**Enterolobium contortisiliquum** Trypsin Inhibitor (EcTI), a Plant Proteinase Inhibitor, Decreases *in Vitro* Cell Adhesion and Invasion by Inhibition of Src Protein-Focal Adhesion Kinase (FAK) Signaling Pathways*\(^\text{a,b}\)*

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**Background:** The effect of *Enterolobium contortisiliquum* trypsin inhibitor (EcTI) on the adhesion, migration, and invasion of gastric cancer cells.

**Results:** EcTI inhibited adhesion, migration, and cell invasion and decreased Src-FAK signaling.

**Conclusion:** EcTI inhibits the invasion of gastric cancer cells through alterations in integrin-dependent cell signaling pathways.

**Significance:** Inhibition of invadopodia formation may be an attractive approach for cancer therapy.

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Tumor cell invasion is vital for cancer progression and metastasis. Adhesion, migration, and degradation of the extracellular matrix are important events involved in the establishment of cancer cells at a new site, and therefore molecular targets are sought to inhibit such processes. The effect of a plant proteinase inhibitor, *Enterolobium contortisiliquum* trypsin inhibitor (EcTI), on the adhesion, migration, and invasion of gastric cancer cells was the focus of this study. EcTI showed no effect on the proliferation of gastric cancer cells or fibroblasts but inhibited the adhesion, migration, and cell invasion of gastric cancer cells; however, EcTI had no effect upon the adhesion of fibroblasts. EcTI was shown to decrease the expression and disrupt the cellular organization of molecules involved in the formation and maturation of invadopodia, such as integrin β1, cortactin, neuronal Wiskott-Aldrich syndrome protein, membrane type 1 metalloproteinase, and metalloproteinase-2. Moreover, gastric cancer cells treated with EcTI presented a significant decrease in intracellular phosphorylated Src and focal adhesion kinase, integrin-dependent cell signaling components. Together, these results indicate that EcTI inhibits the invasion of gastric cancer cells through alterations in integrin-dependent cell signaling pathways.

During cancer progression at primary and secondary sites, the tumor cells invariably interact with extracellular matrix (ECM) components and alter the arrangement of cell-cell and cell-matrix adhesion (1).

Recently, active cellular projections called invadopodia have been described in some types of cancer cells and active macrophages. Invadopodia, protrusions of the plasma membrane, are adhesive actin-rich structures located on the ventral cell surface of invasive tumor cells. In this manner, invadopodia establish focal contact with the substratum and play an intricate role in focal degradation of the extracellular matrix during cell invasion (2–5). Active smooth muscle filamentous actin polymerization, induction of membrane curvature, rapid turnover of cell-matrix adhesions, and local modulation of contractile forces are all likely to play a central role in the promotion of invadopodium formation (6). Invadopodium formation is a sequential step process that begins with the assembly of precursor structures, such as actin, cortactin, and neuronal Wiskott-Aldrich syndrome protein (N-WASP) (7, 8).

The attachment of cells to ECM proteins is mainly mediated by integrins, heterodimeric transmembrane receptors that through focal adhesions connect the ECM to the cellular actin cytoskeleton (9). Besides cell migration, integrins also mediate cell-cell adhesion, cell survival, proliferation, and motility through signaling cascades that they trigger at the attachment sites (10). Moreover, focal adhesions also play a signaling center mediating multiple dynamic protein-protein interactions and consequently regulating the assembly and disassembly of focal adhesions, which are essential for controlling cell movement and migration (11). Integrin clustering induced by cell

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*This article contains supplemental Figs. 1–3.*

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attachment to ECM promotes the formation of cell-matrix adhesion and activation of Src and focal adhesion kinase (FAK). A complex array of proteins is then recruited to the cell membrane and is involved in the site of cell attachment. Catalytic adaptors, such as FAK and Src, facilitate the propagation of signal transduction pathways from adhesion sites (12). Normally, integrins are expressed on the cell surface in an inactive state, unable to bind to their receptors. This inactivity can be an essential attribute because inappropriate integrin activation will lead to excessive activation in adherent cells (13).

FAK and Src are non-receptor tyrosine kinases that control a number of cellular signaling pathways, including cell motility and survival (14). FAK is localized in cell-matrix adhesions and acts as a central regulator of focal adhesion, consequently influencing cell survival, differentiation, proliferation, metastasis, and tissue remodeling (15). In several cell types, FAK directly recruits Src to the focal adhesion sites where Src potentiates activation of FAK through phosphorylation of additional tyrosine residues. Tyrosine phosphorylation of FAK and integrin molecules creates docking sites for other proteins involved in actin cytoskeleton remodeling (16).

Src family kinases represent the largest family of non-receptor tyrosine kinases that interact directly with receptor tyrosine kinases, signal transducers, activators of transcription, and molecules involved in cell adhesion and migration. Aberrant expression or activation of Src family kinases causes perturbations in these activities, leading to transformation and progression of malignant disease (17). Endogenous Src kinases have been shown to promote invadopodium formation in response to growth factors and chemokines. Src phosphorylates several invadopodium components, including cortactin and N-WASP. Cortactin is one of the few cytoskeletal proteins specifically required for the assembly of invadopodia in carcinoma cells, and this protein regulates the formation and maturation of invadopodia (18). Tyrosine phosphorylation of cortactin regulates the recruitment of N-WASP and Arp2/3-dependent actin polymerization at invadopodia. Deacetylation of cortactin by histone deacetylase 6 alters its association with actin, thus modulating cell motility (19). Moreover, subsequent cortactin dephosphorylation promotes invadopodium maturation and matrix metalloproteinase (MMP)-dependent matrix degradation (8).

Several proteolytic enzymes are localized at the tip of invadopodia, including membrane type 1 metalloprotease (MT1-MMP) or MMP-14, secreted MMPs (MMP-2 and MMP-9), and a disintegrin and metalloproteinase-12 (2, 20, 21). To date the major contribution in cancer invasion attributed to MMPs is the degradation of the ECM barrier (1, 22, 23). To carry out such function, MMPs are expected to act at the leading edge of the invading cancer cells. MT1-MMP was identified as the first membrane-anchored type MMP acting as a key enzyme responsible for the degradation of the pericellular ECM (24). MT1-MMP is known to activate MMP-2 and -13 and degrade a wide range of ECM components, including type I collagen, fibronectin, and laminins (25).

Proteinases play a pivotal role in various cell events, and in cancer, proteinase activities are deeply involved in invasion and metastasis and are a hallmark for malignant tumors (26). Many studies suggest the use of proteinase inhibitors in cancer therapy (23, 27–30). Increased proteolysis has been shown to underpin various pathological processes, and as a result, proteinases and proteinase inhibitors have emerged as potential therapeutic targets. There are many examples of therapeutic interventions using proteinase inhibitors: angiotensin-converting enzyme inhibitors for the treatment of hypertension (31), HIV aspartyl proteinase inhibitors to prevent the development of AIDS (30, 32), and application of proteinase inhibitors in the treatment of cancer (23, 33, 34).

Therefore, inhibition of extracellular proteinases and more importantly inhibition of invadopodium formation may be an attractive approach for cancer therapy (35–39). Moreover, considering the complex proteinase network, an inhibitor capable of interfering in invadopodium formation may be useful for the study of tumor progression as well as the study of the involvement of invadopodia in this process. On this note, we studied the effect of Enterolobium contortisiliquum trypsin inhibitor (EcTI), a potent polyspecific Kunitz-type inhibitor (23, 40), on cell adhesion, migration, invasion, and invadopodium formation and consequently analyzed actin remodeling via Src and FAK in an attempt to characterize the intracellular cascades recruited during this signaling. Gastric cancer cells treated with EcTI presented reduced migration and invasion. Treatment with EcTI resulted in a decrease in the organization of invadopodia at the migration front and in a decrease of Src and FAK activation. Our findings suggest EcTI as a potent inhibitor of gastric cancer cell adhesion, migration, and invasion through the inhibition of Src-FAK-mediated signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Inhibitor Purification—EcTI purification procedures were based on previously described methodology (40). Briefly, E. contortisiliquum seeds were swollen in 0.15 m NaCl (1:40, w/v) and homogenized in a blender. The crude extract was centrifuged at 4000 × g; the supernatant was collected, and the proteins were precipitated with acetone (80%, v/v) at 4 °C. The pellet was vacuum-dried and solubilized in 0.05 m Tris/HCl buffer, pH 8.0. This soluble fraction was applied to a DEAE-Sephadex column (2 × 25 cm) pre-equilibrated with 0.1 m Tris/HCl buffer, pH 8.0. After extensive washing with the equilibration buffer, the inhibitor was eluted with 0.15 m NaCl, 0.05 m Tris/HCl buffer, pH 8.0, and the inhibitory activity was measured by the inhibition of trypsin hydrolysis of 1 mM α-benzoyl-DL-arginine p-nitroanilide (Bachem, Bubendorf, Switzerland) as substrate (40). The fractions containing the inhibitory activity were pooled and applied sequentially to a trypsin-Sepharose and a Superdex 75 column equilibrated with 0.05 m Tris/HCl buffer, pH 8.0 coupled to an ÅKTA Purifier (GE Healthcare).

The last step of the purification was a reverse phase chromatography in a C18 protein/peptide column (15 cm × 4.6 mm; Vydac) developed with an acetonitrile gradient (0–100%) in trifluoroacetic acid (TFA) (0.1%, v/v) at room temperature at a flow rate of 0.7 ml/min; the isolated peak was named EcTI. The homogeneity and the molecular weight of the inhibitor were assessed by 8–12% SDS-polyacrylamide gel electrophoresis (40).
Prior to in vitro assays, EcTI was concentrated and buffer-exchanged for 7 mM HEPES, pH 7.4 (vehicle) by centrifugation with an Amicon filter unit (Millipore, Bedford, MA). This same procedure was performed with 7 mM HEPES, pH 7.4 (EcTI vehicle) for the control.

Cell Cultivation and Reagents—The human gastric cancer cell lines Hs746T and MKN28 were purchased from ATCC (Manassas, VA) and cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma), 100 µg/ml streptomycin (Invitrogen), and 100 IU/ml penicillin (Invitrogen) at 37 °C in an atmosphere of 5% CO₂. Fibroblasts isolated from amniotic fluid (WPF5) were kindly supplied by Prof. Walter Pinto, Jr. (Campinas, Brazil) and used between passages 3 and 6 as characterized previously (41). The cells were maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated FCS (Sigma), 2 mM L-glutamine (Sigma), 100 µg/ml streptomycin (Invitrogen), and 100 IU/ml penicillin (Invitrogen) at 37 °C in an atmosphere of 5% CO₂. The cells were incubated with EcTI or 7 mM HEPES, pH 7.4 as vehicle control and 4-amino-5-(4-chlorophenyl)-7-((t-buty1)pyrazolo[3,4-d]pyrimidine (PP2) (Sigma) or 0.1% dimethyl sulfoxide (DMSO; Sigma) as vehicle control during cell proliferation, viability, and confocal assays.

Cell Proliferation Assay—The effect of EcTI on cell proliferation was assayed using a colorimetric bromodeoxyuridine (BrdU) incorporation assay (Kit III, Roche Applied Science) performed according to the manufacturer’s protocol.Briefly, 1 × 10⁵ cells were seeded on a 96-well microplate (Corning Inc., Corning, NY). After 24 h, the medium was removed, and the cells were treated with EcTI (0, 25, 50, 100, and 150 µM) or PP2 (0, 5, 10, 20, and 40 µM) in culture medium with 2% heat-inactivated FCS for 24 or 48 h at 37 °C in an atmosphere of 5% CO₂. After the stated incubation periods, BrdU solution (10 µM) was added to the medium, and the cells were incubated for a further period of 3 h at 37 °C in a humidified atmosphere containing 5% CO₂. Thereafter, the cells were fixed and DNA was denatured in one step by adding the provided nucleoside solution. Incorporated BrdU was detected by an anti-BrdU peroxidase-conjugated antibody. The immune complex was detected by a subsequent substrate colorimetric reaction and quantified by measuring the absorbance at 405 nm. All experimental points were performed in triplicates.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Cell Viability Assay—The viability of the cells treated with EcTI was determined by measuring cell redox activity using the MTT assay. Briefly, 1 × 10⁴ cells were seeded into a 96-well microplate (Corning Inc.). After cell attachment, the medium was removed, and the cells were treated with EcTI (0, 25, 50, 100, and 150 µM) or PP2 (0, 5, 10, 20, and 40 µM) prepared in RPMI 1640 medium supplemented with 2% heat-inactivated FCS for 24 or 48 h at 37 °C in an atmosphere of 5% CO₂. After the stated incubation period, 0.5 mg/ml MTT solution (Sigma) in 10 mM phosphate buffered saline, pH 7.4 (PBS) was added to the medium and incubated for a further period of 2 h at 37 °C in an atmosphere of 5% CO₂. Afterward, the medium was removed, and the cells were washed. The formazan crystals were dissolved in DMSO and measured spectrophotometrically at a wavelength of 540 nm. All experimental points were performed in triplicates.

Immunocytochemistry and Microscopy—Hs746T cells were cultured on coverslips previously coated with a thin film of reconstituted rat tail collagen (collagen type I purified from rat tail tendon by the method described previously (42)). After cell attachment and growth to a confluent monolayer, the cells were treated with 100 µM EcTI for 6 h or 10 µM PP2 for 6 and 24 h. Subsequently, cells were immersion-fixed in 4% paraformaldehyde in PBS for 30 min. After three washes in PBS (15 min each wash), the coverslips were incubated in blocking solution (5% FCS) at room temperature for 1 h and subsequently incubated with primary antibodies (1:50) overnight at 4 °C. Primary antibodies used were rabbit anti-MT1-MMP (H-72, Santa Cruz Biotechnology), mouse anti-integrin β1 (4B7R, Santa Cruz Biotechnology), mouse anti-smooth muscle α-actin conjugated with Cy3 (clone 1A4, Sigma-Aldrich), goat anti-cortactin (G-18, Santa Cruz Biotechnology), and rabbit anti-N-WASP (H-100, Santa Cruz Biotechnology). Afterward, the coverslips were washed three times in PBS and then incubated for 1 h at room temperature with appropriate fluorescent secondary antibodies conjugated to Alexa Fluor® 488 or Alexa Fluor 594 (Molecular Probes/Invitrogen, Eugene, OR). After the secondary antibody incubation period, the coverslips were washed three times with PBS and incubated with 4,6-diamidino-2-phenylindole (DAPI) in PBS (300 ng/ml (Sigma) for 1 h. The cells were washed three times with PBS, mounted on glass slides in Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA), and sealed with nail polish. Negative control immunostaining was performed in blocking solution with omission of each primary antibody and did not yield specific immunostaining (data not shown). Controls consisted of treating cells with 7 mM HEPES, pH 7.4 (EcTI vehicle) or 0.1% DMSO (PP2 vehicle). Coverslips were examined using a Zeiss LSM510 scanning confocal inverted microscope, and images were analyzed using LSM Image Browser 3.2 software (Zeiss, Oberkochen, Germany), and quantification of fluorescence was determined in five random fields using imageJ software.

Protein Preparation and Western Blotting—For the extraction of signaling phosphoproteins (p-Src and p-FAK) a cell suspension was treated with 25 or 100 µM EcTI for 30 min, subsequently seeded onto collagen I-coated culture dishes, and left for 1 h to adhere. Then, the medium was changed to discard any loose cells, and adhered cells were removed from the culture dishes with the aid of a cell scraper in cell lysis buffer (Cell Signaling Technology) containing proteinase inhibitor mixture (Roche Applied Science) and thereafter exposed to 10 freeze/thaw cycles. For the extraction of membrane-bound proteins and invadopodium-related proteins (integrin β1, cortactin, N-WASP, and MT1-MMP), cells in suspension were treated with 25 or 100 µM EcTI for 30 min, subsequently seeded onto collagen I-coated culture dishes, and left to adhere for 6 h. Again, the medium was changed to discard any loose cells, and adhered cells were removed from the culture dishes with the aid of a cell scraper in cell lysis buffer containing proteinase inhibitor mixture and thereafter exposed to 10 freeze/thaw cycles. Controls consisted of treating cells with 7 mM HEPES, pH 7.4 (EcTI vehicle) in culture medium. The total protein content of...
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the cell extracts was measured using the Micro BCA Protein Assay kit (Pierce). The lysate proteins were separated according to molecular mass by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel (Merck) and transferred to polyvinylidene difluoride membranes (Millipore) by 2.5-h electroblotting at 200-mA constant current in blotting buffer (20 mM Tris base, 150 mM glycine, 20% methanol) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were quenched for 1 h with 5% nonfat dry milk in TBST buffer (200 mM Tris/HCl buffer, pH 8.0 containing 150 mM NaCl and 0.05% Tween 20) and then incubated overnight at 4 °C with primary antibodies rabbit anti-phospho-Src (Tyr-416) (Cell Signaling Technology), rabbit anti-Src (Cell Signaling Technology), rabbit anti-phospho-FAK (Tyr-397) (Cell Signaling Technology), rabbit anti-FAK (Cell Signaling Technology), rabbit anti-MT1-MMP (H-72, Santa Cruz Biotechnology), mouse anti-integrin β1 (4B7R, Santa Cruz Biotechnology), goat anti-corticortin (G-18, Santa Cruz Biotechnology), rabbit anti-N-WASP (H-100, Santa Cruz Biotechnology), and anti-β-actin (Sigma) diluted in 1% bovine serum albumin (BSA) in TBST. Thereafter, the membranes were further incubated for 1 h at room temperature with the appropriate secondary antibodies conjugated with horseradish peroxidase (Cell Signaling Technology) diluted in 1% BSA in TBST. After each step, membranes were sequentially washed three times with TBST. Chemiluminescence signal detection was performed using the gel documentation system G:BOX Chemi HR16 (Syngene, Frederick, MD). Densitometric analysis was performed using the Scion Imaging software (Scion Corp.) using β-actin as a control for each sample.

Sample Preparation and Gelatin Zymography—After the incubation of Hs746T cells for 6 h with 25 and 100 μM EcTI or for 24 h with 5 and 10 μM PP2, the culture supernatants were collected by centrifugation. Equal amounts of conditioned medium from each sample were loaded on a 7.5% SDS-polyacrylamide gel co-polymerized with 0.1% gelatin. Electrophoresis was performed under non-reducing conditions at 200 mA for 1 h at room temperature. The gel was washed twice for 30 min each in 2.5% Triton X-100 to remove SDS and incubated in reaction buffer (50 mM Tris/HCl, pH 7.4, 4.5 mM CaCl₂, 2 μM ZnCl₂) for 24 h at 37 °C. The gel was stained with 0.5% Coomasie Brilliant Blue G-250 ( Pierce) for 30 min at room temperature, and excess dye was removed with 3:1:6 ethanol, acetic acid, and water. The presence of MMP-2 was indicated by an unstained proteolytic zone of substrate at 72 kDa (MMP-2 active form).

RNA Extraction and Real Time Reverse Transcription-PCR Analysis—Total RNA was isolated from Hs746T cells after a 6-h treatment with 100 μM EcTI using TRIzol® reagent (Invitrogen). The concentration and purity of the RNA in each sample were determined using a spectrophotometer at 260 and 280 nm. First strand cDNA was reverse transcribed using 1 μg of total RNA and the ImProm-II™ reverse transcription kit (Promega, Madison, WI), according to the manufacturer’s protocol. Quantitative RT-PCR amplification was performed on 2 μl of the cDNA (1:5) with specific primers for MT1-MMP and integrin β1 and the SYBR Green Master Mix kit (Applied Biosystems, Foster City, CA) in a 7500 Real-Time PCR System (Applied Biosystems, Warrington, UK) using an activation cycle of 95 °C for 10 min and 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 30 s. The specificity of the amplified products was analyzed through dissociation curves generated by the equipment yielding single peaks. Negative controls were used in parallel to confirm the absence of any form of contamination in the reaction. Analysis of the data was carried out using the 2−ΔΔCt method (43) using the 7500 Real-Time PCR System software. The primer combinations used were as follows: for MT1-MMP, forward, 5'-TGTAGGATGGATACCCAATGCCCATA-3'; reverse, 3'-TTCTGAACCCACAACTACTCCGCG-5', for integrin β1, forward, 5'-TTGTTGTGTGACGTGTGTTGTTTGTG-3'; reverse, 3'-GGCTTCAAAGTTCCTCGATTCAACA-5'. Expression values were normalized to the housekeeping gene ribosomal protein S29 using the following primer combination: forward, 5'-CCTGGAAGGAGAAAGHAAAGAGAGACAGACACGTTAAGCTTTCAACA-5'; reverse, 3'-TTGAGAACCCTCTGTGTATTTGTCA-5'.

Adhesion Assay—The effect of EcTI upon the adhesion of gastric cancer cells and fibroblasts was tested through an adhesion assay. First, cells were detached from culture dishes with trypsin, and subsequently, 5 × 10⁵ cells were incubated with 5, 10, 25, 50, and 100 μM EcTI for 30 min after which the cells were seeded in a 96-well microplate (Corning Inc.) coated with 10 μg of fibronectin (Sigma), laminin (Sigma), collagen I (purified from rat tail tendon by the method described previously (42)), or collagen IV (Sigma) and left to adhere for 4 h in a humidified atmosphere at 37 °C and 5% CO₂. The cells were then washed three times with sterile PBS to remove any detached cells. The adhered cells were stained with 1% toluidine blue (Sigma) for 5 min, and after that, cells were solubilized with 1% SDS for 30 min at 37 °C. The absorbance was measured at 540 nm. Controls consisted of treating cells with 7 mM HEPES, pH 7.4 (EcTI vehicle) in culture medium. The experiment was performed in triplicate.

Monolayer Wound Healing Assay—Hs746T cells were seeded in a 24-well plate (Corning Inc.). After cell attachment and growth to a confluent monolayer, the medium was replaced with serum-free RPMI 1640 medium, and a scratch wound was made with a sterilized p-1000 pipette tip (Axygen) down the center of each well. The plates were washed to remove any loose cells, 25 or 100 μM EcTI in RPMI 1640 medium with 2% heat-inactivated FCS was added to each well, and the cells were left for 24 h. Controls consisted of treating cells with 7 mM HEPES, pH 7.4 (EcTI vehicle) in culture medium. Thereafter, the cells were observed under a phase-contrast microscope every 8 h, and images were captured with a Sony Cyber-shot camera. The cell migration distance was determined by measuring the width of the wound and subtracting half this value from the initial half-width value of the wound. The average wound width could be obtained from the wound area by dividing the area by the length of the analyzed region. The obtained wound widths were plotted against time in Microsoft Excel software (Redmond, WA), and a linear fit was generated for each data set. The slope of the linear fit was used as a measure of cell migration. Each experiment was performed in triplicate.

Invasion on Matrigel and Collagen Type I Assay—The invasion ability of Hs746T cells was tested using Millicell cell culture inserts of 8-μm pore size (Millipore). Briefly, 60 μl of
Matrigel (BD Biosciences) diluted in serum-free medium (1:12) or 30 μg of collagen type I in cold PBS was added to the upper chambers of Transwell inserts and left for 30 min at 37 °C to enable gel-like formation. A volume of 500 μl containing 2 × 10⁵ cells pretreated with EcTI prepared in serum-free RPMI 1640 medium was added to the upper chamber. Controls consisted of treating cells with 7 mM HEPES, pH 7.4 (EcTI vehicle) in culture medium. The lower chamber of the Transwell unit was filled with 500 μl of RPMI 1640 medium supplemented with 10% heat-inactivated FCS to serve as a chemoattractant for cell migration to the lower chamber. Plates were incubated for 24 h at 37 °C in an atmosphere of 5% CO₂. The cells were then fixed for 30 min with methanol (Merck), and cells in the upper chamber were removed with a cotton swab. Transwell units were removed from 24-well plates and stained with 1% toluidine blue (Sigma) for 15 min. The cells that migrated to the lower chamber were counted under a light microscope by two independent observers.

Invasion through Three-dimensional Fibroblast Network—Hs746T GFP-transfected cells were seeded upon a three-dimensional fibroblast network according to Coulson-Thomas et al. (41) to assay the effect of EcTI on cell invasion. Briefly, Hs746T cells were seeded in 6-well plates (Corning Inc.) and incubated at 37 °C. At 80–90% confluence, the cells were washed with PBS and incubated with serum-free RPMI 1640 medium for 6 h. The cells were then incubated with plasmid pEGFP-N1 (U55762, Clontech) in Opti-MEM® I reduced serum medium (Invitrogen) with Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer’s protocol. After a 20-h transfection period at 37 °C in an atmosphere of 5% CO₂, the cells were washed, the GFP fluorescence was analyzed by fluorescence microscopy, and thereafter, cells were maintained in RPMI 1640 medium supplemented with 2% heat-inactivated FCS at 37 °C in an atmosphere of 5% CO₂.

Confluent fibroblasts seeded on coverslips were treated with fresh 50 μg/ml ascorbic acid every other day for 10 days to produce the three-dimensional fibroblast network. Approximately 5 × 10⁵ GFP-positive Hs746T cells were treated with 100 μM EcTI for 30 min, added onto the three-dimensional fibroblast network, and left to invade for 6 h at 37 °C in an atmosphere of 5% CO₂. Controls consisted of treating cells with 7 mM HEPES, pH 7.4 (EcTI vehicle) in culture medium. The cells were observed under a fluorescence microscope (Axioplan 2, Zeiss) every 2 h to evaluate the efficiency of the invasion. After the 6-h invasion period, the coverslips were immersion-fixed in 4% paraformaldehyde in PBS for 60 min, and immunocytochemistry was performed as described above. Goat anti-GFP (I-16, Santa Cruz Biotechnology) and mouse anti-smooth muscle α-actin conjugated with Cy3 (clone 1A4, Sigma) were used to stain the invading cells and fibroblast three-dimensional network, respectively. The invasion efficiency of the GFP-positive cells through the fibroblast three-dimensional network was determined by capturing z-stack images using a Zeiss LSM510 scanning confocal inverted microscope and analyzed using LSM Image Browser 3.2 software (Zeiss). All experimental points were performed in quadruplicate.

Statistical Analysis—Statistical significance was determined by paired t test in Microsoft Excel. All experiments were performed in triplicate unless stated otherwise. Reproducible results were obtained, and representative data are shown. p values <0.05 were considered significant.

RESULTS

Purification of EcTI—Modifications of the previously described EcTI purification procedures improved the overall yield so that enough material was achieved to study the effects of the Kunitz-type inhibitor EcTI from seeds of E. contorsilquium on human gastric cancer cell lines. The purity of the preparation was assessed by reversed-phase chromatography (supplemental Fig. 1A) and by 8–12% SDS-PAGE (supplemental Fig. 1B). The reversed-phase chromatography yielded one main peak, which was collected and applied to SDS-PAGE. The SDS-PAGE revealed the presence of one band, indicating the occurrence of solely one protein population in the collected peak. The band revealed by SDS-PAGE was 20 kDa, thereby representing the purified EcTI. The conserved activity of the purified inhibitor was confirmed by its ability to block trypsin activity in vitro (results not shown).

Effect of EcTI on Cell Viability and Proliferation—Many reports have described inhibitory actions of plant inhibitors on tumor development (23, 44–50). Therefore, at first, we investigated the activity of EcTI on cell viability of the Hs746T and MKN28 gastric cancer cell lines using the MTT assay based on the ability of the dehydrogenases of viable cells to reduce the MTT reagent to formazan. The effect of EcTI was also assayed on a non-carcinogenic cell line (fibroblasts) to assess the specificity of the EcTI to target cancer cells. EcTI slightly decreased the viability of the gastric cancer cells at the highest concentrations tested (100–150 μM EcTI) after 24 h of treatment; 7 and 6% inhibitions of Hs746T and MKN28 cell viability, respectively, were observed with 150 μM EcTI (Fig. 1, C and E). After 48 h of treatment, there was a decrease in Hs746T cell viability at all EcTI concentrations (5–150 μM) assayed when compared with the control vehicle; 37% inhibition of cell viability was observed with 150 μM EcTI (Fig. 1D), and a 16.5% decrease of cell viability was observed for MKN28 cells (results not shown). The cell viability of the gastric cancer cells was significantly decreased in a time- and dose-dependent manner. EcTI decreased the viability of the fibroblasts solely at the highest concentrations tested (14 and 17% decrease for 150 μM EcTI after 24 and 48 h of treatment, respectively) (Fig. 1, A and B).

Taking into account the subtle effect that EcTI had upon cell viability, we assayed the possible effects these inhibitors could have upon cell proliferation. Cell proliferation was measured using a bromodeoxyuridine-based assay in which BrdU incorporation of cells treated with different concentrations of EcTI was measured. No significant decrease in cell proliferation was observed after cells were exposed to EcTI, indicating that EcTI presents no antiproliferative activity at the concentrations used (supplemental Fig. 1).

Adhesion Molecules Targeted by EcTI—The ability of the Hs746T cells treated with different concentrations of EcTI to adhere to different adhesion molecules (fibronectin, laminin, collagen I, and collagen IV coats) was investigated. EcTI presented an impressive dose-dependent inhibitory effect toward...
gastric cell adhesion on laminin and collagen I at concentrations ranging between 5 and 150 μM (Fig. 2B). An approximate decrease of 40% in adhesion was observed when Hs746T cells treated with 150 μM EcTI were seeded on either laminin or collagen I. A slight dose-dependent decrease in the adhesion of gastric cancer cells was also observed when the cells were seeded on collagen IV. The inhibitory effect of EcTI regarding fibronectin was more subtle (5.7% at 5 μM and 11.5% at 150 μM). Interestingly, fibroblast adhesion to the different coats was not inhibited by EcTI at any of the concentrations evaluated. In fact, at some EcTI concentrations, a slight increase in fibroblast adhesion was observed (Fig. 2A). Therefore, our data suggest that EcTI inhibits gastric cancer adhesion possibly through collagen I- and laminin-mediated pathways.

Effect of EcTI on Gastric Cancer Cell Migration and Invasion—EcTI inhibited gastric cancer cell adhesion, and therefore a possible inhibitory effect on gastric cancer cell migration was investigated. A wound healing assay was performed, revealing a 30% decrease in cell migration when gastric cancer cells were preincubated with 100 μM EcTI (Fig. 3A). Thereafter, the inhibitory activity of EcTI on Hs746T cells invasion was assessed through matrigel and collagen type I matrices (Fig. 3, B and C) as well as through a three-dimensional fibroblast network (Fig. 3D).

A drastic 60% reduction of gastric cell invasion through a collagen I matrix was observed at 25 μM, and 86.5% reduction was observed at 100 μM (Fig. 3C). Therefore, the combined data suggest that EcTI inhibits collagen I-mediated adhesion and invasion.

Thereafter, a fibroblast based three-dimensional matrix was produced in vitro to mimic an in vivo environment. GFP-positive gastric cancer cells treated or not with EcTI were seeded upon this three-dimensional stroma and left for 6 h. EcTI treatment limited the ability of gastric cancer cells to invade and therefore reduced the number of EcTI-treated gastric cancer cells within the fibroblast based three-dimensional matrix (Fig. 3D, panels iii and iv) when compared with vehicle controls (Fig. 3D, panels i and ii). Moreover, GFP-positive cells treated with EcTI presented drastic changes in cell morphology, showing a more rounded shape and reduced projections (Fig. 3D, panel iii compared with panel i).

Immunocytochemistry of Migrating Cells Treated with EcTI—Invadopodia are protrusions of the plasma membrane characterized by the presence of cortactin, N-WASP, and MT1-MMP; they establish focal contact with the substratum and play an intricate role in the focal degradation of the ECM during cell invasion. These specialized cellular protrusions have been described in macrophages and invasive cancer cell lines (4). Our data show that EcTI has a strong inhibitory effect upon collagen I-mediated gastric cancer cell adhesion, migration, and invasion.

Accordingly, changes in gastric cancer cell morphology, including the loss of cellular projections, were observed in gastric cancer cells treated with EcTI while invading three-dimensional stroma (Fig. 3D). Invadopodia preferentially form at the

**FIGURE 1. Effect of EcTI on cell viability.** Fibroblast (A and B), Hs746T (C and D), and MKN28 cells (E and F) were treated with increasing concentrations (0–150 μM) of EcTI for 24 and 48 h. Cell viability was measured using the MTT assay. Controls consisted of treating cells with medium with 7 mM HEPES, pH 7.4 (EcTI vehicle). The percentage of viable cells was calculated as the ratio of treated cells to control cells. Error bars indicate S.D. of triplicate samples. *, p < 0.05 versus control (0 μM EcTI; vehicle only).
cell surface of monolayer cell cultures when grown upon a collagen I coat. Therefore, we studied the effect of EcTI on the formation and morphology of cellular projections, more precisely invadopodia, in Hs746T cells through immunocytochemistry of migrating gastric cancer cells upon a collagen I substratum.

Strong staining for integrin α1 was observed at the cell membrane in control gastric cancer cells and co-localized with α-actin, which is characteristic of invadopodia (Fig. 4A). On the other hand, intense integrin α1 staining at the cell membrane was not observed when the gastric cancer cells were treated with EcTI as shown by densitometric analysis (Fig. 4D). Moreover, MT1-MMP was also concentrated at specific sites of the cell membrane and co-localized with an integrin α1-rich area in control gastric cancer cells. There was an overall drastic decrease in MT1-MMP immunolocalization that disappeared at the cell membrane of gastric cancer cells treated with EcTI (a decrease of fluorescence of 76% compared with control) (Fig. 4A). Cortactin is a protein involved in α-actin polymerization and thus is vital for invadopodium formation. Control gastric cancer cells presented intense organized cortactin immunostaining throughout the cytoplasm; however, in gastric cancer cells treated with EcTI, cortactin immunostaining was present in 22% of the cells and was restricted to the perinuclear area (Fig. 4B). N-WASP is a substrate for cortactin, essential for α-actin polymerization, and pivotal for invadopodium formation. The control cells presented N-WASP in the cytoplasm of...
Expression of Integrin β1, Cortactin, N-WASP, and MT1-MMP in Gastric Cancer Cells Treated with EcTI—Through Western blotting, a 61% decrease of integrin β1 in gastric cancer cells treated with 100 μM EcTI and subsequently seeded on collagen I was observed, therefore corroborating the immunocytochemistry (Fig. 5B). When treated gastric cancer cells were subsequently seeded on uncoated culture dishes, a more subtle inhibitory effect, a 35% decrease in integrin β1 when treated with 100 μM EcTI compared with untreated controls, was observed. A decrease in the expression of cortactin (50% at 100 μM) in gastric cancer cells treated with EcTI was also observed. The decrease in cortactin expression was similar in gastric cancer cells treated with EcTI seeded on collagen coats or uncoated culture dishes. An impressive decrease of N-WASP was also observed for gastric cancer cells treated with EcTI when compared with controls; a 41% decrease of N-WASP was detected in cells treated with 100 μM EcTI and seeded on collagen I, whereas only a 15% decrease was detected when cells were seeded on uncoated culture dishes. Dose-dependent inhibition of MT1-MMP was observed in EcTI-treated gastric cancer cells seeded on collagen I (a 40% decrease when treated with 25 μM and a 73% decrease with 100 μM); however, only a 2% decrease in the expression of this enzyme was observed when EcTI-treated gastric cancer cells were seeded on uncoated culture dishes.

To further elucidate the effect of EcTI on integrin β1 and MT1-MMP, the gene expression levels were analyzed using quantitative RT-PCR. A decrease in the expression of both integrin β1 and MT1-MMP (23% and 63.9%, respectively) was observed when cells were treated with EcTI (Fig. 5, E and F). Therefore, EcTI affects the cellular disposition of integrin β1 and MT1-MMP as well as the expression of these components.

Activity of MMP-2 in Gastric Cancer Cells Treated with EcTI—MMP-2 is also secreted at the tip of the invadopodia and is up-regulated in a myriad of cancer types. Therefore, zymography was performed to determine whether EcTI also affects the activity of MMP-2. Indeed, gelatinolytic activity of secreted MMP-2 from Hs746T cells seeded on collagen I coats was affected by EcTI (Fig. 5C). A 40% decrease in MMP-2 activity was observed in gastric cancer cells treated with 100 μM EcTI compared with the control.

Src-FAK Signaling Mediates Inhibitory Events Evoked by EcTI—The increase in phosphorylation of Src has been correlated to the metastatic potential of different cancers. Src family kinase and FAK activity is required for cell adhesion, spread, and migration (14–17). Given the potent effect of EcTI upon cell adhesion, migration, and invasion, we examined SrcTyr-416 and FAKTyr-397 pathways to elucidate their participation in these events. The signaling pathways were analyzed primarily through Western blotting of cell extracts. Quantitative densitometry enabled the quantification of the levels of SrcTyr-416, FAKTyr-397, and their phosphorylated forms, thereby determining the level of activation of the signaling pathways (Fig. 6B). Treatment of gastric cancer cells with EcTI led to an inhibition of SrcTyr-416 and FAKTyr-397 activity in a dose-dependent manner. Moreover, the decrease of both SrcTyr-416 and FAKTyr-397 activity was enhanced when the treated cells were seeded on collagen I. Gastric cancer cells treated with 25 μM EcTI showed a 2 and
20% decrease of SrcTyr-416 activation when treated cells were seeded on uncoated and collagen I-coated culture dishes, respectively, and treatment with 100 μM resulted in a 12 and 67% decrease in SrcTyr-416 activation, respectively. Gastric cancer cells treated with 25 μM EcTI showed a 26 and 60% decrease of FAKTyr-397 activation when treated cells were seeded on uncoated and collagen I-coated culture dishes, respectively, and treatment with 100 μM resulted in a 36 and 80% decrease in FAKTyr-397 activation, respectively.

Effect of Src Tyrosine Kinase Inhibitor on Gastric Cancer Cells—EcTI clearly led to the disruption of invadopodium formation, a decrease in migration and invasion, and a decrease in Src and FAK activation. To determine whether the decrease in Src and FAK activation mediates the inhibition of invadopodium formation, we analyzed the effect of PP2, a Src tyrosine kinase inhibitor, on invadopodium formation in Hs746T cells. When Hs746T cells seeded on collagen I coats were treated with 10 μM PP2 for 24 h, cortactin immunostaining was decreased and restricted to the perinuclear area (Fig. 6C). These results reproduced those observed when Hs746T cells seeded on collagen I coats were treated with 10 μM PP2 (Fig. 6, D and E). Therefore, by specifically targeting the Src signaling pathway in the gastric cancer cells with a Src tyrosine kinase inhibitor, we were able to reproduce the same downstream events observed with EcTI, suggesting that EcTI inhibits invadopodium formation through the Src-FAK signaling pathway.

The effect of the Src tyrosine kinase inhibitor on the cell viability and proliferation of Hs746T and MKN28 gastric cancer cells was also studied. After 24 h of treatment with 40 μM PP2, only a 15 and 12% inhibition of cell viability was observed with Hs746T and MKN28 cells, respectively (supplemental Fig. 3, A and E). After 48 h, a 29 and 40% decrease in cell viability was observed for Hs746T and MKN28 cells, respectively (supplemental Fig. 3F). No significant decrease in gastric cancer cell proliferation was observed when cells were exposed to PP2 at the concentrations utilized in these experiments (supplemental Figs. 1 and 3). The effects of the Src tyrosine kinase inhibitor PP2 on gastric cancer cell viability and proliferation are very similar to those observed for EcTI.

DISCUSSION
The use of proteinase inhibitors of plant origin has traditionally been limited to the action of these inhibitors on proteolytic enzymes in vitro. However, inhibitors of the Kunitz family have been shown to present further activities, such as affecting signaling
Several studies have focused on the effect of these macromolecules on tumor cells, exploring the possibility of proteinase inhibitors as possible therapeutic targets (23, 44–50). In the present study, we investigated the effects of EcTI, a Kunitz-type inhibitor purified from seeds of *E. contortisiliquum*, on gastric cancer cells.

EcTI presented no effect upon the proliferation of both gastric cancer cell and fibroblasts, and a subtle decrease of cell viability was observed. However, EcTI-treated gastric cancer cells presented altered morphology and compromised adherence to ECM-coated culture dishes. Thereafter, the effect of EcTI on gastric cancer cell adherence, migration, and invasion was investigated. EcTI presented an impressive limiting effect upon gastric cancer cell adhesion and invasion, and these events were not observed in fibroblasts. Interestingly, the restricting events on cell invasion were more prominent when gastric cancer cells were seeded upon collagen I coats, providing evidence that the collagen I adhesion pathways play a pivotal role in the events affected by EcTI.

Integrins on the cell surface classically bind to collagen I in the ECM to form adhesion complexes. These focal adhesion complexes mediate cell adhesion and spread (16, 55–57). During cell spread, a complex array of proteins is recruited at the cell membrane, such as proteins involved in the assembly of the actin cytoskeleton. Integrins cluster at the cell membrane after contact with specific ECM ligands and recruit through their intracellular domains either adapter proteins or signal proteins, resulting in phosphorylation and dephosphorylation signals within the cell. EcTI strongly inhibited the adhesion of gastric cancer cells on collagen I and laminin coats in a dose-dependent manner, whereas a more subtle inhibitory effect was observed with fibronectin and collagen IV. Thus, the EcTI inhibitory effect on gastric cancer cell adhesion is dependent on the ECM to which these cells adhere. Events downstream of these focal adhesion complexes were then studied. Gastric cancer cells treated with EcTI presented a decrease in integrin β1 expression, and such effect would compromise the adhesion of these cells to collagen I. Antibody inhibition of integrin β1 blocks prostate cancer tumor cell binding to bone stroma, indicating necessary roles for integrin β1 in prostate tumor cells binding to bone stroma (58). A β1 family member, α2β1, which is a collagen type I receptor, is expressed by tumor cells, and its activity promotes invasion and adherence to the bone stroma (59).

**FIGURE 6.** Effect of EcTI on Src-FAK signaling and effect of PP2 on invadopodia. A, Hs746T cells pretreated with EcTI for 30 min were plated on a tissue culture plastic (No coat) or on collagen I for 1 h, and lysate proteins were separated by 10% SDS-PAGE and electrotransferred to PVDF membrane. Membranes were blocked and incubated with anti-phospho-Src, anti-phospho-FAK, anti-Src, anti-FAK, and anti-β-actin (loading control) antibodies. Antibody binding was visualized by chemiluminescence, and the relative phosphorylation levels of these proteins were determined by densitometric analysis (B). C, immunolocalization of cortactin after treatment of Hs746T cells with 10 μM PP2. Cells were seeded on collagen I and allowed to migrate for 18 h. The immunofluorescence images of cells migrating were captured using confocal microscopy. Controls consisted of treating cells with medium with 0.1% DMSO (PP2 vehicle). Cortactin staining is red. Nuclei (blue) were stained with DAPI. Co-localization of images is also shown (Merge). The scale bar represents 20 μm. D, the relative fluorescence levels of cortactin were determined by densitometric analysis. Error bars indicate S.D. of five fields. *, p < 0.05 versus control (PP2 vehicle). E, cells plated on collagen I were treated with 10 μM PP2 for 18 h, and the activity of MMP-2 in the supernatant was revealed by gelatin zymography. F, densitometric quantitation analysis of the active MMP-2 band. The percentage was calculated as the ratio of treated cells to control cells. col I, collagen I.

pathways and influencing biological processes (51–54). Several studies have focused on the effect of these macromolecules on tumor cells, exploring the possibility of proteinase inhibitors as possible therapeutic targets (23, 44–50). In the present study, we investigated the effects of EcTI, a Kunitz-type inhibitor purified from seeds of *E. contortisiliquum*, on gastric cancer cells.

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 Src-FAK Signaling Inhibition by Plant Proteinase Inhibitor

The ECM-integrin binding evokes molecular signaling cascades. FAK and Src signaling pathways are important signaling pathways involved in the cross-talk between cells and their surrounding ECM, and more importantly they mediate collagen I/integrin signaling, referred to as the integrin β1-FAK-Src signaling pathways (60). Moreover, the catalytic activity of FAK and Src plays a pivotal role in recruiting the machinery necessary for cell migration (16). FAK is known to bind integrin β1, which consequently leads to FAK activation and autophosphorylation. This phosphorylated tyrosine provides a docking site for Src. The tyrosine kinase Src then phosphorylates additional sites on FAK, which leads to further increased activity of FAK, formation of an activated FAK-Src complex, and the recruitment of proteins that contain Src homology 2 domains (55, 61, 62). A significant decrease in Src and FAK phosphoproteins was observed in gastric cancer cells treated with EcTI. Therefore, the reduced expression of integrin β1 led to a reduced number of adhesion complexes and thereby reduced the overall FAK and Src activation. FAK and Src activation at focal adhesion sites enhances cytoskeletal reorganization, cellular adhesion, migration, and invasion. In addition to a decrease in expression, changes in the distribution and arrangement of integrin β1 at the cell surface were also observed in gastric cancer cells treated with EcTI.

Several studies have indicated that FAK may have a direct role in cell survival (63–66); however, there are also published data showing that inhibition of the activation of FAK does not affect cell proliferation (16, 67–69). EcTI did not alter cell cycle progression (data not shown). PP2 does not affect the cell cycle progression of pancreatic endocrine tumors cells; however, it does inhibit the adhesion, spreading, and migration of these cells (16). Morelloflavone blocks the migration of vascular smooth muscle cells and inhibits the phosphorylation of FAK and Src without causing apoptosis or cell cycle arrest (70). Moreover, dasatinib had no effect on the cell cycle of A549 cells when several head and neck squamous cell carcinomas were analyzed (71).

Recently, invadopodia, cellular protrusions of the cell membrane specialized in focal adhesion, degradation, and subsequent invasion, have been described. These structures are rich in integrin and present actin-rich structures on the ventral cell surface. Through immunocytochemistry we were able to demonstrate that EcTI prevented the formation of invadopodia in the gastric cancer cells. Phosphorylated Src activates cortactin, which subsequently binds to N-WASP, F-actin, and Arp2/3, inhibiting actin depolymerization and more importantly enabling actin assembly, which is the first step in invadopodium formation (72). Therefore, the decrease in integrin β1 expression and subsequent decrease in Src and FAK activation in gastric cancer cells treated with EcTI could lead to the absence of invadopodia in these cells. The increased expression or activity of FAK and/or Src in tumors is associated with a more invasive and aggressive phenotype and has led to the development of Src and FAK inhibitors as new anticancer drugs (55, 62, 73). To clarify whether the Src-FAK signaling pathway could potentially mediate the events observed, gastric cancer cells were treated with a Src tyrosine kinase inhibitor (PP2). Interestingly, gastric cancer cells treated with this inhibitor presented the same changes in the arrangement of cortactin as observed with EcTI. Therefore, inhibition of the Src-FAK signaling pathway in gastric cancer cells disrupted invadopodium formation. Our findings suggest that EcTI inhibits invadopodium formation by interfering with the integrin-mediated Src-FAK signaling pathway. The Src signaling pathway mediates invadopodium formation by decreasing F-actin and cortactin expression in breast cancer cells, which culminates in blocking focal degradation of ECM (3). Moreover, Lauzier et al. (74) have demonstrated that inhibition of invadopodium formation by PP2 in arthritic synovial cells leads to a direct effect on extracellular matrix degradation in vitro and in vivo, supporting the notion that invadopodia are a relevant therapeutic target. Therefore, EcTI prevents the activation of Src and subsequently triggers signaling pathways that ultimately disrupt invadopodium formation. Invadopodia are primarily responsible for focal degradation, which precedes the invasion of cells through the surrounding stroma. Therefore, this specialized structure concentrates both MT-MMPs and soluble MMPs at the tips of the invadopodia. The physiological function of invadopodia involves a four-step process: localization of cortactin on the site of the protrusion formation, recruitment of MT1-MMP, degradation of the matrix, and dissociation of the cortactin from the cell membrane (18). Cortactin is indispensable for invadopodium formation, and MT1-MMP is fundamental for focal degradation (18, 72). Cortactin is an essential scaffold that regulates the activities of N-WASP, which binds to the Arp2/3 complex and initiates actin branching at the cell membrane and hence the formation of invadopodia (18, 75, 76). MT1-MMP drives three-dimensional ECM invasion through the cleavage of cell surface molecules, such as E-cadherin, integrin, CD44, syndecan-1, collagen type I, and laminin (77) as well as degradation of ECM components, such as fibronectin, collagen type I, collagen type IV, and laminin (75). EcTI-treated gastric cancer cells presented unorganized cortactin, N-WASP, and MT1-MMP cell distribution. Control gastric cancer cells presented a traditional invadopodium distribution of cortactin, N-WASP, and MT1-MMP where these components concentrate at specific sites of the cell membrane. After EcTI treatment, these cells presented an overall decrease in the expression of these components, and these components attained a pericellular distribution. MT1-MMP is an important enzyme involved in degradation of the pericellular ECM, and it acts in the activation of MMP-2, another important enzyme participant in this process (25). The decrease of MT1-MMP expression indicates a decrease in functional activity of invadopodia that will result in inhibition of cell invasion. EcTI drastically disrupted the cellular arrangement of MT1-MMP and decreased MT1-MMP expression and MMP-2 activity. Moreover, directly inhibiting the Src-FAK signaling pathway also led to a decrease in MMP-2 activity. This finding corroborates the notion that the decrease in MMP activity at the cell surface is an event downstream of EcTI affecting invadopodium formation and not due to a direct inhibitory activity of EcTI. Various researchers dedicated to the study of inhibiting MT1-MMP in tumor cells have shown that imatinib inhibits the expression of MT1-MMP and pro-MMP-2 activation of colorectal cancer cells (78), all-trans-retinoic acid inhibits MT1-MMP of human breast cancer cells (79), and capsaicin suppresses EGF-induced MT1-MMP of fibrosarcoma cells (80). Likewise, EcTI effectively inhibited the formation of inva-
dopodia through integrin-mediated activation of Src-FAK and therefore decreased the proteolytic activity and invasive potential of the gastric cancer cells (Fig. 7).

Modern therapeutic strategies for cancer chemotherapy involve the administration of drug mixtures, enabling the administration of lower doses of each drug than if applied individually. Moreover, a new strategy is being developed that involves personalized chemotherapy, which has recently grown in popularity. Personalized chemotherapy involves the administration of chemotherapy based on the characteristics of the individual tumor. Novel antitumor agents that have the ability to inhibit the invasion of certain aggressive metastatic tumors are interesting candidates for personalized treatment if the cancer cells prove to be responsive. Our study, although still at an experimental level, provides a possible candidate for combined therapy against aggressive tumors and an interesting approach for researching new potential drugs.

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FIGURE 7. Schematic mechanism of action of EcTI on invadopodium formation. A, the integrin activation leads to invadopodium formation through FAK<sup>Tyr-397</sup> and Src<sup>Tyr-416</sup> signaling pathways. B, the inhibition of activated integrin β1 expression by EcTI leads to a decrease of FAK<sup>Tyr-397</sup> activation followed by a decrease of Src<sup>Tyr-416</sup> activation, preventing the recruitment of proteins important for the formation of invadopodia, such as cortactin and N-WASP. Because the formation of invadopodia is impaired, the proteolytic activity of the cell is decreased, which is reflected in the inhibition of ECM degradation.

REFERENCES
1. Kessenbrock, K., Plaks, V., and Werb, Z. (2010) Cell 141, 52–67
2. Albrechtsen, R., Stautz, D., Sanjay, A., Kveiborg, M., and Wewer, U. M. (2011) Exp. Cell Res. 317, 195–209
3. Balzer, E. M., Whipple, R. A., Thompson, K., Boggs, A. E., Slovic, J., Cho, E. H., Matrone, M. A., Yoneda, T., Mueller, S. C., and Martin, S. S. (2010) Oncogene 29, 6402–6408
4. Blouw, B., Seals, D. F., Pass, L., Diaz, B., and Courtneidge, S. A. (2008) Eur. J. Cell Biol. 87, 555–567
5. Yamamoto, H., Sutoh, M., Hatakeyama, S., Hashimoto, Y., Yonezama, T., Koie, T., Saitoh, H., Yamaya, K., Fuyu, T., Nakamura, T., Ohyama, C., and Tsuiboi, S. (2011) J. Urol. 185, 1930–1938
6. Albigez-Rizo, C., Destaing, O., Fournade, B., Planus, E., and Block, M. R. (2009) J. Cell Sci. 122, 3037–3049
7. Desmarais, V., Yamaguchi, H., Oser, M., Soon, L., Bouneimine, G., Sarmiento, C., Eddy, R., and Condeelis, J. (2009) Cell Motil. Cytoskeleton 66, 303–316
8. Smith-Pearson, P. S., Greuber, E. K., Yogalingam, G., and Pendergast, A. M. (2010) J. Biol. Chem. 285, 40201–40211
9. Misra, A., Lim, R. P., Wu, Z., and Thanabalu, T. (2007) Biochem. Biophys. Res. Commun. 364, 908–912
10. Schneider, J. G., Amend, S. R., and Weillbaecher, K. N. (2011) Bone 48, 54–65
11. Xu, Y., Bismar, T. A., Su, J., Xu, B., Kristiansen, G., Varga, Z., Teng, L., Inger, D. E., Mammo, A., Kumar, R., and Alaou, Jamili, M. A. (2010) J. Exp. Med. 207, 2421–2437
12. Legate, K. R., and Fässler, R. (2009) J. Cell Sci. 122, 187–198
13. Anthis, N. J., and Campbell, I. D. (2011) Trends Biochem. Sci. 36, 191–198
14. Sanchez, A. M., Flamini, M. I., Baldacci, C., Goglia, L., Genazzani, A. R., and Simoncini, T. (2010) Mol. Endocrinol. 24, 2114–2125
15. Lee, S. H., Lee, Y. J., Park, S. W., Kim, H. S., and Han, H. J. (2011) J. Cell. Physiol. 226, 1850–1859
16. Di Florio, A., Capurso, G., Milione, M., Panzuto, F., Geremia, R., Delle Fave, G., and Sette, C. (2007) Endocr. Relat. Cancer 14, 111–124
17. Caccia, D., Miccichè, F., Cassinelli, G., Mondellini, P., Casalin, P., and Bongarzone, I. (2010) Mol. Cancer 9, 278
18. Artyom, V. V., Zhang, Y., Seifler-Moiseiwitsch, F., Yamada, K. M., and Mueller, S. C. (2006) Cancer Res. 66, 3034–3043
19. Zhang, Y., Yuan, Z., Zhang, Y., Song, S., Salas-Burgos, A., Koomen, J., Oliashaw, N., Parsons, I. J., Ysim, Y., Jent, S. R., Yao, T. P., Lane, W. S., and Seto, E. (2007) Mol. Cell 27, 197–213
20. Poincloux, R., Lizzárraga, F., and Chavrier, P. (2009) J. Cell Sci. 122, 3015–3024
21. Steffen, A., Le Des, G., Poincloux, R., Recchi, C., Nassy, P., Rottner, K., Galli, T., and Chavrier, P. (2008) Curr. Biol. 18, 926–931
22. Nagase, H., Visse, R., and Murphy, G. (2006) Cardiovasc. Res. 69, 562–573
23. Nakahata, A. M., Mayer, B., Ries, C., de Paula, C. A., Karow, M., Neth, P., Sampao, M. U., Jochem, M., and Oliava, M. L. (2011) Biol. Chem. 392, 327–336
24. Seiki, M., Koshikawa, N., and Yano, I. (2003) Cancer Metastasis Rev. 22, 129–143
25. Takino, T., Tsumiho, H., Ozawa, T., and Sato, H. (2010) Biochem. Biophys. Res. Commun. 396, 1042–1047
26. Miyata, S., Fukushima, T., Kohama, K., Tanaka, H., Takeshita, H., and Kataoka, H. (2007) Hum. Cell 20, 100–106
27. Armstrong, A. J., Netto, G. J., Rudek, S. A., Hashi, S., Wood, D. P., Creel, P. A., Mundy, K., Davis, S. L., Wang, T., Albadine, R., Schultz, L., Partin, A. W., Jimeno, A., Fedor, H., Febbo, P. G., George, D. J., Gurganus, R., De Marzo, A. M., and Carducci, M. A. (2010) Clin. Cancer Res. 16, 3057–3066
28. Bergeron, S., Lempieux, E., Durand, V., Cagnol, S., Carrier, J. C., Lussier, J. G., Boucher, M. J., and Rivard, N. (2010) Mol. Cancer 9, 271
29. Donia, M., Maksimovic-Ivanic, D., Mijatovic, S., Miljkovic, D., Timotijevic, G., Bagone, P., Caponnetto, S., Al-Bed, Y., McCubrey, J., Stosic-Grujicic, S., and Nicoletti, F. (2011) Cell Cycle 10, 492–499
30. See, A. P., Zeng, J., Tran, P. T., and Lim, M. (2011) Radiat. Oncol. 6, 25
31. Abbenante, G., and Fairlie, D. P. (2005) Med. Chem. 1, 71–104
32. Korting, H. C., Schaller, M., Eder, G., Hamm, G., Böhmer, U., and Hube, B.
Src-FAK Signaling Inhibition by Plant Proteinase Inhibitor

(1999) Antimicrob. Agents Chemother. 43, 2038–2042

33. Ateeq, B., Tomlins, S. A., Laxman, B., Asangani, I. A., Cao, Q., Cao, X., Li, Y., Wang, X., Feng, F. Y., Pienta, K. J., Varambally, S., and Chinnaiyan, A. M. (2011) Sci. Transl. Med. 3, 72ra17

34. Donkor, I. O. (2011) Expert Opin. Ther. Pat. 21, 601–636

45. Chuu, C. P. (2011)

47. Pick, A., Müller, H., Mayer, R., Haenisch, B., Pajeva, I. K., Weigt, M., Sumikawa, J. T., Brito, M. V., Macedo, M. L., Uchoa, A., Miranda, A., Macedo, M. L., de S., Wilken, R., Veena, M. S., Wang, M. B., and Srivatsan, E. S. (2011)

48. Shin, J. S., Hong, S. W., Lee, J. G., Lee, Y. M., Kim, D. W., Kim, J. E., Jung, ós, V., Gasent, J. M., L, and Grande, E. (2010) Onco Targets Ther. 3, 83–97

56. Cavallaro, U., and Christofori, G. (2004) Nat. Rev. Cancer 4, 118–132

57. Malliri, A., and Collard, J. G. (2003) Curr. Opin. Cell Biol. 15, 583–589

58. Van der Velde-Zimmermann, D., Verdaasdonk, M. A., Rademakers, L. H., De Weger, R. A., Van den Tweel, J. G., and Joling, P. (1997) Exp. Cell Res. 230, 111–120

59. Lang, S. H., Clarke, N. W., George, N. J., and Testa, N. G. (1997) Clin. Exp. Metastasis 15, 218–227

60. Bouchard, V., Harnois, C., Demers, M. J., Thibodeau, S., Laquerre, V., Gauthier, R., Vézina, A., Noël, D., Fujita, N., Tsuruo, T., Arqiuin, M., and Vachon, P. H. (2008) Apoptosis 13, 531–542

61. Gjorevski, N., and Nelson, C. M. (2009) Cytokine Growth Factor Rev. 20, 459–465

62. Halder, J., Lin, Y. G., Merritt, W. M., Spannuth, W. A., Nick, A. M., Honda, T., Kama, A. A., Han, L. Y., Kim, T. J., Lu, C., Tari, A. M., Bornmann, W., Fernandez, A., Lopez-Berestein, G., and Sood, A. K. (2007) Cancer Res. 67, 10976–10983

63. Aguirre Ghiso, J. A. (2002) Oncogene 21, 2513–2524

64. Hecker, T. P., Grammer, J. R., Gillespie, G. Y., Stewart, J., Jr., and Gladson, C. L. (2002) Cancer Res. 62, 2699–2707

65. Lu, H., Murtagh, J., and Schwartz, E. L. (2006) Mol. Pharmacol. 69, 1207–1215

66. Tanjoni, I., Walsh, C., Uryu, S., Tomar, A., Nam, J. O., Mielgo, A., Lim, S. T., Liang, C., Koenig, M., Sun, C., Patel, N., Kwock, C., McMahon, G., Stupack, D. G., and Slapapfer, D. D. (2010) Cancer Biol. Ther. 9, 764–777

67. Canel, M., Secades, P., Garzón-Arango, M., Allonca, E., Suarez, C., Serrels, A., Frame, M., Brunton, V., and Chiara, M. D. (2008) Br. J. Cancer 98, 1274–1284

68. Kwiatkowska, A., Kijewska, M., Lipko, M., Hibern, U., and Kobams, B. (2011) Biochm. Biophys. Acta 1813, 655–667

69. Patsenker, E., Popov, Y., Wieser, M., Goodman, S. L., and Schuppan, D. (2007) J. Hepatol. 46, 878–887

70. Pinkaw, D., Cho, S. G., Hui, D. Y., Wiktorowicz, J., E., Hutadiloko-Towata, N., Mahabuburamin, W., Tonganunt, M., Mastro, N. J., Phongdara, A., Liu, M., and Fujise, K. (2009) Biochim. Biophys. Acta 1790, 31–39

71. Johnson, F. M., Saial, B., Talpaz, M., and Donato, N. J. (2005) Clin. Cancer Res. 11, 6924–6932

72. Bozzuto, G., Ruggieri, P., and Molinar, A. (2010) Ann. Int. Super. Sanita 46, 66–80

73. McLean, G. W., Carragher, N. O., Avizinjeyete, E., Evans, J., Brunton, V. G., and Frame, M. C. (2005) Nat. Rev. Cancer 5, 505–515

74. Lauzier, A., Barbonneau, M., Harper, K., Jilaveneal-Pelms, M., and Dubois, C. M. (2011) Arthritis Rheum. 63, 1591–1602

75. Artyym, V. V., Matsumoto, M., Mueller, S. C., and Yamada, K. M. (2011) Eur. J. Cell Biol. 90, 172–180

76. Oser, M., Yamaguchi, H., Mader, C. C., Bravo-Corder, J. J., Arias, M., Chen, X., Desmarais, V., Van Rheeen, J., Kolesar, A. J., and Condeelis, J. (2009) Cell Biol. 186, 571–587

77. Rowe, R. G., and Weiss, S. J. (2009) Annu. Rev. Cell Dev. Biol. 25, 567–595

78. Stathai, X. N., Roussis, A. E., Kanakis, I., Tzanakakis, G. N., Chalkiadaki, G. M., Mavrodil, D., Kletas, D., and Karanam, N. K. (2007) Int. J. Cancer 121, 2808–2814

79. Dutta, A., Sen, T., Banerji, A., Das, S., and Chatterjee, A. (2009) J. Oncol. 2009, 627840

80. Hwang, Y. S., Park, K. K., and Chung, W. Y. (2011) Arch. Oral Biol., in press