cell lines including MCF-7, SKBR3 and 4T-1 breast cancer cells (Figure 1A). Consistently, our confocal immunofluorescence analysis showed the similar results (Supplementary Figure 1). Next, we investigated whether CXCR4 inhibitor, GST-NT21MP, could inhibit cell growth of breast cancer cells.

GST-NT21MP inhibits cell proliferation of breast cancer cell lines. To determine whether GST-NT21MP could exert its antitumour activity in breast cancer cells, we measured cell growth inhibitory effects of GST-NT21MP using the MTT assay. As illustrated in Figure 1B, treatment with SDF-1 α significantly

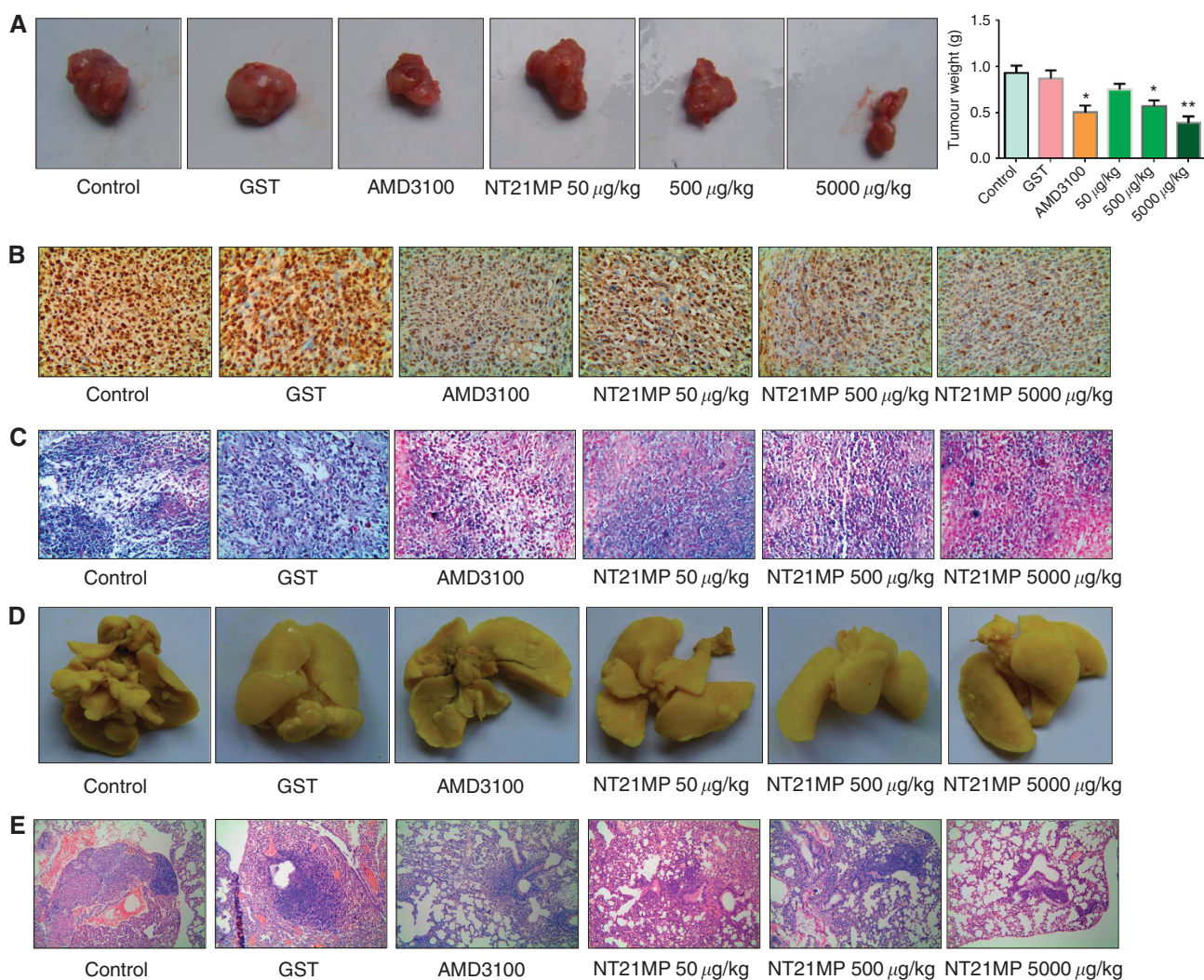


Figure 4. GST-NT21MP inhibited tumour growth and induced apoptosis as well as suppressed tumour metastasis *in vivo*. (A) GST-NT21MP retarded the growth of 4T-1 cells in mice. Left panel: photographs of tumour size at the time of killing. Right panel: total tumour weights in mice were measured at the time of killing. * $P < 0.05$, ** $P < 0.01$, compared with control. (B) The expression of PCNA was assessed by immunohistochemistry. Tumours were resected and 5 μ m tissue sections were stained with antibody to PCNA. (C) Immunohistochemical demonstration of apoptosis by TUNEL in tissues harvested from mice. Significant differences in percentage of TUNEL-positive cells were noted in tumours derived from the GST-NT21MP treatment group relative to control group. (D) Lung tissues in mice were immersed and fixed in Bouin compound for 24 h and destained with absolute ethanol for detecting the metastatic nodules on the lung surface of mice. (E) Metastatic Lewis lung tumours were inhibited in GST-NT21MP-treated group under light microscope.

increased cell viability, whereas GST-NT21MP inhibited cell growth in a dose-dependent manner in all three breast cancer cells including MCF-7, SKBR3 and 4T-1 cells (Figure 1B, Supplementary Figure 2). To further confirm these results, we performed clonogenic assay to detect the effects of GST-NT21MP on cell survival as shown below.

Inhibition of cell survival by GST-NT21MP using clonogenic assay. The effect of cell survival by GST-NT21MP was measured by soft agar colony formation assay. Consistent with our MTT result, we found that treatment with GST-NT21MP significantly inhibited the colony formation compared with SDF-1 α -treated groups (Figures 1C–E), suggesting that GST-NT21MP inhibits the growth of breast cancer cells. Moreover, we evaluated the effects of GST-NT21MP treatment on apoptosis in breast cancer cells.

GST-NT21MP induced apoptosis in breast cancer cell lines. MCF-7, SKBR3 and 4T-1 cells were treated with GST-NT21MP for 72 h. By staining cells with annexin V-FITC and PI, FACS was used to distinguish and quantitatively determine the percentage of dead, viable and apoptotic cells after treatment. We found that SDF-1 α treatment resulted in a significant decrease in apoptosis. GST-NT21MP significantly attenuated the anti-apoptotic effects of SDF-1 α in a dose-dependent manner (Figure 2), indicating that GST-NT21MP could induce apoptosis in breast cancer cells.

GST-NT21MP decreased breast cancer cell adhesion and invasion. As CXCR4 has a critical role in cancer metastasis, we tested the effects of GST-NT21MP on breast cancer cell adhesion and invasion. As expected, we observed that SDF-1 α promoted cell adhesion to fibronectin (Figures 3A–C). Furthermore, GST-NT21MP treatment led to decreased SDF-1 α -induced penetration of breast cancer cells through the matrigel-coated membrane compared with the control cells (Figure 3D). These results indicate that GST-NT21MP could inhibit invasion of breast cancer cells.

GST-NT21MP inhibited breast tumour growth and induced apoptosis *in vivo*. To determine whether systemic therapy with GST-NT21MP could stunt tumour growth in animals, we established 4T-1 breast cancer mouse model. We injected 4T-1 cells in the second right mammary gland in mice. As shown in Figure 4A, GST-NT21MP treatment significantly inhibited tumour growth compared with untreated control. It is important to note that 5000 g kg⁻¹ GST-NT21MP did not cause any toxicity or loss of body weight during the course of the treatment and up to 4 weeks. We also detected the PCNA expression by immunohistochemical staining. Notably, we observed a decreased PCNA in the GST-NT21MP-treated tumours compared with control tumours (Figure 4B), demonstrating that GST-NT21MP inhibited tumour cell proliferation. Tumour apoptosis was evaluated by TUNEL.

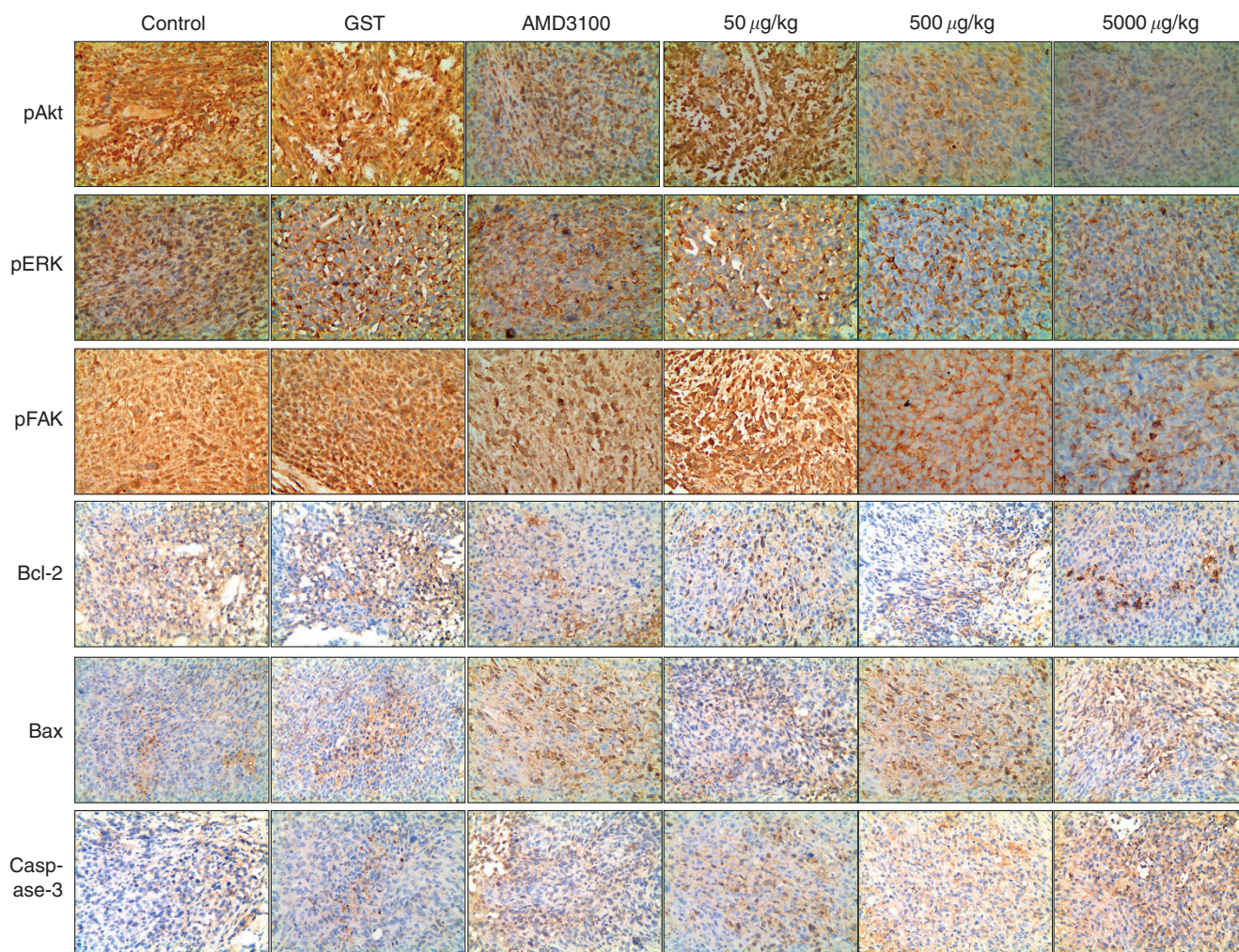


Figure 5. Immunohistochemical staining of tumour tissue sections with antibodies to pAkt, p-ERK, p-FAK, Bcl-2, Bax and caspase-3. The expression of pAkt, p-ERK, p-FAK, Bcl-2, Bax and caspase-3 was assessed by immunohistochemistry in tumours from control, GST, AMD3100 and GST-NT21MP-treated groups.

We found that the apoptosis of tumour cells were increased by GST-NT21MP treatment compared with the control group (Figure 4C).

Inhibitory effect of GST-NT21MP on metastasis *in vivo*. The mice were killed in the fourth week and lung tissues were immersed and fixed in Bouin compound for 24 h and destained with absolute ethanol for detecting the metastatic nodules in the lung surface of mice. We found that the lung metastatic nodules were significantly decreased after GST-NT21MP treatment compared with control groups (Figure 4D). Specifically, no obvious metastatic nodules were seen with visual observation in 5000 g kg⁻¹ GST-NT21MP-treated group. Under light microscope, metastatic Lewis lung tumours were inhibited in GST-NT21MP-treated groups (Figure 4E), suggesting that GST-NT21MP could inhibit metastasis in breast cancer.

GST-NT21MP downregulated Akt, ERK1/2, FAK, Src and Bcl-2 *in vivo*. To determine whether the antitumour activity of GST-NT21MP is due to inhibition of CXCR4 target genes, we measured the expression of Akt, ERK and FAK in mouse tumours by immunohistochemistry and western blot analysis, respectively. As expected, we found that GST-NT21MP treatment resulted in the reduction of pAkt, pERK and pFAK levels (Figures 5, 6A and B), but not total levels of Akt, ERK and FAK (Supplementary Figure 3). It has been reported that Src kinase activation is involved in SDF-1 α stimulation of CXCR4 in breast cancer cells (Cabioglu *et al*, 2005). To explore whether GST-NT21MP could regulate the activation of Src, we measured the expression of Src in mouse tumours. We observed that the expression of p-Src was significantly decreased after

GST-NT21MP treatment (Figure 6A). To further elucidate whether GST-NT21MP could regulate downstream genes of pAkt and ERK, we assessed the expression of Bcl-2, Bax and caspase-3 in mouse tumours. We observed that GST-NT21MP treatment down-regulated Bcl-2 and upregulated Bax as well as activated caspase-3 (Figures 5, 6C and D). Therefore, our results suggest that GST-NT21MP is a powerful agent for the inhibition of breast cancer cell growth and metastasis, which is mediated by suppression of Akt, ERK and FAK pathways.

DISCUSSION

Multiple studies have demonstrated that SDF-1/CXCR4 has pivotal roles in tumorigenesis (Liekens *et al*, 2010; Domanska *et al*, 2013). The activation of CXCR4 has been reported to promote tumour growth, motility, invasion and metastasis in various human cancers (Domanska *et al*, 2013). Therefore, development of an inhibitor targeting CXCR4 could provide a therapeutic benefit for human cancers (de Nigris *et al*, 2012; Peled *et al*, 2012). To achieve better treatment of human cancer, we recently developed GST-NT21MP, which is capable of antagonising the function of CXCR4 pathway. This study demonstrates that GST-NT21MP inhibits tumour growth and metastasis of breast cancer cells, which was in part mediated through inactivation of Src, Akt, ERK, FAK and their downstream genes such as Bcl-2.

SDF-1/CXCR4 axis has been reported to enhance cell proliferation in a variety of human cancers including breast cancer (Berghuis *et al*, 2012; Wang *et al*, 2012a; Ling *et al*, 2013). Consistent with this

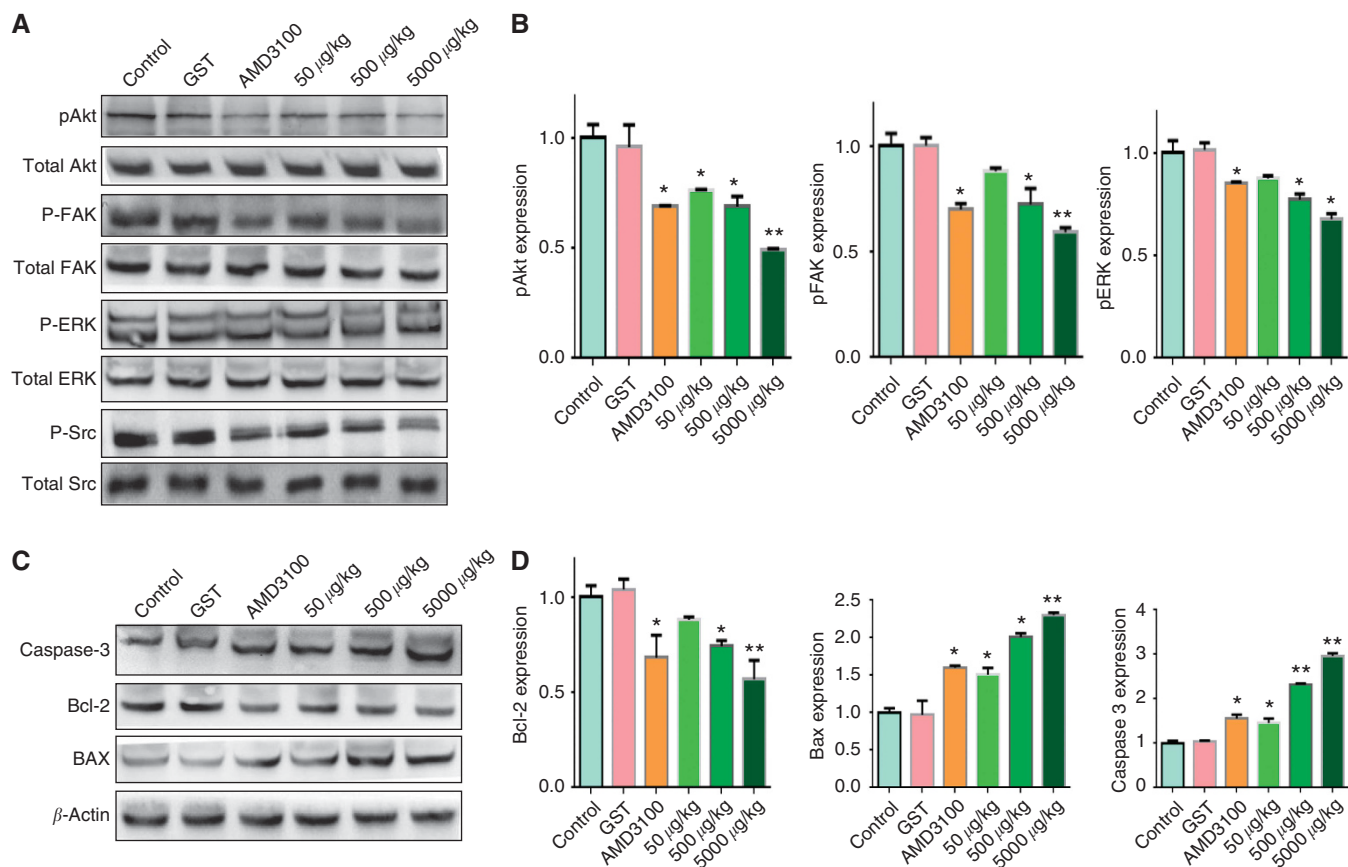


Figure 6. GST-NT21MP inhibits the activation of Akt, ERK, FAK and expression of Bcl-2 *in vivo*. (A) The activation of Akt, ERK, Src and FAK in tumour tissue extracts was detected by western blotting. (B) Quantitative analysis of pAkt, pERK and pFAK in panel A. * $P < 0.05$, ** $P < 0.01$, compared with control. (C) The expression of Bcl-2, Bax and caspase-3 in tumour tissue extracts was detected by western blotting. (D) Quantitative analysis of Bcl-2, Bax and caspase-3 in panel C. * $P < 0.05$, ** $P < 0.01$, compared with control.

notion, we found that SDF-1 α promoted breast cancer cell growth whereas GST-NT21MP was capable of inducing growth inhibition in breast cancer cells with highly expressing CXCR4 as detected by MTT assay and clonogenic assay. As CXCR4 is involved in cell apoptosis (Rubin *et al*, 2003; Hattermann *et al*, 2012; Yu *et al*, 2012), we measured the apoptosis in breast cancer cells after GST-NT21MP treatment. We observed that GST-NT21MP treatment led to the induction of apoptosis in breast cancer cells, suggesting that blockade of CXCR4 pathway is sufficient to trigger apoptosis in breast cancer.

It is well known that SDF-1/CXCR4 promotes metastasis through stimulating cell migration and invasion in breast cancer (Drury *et al*, 2011; Hawkins and Richmond, 2012; Luker *et al*, 2012). To support this notion, the expression levels of CXCR4 in human breast cancer cells and metastases are significantly higher than that in normal breast epithelial cells (Muller *et al*, 2001). Correspondingly, SDF-1 is highly expressed in typical target organs of breast cancer metastasis such as lungs, livers, bone marrow and lymph nodes (Muller *et al*, 2001). Thus, to further explore the anti-metastasis activity of GST-NT21MP, we detected the cell invasion in breast cancer cells after GST-NT21MP treatment. We observed a marked decrease in cell invasion in GST-NT21MP-treated cells. These results suggest that GST-NT21MP is a potential agent to inhibit cell growth and invasion in breast cancer cells.

Although our cell-based assays showed that GST-NT21MP is an antitumour agent in breast cancer, the major challenge in developing a therapeutic agent is to show efficacy against breast cancer *in vivo*. Therefore, we tested the antitumour activity of GST-NT21MP against 4T-1 cells in a mouse model. We found that GST-NT21MP treatment significantly retarded tumour growth compared with control group, which could in part be attributed to decreased proliferation as evidenced by reduced PCNA immunoreactivity in the tumours of GST-NT21MP-treated animals. Moreover, we demonstrated that GST-NT21MP induced apoptosis *in vivo*. Furthermore, we observed that GST-NT21MP inhibited formation of metastatic lung nodules. Therefore, our CXCR4 inhibitor could be a potential agent for treatment of breast cancer.

Recent reports have shown that SDF-1/CXCR4 axis has an oncogenic role by regulating important proteins in the survival pathway such as Akt, ERK and FAK (Chetram *et al*, 2011; Ping *et al*, 2011; Singh *et al*, 2012). For example, the combination of SDF-1 α and CXCR4 activates various downstream genes including Akt, ERK and FAK, which subsequently stimulates expression of some proteins that are related to proliferation, migration, invasion and metastasis of cancer cells (Liang *et al*, 2007a, b; Zhao *et al*, 2008; Shen *et al*, 2010). In this study, we further tested whether GST-NT21MP could regulate these CXCR4 downstream genes. Indeed, we found that GST-NT21MP inhibited the phosphorylation of Akt, ERK and FAK *in vivo* in breast cancer. We also found that GST-NT21MP inhibited the expression of Akt target gene Bcl-2. Moreover, we observed that GST-NT21MP increased Bax and activated caspase-3. It has been documented that SDF-1 α /CXCR4 could regulate the activation of Src in breast cancer (Cleardin, 2011). Moreover, Src kinase has been found to regulate activation of Akt, FAK1 and ERK in human cancers including breast cancer (Reynolds *et al*, 2013; Zhang *et al*, 2013). In line with this, we found that GST-NT21MP significantly inhibited activation of Src, suggesting that GST-NT21MP could exert its antitumour activity through Src-mediated pathway and its downstream genes such as Akt, FAK1 and ERK. Our results indicate that blocking CXCR4 pathway by GST-NT21MP led to the inactivation of Src and subsequent inhibition of Akt, ERK and FAK as well as Bcl-2, which are believed to be mechanistically linked with GST-NT21MP-mediated antitumour activity.

In summary, we presented experimental evidence in support of the antitumour activity of GST-NT21MP and thus it could be a

novel agent for the treatment of breast cancer. On the basis of our results, we propose a hypothetical pathway by which GST-NT21MP inhibits tumour growth of breast cancer partly through inactivation of Src and subsequent inhibition of Akt, ERK and FAK signalling pathways. However, further investigation is required to explore the precise molecular mechanisms underlying GST-NT21MP-mediated antitumour activity before its use for the treatment of human breast cancer patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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