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Butein downregulates chemokine receptor CXCR4 expression and function through suppression of NF-κB activation in breast and pancreatic tumor cells

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ABSTRACT

The CXC chemokine receptor-4 (CXCR4), a Gi protein-coupled receptor for the ligand CXCL12/stromal cell-derived factor-1α (SDF-1α), is known to be expressed in various tumors. This receptor mediates homing of tumor cells to specific organs that express the ligand CXCL12 for this receptor and plays an important role in tumor growth, invasion, metastasis, and angiogenesis. Thus, *a priori*, agents that can downregulate CXCR4/CXCL12 signaling cascade have potential against cancer metastasis. In this study, we report the identification of butein (3, 4, 2′, 4′-tetrahydroxychalcone) as a novel regulator of CXCR4 expression and function. We found that butein downregulated the expression of CXCR4 in HER2-overexpressing breast cancer cells in a dose- and time-dependent manner. The decrease in CXCR4 expression induced by butein was not cell type-specific as the inhibition also occurred in pancreatic, prostate, multiple myeloma, head and neck, and hepatocellular cancer cell lines. When investigated for the molecular mechanism(s), it was found that the downregulation of CXCR4 was not due to proteolytic degradation but rather to transcriptional regulation as indicated by downregulation of mRNA expression, inhibition of NF-κB activation evident by both DNA binding, and reporter assays, and suppression of chromatin immunoprecipitation activity. Suppression of CXCR4 expression by butein correlated with the inhibition of CXCL12-induced migration and invasion of both breast and pancreatic cancer cells. Overall, our results demonstrate for the first time that butein is a novel inhibitor of CXCR4 expression and thus has a potential in suppressing metastasis of cancer.

1. Introduction

Metastasis which accounts for >90% of cancer-related mortality is a highly systemic, non-spontaneous and organ-selective process [1].There are currently no approved drugs that specifically target the metastatic process [2]. Three major theories have been proposed in an attempt to explain the organ-specific spread of tumor cells [3–5]. The first theory attributed the organ-specific spread to the presence of the appropriate growth factors within the target organs whilst the second theory proposed that the bias in metastatic pattern is the result of the expression of specific adhesion molecules on the endothelial cells lining the blood vessels in the target organs [3,6]. The third, often referred to as the chemoattractant theory, suggested that chemoattractants in target organs and their respective chemoattractant receptors on tumor cells are responsible for the organ-selective metastatic spread [7]. The identification of such paired chemokines in target organs and chemokine receptors on tumor cells gave credence to the chemoattractant theory [7,8].

Chemokines are a large family of small (7–15 kDa), structurally related, heparin-binding proteins that govern multiple aspects of host defense and inflammation such as hematopoiesis, leukocyte trafficking, adhesion and angiogenesis [8–10]. They are produced locally in various tissues and act on leukocytes via selective membrane-bound G protein-coupled receptors, the two major subfamilies of which are CCR and CXCR [8,11]. One of the most extensively studied chemokines is stromal cell-derived factor-1α (SDF-1α, also known as CXC chemokine ligand 12; CXCL12), and its receptor, CXCR4 [12,13]. The chemotactic properties of SDF-1α have

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been widely associated with metastasis of several epithelial and hematopoietic cancers including breast, prostate, ovary, and lung cancers [14,15]. CXCR4 has been linked with leukocyte trafficking [16], B cell lymphopoiesis and myelopoiesis [17], neuronal cell migration [18] and HIV invasion of host cells [19]. In addition, CXCR4/SDF-1α interaction plays an important role in the targeted metastasis of breast cancer [13]. The SDF-1α/CXCR4 attraction leads breast cancer cells to leave the circulation and migrate into organs that express large amounts of chemokines, where the cancer cells proliferate, induce angiogenesis and form metastatic tumors [12,13]. The receptor tyrosine kinase, HER2, has been shown to induce the upregulation of CXCR4, leading to breast cancer invasion [20]. As CXCR4 expression has been correlated with poor overall survival rate in patients with breast cancer [21], and colon cancer [22], CXCR4 has been cited as a probable therapeutic target for preventing cancer metastasis [23].

Anticancer drugs derived from natural sources, generally regarded as safe, have been shown to mediate antitumor activities against a variety of cell types [24]. Butein, a tetrahydroxychalcone derived from numerous plants, including the stembark of cashews (*Semecarpus anacardium*) and the heartwood of *Dalbergia odorifera*, has been shown to exhibit substantial antitumor activities, as indicated by inhibition of proliferation of a wide variety of tumor cells [25,26], suppression of phorbol ester-induced skin tumor formation [27], and inhibition of carrageenan-induced rat paw edema [28]. As CXCR4 is known to mediate growth and metastasis of tumors, we hypothesized that butein may modulate the expression of CXCR4 and inhibit tumor cell invasion. Our results demonstrate, for the first time, that butein can downregulate CXCR4 expression in various tumor cells, including HER2-over-expressing breast cancer cells, through suppression of NF-κB activation. We also found that butein can inhibit CXCL12-induced invasion of breast and pancreatic tumor cells.

2. Materials and methods

2.1. Reagents

Butein, Tris, glycine, NaCl, SDS, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN), chloroquine and McCoy's 5A medium were purchased from Sigma–Aldrich (St. Louis, MO, USA). Butein was dissolved in dimethylsulfoxide as a 20 mM stock solution and stored at 4 °C. Further dilution was done in cell culture medium. RPMI 1640, DMEM, fetal bovine serum (FBS), 0.4% trypan blue vital stain, antibiotic–antimycotic mixture, and HRP-conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, CA, USA). Antibodies against CXCR4 and HER2 were obtained from Abcam (Cambridge, MA, USA). CXCL12 was purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel).

2.1.1. Cell Lines

Human breast cancer cells MCF-7, MDA-MB-231, SKBr3, BT474, and hepatocellular carcinoma HepG2 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Prostate cancer DU145 cells were kindly provided by Prof. Shazib Pervaiz of our institute. U266 (multiple myeloma), SCC-38 (squamous cell carcinoma), and AsPC-1 (pancreatic adenocarcinoma) were kindly provided by Prof. Bharat B. Aggarwal, M.D. Anderson Cancer Center, Houston, TX, USA. PANC-28 and MIA PaCa-2 cells were a kind gift from Dr. Kanaga Sabapathy, National Cancer Centre Singapore. MCF-7, MDA-MB-231, PANC-28, MIA PaCa-2, AsPC-1, HepG2, DU145, and U266 cells were cultured in RPMI 1640 medium with 10% FBS. SKBr3 and BT474 cells were cultured in McCoy's 5A medium with 10% FBS. SCC-38 cells were cultured in DMEM containing 10% FBS, 100 μM non-essential amino acids, 1 mM pyruvate, 6 mM L-glutamine, and 1× vitamins. Culture media were also supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin and cells were maintained at 37 °C in an atmosphere of 5% CO_2–95% air.

2.1.2. Western blot analysis

For detection of CXCR4 and HER2, butein-treated whole-cell extracts were lysed in lysis buffer (20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/mL aprotinin, 0.005 mg/mL leupeptin, 0.4 mM PMSF, and 4 mM $NaVO_4$). Lysates were then spun at 14,000 rpm for 10 min to remove insoluble material and resolved on a 10% SDS gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% non-fat milk to minimize non-specific binding, and probed with anti-CXCR4 or HER2 antibodies (1:3000) overnight at 4 °C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 2 h, and the CXCR4/HER2 expression was detected by chemiluminescence emission (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

2.1.3. Nuclear extract preparation

Nuclear extracts were prepared at various time points after treatment for subsequent NF-κB DNA-binding activity assay. Cell nuclear fractions were extracted using a nuclear extraction kit (Active Motif, Carlsbad, CA, USA). Briefly, cells were washed, collected in ice-cold PBS in the presence of phosphatase inhibitors, and then centrifuged at $300 \times g$ for 5 min. Cell pellets were resuspended in a hypotonic buffer, treated with detergent, and centrifuged at $14{,}000 \times g$ for 30 s. After collection of the cytoplasmic fraction, the nuclei were lysed, and nuclear proteins were solubilized in lysis buffer and protein concentrations were determined by the Bradford protein assay (Bio-Rad Laboratories, Hercupetins, CA, USA).

2.1.4. CXCR4 reporter assay

SKBr3 cells were plated in 96-well plates with 1×10^4 cells per well in 10% FBS-containing McCoy's 5A medium. After overnight incubation, cells were transfected with a CXCR4 luciferase plasmid. This plasmid was a kind gift from Dr. Joseph Sodroski, Dana-Farber Cancer Institute, Boston, Massachusetts, USA and has been characterized previously [29]. The NF-κB binding site has already been identified in the proximal region of the CXCR4 promoter and is reported to regulate CXCR4 expression in breast cancer cells [30]. Transfections were done according to the manufacturer's protocols using FuGENE® 6 obtained from Roche (Indianapolis, IN, USA). At 24 h post-transfection, cells were treated with indicated concentrations of butein for 8 h and then washed and lysed in luciferase lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured with a luminometer by using a luciferase assay kit (Promega) and was normalized to β-galactosidase activity. All luciferase experiments were done in triplicates and repeated three or more times.

2.1.5. NF-κB DNA-binding activity assay

NF-κB DNA-binding activity was analyzed using the TransAM NF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA, USA), following the manufacturer's instructions. Briefly, nuclear extracts (5 μg) from butein-treated SKBr3 cells were incubated in a 96-well plate coated with oligonucleotide containing the NF-κB consensus-binding sequence 5′-GGGACTTTCC-3′. Bound NF-κB was then detected by a specific primary antibody. An HRP-conjugated secondary antibody was then applied to detect the bound primary antibody and provided the basis for colorimetric quantification. The enzymatic product was measured at 450 nm with a microplate reader (Tecan Systems, San Jose, CA, USA). Specificity of this assay was tested by the addition of wild-type or mutated NF-κB consensus oligonucleotide in the competitive or

mutated competitive control wells before the addition of the nuclear extracts.

2.1.6. NF-κB luciferase reporter assay

SKBr3 cells were plated in 96-well plates with 1×10^4 cells per well in 10% FBS-supplemented McCoy's 5A medium. After overnight incubation, the cells were transfected with the NF-κB reporter plasmid linked to a luciferase gene or with the dominant-negative IκBα (IκBα-DN) plasmid. NF-κB luciferase plasmid was obtained from Stratagene (La Jolla, CA, USA). Transfections were done according to the manufacturer's protocols using Fugene-6 (Roche). At 24 h post-transfection, cells were treated with indicated concentrations of butein for 4 h and then washed and lysed in luciferase lysis buffer (Promega), and luciferase activity measured, as described for the CXCR4 reporter assay (*vide supra*).

2.1.7. RNA extraction and Real-time PCR analysis

Total RNA was extracted using the Trizol reagent (Invitrogen (Carlsbad, CA, USA), according to the manufacturer's instructions. Reverse transcription (RT) was then carried out at 37 °C for 1 h. Each RT reaction contains 1 μg of total RNA, 1X RT buffer, 5 mM $MgCl_2$, 425 μM each of dNTPs, 2 μM random hexamers, 0.35U/μL RNase inhibitor, 1.1 U/μL MultiScribe reverse transcriptase and made up to 10 μL with sterile water. The relative expression of CXCR4 and CXCR7 was then analyzed using quantitative RT-PCR (ABI PRISM 7500, Applied Biosystems, Foster City, CA, USA) with 18sRNA as an internal control. Primers and probes for human CXCR4, CXCR7 and 18sRNA were purchased as Assays-on-Demand kits (Applied Biosystems).

2.1.8. Chromatin immunoprecipitation (ChIP) assay

The cells were processed for the ChIP assay as per the protocol described by Saccani et al., 2002 [31]. The antibody used for the ChIP was NF-κB (p65) Ab Santa Cruz Biotechnology (Santa Cruz, CA, USA). The sequence for human CXCR4 gene promoter was as follows: sense primer, 5′-ACAGAGAGACGCGTTCCTAG-3′ and anti-sense primer, 5′-AGCCCAGGGGACCC TGCTG-3′. The PCR products were analyzed on 2% agarose gel electrophoresis and documented.

2.1.9. Wound healing assay

SKBr3 and AsPC-1 cells were treated, as described above. Before plating the cells, two parallel lines were drawn at the underside of

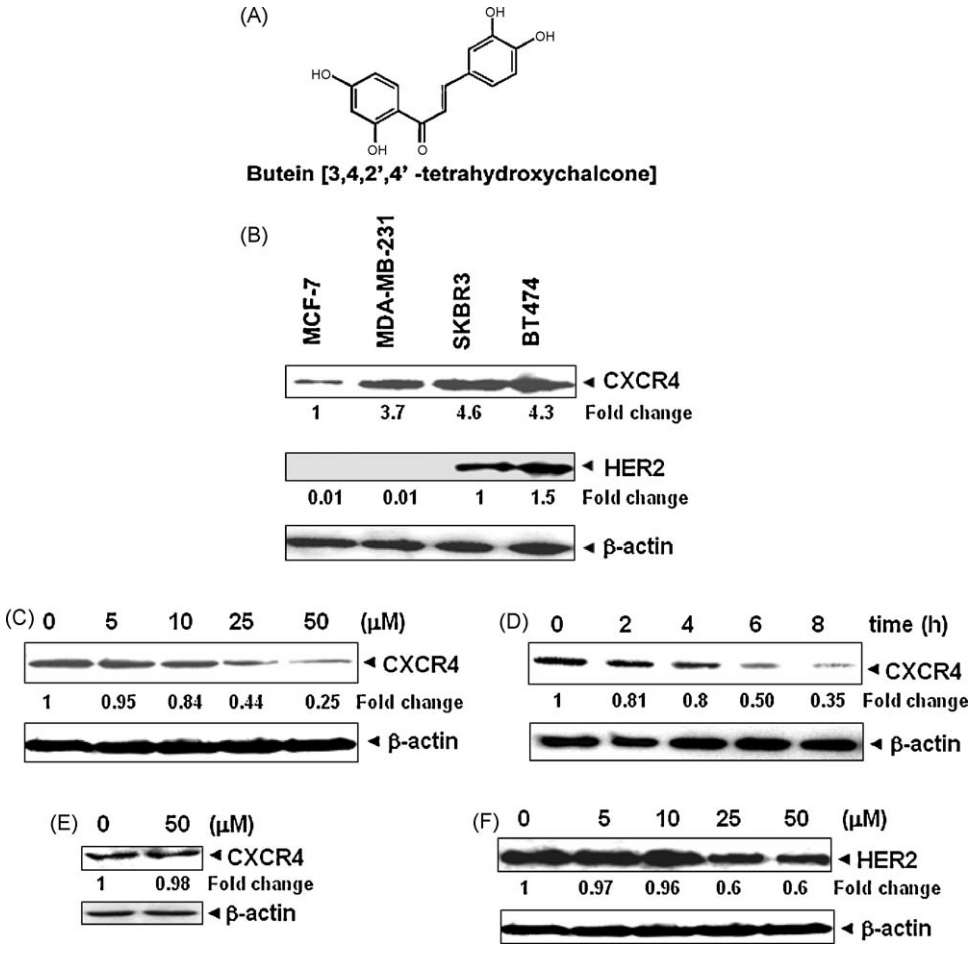

Fig. 1. Butein suppresses CXCR4 in breast cancer SKBr3 cells. (A) The chemical structure of butein. (B) Western blot analysis of CXCR4 expression. Whole-cell extracts of MCF-7, MDA-MB-231, SKBr3 and BT474 (30 μg) were resolved on SDS-PAGE gel and probed with anti-CXCR4 and HER2 antibodies. As a loading control, stripped membrane was probed with β-actin antibodies. (C) Butein suppresses CXCR4 levels in a dose-dependent manner. SKBr3 cells (1×10^6) were treated with the indicated concentrations of butein for 8 h. Whole-cell extracts were then prepared, and 30 μg of protein was resolved on SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed for CXCR4. (D) Butein suppresses CXCR4 levels in a time-dependent manner. SKBr3 cells (1×10^6) were treated with 50 μM butein for the indicated times, after which Western blotting was done as described above. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. (E) Butein did not affect CXCR4 expression in MDA-MB-231 cells. MDA-MB-231 cells (1×10^6) were treated with 50 μM butein for 8 h, after which Western blotting was done as described above. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. (F) Effect of butein on HER2 expression in SKBr3 cells. SKBr3 cells (1×10^6) were treated with the indicated concentrations of butein for 8 h, after which Western blotting for HER2 was done as described above. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. The densitometric analysis of the scanned blots was done using Image J software and the results are expressed as fold change relative to the control. The representative results of three independent experiments are shown.

the wells, to serve as fiducial marks demarcating the wound areas to be analyzed. Prior to inflicting the wound, the cells should be fully confluent. The growth medium was aspirated off and replaced by calcium-free PBS to prevent killing of the cells at the edge of the wound by exposure to high calcium concentrations before two parallel scratch wounds were made perpendicular to the marker lines with a sterile 1000-μL automated pipette tip. Thereafter, the calcium-free medium was then changed to medium with or without butein. After incubation for 6 h, the growth medium was then changed to basal medium with or without CXCL12. 48 h later, the wounds were observed using bright field microscopy and multiple images were taken at areas flanking the intersections of the wound and the marker lines at the start and end of the experiment. Gap distance of the wound was measured at three different sites using Photoshop software, and the data were normalized to the average of the control. Graphs were plotted against the percentage of migration distance the cells moved before and after treatment, normalized to control.

2.2. Invasion assay

The *in vitro* invasion assay was performed using Bio-Coat Matrigel invasion assay system (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. SKBr3 and AsPC-1 cells (2×10^5 cells) were suspended in serum-free medium (RPMI medium for AsPC-1 and McCoy's 5A medium for SKBr3) and seeded into the Matrigel transwell chambers consisting of polycarbonate membranes with 8-μm pores. After pre-incubation with or without butein for 6 h, the transwell chambers were then placed into appropriate wells of a 24-well plate, in which either the basal medium only or basal medium containing CXCL12 had been added. After incubation for 24 h, the upper surfaces of the transwell chambers were wiped with cotton swabs and the invading cells were fixed and stained with crystal violet solution. The invading cell numbers were counted in five randomly selected microscope fields (200×).

2.2.1. Statistical analysis

The experiments were carried out in triplicates and repeated twice. The *p* value was obtained with ANOVA and SNK statistical analyses.

3. Results

The present study was designed to investigate the effect of butein (with structure shown in Fig. 1A) on both constitutive and inducible CXCR4 expression in tumor cells, more specifically, to monitor its influence on the critical role CXCR4 plays in tumor cell invasion and metastasis. We also investigated the effect of butein on CXCR4 expression in various tumor cell types and its effect on invasion in breast and pancreatic cancer cell lines.

3.1. Butein suppresses the expression of CXCR4 protein in HER2-overexpressing breast cancer SKBr3 cells

HER2 overexpression has been linked with metastasis of breast cancer [20]. Furthermore, HER2 has been shown to induce the expression of CXCR4 in breast cancer cells [20]. Fig. 1B shows the relative expression of CXCR4 and HER2 in four different breast cancer cells and it is clearly evident that expression of CXCR4 in SKBr3 and BT474 cells increases with endogenous expression of HER2 in these cell lines as compared to very low and relatively low expression in HER2-lacking MCF-7 and MDA-MB-231 cells. Hence, we decided to investigate the effect of butein on CXCR4 expression in detail in HER2-overexpressing SKBr3 cells. When SKBr3cells were incubated either with different concentrations of butein for 8 h or with 50 μM of butein for different times, butein suppressed the expression of CXCR4 in a dose- (Fig. 1C) and time- (Fig. 1D) dependent manner. The exposure of cells to 50 μM butein for 8 h significantly inhibited the CXCR4 expression, as evident by time kinetics study in Fig. 1D. This downregulation was not due to decrease in cell viability as 90% of cells were viable under these conditions (data not shown). Moreover, we found that butein had a minimal effect on the expression of CXCR4 in HER2-lacking MDA-MB-231 cells (Fig. 1E). Since HER2 enhances the expression of CXCR4 by stimulating CXCR4 translation and attenuating CXCR4 degradation [32], we also examined whether butein down-regulates CXCR4 expression through regulation of HER2 expression. For this, HER2-overexpressing SKBr3 cells were incubated with different concentrations of butein for 8 h and then examined for HER2 expression by Western blot analysis using specific antibodies. We found that HER2 expression was partially affected after butein treatment (Fig. 1F), thus suggesting that down-regulation of CXCR4 expression by butein is not completely due to modulation of HER2 expression.

3.1.1. Butein down-modulates CXCR4 in different cell types

Up to this point, all of the afore-mentioned studies were carried out with HER2-overexpressing breast cancer SKBr3 cells. However, CXCR4 is known to be overexpressed in a wide variety of tumor cells [33]. Hence, we carried out the same experiment to find out whether butein downregulates expression of CXCR4 in pancreatic (PANC-1, PANC-28 and MIA PaCa-2) cancer cell lines, which had thus far not been reported as yet. Cells were treated with 50 μM butein for 8 h before assessing the resultant effect on CXCR4 expression. Fig. 2 clearly demonstrates that butein substantially downregulated CXCR4 expression in all three pancreatic cancer cell lines. Upon further extension of studying the effect of butein on CXCR4 expression in prostate cancer (DU145), multiple myeloma (U266), head and neck squamous cell carcinoma (SCC-38), hepatocellular carcinoma (HepG2) cancer cell lines, we also found that butein dramatically downregulated the CXCR4 expression in

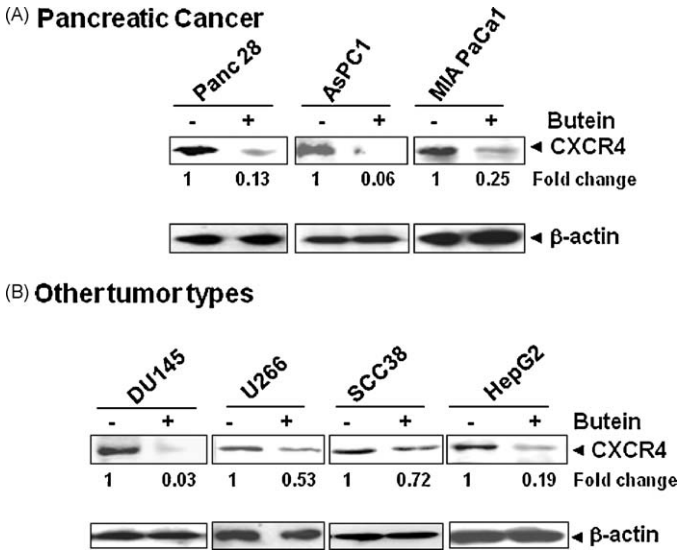

Fig. 2. Butein downregulates CXCR4 in different tumor cell types. Pancreatic cancer (PANC 28, AsPC1, MiaPaca1), prostate cancer (DU145), multiple myeloma (U266), head and neck squamous cell carcinoma (SCC38) and hepatocellular carcinoma (HepG2) cells were incubated with 50 μM butein for 8 h. Whole-cell extracts were prepared and analyzed by Western blot analysis using antibody against CXCR4. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. The densitometric analysis of the scanned blots was done using Image J software and the results are expressed as fold change relative to the control. Representative results of three independent experiments are shown.

all these cell lines (Fig. 2). This showed convincingly that CXCR4 downregulation by butein is not cell type-specific.

3.1.2. Downregulation of CXCR4 expression by butein is not mediated through its degradation

Because butein could downregulate CXCR4 expression by enhancing its degradation, and CXCR4 has been shown to undergo ubiquitination at its lysine residue followed by degradation [34,35], we first explored the possibility that butein may enhance the rate of CXCR4 degradation via the activation of proteasomes. To determine this, we examined the ability of ALLN, a proteasome inhibitor, to block butein-induced degradation of CXCR4 in SKBr3 cells. Cells were pretreated with ALLN for 1 h before being exposed to butein. As shown in Fig. 3A, ALLN had no effect on butein-

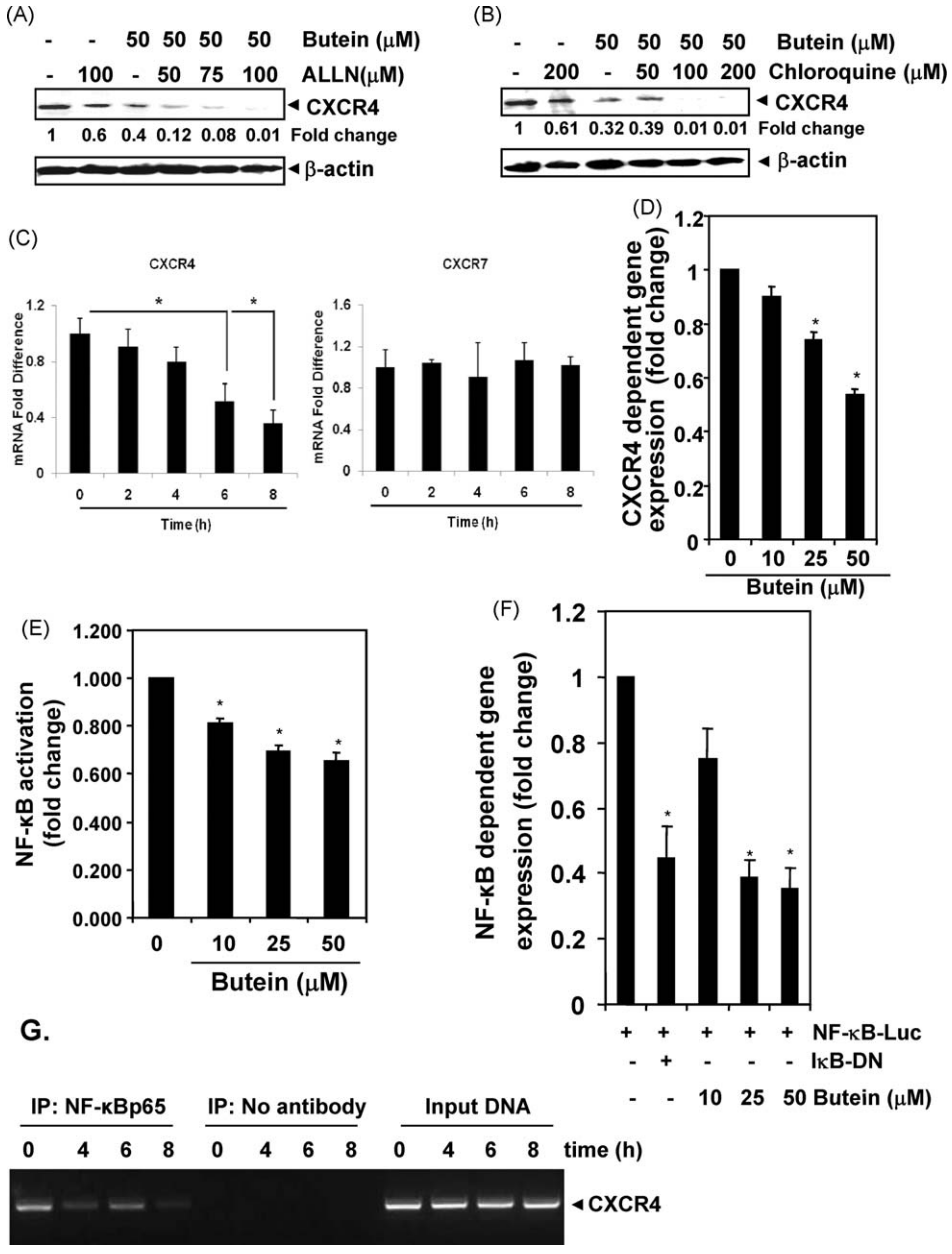

Fig. 3. Butein suppresses CXCR4 through mRNA level. (A and B) Butein suppresses CXCR4, through lysosomal but not proteosomal degradation. Cells were treated with indicated concentrations of ALLN or chloroquine for 1 h at 37 °C, followed by treatment of 50 μM butein for 8 h. Whole-cell extracts were prepared and analyzed by Western blot analysis using antibodies against CXCR4. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. Representative results of three independent experiments are shown. The densitometric analysis of the scanned blots was done using Image J software and the results are expressed as fold change relative to the control. (C) Butein suppresses expression of CXCR4 mRNA without any effect on CXCR7. Cells were treated with 50 μM butein for indicated times. Total RNA was isolated and analyzed by RT-PCR assay as described in Materials and Methods. 18S was used to show equal loading of RNA. Representative results of three independent experiments are shown. (D) SKBr3 cells were transiently transfected with a CXCR4-luciferase plasmid and then treated with the indicated concentrations of butein for 8 h. Cell supernatants were thereafter collected and assayed for luciferase activity as described in Materials and Methods. Results are expressed as fold activity over the activity of the vector control. Bars indicate standard deviation. * indicates p value <0.05). (E) Butein inhibits NF-κB activation in HER2-overexpressing breast cancer cells. SKBr3 cells were incubated with indicated concentrations of butein for 4 h. The nuclear extracts were assayed for NF-κB activation by TransAM p65 transcription factor assay kit. (F) SKBr3 cells were transiently transfected with an NF-κB-luciferase plasmid and then treated with the indicated concentrations of butein for 4 h. Cell supernatants were thereafter collected and assayed for luciferase activity as described in Section 2.1.6. Representative results of three independent experiments are shown. Results are expressed as fold activity over the activity of the vector control. Bars indicate standard deviation. * indicates p value <0.05). (G) Butein inhibits binding of NF-κB to the CXCR4 promoter. SKBr3 cells were treated with 50 μM butein for indicated time intervals and the proteins were cross-linked with DNA by formaldehyde and then subjected to ChIP assay using an anti-p65 antibody with the CXCR4 primer. Reaction products were resolved by electrophoresis.

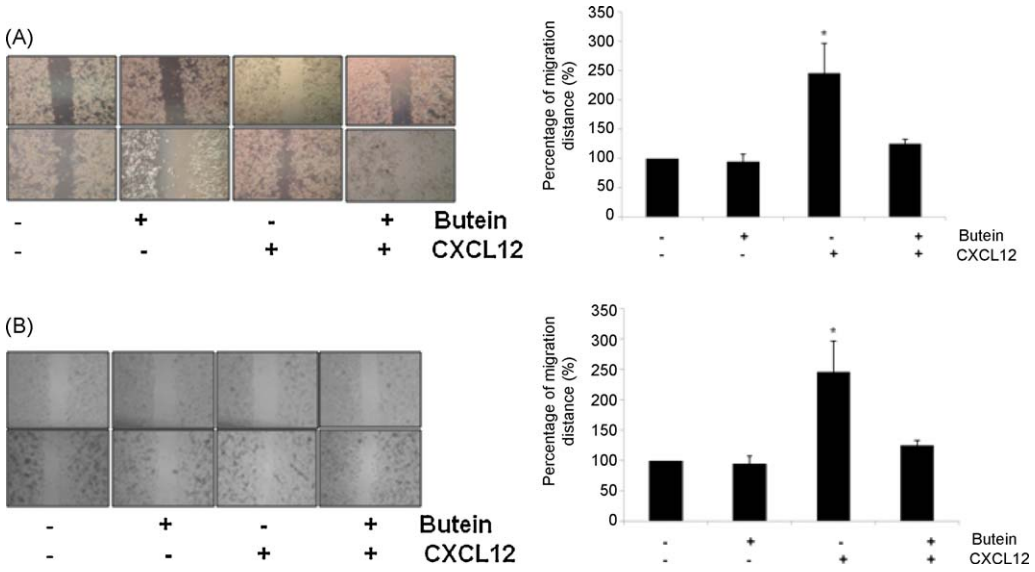

Fig. 4. Butein suppresses migration of breast and pancreatic cancer cells. (A) The wound-healing assay for evaluating the inhibitory effect of butein on SKBr3 cell migration. Confluent monolayers of SKBr3 cells were scarred, and repair was monitored microscopically after 6 h of pre-treatment with 50 μM butein before being exposed to 200 ng/mL CXCL12 for 48 h. Width of wound was measured at time zero and 48 h of incubation with and without butein in the absence or presence of CXCL12. The representative photographs showed the same area at time zero and after 48 h of incubation. Graphs, mean ($n = 3$); bars, SE. $^*p < 0.05$. (B) The wound-healing assay for evaluating the inhibitory effect of butein on AsPC1 cell migration. Confluent monolayers of AsPC1 cells were scarred, and repair was monitored microscopically after 6 h of pre-treatment with 50 μM butein before being exposed to 200 ng/mL CXCL12 for 48 h. Width of wound was measured at time zero and 48 h of incubation with and without butein in the absence or presence of CXCL12. The representative photographs of three independent experiments showed the same area at time zero and after 48 h of incubation. Graphs, mean ($n = 3$); bars, SE. $^*p < 0.05$.

induced degradation of CXCR4, suggesting that this is an unlikely basis for the suppression of butein on CXCR4 expression.

We next examined the ability of chloroquine, a lysosomal inhibitor, to block butein-induced degradation of CXCR4, as CXCR4 has been shown to undergo ligand-dependent lysosomal degradation [35]. The cells were pretreated with chloroquine for 1 h before exposure to butein. Our results showed that chloroquine at 200 μM only slightly prevented the degradation of CXCR4 (Fig. 3B), suggesting that this was arguably not the primary pathway for suppression of expression of CXCR4.

3.1.3. Downregulation of CXCR4 by butein occurs at the transcriptional level

Since butein did not downregulate CXCR4 expression by enhancing its degradation, we investigated whether suppression occurs at the transcriptional level instead. Cells were treated with butein for different times and then extracted the mRNA for analysis by real-time PCR. As shown in Fig. 3C, butein induced the downregulation of CXCR4 mRNA in a time-dependent manner. Interestingly, butein had no effect on the mRNA of another chemokine receptor, CXCR7, thus indicating that the effects are specific for CXCR4. We also examined whether butein modulates constitutive CXCR4-dependent reporter activity in SKBr3 cells. When SKBr3 cells, after transfection with the CXCR4-luciferase plasmid, were incubated with different concentrations of butein for 8 h, this chalcone suppressed constitutive CXCR4 reporter activity in a dose-dependent manner (Fig. 3D).

3.1.4. Butein suppresses constitutive activation of NF-κB in SKBr3 cells

To follow up on a previously reported finding that HER2 expression regulates NF-κB activation in breast cancer [36], we used a DNA-binding assay to explore whether overexpression of HER2 affects NF-κB activation in SKBr3 cells, and found that HER2-overexpressing SKBr3 cell line showed constitutive NF-κB activation and treatment with butein downregulated NF-κB activation in a dose-dependent manner (Fig. 3E). These results suggest that butein may, at least in part, downregulate CXCR4 expression through suppression of NF-κB activation. However, DNA binding alone is not always associated with NF-κB-dependent gene transcription [37], suggesting that additional regulatory steps are involved. Subsequent results also indicated that butein inhibited NF-κB reporter activity in a dose-dependent manner in SKBr3 cells (Fig. 3F).

3.1.5. Butein inhibits binding of NF-κB to the CXCR4 promoter

Whether the downregulation of CXCR4 by butein in SKBr3 cells was due to suppression of NF-κB activation *in vivo* was examined by a ChIP assay targeting NF-κB binding in the CXCR4 promoter. We found that butein suppressed the NF-κB binding to the CXCR4 promoter (Fig. 3G), thereby indicating that butein inhibits CXCR4 expression by suppressing NF-κB binding to the CXCR4 promoter.

3.1.6. Butein suppresses CXCL12-induced breast cancer cell migration and invasion

Several lines of evidence implicate the role of CXCR4 in breast cancer metastasis [7,13]. Muller et al. found that motility and migration of breast cancer cells can be induced when they are exposed to their ligand, CXCL12 [7]. Also, breast cancer metastasis can be inhibited by silencing CXCR4 [38]. Whether downregulation of CXCR4 by butein correlates with breast cancer cell migration was examined using an *in vitro* wound healing assay. We found that breast cancer cells migrated faster under the influence of CXCL12 and this effect was abolished on treatment with butein (Fig. 4A). Using an *in vitro* invasion assay, we found that CXCL12 induced the invasion of breast cancer SKBr3 cells and that butein effectively abrogated the invasion (Figure 5A and B).

3.1.7. Butein inhibits CXCL12-induced pancreatic cancer cell migration and invasion

In addition, the CXCL12/CXCR4 signaling has been shown to play a critical role in pancreatic cancer metastasis [39]. Using an *in vitro* wound healing assay, we found that pancreatic cancer AsPC-1 cells migrated faster under the influence of CXCL12 and this effect was abolished on treatment with butein (Fig. 4B). To elucidate

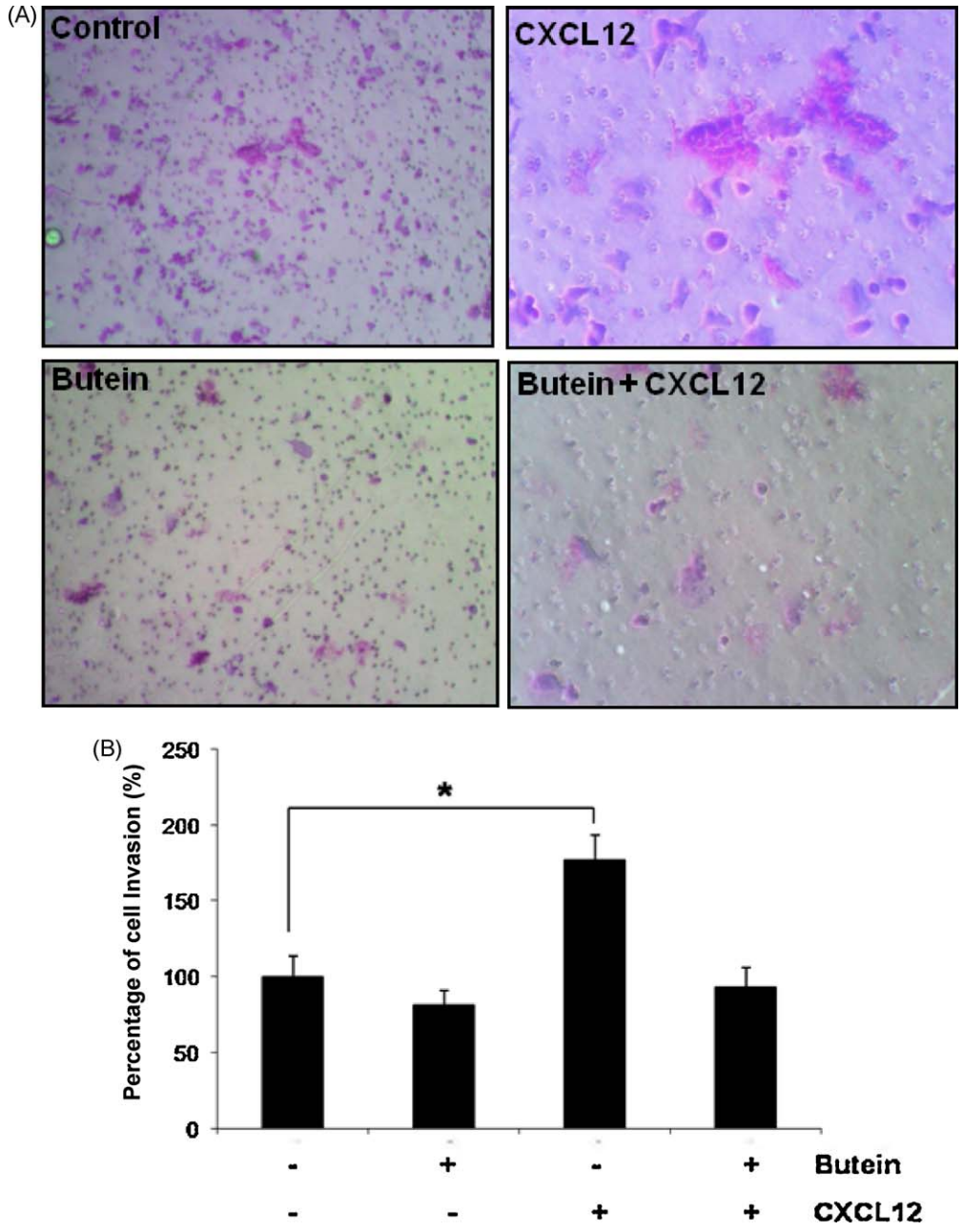

Fig. 5. Butein suppresses invasion in breast cancer cells. (A) SKBr3 (2×10^5 cells) were seeded in the top-chamber of the Matrigel. After pre-incubation with or without butein (50 μM) for 6 h, transwell chambers were then placed into the wells of a 24-well plate, in which we had added either the basal medium only or basal medium containing 200 ng/mL CXCL12 in a predetermined arrangement. After incubation, they were assessed for cell invasion as described in Section 2. (B) Columns, mean number of invaded cells; bars, SE. $^*p < 0.05$. Representative results of three independent experiments are shown.

further the effect on butein on CXCL12-induced cell invasion, we investigated and found that treatment of butein suppressed CXCL12-induced invasion of pancreatic cancer cells AsPC-1 (Fig. 6A and B). We also found that butein downregulated the expression of both mRNA (Fig. 6C) and protein (Fig. 6D) for CXCR4 in a time-dependent manner.

4. Discussion

The goal of the present study was to determine whether butein, a component of cashews that has been linked with anticancer activities, can suppress the expression and function of CXCR4, a chemokine receptor that has been closely linked with cancer cell growth, invasion, angiogenesis, and metastasis. Our results showed for the first time that butein downregulated the expression of CXCR4 in different types of tumor cells, irrespective of the cell type. For example, butein was found to suppress CXCR4 expression in HER2-overexpressing SKBr3 breast cancer cells. Our results showed that downregulation of CXCR4 did not occur through proteolytic degradation of the receptor but rather through downregulation of the transcript. Furthermore, suppression of receptor expression led to downregulation of migration and invasion induced by the ligand CXCL12 in both pancreatic and breast cancer cells.

The CXCR4 chemokine receptor has been found to be over-expressed in different tumors, including breast cancer, ovarian cancer, glioma, pancreatic cancer, prostate cancer, acute myeloid leukemia, chronic lymphoblastic leukemia (CLL), B-CLL, melanoma, cervical cancer, colon carcinoma, rhabdomyosarcoma, astrocytoma, small-cell lung carcinoma, renal cancer, and non-Hodgkin's

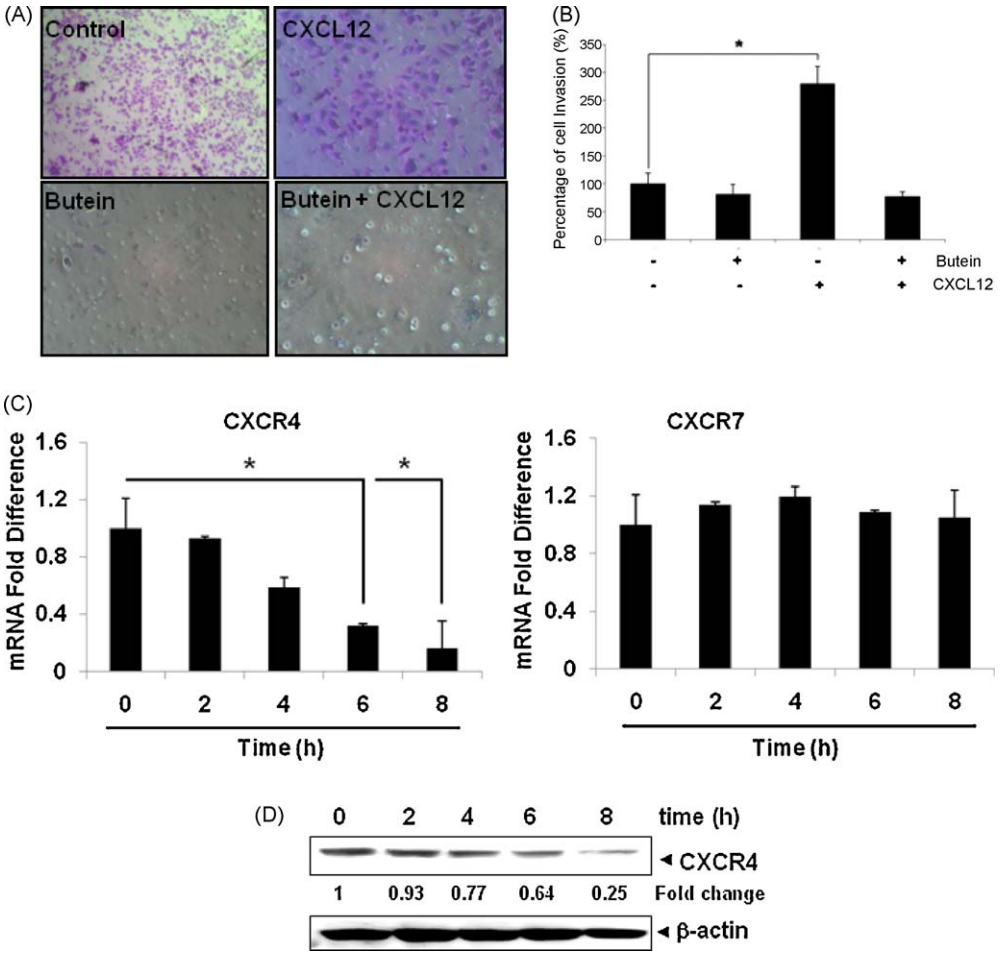

Fig. 6. Butein suppresses invasion and CXCR4 expression in pancreatic cancer cells. (A) AsPC-1 (2×10^5 cells) were seeded in the top-chamber of the Matrigel. After pre-incubation with or without butein (50 μM) for 6 h, transwell chambers were then placed into the wells of a 24-well plate, in which we had added either the basal medium only or basal medium containing 200 ng/mL CXCL12, in a predetermined arrangement. After incubation, the chambers were assessed for cell invasion as described in Section 2. (B) Columns, mean number of invaded cells; bars, SE. $^*p < 0.01$. Representative results of three independent experiments are shown. (C) Butein suppresses expression of CXCR4 mRNA without any effect on CXCR7 mRNA. AsPC-1 cells were treated with 50 μM butein for indicated times. Total RNA was isolated and analyzed by RT-PCR assay as described in Section 2. 18S was shown to equal loading of total RNA. Representative results of three independent experiments are shown. (D) Cells were incubated with 50 μM butein for indicated times. Whole-cell extracts were prepared and analyzed by Western blot analysis using antibodies against CXCR4. The same blots were stripped and reprobed with β-actin antibody to show equal protein loading. The densitometric analysis of the scanned blot was done using Image J software and the results are expressed as fold change relative to the control. Representative results of three independent experiments are shown.

lymphoma, as compared to normal cells which show little or no CXCR4 expression [23,32,40–43]. Although it is still unclear what leads to the overexpression of CXCR4 in cancer cells, studies point to genetic and microenvironmental factors [44]. PAX3- and PAX7-FKHR gene fusion [45], mutations in the von Hippel Lindau tumor suppressor gene [46], hypoxia in the tumor microenvironment [47], NF-κB [30], and inflammatory cytokines such as vascular endothelial growth factor [48] and tumor necrosis factor alpha [44], have all been implicated in CXCR4 overexpression. Recently, the epidermal growth factor receptor, c-erbB2, and its encoding gene, HER2/*neu*, have also been implicated in the positive regulation of CXCR4 expression at the post-transcriptional level [49,50]. Given that CXCR4 has been linked with the metastasis of various cancers and CXCR4 expression has been correlated with poor prognosis and poor overall patient survival [51], CXCR4 appears an ideal therapeutic target for the investigation of novel therapeutic interventions for the prevention of metastatic cancer.

Our results clearly indicate that butein suppressed CXCR4 expression in HER2-overexpressing breast cancer cells in a dose- and time-dependent manner, but had minimal effect on CXCR4 expression in HER2-lacking breast cancer cells. Our data also showed that butein suppressed CXCR4 expression on various tumor cell lines including pancreatic cancer, prostate cancer, multiple myeloma, head and neck squamous cell carcinoma and hepatocellular carcinoma, thereby indicating that the effect of butein on CXCR4 is not limited to a single cell type. The ligand-dependent down-regulation of the CXCR4 receptor by lysosomal degradation is well documented [32]. Recent reports suggest that degradation involves atrophin-interacting protein (AIP)-4 mediated ubiquitination and degradation [35]. However, our data indicates that butein does not downregulate the CXCR4 through this mechanism. As such, with downregulation of CXCR4 by butein arguably not occurring at the post-translational level, we postulated that the inhibition of CXCR4 expression by butein could occur at the transcriptional level. Indeed, we found that butein downregulated the expression of CXCR4 mRNA without having any effect on the expression of CXCR7 mRNA in breast and pancreatic cancer cells.

Butein has been previously reported to downregulate NF-κB activation in various tumor cells [25]. Interestingly, the NF-κB binding site has also been identified in the proximal region of the CXCR4 promoter and postulated to play a role in CXCR4 expression in human breast cancer cells [30]. Therefore, it is possible that downregulation of CXCR4 by butein occurs via the suppression of NF-κB. Indeed, we found that inhibition of constitutive NF-κB

activation by butein leads to downregulation of CXCR4 in SKBr3 cells. Our results are in agreement with those of Li et al. who showed that HER2-induced CXCR4 expression required NF-κB activation [20]. Whether mechanism(s) other than suppression of NF-κB activation are involved in downregulation of CXCR4 by butein, cannot currently be confirmed or ruled out. Furthermore, we observed that butein specifically downregulated the expression of CXCR4 by inhibiting NF-κB but not CXCR7. Whether activation of NF-κB also plays a significant role in CXCR7 expression in HER2 overexpressing breast cancer cells requires further investigation. Moreover, besides CXCR4, the activation of NF-κB also induces the expression of various molecules including cyclooxygenase-2, matrix metallopeptidase-9, and adhesion molecules such as intracellular adhesion molecule 1, vascular cell adhesion molecule 1, and endothelial-leukocyte adhesion molecule 1, all of which have been linked with cancer cell invasion and metastasis [52]. Because butein can inhibit both DNA binding ability and transcriptional activation of NF-κB, as shown in this study, it is possible that butein can suppress the expression of other NF-κB regulated molecules as well in SKBr3 cells.

We further investigated the effect of butein on CXCL12-induced migration and invasion of both breast and pancreatic cancer cells. CXCL12 not only binds to CXCR4 but also to CXCR7 [53]. We found that although butein did not affect the expression of CXCR7, preincubation of cells with butein completely blocked CXCL12-induced cell migration and invasion. Although the role of CXCL12 in the promotion of invasive growth is well documented and the intracellular signals triggered by CXCR4 activation have been extensively studied, the published evidence is not consistent on whether CXCR7 expression consistently contributes to tumor growth, invasion and metastasis [54]. This argues in favor of the critical role of the CXCR4 receptor in tumor invasiveness and progression and the potential of butein in downregulating CXCL12-induced migration and invasion of both breast and pancreatic cancer cells.

Elevated levels of CXCR4 have been reported with nodal metastasis of human breast cancer [55]. Butein has been shown to inhibit tumor initiation and promotion [25,26,56–63], to suppress phorbol ester-induced skin tumor formation [27], and to inhibit carrageenan-induced rat paw edema [28]. Our data show that butein downregulates expression of CXCR4, a key receptor involved in the cross-talk between tumor cells and its microenvironment, and thus, that some of the anti-tumor effects of butein are possibly mediated through CXCR4 regulation. Further *in vivo* studies are needed to demonstrate the relevance of these observations to cancer treatment.

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