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Denbinobin suppresses breast cancer metastasis through the inhibition of Src-mediated signaling pathways☆

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Abstract

Denbinobin (5-hydroxy-3,7-dimethoxy- 1,4-phenanthraquinone), a biologically active chemical isolated from *Ephemerantha lonchophylla*, has been demonstrated to display anti-cancer activity. Breast cancer is the leading cause of female mortality, and the high mortality is mainly attributable to metastasis. Src kinase activity is elevated in many human cancers, including breast cancer, and is often associated with aggressive disease. In the present study, we examined the anti-metastatic effects of denbinobin through decreasing Src kinase activity in human and mouse breast cancer cells. Denbinobin caused significant block of Src kinase activity in both human and mouse breast cancer cells. Moreover, phosphorylation of the signaling molecules focal adhesion kinase, Crk-associated substrate and paxillin downstream of Src was also inhibited by denbinobin. Furthermore, denbinobin inhibited the in vitro migration, invasion and in vivo metastasis of breast cancers in a mouse metastatic model. The denbinobin-treated group showed a significant reduction in tumor metastasis, orthrotopic tumor volume, and spleen enlargement compared to the control group. In addition, transfection of breast cancer cells with a plasmid coding for a constitutively active Src prevented the denbinobin-mediated phosphorylation of Src and downstream molecules and cell migration. Our findings provide evidences that denbinobin inhibits Src-mediated signaling pathways involved in controlling breast cancer migration and metastasis, suggesting that it has therapeutic potential in breast cancer treatment.

Keywords: Denbinobin; Natural products; Breast cancer; Src; Migration; Metastasis

1. Introduction

Invasion and metastasis are the main clinical phenomena that distinguish malignant from benign tumors and are the leading causes of death in cancer patients. Metastasis is a complicated process involving the degradation of the basement membrane, invasion of the stroma, adhesion, angiogenesis, cell proliferation and migration [1]. Many factors in tumor metastasis need further investigation, as a variety of modulators may be involved [2], and current clinical methods cannot accurately identify which patients will develop metastasis [3]. In addition, there are few treatments that can significantly suppress tumor metastasis, so the development of more effective therapeutic agents is a high priority.

Src family kinases comprise a subclass of membrane-associated non-receptor tyrosine kinases involved in a variety of cellular signal transduction pathways. Src family kinases include Src, Yes, Fyn, Lyn, Hck, Blk, Brk, Fgr, Frk, Srm and Yrk [4]. Although most Src family members are expressed primarily in cells of hematopoietic origin, Src displays a more ubiquitous pattern of expression, with high levels in some epithelial tissue [4]. Under normal physiological condition, 90-95% of Src is in an inactive conformation [5]. However, activated Src is frequently reported in human cancers, including colon adenocarcinoma [6], breast cancer [7] and pancreatic cancer [8]. In the case of breast cancer, increased Src activity is believed to play an important role in development and progression [4,5]. When acted upon by upstream signals, such as growth factors or cytoplasmic proteins, Src undergoes a conformational change resulting in activation of its kinase activity [9]. Activated Src coordinates multiple signaling pathways involved in tumor progression, such as proliferation, survival, motility, angiogenesis, and invasion [10]. In addition, its ability to promote tumor cell invasion can lead to the development of tumor metastasis [11]. A recent study using $Src^{-/-}$ mice demonstrated that abolishing Src expression/activity decreases tumor cell extravasation and subsequently decreases experimental metastasis [12]. Thus, Src is a potential therapeutic target of human cancer, including breast cancer.

Increased Src activity can be caused by increased transcription or by deregulation due to overexpression of upstream growth factor receptors, such as epidermal growth factor receptor or platelet-derived growth factor receptor, or by cytoplasmic proteins, such as

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ephrins and integrin [13,14]. Src kinases phosphorylate tyrosyl residues of critical cellular substrates, resulting in the activation of oncogenic signal transduction pathways [15]. Focal adhesion kinase (FAK), one such substrate of Src, plays an important role in integrin signaling and is highly expressed in many tumor cells [16]. A study using an inducible dominant-negative Src demonstrated that Src suppression significantly reduces the migration, attachment, and spreading of MCF-7 cells through changes in FAK activation and the interaction of CRK-associated substrate (p130Cas) with FAK [17]. The Src-FAK signaling complex recruits and phosphorylates a number of signaling proteins and is involved in adhesion regulation and the motile and invasive phenotype [5].

Denbinobin (5-hydroxy-3,7-dimethoxy- 1,4-phenanthraquinone) is a biologically active chemical isolated from *Ephemerantha lonchophylla*. Previous studies have shown that it acts as an antioxidant and anti-tumorigenesis agent [18–23]. In this study, we evaluated its effects on Src kinase inhibition and breast cancer cell migration in vitro and on tumor metastasis in vivo.

2. Materials and methods

2.1. Materials

Denbinobin was extracted and purified by one of our colleagues (Dr. Chien-Chih Chen) to a purity of greater than 98%, as shown by high-performance liquid chromatography and nuclear magnetic resonance [19]. Rabbit polyclonal antibodies against human phospho-FAK (Tyr576/577), phospho-FAK (Tyr925), phospho-p130Cas (Tyr410), and phospho-paxillin (Tyr118) and monoclonal antibodies against human Src were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal anti-human phospho-Src (Tyr416) antibody was purchased from Millipore (Billerica, MA, USA). Rabbit polyclonal anti-human FAK, p130Cas, or Glyceraldehyde 3-phosphate dehydrogenase antibodies and protein A/G-PLUS agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horse radish peroxidase (HRP)- or fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse or anti-rabbit IgG antibodies were obtained from Jackson ImmunoResearch (Cambridgeshire, UK). Constitutively active c-Src (CA-Src) plasmid, which contains a tyrosine-to-phenylalanine substitution (Y529F), was obtained from Upstate (Lake Placid, NY, USA), and the pGL4.74[hRluc/TK] vector was obtained from Promega (Madison, WI, USA). Lipofectamine 2000 reagent was purchased from Gibco Laboratories Inc. (Palo Alto, CA, USA). The blind well chemotaxis chamber was obtained from Neuro Probe (Gaithersburg, MD, USA) and the Nuclepore Track-etch polycarbonate membrane from Whatman (Maidstone, Kent, UK). Recombinant human EGF was purchased from PeproTech Asia (Rehovot, Israel). The protein tyrosine kinase assay kit and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Mouse breast tumor 4T1-Luc cell lines cotransfected with the firefly luminance-containing vector and selected in G418 were kindly provided by Dr. Min-Liang Kuo (Institute of Pathology, College of Medicine, National Taiwan University) and human breast adenocarcinoma MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Both were cultured in the media recommended by the supplier (high glucose DMEM medium for 4T1-Luc cells and RPMI-1640 medium for MDA-MB-231 cells) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel) at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.3. Cell viability assays

Cells (1×10^4) in 100 μl of medium in 96-well plates were incubated with vehicle or test compound for 48 h, then 25 μl of 1 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added, and the plates incubated at 37°C for 2 h. The cells were then pelleted and lysed in 100 μl of dimethyl sulfoxide and the absorbance at 550 nm measured on a microplate reader.

2.4. Immunoblot analysis

Cells (1×10^6) were lysed in a lysis buffer and whole-cell extracts (60 μg) were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrohporesis and blotted onto nitrocellular membranes. Immunoblot detection was performed with the corresponding antibodies using an ECL detection kit and exposure to photographic film.

2.5. Src kinase assays in vitro

Cells were lysed in 50 mM HEPES, pH 7.4, containing 0.1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM benzamidine, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, and 2 μg/ml of pepstatin A and the lysates were clarified by centrifugation at 17,000 g for 30 min at 4°C, then active Src was precipitated using 1 μg of anti-c-Src antibody and protein A/G-agarose beads at 4°C overnight. The precipitated beads were washed three times with 1 ml of ice-cold cell lysis buffer and used in the kinase reaction. The Src kinase assay was performed using the supplier's protocol (Sigma-Aldrich). The absorbance was measured at 492 nm.

2.6. Transient transfection with a constitutively activated c-Src mutant

1×10^6 cells were seeded in 6-well plates in 1 ml of serum-free medium one day before transfection. Following the manufacturer's protocol, 10 μl of Lipofectamine 2000 (Invitrogen) was mixed for 5 min with 50 μl of Opti-MEMI reduced serum medium, then 10μg of CA-Src (Y529F) plasmid DNA and 10μg of pEGFP-N1 plasmid in 50 μl of Opti-MEMI reduced serum medium were added. The mixture was incubated for 20 min at room temperature, then was added to the cells, and the mixture incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air. Transfection efficiency, determined by fluorescence microscopy, was >60% in all experiments.

2.7. Cell migration and invasion assays

The migration assays were measured using a blind well chemotaxis chamber. Cells (1×10^5 in 100 μl of culture medium containing 0.5% FBS) or 100 μl of culture medium containing 10% FBS was added to the top or bottom wells of the chamber, respectively. The chambers were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air, then non-migrated cells on the top of the filters were wiped off with cotton swabs and the migrated cells attached to the bottom of the filters were fixed and stained with 1% crystal violet, counted in microscope and the absorbance at 550 nm measured. In invasion assays, the filter was coated with Matrigel (BD Biosciences, Bedford, MA) at a concentration of 125 μg/cm^2, then followed protocols were performed as above described.

2.8. Metastatic assays in vivo

4T1-Luc cells (2×10^5 in 0.1 ml of medium) were injected into the left lower mammary fat pad of 5-week-old female BALB/c mice. Tumor growth was measured weekly and the volume (mm^3) was determined by measuring the largest diameter (l) and smallest diameter (s), using the equation volume$=0.5\ ls^2$. The animals were also weighed weekly. After the orthotopic tumor volume reached 50 mm^3 (14 days after tumor cell injection), the mice were either left untreated or were injected intraperitoneally with either paclitaxel (20 mg/kg) every 4 days or denbinobin (10 mg/kg) 3 times a week. An IVIS 200 biophotonic imager (Xenogen) was used to monitor metastasis of the breast carcinoma cells. Briefly, the animals were anesthetized by isoflurane inhalation and injected intraperitoneally with 100 μl of 150 mg/ml of D-luciferin (Xenogen) in phosphate-buffered saline (PBS), and bioluminescence imaging with a CCD camera (IVIS, Xenogen) was initiated 10 min after injection. At the experimental endpoint of day 40 after tumor injection, when lung metastasis was observed in the control group by bioluminescence imagery, the animals were sacrificed and imaged within 15 min after injection. All animal studies were performed using protocols approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University.

2.9. Immunocytochemistry and microscopy

Cells or removed orthotopic tumors were fixed in 4% paraformaldehyde in PBS. After fixation, the orthotopic tumors were embedded in paraffin and cut into 5-μm sections. Nonspecific binding sites in the fixed cultures or tumor slices were blocked by overnight incubation with 5% bovine serum albumin (BSA) in PBS overnight, then the samples were incubated with primary antibodies (1:100) in 0.5% BSA for 60 min at room temperature. After 3×10-min washes in PBS, the cells or tumor sections were stained for another 60 min with FITC-conjugated goat anti-mouse or anti-rabbit IgG antibodies (1:100 dilution in PBS) then were viewed and photographed under a Leica TCS SP5 confocal laser-scanning microscope using appropriate fluorescence filters.

2.10. Data analysis

The data are expressed as the mean±S.E.M. and were analyzed statistically using one-way analysis of variance (ANOVA). When ANOVA showed significant differences between groups, Tukey post hoc test was used to determine the specific pairs of groups showing statistically significant differences. A P value of less than .05 was considered statistically significant.

3. Results

3.1. Denbinobin inhibits the kinase activity of Src and its downstream signaling pathways in vitro

Src-mediated downstream signaling pathways involving FAK, p130Cas, and paxillin are reported to play an essential role in tumor cell migration and metastasis [24–30]. EGF treatment induced Src phosphorylation at Tyr416 within 5 min and this was maintained for at least 1 h in both 4T1-Luc (Fig. 1A) and MDA-MB-231 (Fig. 1B) breast cancer cells. Furthermore, following Src activation, FAK was phosphorylated at Src-dependent phosphorylation sites (Tyr576 and Tyr925) and p130Cas was phosphorylated at Tyr410 (Fig. 1A). Denbinobin treatment for 30 min not only significantly reduced Src Tyr416 phosphorylation but also reduced phosphorylation of FAK Tyr576 and Tyr925, p130Cas Tyr410, and paxillin Tyr118 in a concentration-dependent manner in both cell types (Fig. 1C, D). This inhibition were not due to decreased protein levels, since total Src and FAK levels were unchanged and none of the treatments had any significant effect on cell viability at 6 or 12 h, assessed using the MTT assay (Fig. 1E, F). In order to directly examine the role of denbinobin in Src activation, both types of breast cancer cells were transfected for 24 h with 10μg of plasmid CA-Src coding for a constitutively activate Src and phosphorylation of Src, FAK, p130Cas, and paxillin was found to be significantly increased, reversing the inhibition by denbinobin (Fig. 1C, D). These data clearly demonstrate that denbinobin inhibits the phosphorylation of Src and associated downstream molecules.

We next asked whether denbinobin influenced Src kinase. To address this question, we treated both cell lines with denbinobin and evaluated the denbinobin-mediated Src kinase inhibitory effect using the Src kinase assay as described in the Materials and Methods section. As shown in Fig. 2, 10μM of denbinobin resulted in marked inhibition of Src kinase to less than 20% of control levels in both cell lines.

3.2. Denbinobin inhibits the migration and invasion of breast cancer cells in vitro

Since Src kinase plays an important role in cell migration, we next examined the effect of denbinobin on cell migration and invasion in a chemotaxis chamber assay. MDA-MB-231 cells and 4T1-Luc cells are highly invasive breast cancer cell lines. EGF treatment increased the migration and invasion of both cell types, and these effects were significantly inhibited by 3 μM denbinobin to less than 35% of the levels in the EGF control group, respectively (Fig. 3A, B). Furthermore, after transfection with CA-Src, the constitutively active Src increased the migratory and invasion motility of cancer cells with or without EGF treatment and prevented inhibition by denbinobin (3 μM) (Fig. 3A, B). These results confirmed our previous argument that denbinobin exerts its anti-migratory effect via Src inactivation in breast cancer cells.

In order to further characterize the molecules involved in the inhibitory effect of denbinobin, we labeled cells on coverslips with FITC-conjugated antibodies against phosphorylated Src, FAK, or paxillin and examined the results on a confocal laser-scanning microscope. As shown in Fig. 4, p-Src was found to be localized in the cytoplasm in cells with or without treatment with 3 μM denbinobin for 1 h. However, the denbinobin-treated cells were rounder in shape and the intensity of the p-Src labeling was significantly reduced (Fig. 4A, B). Likewise, labeling for FAK phosphorylated at Tyr576/577 and paxillin phosphorylated at Tyr118 was decreased by the same treatment (Fig. 4A, B), supporting our results obtained by immunoblot analysis (Fig. 1) and Src kinase assay (Fig. 2). These findings clearly demonstrate that denbinobin inhibits breast cancer cell migration by inhibiting Src-FAK signaling.

3.3. Denbinobin inhibits breast cancer cell metastasis in vivo

We next used a metastatic animal model to evaluate whether denbinobin inhibited breast cancer cell metastasis. When introduced orthotopically, the 4T1-Luc mammary carcinoma cells can metastasize to several organs, including the lung, liver, and spleen. We used an IVIS biophotonic imager to monitor the luminance of 4T1-Luc metastasis and an end point of 40 days, when all control mice showed lung metastasis. At 40 days after injecting BALB/c mice with 2×10^5 mouse breast cancer cells, strong luminance was observed in the lung (Fig. 5A). Luminance could be detected in the removed organs (lung, liver and spleen) and spleen enlargement was also observed, indicating that the orthotopic 4T1-Luc cells had metastasized to the organs. Paclitaxel treatment reduced the luminance in the lung, and less luminance was seen in the removed lung and none in the liver (Fig. 5A). In the denbinobin (10 mg/kg)-treated group, no detectable luminance was seen in the lung in the living animals (Fig. 5A). Furthermore, in order to investigate the signaling molecules involved in the metastatic model, immunofluorescence analysis was performed on paraffin-embedded tumor tissues. Immunofluorescence images of the orthotopic tumors again revealed that denbinobin treatment significantly inhibited phosphorylation of Src, FAK, and paxillin (Fig. 5B). These results suggest that denbinobin inhibits breast cancer metastasis by suppressing the activity of Src-activated downstream molecules.

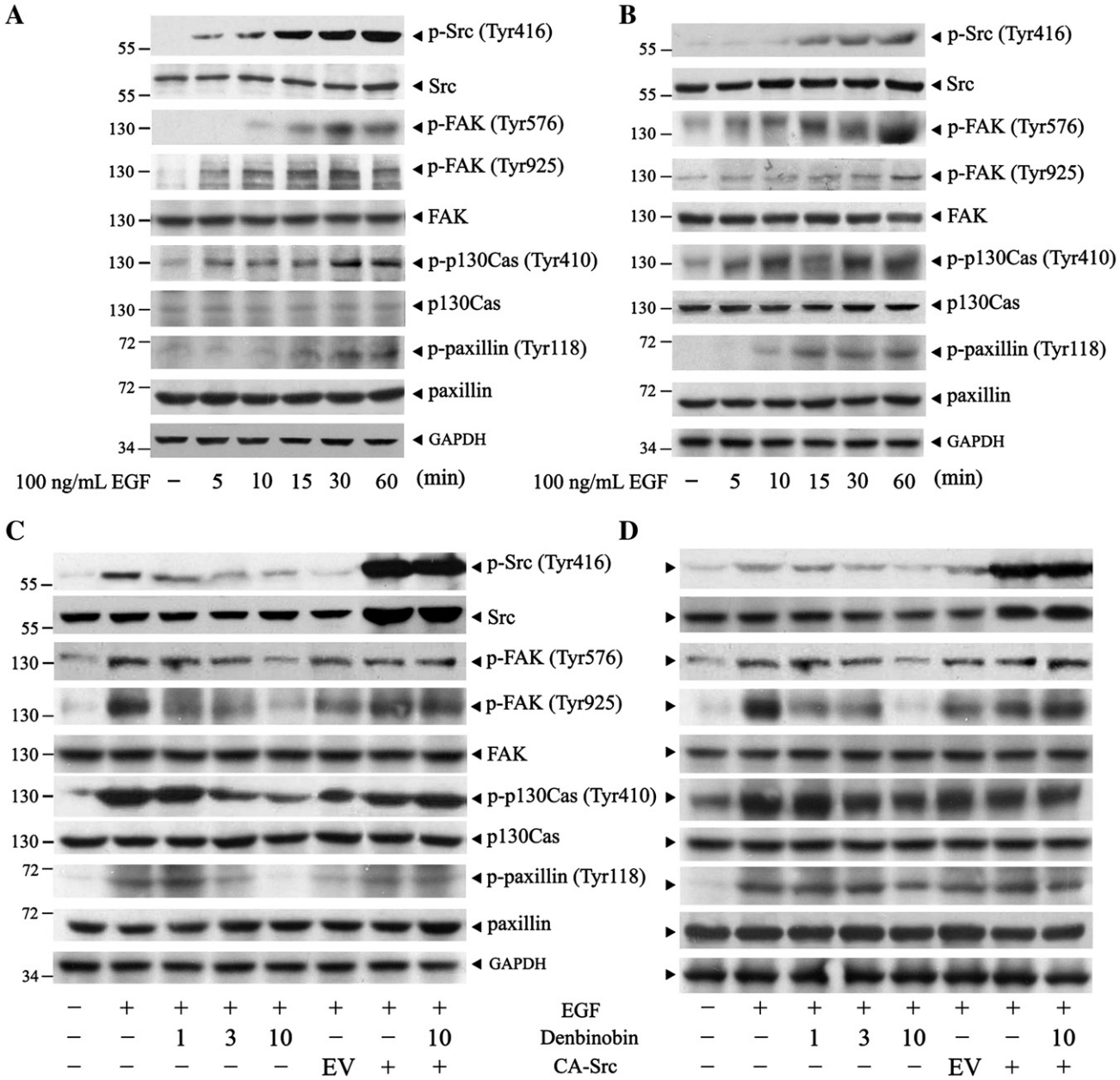
Together, these results demonstrate that denbinobin significantly inhibits the kinase activity of Src and that its inhibitory effect on Src-mediated signals causes suppression of breast cancer migration and metastasis.

4. Discussion

Elevated Src expression and Src kinase activity have been seen in many solid tumors, including breast cancer [5,7]. Src kinases are transducers of signals and are activated by various cell-surface receptors and interact with numerous substrates, mediating a wide range of biological events, such as growth [25], proliferation [31], angiogenesis [32], invasion [33], metastasis [12,34], chemoresistance [35] and bone turnover [36]. The abolition of Src signals using $Src^{-/-}$ mice or Src inhibitors significantly inhibits in vitro or in vivo tumor progression, suggesting Src as a therapeutic target for cancer therapy [4,5,27]. Using several small-molecule Src inhibitors, inhibition of cell migration and invasion is consistently seen [17,34,37]. Recent studies have suggested that cancer cells may regulate their own adhesion to matrix proteins by intracellular signals that alter the binding affinity for matrix receptors [38–40].

In the present study, we demonstrated that denbinobin markedly inhibited Src kinase and blocked the activity of Src-FAK signaling pathways. FAK is phosphorylated by Src on a number of

Fig. 1. Denbinobin inhibits the kinase activity of Src and its downstream signaling pathways in vitro. In (A) and (B), 1×10^6 4T1-Luc cells (1×10^6) (A) or MDA-MB-231 cells (B) were incubated with EGF (100 ng/ml) for 0–60 min, then the cells were harvested and whole cell extracts prepared for Western blot analysis for the indicated proteins. In (C) and (D), 4T1-Luc cells (1×10^6) (C) or MDA-MB-231 cells (1×10^6) (D) were incubated with 10 μg empty vector (EV) or constitutively active Src plasmid (CA-Src) for 24 h, denbinobin (1,3,10 μM) for 30 min, and 100 ng/ml EGF for 1h as indicated, then whole cell extracts were prepared for Western blot analysis for the indicated proteins. Viability of 4T1-Luc cells (E) or MDA-MB-231 cells (F) treated with 0.3-10 μM denbinobin for 6 or 12 h compared to that of the control group estimated using the MTT assay. The data are the mean±S.E.M. for four separate experiments.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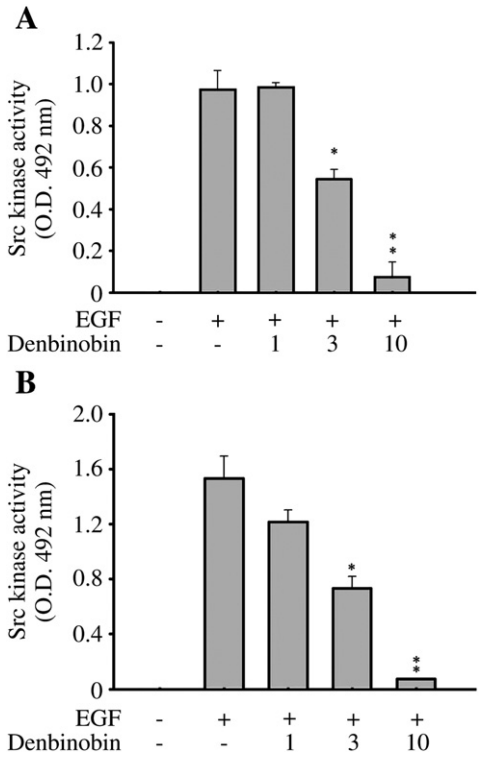

Fig. 2. In vitro inhibition of Src kinase by denbinobin. 10^6 4T1-Luc cells (A) or MDA-MB-231 cells (B) were incubated with 0–10 μM denbinobin for 30 min in the presence of EGF (100 ng/ml) for another 1 h, then equal amounts of total cell lysates were immunoprecipitated with 1μg of anti-Src antibody, followed by kinase assay using a tyrosine kinase kit. The absorbance was measured at 492 nm. The data represent the mean±S.E.M. for four replicates. *P<.05 and **P<.01 compared to the control group, respectively. The experiment was performed four times with similar results.

tyrosine residues and, like Src, is associated with malignant progression of breast cancer [28,37,41]. Our data showed that decreased phosphorylation of FAK on Tyr925 was seen following denbinobin treatment and that this correlated with a reduction in motility. These results agree with previous finding indicating that the Src kinase-dependent phosphorylation of Tyr925 in FAK is important in controlling the extension and retraction of cell protrusions or adhesion turnover [42]. Another substrate of Src, p130Cas, is also involved in cell spreading, focal adhesion formation, and invasion, and its phosphorylation was also decreased following denbinobin treatment. A recent report suggested that FAK promotes assembly of a Src-p130Cas-CRK-DOCK180 complex, which activates Rac1 and subsequently promotes matrix metalloproteinase-mediated matrix degradation [28]. Another study demonstrated that Src kinase can directly phosphorylate paxillin or promote paxillin phosphorylation indirectly by activation of FAK/cell adhesion kinase-β [43,44]. Paxillin functions as an adaptor protein that facilitates the assembly of multi-protein complexes to regulate the active state of Rho family proteins, e.g., Rac, and, thus, regulate the dynamics of the actin cytoskeleton [44]. Our Western blot (Fig. 1) and immunofluorescence (Fig. 4) results showed that denbinobin treatment decreased cell migration at concentrations resulting in detectable inhibition of phosphorylation of Src, FAK, p130Cas, and paxillin, suggesting that denbinobin causes its migration inhibitory effect by suppressing Src-FAK signals.

The 4T1 metastatic model is a suitable model for evaluating the metastatic process in vivo [45]. The xenograft models, in which human tumor cells are introduced into immunocompromised mice, have been used extensively for the study of tumor growth and metastasis [45,46]. However, while some human xenograft models can approximate primary tumor growth in mice, replication of tumor metastasis is rather problematic. For instance, human tumor cells generally metastasize poorly in mice, and even when metastasis does occur, unexpected and uncharacteristic conditions are often observed [45,46]. On the other hand, the 4T1 model, a syngeneic mouse model, has been shown to be more appropriate for the evaluation of in vivo metastatic process [45]. Such models are particularly useful as that the metastatic tissues, the tumor microenvironment and the host are from the same species and can therefore faithfully represent the typical metastatic characteristics similar to those observed in cancer patients [45,47]. This is important especially when considering the significance of the microenvironment and tumor-host interactions to tumor cell development. The 4T1 model also poses an important advantage of allowing analyses to be carried out on animals with normal immune function [45]. Since the immune system plays an important role in the development and progression of cancer, models that can be applied on immunocompetent mice are essential for analysis of cancer progression and evaluation of therapeutics for cancer treatment [45].

Denbinobin, known as Shi-Hu in Chinese medicine, has been used to treat the common cold and fever for centuries in the practice of traditional medicine in China. It has been reported that denbinobin exhibits anti-oxidant effect and cytotoxicity against human lung carcinoma [19,22], colorectal cancer [23], and myelogenous leukemia cell lines [21]. In the present study, we observed that denbinobin suppressed breast cancer metastasis significantly. This novel observation allows us to hypothesize that more potent drugs for suppressing breast cancer metastasis could be developed from compounds sharing similar structural features with denbinobin with proper chemical modifications.

In summary, our study demonstrates that denbinobin significantly inhibits Src kinase activity and the Src-mediated phosphorylation of downstream molecules in two types of breast cancer cells. This inhibitory effect results in a decrease in breast cancer cell migration in vitro and metastasis in vivo. These results indicate that denbinobin has potential application in the treatment of breast cancer metastasis.

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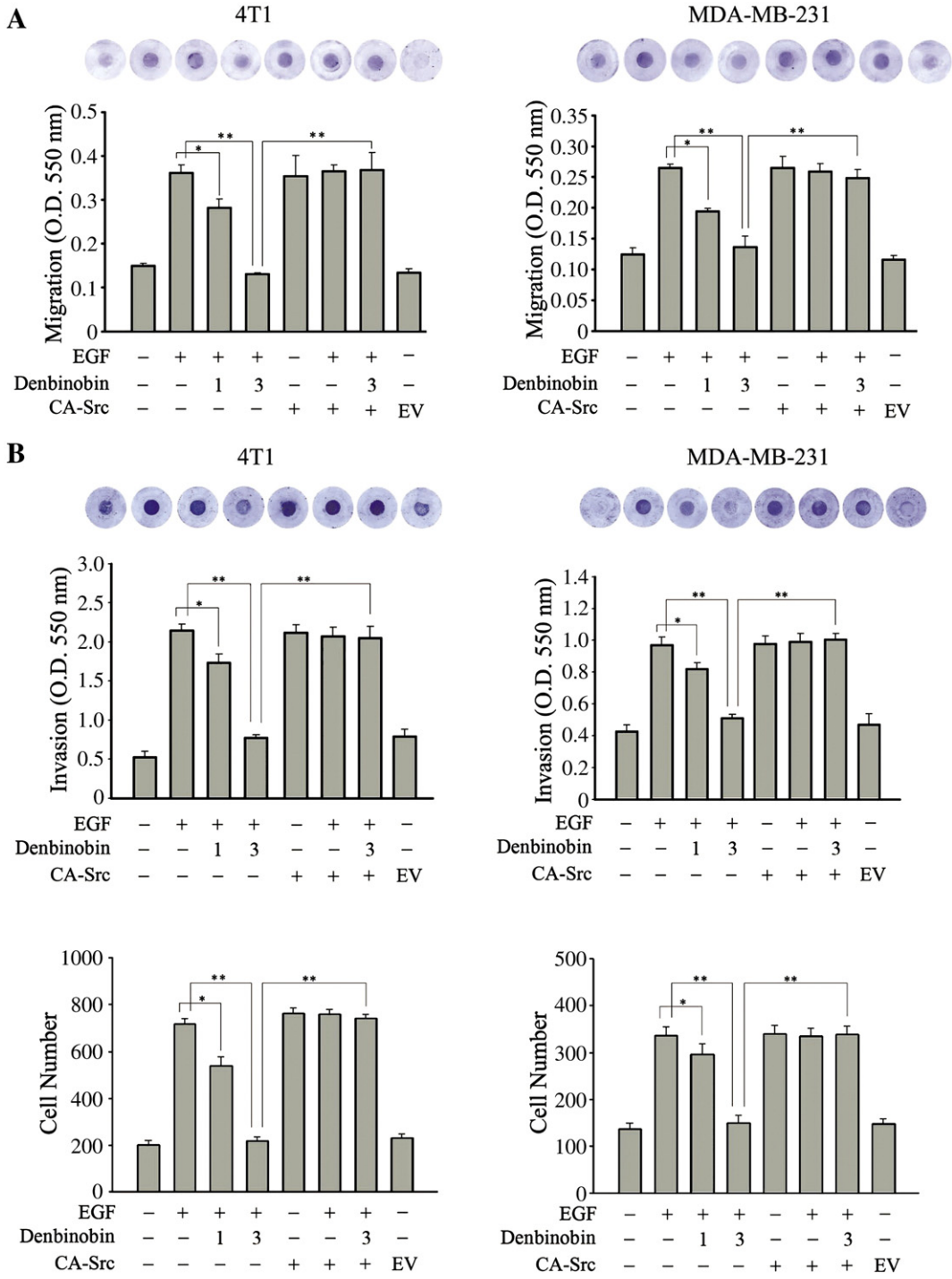

Fig. 3. Inhibitory effects of denbinobin on breast cancer cell migration and invasion. (A) 2×10^5 4T1-Luc cells or 1×10^5 MDA-MB231 cells were incubated with 1, 3 μM denbinobin for 30 min in the presence of EGF (100 ng/ml) for another 1 h, or transfected with 10 μg of empty vector (EV) or CA-Src plasmid for 24 h, then cells harvested and placed in the top compartment of chemotaxis chamber with indicated treatment. Culture medium containing 10% FBS was added to the bottom compartment of the chamber. After 24 h, cell migration was measured as described in the Materials and Methods section. (B) Cells were seeded onto the upper chamber consisting of 8 μm pore-size filters coated with Matrigel, then treated without or with denbinobin (1, 3μM) for 24 h in the absence or presence of EGF (100 ng/ml). Cells that invaded the filter were measured or counted in microscope. The data represent the mean±S.E.M. for three replicates. *P<.05 and **P<.01 comparing the indicated groups. The results shown are representative of those obtained in three independent experiments.

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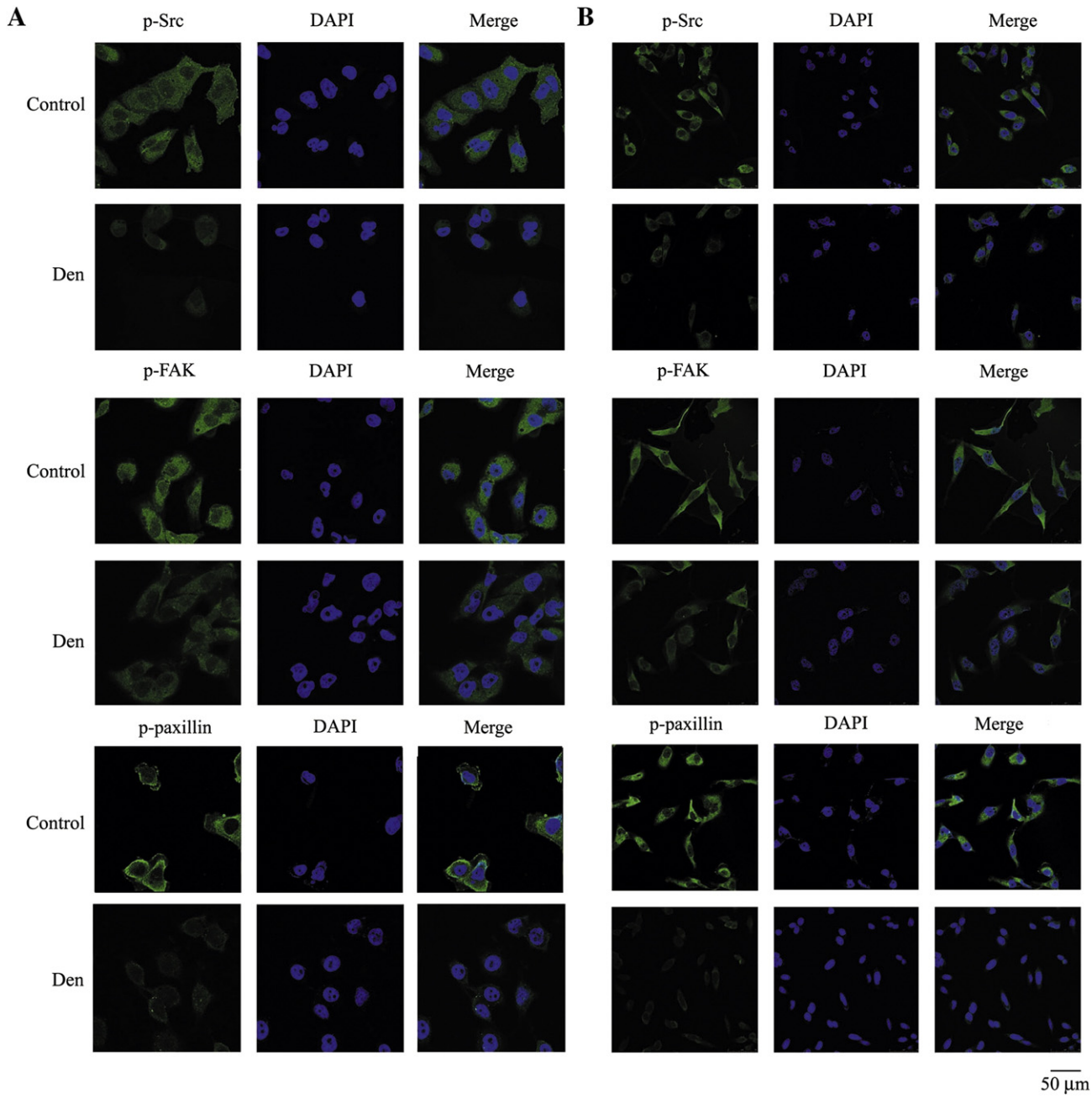

Fig. 4. Denbinobin inhibits the phosphorylation of Src and downstream proteins in breast cancer cells. 1×10^5 4T1-Luc cells (A) or MDA-MB-231 cells (B) were cultured on coverslips for 1 h in the presence or absence of 3 μM denbinobin (Den), then were fixed and stained overnight with the indicated antibodies at 4°C. The proteins are labeled with FITC (green, left panels), the nuclei are stained with DAPI (blue, center panels), and the merged images are shown on the right. The results shown are representative of those obtained in four independent experiments. Scale bar=50μm.

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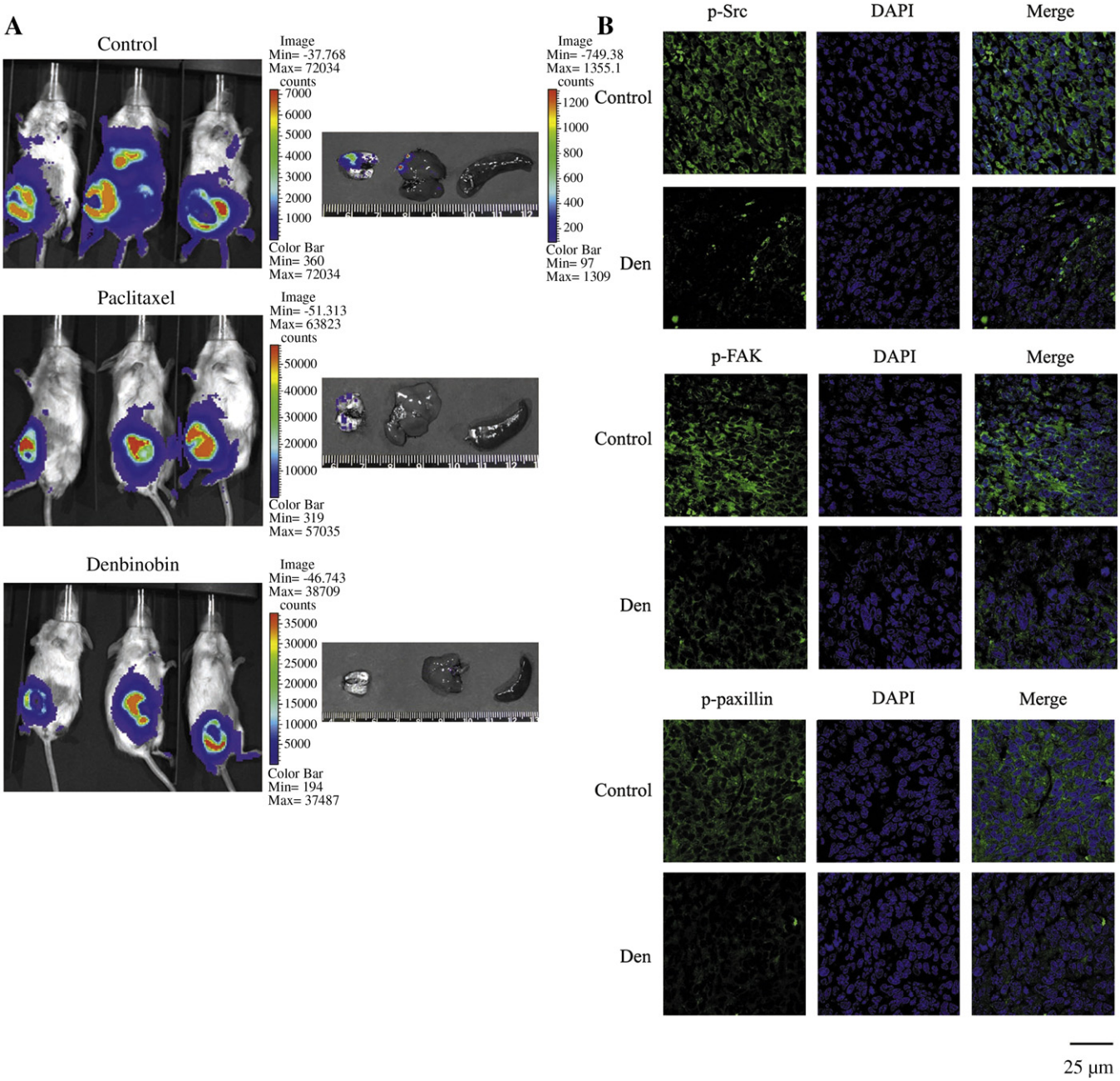

Fig. 5. Denbinobin suppresses breast cancer metastasis in vivo. (A) 4T1-Luc cells (2×10^5 in 0.1 ml of medium) were injected into the left lower mammary fat pad of five-week-old female BALB/c mice (five animals per group). After the orthotopic tumor volume reached 50 mm^3 (14 days after tumor cell injection), the mice were left untreated or were injected intraperitoneally with paclitaxel (20 mg/kg) every 4 days or denbinobin (10 mg/kg) 3 times a week. An IVIS 200 biophotonic imager (Xenogen) was used to monitor metastasis of the breast carcinoma. At 40 days after implantation, the whole animals were imaged as described in the Materials and Methods section. The experiment was performed three times with similar results. (B) After 40 days implantation, the orthotopic tumors were removed, embedded in paraffin, and cut into 5-μm sections, which were treated with the indicated antibodies for 24 h at 4°C. The specific proteins are labeled by FITC (green) and the nuclei are stained with DAPI (blue). All the stained slides were viewed and photographed under an SP5 confocal laser-scanning microscope. The results shown are representative of those obtained in four independent experiments. Scale bar=25 μm.

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