

Recombinant anthrax lethal toxin inhibits cell motility and invasion in breast cancer cells through the dysregulation of Rho GTPases

DANA EL-CHAMI, MARIA AL HADDAD, RALPH ABI-HABIB and MIRVAT EL-SIBAI

Department of Natural Sciences, School of Arts and Sciences, Lebanese American University, Beirut 1102 2801, Lebanon

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Abstract. Breast cancer is the leading cause of cancer-associated death among women worldwide. Targeting breast cancer cell metastasis is an important therapeutic approach. The MAPK pathway is a key cell signaling pathway that plays a pivotal role in cellular invasion and migration. Numerous studies have identified the MAPK pathway as a way to target cell survival and motility. The present study treated MBA-MD-231 breast cancer cells with anthrax lethal toxin (LeTx), a potent MAPK inhibitor that selectively cleaves and inactivates all MEKs, as a potential therapeutic method to inhibit breast cancer cell migration. LeTx has been demonstrated to affect breast cancer cell migration. Cells treated with LeTx showed a significant decrease in motility, as observed using wound healing and random 2D motility assays. Additionally, cells treated with LeTx showed an increase in adhesion, which would explain the decrease in migration. Pull-down assays examining the activation status of the members of the Rho family of GTPases revealed an increase in RhoA activation accompanied by a decrease in Cdc42 activation following LeTx treatment. Finally, LeTx mediated a decrease in invasion using a Boyden chamber assay, which could be a result of the decrease in Cdc42 activation. The present study reported the effect of LeTx treatment on the migration, adhesion and invasion of breast cancer cells, demonstrating that this effect was associated with the dysregulation of the Rho GTPases, RhoA and Cdc42.

Introduction

Breast cancer is the most common cancer diagnosed among women in the US and the second leading cause of cancer death among women around the world (1). Breast tumors are heterogenous, exhibiting notable phenotypic diversity.

Through molecular analysis and gene profiling, breast tumors are subclassified into three types: HER2 Positive, estrogen receptor (ER) positive and basal-like breast cancer (2).

HER2, encoded by the ERBB2 gene, is a member of the human epidermal growth factor receptor family of proteins (3). HER2, a receptor tyrosine kinase, regulates internal cell activities such as cell proliferation and survival (4). When HER2 is overexpressed, it leads to the overactivation of cellular pathways such as the PI3K and the MAPK pathways, which are known to be involved in tumorigenesis (5). As for the estrogen receptor, once activated, it functions as a transcription factor leading to the transcription of a number of genes involved in cell proliferation and survival, including c-fos, insulin-like growth factor binding protein 4 and E2F1 (6). Lastly, progesterone increases breast cell proliferation through the activation of the DNA replication machinery (7). All of these characteristics have led to the high occurrence of aggressive, invasive and metastatic profiles of breast cancer, with limited targeted therapeutic options (8).

Cellular motility is a structured process involved in inflammation, embryogenesis, migration and invasion of cells (9). Being a vital process, cell motility is tightly regulated by several proteins, including the Rho family of small GTPases. These consist of 22 members grouped into subfamilies according to their sequence homology (10,11). Rho GTPases are molecular switches that play an important role in regulating the dynamics of the actin cytoskeleton, impacting cellular polarity, adhesion and invasion (12,13).

Rho GTPases switch between an active GTP-bound form and an inactive GDP-bound form. This is regulated by upstream effectors such as guanine exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs are nucleotide exchange factors that catalyze the dissociation of GDP and its exchange to GTP, leading to the activation of the Rho GTPase. GAPs are GTPase-activating proteins, which activate the intrinsic GTPase activity of the Rho GTPase, leading to the inactivation of the protein (14,15).

The MAPK family of proteins includes three kinase types: Extracellular regulated kinases (ERKs), the stress-activated protein kinases p38 and JNKs (16). The Raf/MEK/ERK pathway is a signal transduction pathway that relays signals from cell surface receptors to transcription factors, therefore regulating gene expression. After activation, the small GTPase Ras recruits and phosphorylates Raf (MAP3K) (17).

Correspondence to: Dr Mirvat El-Sibai, Department of Natural Sciences, School of Arts and Sciences, Lebanese American University, P.O. Box 13-5053, Chouran, Beirut 1102 2801, Lebanon
E-mail: mirvat.elsibai@lau.edu.lb

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Successively, Raf phosphorylates a second kinase, MEK (MAP2K), which then phosphorylates two proteins, ERK1 and ERK2; after phosphorylation, ERK1/2 are translocated to the nucleus, where they phosphorylate different transcription factors, altering gene expression (17). The translocation of ERK into the nucleus affects numerous cell processes, such as proliferation, cell cycle progression, adhesion, invasion, survival, metabolism and differentiation (17). The most notable targets of ERK1/2 are c-Myc, c-Fos, Elk1 and c-Jun (16,17).

Anthrax lethal toxin (LeTx) is a binary toxin produced by the Gram-positive bacteria, *Bacillus anthracis* (18). *B. anthracis* contains two virulence encoding plasmids: pXO1 and pXO2. pXO1 encodes for three factors: Protective agent (PA), lethal factor (LF) and edema factor. Although separately non-toxic, a combination of PA and LF generates LeTx (19). PA (83 kDa) binds to the host cell surface receptors, tumor endothelial marker 8 (TEM8) or capillary morphogenesis gene 2 (CMG2), with TEM8 demonstrating increased expression in breast tumor tissue (18,19). Upon binding to TEM8 or CMG2, PA is cleaved by furin-like proteases, releasing a 20 kDa amino-terminal fragment and yielding a 63-kDa active PA fragment (PA63) (18,19). PA63 then oligomerizes, forming a ring-shaped, pre-pore heptameric or octameric structure (18). The formation of the PA63 pre-pore complex allows the binding of three to four LF molecules, depending on whether the PA63 pre-pore complex is in the heptameric or octameric form, respectively (18,19). Binding of LF initiates internalization of the complex into the cell through receptor-mediated endocytosis; upon acidification of the endosome, PA63 undergoes a conformational change into a mature pore complex that subsequently translocates LF into the cytosol (18-20). LF is a matrix metalloproteinase that cleaves and inactivates all MEKs, thus inhibiting all three branches of the MAPK pathway (20).

Previous studies have investigated LeTx as a potential therapeutic target and abundant literature exists describing the selective antitumor potential of LeTx in a number of tumor types, including melanoma and acute myeloid leukemia, both *in vitro* and *in vivo* (21-26). The tumor selectivity of LeTx derives from the fact that the majority of normal cells, with the exception of endothelial cells and macrophages, are not sensitive to the inhibition of the MAPK pathway (25). Hence, this pathway is not essential for the survival of the majority of normal cells. Moreover, not all cancer cells are sensitive to the inhibition of the MAPK pathways. For example, several studies have shown that melanoma cells that carry N-Ras mutations are not sensitive to the LF-mediated inhibition of the MAPK pathway, while those carrying a V600E B-Raf mutation are sensitive to the inhibition of this pathway (25,27). Previously, it was also demonstrated that LeTx successfully decreased cellular motility and invasion of glioblastoma cells by increasing their adhesion via an increase in RhoA activity (28).

The aim of the present study was to investigate the effect of LeTx, a MEK inhibitor, on breast cancer cell motility and invasion. First, the effect of LeTx on the MAPK pathway was examined through analyzing the phosphorylation status of ERK. Furthermore, the effect of LeTx on the migration, adhesion and invasion of MDA-MB-231, a highly aggressive and invasive type of breast cancer cell line, was studied.

Materials and methods

Cell culture. The human epithelial triple negative breast cancer cell line MDA-MB-231 was cultured adherently in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units penicillin/streptomycin (all Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO₂ in a humidified chamber.

Drug concentrations. We have previously shown that the MDA-MB-231 cell line was not sensitive to LeTx, hence its viability and proliferation were not affected by the maximum LeTx concentration used in the present assay (Abi-Habib *et al.*, unpublished data). Therefore, a concentration of LeTx consisting of 10⁻⁸ M PA and 10⁻⁹ M LF was used for cell treatment for 24 h. Cells were also treated with the MEK1/2 inhibitor U0126 (Sigma-Aldrich; Merck KGaA) at a final concentration of 50 µM for 24 h.

Pull-down assay. Cell lysates were collected from breast cancer cells following treatment with LeTx (10⁻⁸ M PA/10⁻⁹ M LF) or U0126 (50 µM) for 24 h. The RhoA/Rac1/Cdc42 Activation Assay Combo kit (cat. no. STA-405; Cell Biolabs, Inc.) was used for a pull-down assay following the manufacturer's instructions. Briefly, the cells were lysed with lysis buffer (25 mM HEPES, 1% Igepal, 150 mM NaCl, 10 mM MgCl₂, 10% glycerol, 1 mM EDTA, 1 mM NaVO₄, 20 mM NaF, 1 mM PMSF, 100 lg/ml aprotinin and 5 IM leupeptin). Lysates were cleared by centrifugation for 1 min at 1,500 x g at 4°C and incubated with GST-RBD beads (20 µg) or GST-CRIB beads (20 µg) provided in the aforementioned kit for 1 h at 4°C with gentle shaking. Then, samples were centrifuged for 2 min at 1,000 x g at 4°C, and the pellet was washed 3 times with PBS. Beads alone samples (without cell lysate) were used as a negative control and the total cell lysate (before incubation with the beads) were blotted for total RhoA, Cdc42 and β-actin.

Western blotting. Proteins were extracted using the 1X lysis buffer (25 mM HEPES, 1% Igepal, 150 mM NaCl, 10 mM MgCl₂, 10% glycerol, 1 mM EDTA, 1 mM NaVO₄, 20 mM NaF, 1 mM PMSF, 100 lg/ml aprotinin and 5 IM leupeptin). Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Inc.). Proteins (25 µg/ml) were separated by 9% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) in PBS for 1 h at room temperature and then incubated with the corresponding primary antibodies overnight at 4°C and HRP-conjugated secondary antibodies for 1 h at room temperature the following day. GTP-RhoA and GTP-Cdc42 were detected using anti-RhoA (1:500) or anti-Cdc42 (1:500), provided in the aforementioned RhoA/Rac1/Cdc42 Activation Assay Combo kit. Mouse monoclonal anti-ERK (1:200; cat. no. ab54230), mouse monoclonal anti-phospho-Erk1 (pT202/pY204) + phospho-Erk2 (pT185/pY187) (1:200; cat. no. ab50011) and rabbit polyclonal anti-β-actin antibodies (1:500; cat. no. ab8227) were purchased from Abcam. Anti-rabbit (cat. no. W4011) and anti-mouse (cat. no. W4021) HRP-conjugated secondary antibodies (both 1:1,000) were obtained from Promega Corporation. Finally, the bands were visualized with chemiluminescent reagent ECL

(GE Healthcare Life Sciences) using the Chemidoc imaging system (Bio-Rad Laboratories, Inc.). The protein expression levels were compared by densitometry using ImageJ v1.51k (National Institutes of Health) (29).

Wound healing. Cells were cultured to confluence on culture plates. After 24 h, a wound was made in the monolayer with a sterile pipette tip. Cells were then washed twice with PBS to remove debris and new medium was added. Images were captured at 0 and 72 h. Wound widths were measured at 13 different points for each wound, and the average rate of wound closure was calculated (in $\mu\text{m}/\text{h}$) using ImageJ (29). The assay was done using infinity-corrected optics on a Zeiss Observer Z1 microscope supplemented with a computer-driven Roper cooled CCD camera and operated by Zen Blue 2.5 software (all Zeiss AG).

Random cell motility assay (time-lapse). Cells treated as indicated were imaged randomly moving in DMEM (with 10% FBS and 1% Penicillin/Streptomycin) in their respective plates that were placed on a heated stage (37°C) with controlled CO_2 levels (5%). Cell images were collected every min for 2 h using a 20x objective lens on the Zeiss Observer Z1 microscope. The total distance traveled by the cells was quantified using the ROI tracker plugin in ImageJ (29). The rate ($\mu\text{m}/\text{min}$) of at least 10 randomly selected cells per condition was then calculated by dividing the total distance traveled over time. Finally, the difference in cell motility was also expressed as fold change of the treated cells normalized to the control (29).

Adhesion assay. Collagen Solution, Type I (Sigma-Aldrich; Merck KGaA) was used to coat 96-well plates overnight at 37°C then washed with washing buffer (0.1% BSA in RPMI-1620 AQ media). The plates were then blocked with 0.5% BSA in RPMI-1620 AQ media at 37°C in a CO_2 incubator for 1 h. This was followed by washing the plates and chilling them on ice. Meanwhile, the cells were trypsinized and counted to 4×10^5 cells/ml. In total, 50 μl of cells were added in each well and incubated at 37°C in a CO_2 incubator for 30 min. The plates were then shaken and washed three times with PBS. Cells were then fixed with 4% paraformaldehyde at room temperature for 10 min, washed and stained with crystal violet (5 mg/ml in 2% ethanol) for 10 min at room temperature. Following the staining, the plates were washed extensively with water and left to dry completely. Crystal violet was solubilized by incubating the cells with 2% SDS for 30 min at room temperature. The absorption of the plates was read at 550 nm using a Varioskan Flash Multimode reader (Thermo Fisher Scientific, Inc.).

Invasion assay. Cells were treated with LeTx or left untreated as control, and the invasion assay was performed following the treatment period using the collagen-based invasion assay kit (cat. no. ECM551; EMD Millipore) according to the manufacturer's instructions. Briefly, 24 h prior to the assay, cells were starved with serum-free medium. Cells were harvested, centrifuged at $600 \times g$ for 5 min at 4°C and then resuspended in quenching medium (without serum). Cells were then counted using a hemocytometer and brought to a concentration of 1×10^6 cells/ml. In the meantime, the kit inserts (collagen-coated 8- μm pore size polycarbonate membrane) were rehydrated

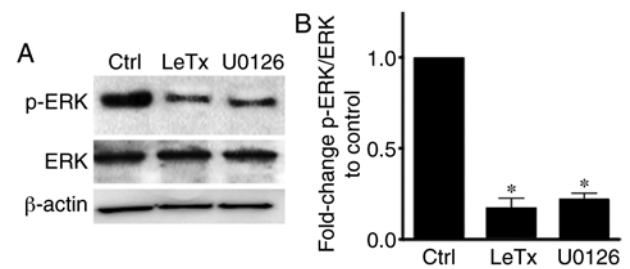


Figure 1. LeTx treatment leads to a decrease in p-ERK in breast cancer cells. (A) MDA-MB-231 cells were either treated with recombinant LeTx or U0126 for 24 h. Cells were lysed and western blot was conducted using p-ERK, ERK or β -actin antibodies. (B) Level of expression were quantified using ImageJ and represented by fold-change. * $P < 0.05$ vs. Ctrl. LeTx, anthrax lethal toxin; p-, phosphorylated; Ctrl, control.

with prewarmed 300 μl of serum-free medium for 30 min at room temperature. After rehydration, 250 μl of medium was removed from the inserts, and 250 μl of cell suspension was added. Inserts were then placed in a 24-well plate, and 500 μl of complete medium (with 10% serum) was added to the lower wells. Plates were incubated for 48 h at 37°C in a CO_2 incubator. Following the incubation period, inserts were stained for 20 min at room temperature with 400 μl of cell stain provided with the kit. The stain was then extracted with extraction buffer (also provided). The extracted stain (100 μl) was then transferred to a 96-well plate suitable for colorimetric measurement using a plate reader. Optical density was then measured at 560 nm.

Statistical analysis. The results reported represent mean values from three independent experiments. The error estimates are given as \pm SEM. The P-values were calculated by a one-way ANOVA or unpaired t-test. Tukey's post hoc test was used for comparing all possible group pairings to check if the changes observed in the results were significant. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

LeTx treatment leads to a decrease in phosphorylated (p-)ERK in breast cancer cells. In the ERK MAPK module, MEK1/2 (a MAPK kinase) phosphorylates and subsequently activates ERK (a MAP kinase) (20,21). LeTx is known to cleave MEK by degrading it in order to inhibit the MAPK pathway in cells (26). The present results showed a decrease in the level of p-ERK upon treatment of MBA-MD-231 cells with LeTx and the MEK1/2 inhibitor U0126 in comparison with control, while leaving the total ERK expression intact (Fig. 1).

LeTx treatment leads to a decrease in migration in breast cancer cells. In order to study the effect of LeTx on MDA-MB-231 cell migration, a 2D wound closure assay was performed. The rate of wound closure was calculated over the span of 72 h. Treatment with LeTx caused a decrease in the rate of wound closure from 3.8 to 0.9 $\mu\text{m}/\text{h}$ (~ 2.4 -fold decrease in wound closure rate) (Fig. 2A and B).

In order to eliminate the confounding effect of cell proliferation, a time lapse examination of individual cells undergoing random migration in serum was also performed (29).

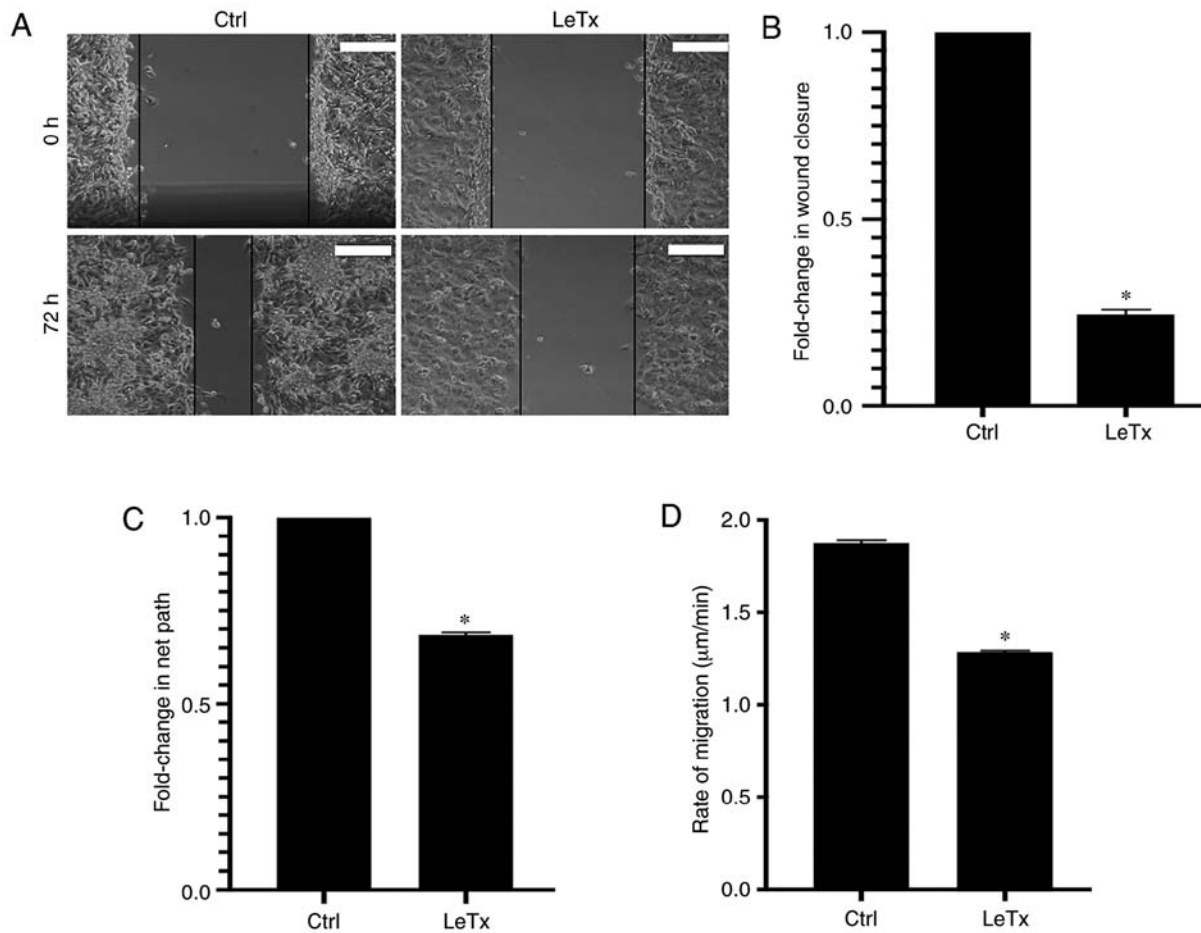


Figure 2. LeTx treatment leads to a decrease in migration in breast cancer cells. Monolayers of cells were wounded and images were captured at 0 and 72 h. (A) Representative wound closure images. Scale bar, 100 μm . (B) Frames were quantitated using ImageJ. (C) Random 2D migration quantitation showing net path of LeTx treated cells and control cells represented. (D) Rate of migration of treated cells in comparison with Ctrl cells. * $P < 0.05$ vs. Ctrl. LeTx, anthrax lethal toxin; Ctrl, control.

Consistently with the results obtained in the wound healing, a 0.75-fold decrease in the net path and rate of migration was observed in cells treated with LeTx (Fig. 2C and D; Videos S1 and S2).

LeTx treatment leads to an increase in adhesion in breast cancer cells. Since treated cells exhibited a reduction in total net path and rate of migration, it was hypothesized that this reduction in migration may be due to an increase in MDA-MB-231 adhesion to the underlying matrix, as previously seen in another tumor model (28). Indeed, cells treated with LeTx displayed a ~0.7-fold increase in adhesion (Fig. 3).

LeTx treatment leads to an alteration of Rho GTPase activation. Since LeTx-treated MBA-MD-231 cells exhibited an increase in cell adhesion, it was analyzed whether this was due to an increase in RhoA activation. RhoA at the leading edge of cells is needed for the maturation of the focal adhesions that anchor the cell to the underlying extracellular matrix (29). Performing a pull-down assay, using GST-RBD and GST-PAK beads, revealed an increase in RhoA activation in the LeTx-treated cells, which is consistent with the increase in total adhesion (Fig. 4A). In addition, there was a decrease in Cdc42 activation, which might additionally explain the decrease in cell migration in response to LeTx treatment (Fig. 4B).

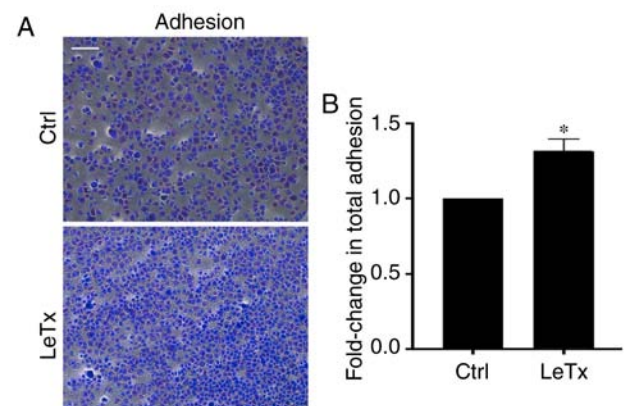


Figure 3. LeTx treatment leads to an increase in adhesion in breast cancer cells. (A) Representative micrographs of LeTx treated cells and control cells. Scale, 100 μm . (B) Quantitation of adhesion changes. * $P < 0.05$ vs. Ctrl. LeTx, anthrax lethal toxin; Ctrl, control.

LeTx treatment leads to a decrease in breast cancer invasion. Cdc42 is a potent regulator of invadopodia and cancer cell invasion (30). Accordingly, having detected an inhibition of Cdc42 in response to LeTx treatment in these cells, a decrease in invasion was expected. Indeed, the results showed a decrease in invasion upon treating cells with LeTx (Fig. 5A and B).

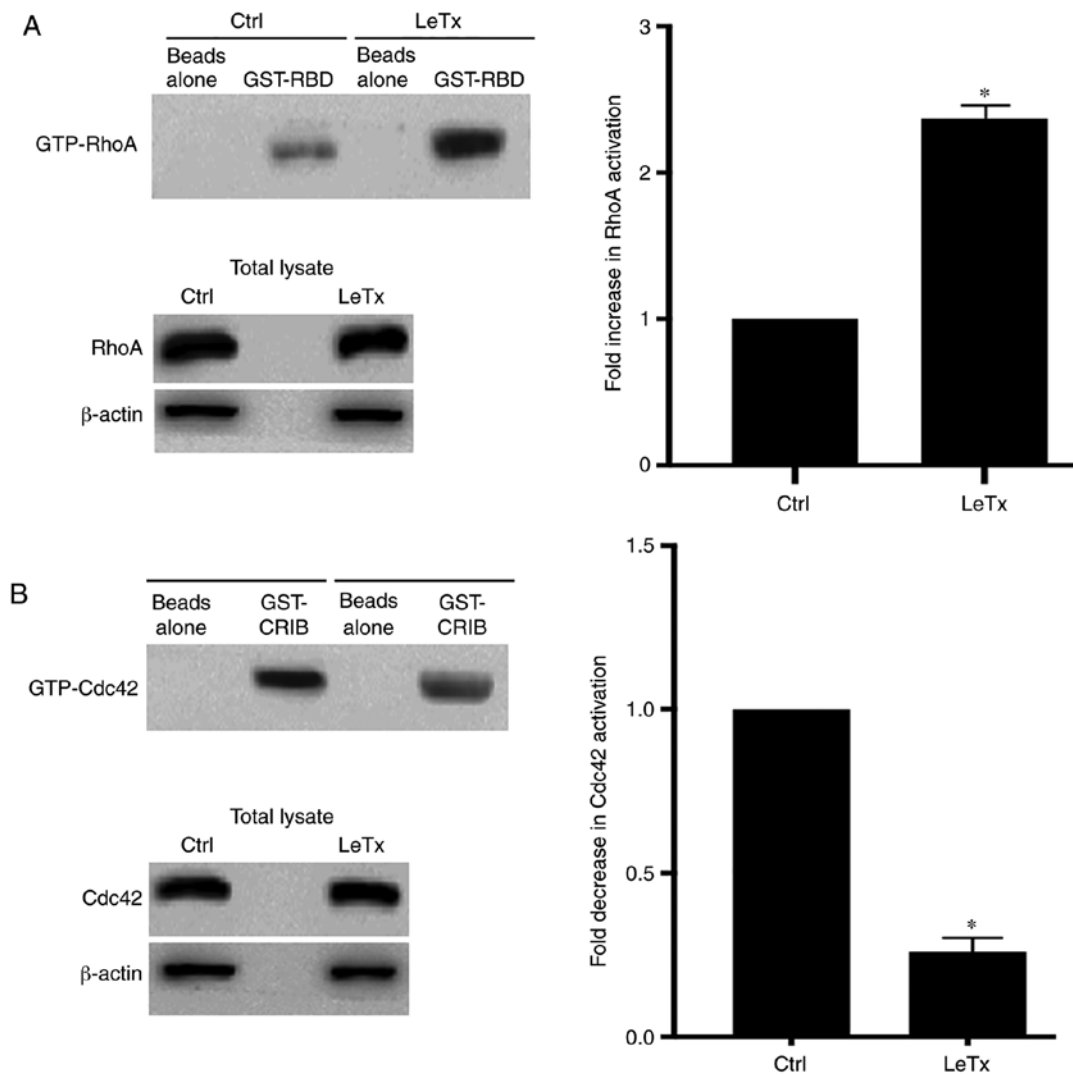


Figure 4. LeTx treatment leads to an alteration of Rho GTPase activation. (A) Pull-down assay of active RhoA. Samples were immunoblotted with RhoA or β -actin antibodies. Graphs show quantitation of the gels using the ImageJ software. Bands of active RhoA were normalized to the amount of total proteins. (B) Pull-down assay of active Cdc42. Samples were immunoblotted with Cdc42 or β -actin antibodies. Graphs show quantitation of the gels using the ImageJ software. Bands of active Cdc42 were normalized to the amount of total proteins. * $P < 0.05$ vs. Ctrl. LeTx, anthrax lethal toxin; Ctrl, control.

Collectively, these results consolidated LeTx as a potential potent therapeutic agent to target breast cancer migration and invasion through the inhibition of cell migration regulators.

Discussion

Metastatic lesions attack vital organs leading to a poor prognosis of patients with breast cancer (31). The MAPK pathway has been described to play a vital role in cellular proliferation, survival and migration. ERK, a member of the MAPK cascade has been shown to lead to an increased cell proliferation upon overactivation in a number of different cancer types, including pancreatic cancer, colon cancer, melanoma and breast cancer (32,33). Previous studies have established that inactivation of the MAPK pathway could lead to the inhibition of cancer cell survival as well as the inhibition of migration and invasion (28,34).

LeTx is a binary toxin composed of two proteins: PA And LF. LF is a zinc-activated metalloprotease that inhibits the MAPK pathway by cleaving MEKs (35,36). Based on the

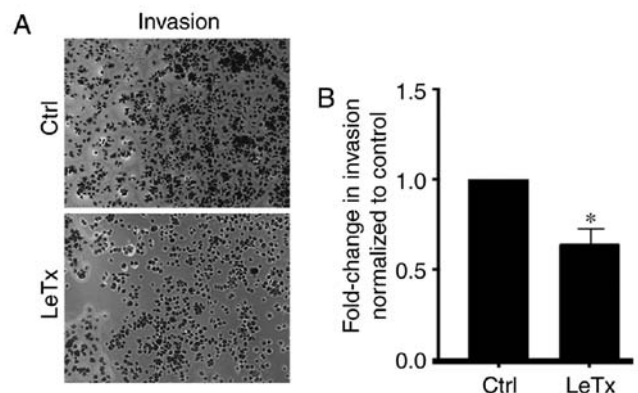


Figure 5. LeTx treatment leads to a decrease in breast cancer invasion and invadopodia formation through Cdc42 inhibition. (A) Cells were treated with LeTx or untreated. (B) Quantified invasion data. * $P < 0.05$ vs. Ctrl. LeTx, anthrax lethal toxin; Ctrl, control.

ability of LeTx to cause cell death or inhibition of migration and invasion due to MAPK inhibition, breast cancer cells were

treated with LeTx, and the migratory and invasive capabilities of the cells were examined in the present study.

Previous research has shown the ability of LeTx to impair migration of cells and disrupt their polarity (37). The present study treated breast cancer cells with LeTx, which led to a decrease in 2D cell motility as shown in the quantification of the wound healing assay. Consequently, it was suspected that there was a relationship between LeTx and an increase in cellular adhesion resulting in a decreased net path and rate of migration. After treatment with the toxin, cells exhibited an increase in adhesion as shown in the adhesion assay. We have previously shown that overexpression of RhoA leads to an increase in cell adhesion in breast cancer cells, among other tumor types (28,29,38-41). RhoA regulates focal adhesion dynamics via its downstream effector Rho-associated protein kinase 1 and the formation of stress fibers needed for migration (28,38-41). At focal contact points with the underlying extracellular matrix, cells recruit RhoA, which in turn recruits actin to form premature adhesions (28,40-43). RhoA activity then mediates the maturation of these contacts into mature adhesions (40). In parallel, as previously mentioned, ERK, through Fos-related antigen 1, leads to the inhibition of RhoA (31,42). Thus, the disruption of the inhibition of the MAPK pathway via LeTx should relieve inhibition of RhoA activation. The current study reported that, indeed, the increased adhesion phenotype was due to an increase in RhoA activation upon LeTx treatment, in addition to a decrease in Cdc42 activation, which might be the mechanism behind the decrease in cell migration.

Lastly, the present study explored the effect of LeTx on invasion of MDA-MB-231 cells. Treating the cells with LeTx led to a decrease in invasion. Cdc42 is known to play a role in invasion of cells by contributing to the formation of invadopodia by activating actin-related protein 2/3 via Neural Wiskott-Aldrich syndrome protein and by exerting an effect on matrix metalloproteinases, which is necessary for their translocation to invadopodia (43,44). Having observed decreased Cdc42 activation in treated cells in pull-down assays, it was hypothesized that the effect exerted by LeTx on invasion was through Cdc42.

Overall, the present study has demonstrated the effect of LeTx on the migration and invasive capabilities of breast cancer cells via the MAPK pathway. By inhibiting MAPK, cells demonstrated decreased motility, increased adhesion and decreased invasion, which are characteristics attributed to cancer metastasis (45). The current data has been indicative of the importance of Rho GTPases in these cellular processes and offers an insight on the potential use of LeTx in therapeutic approaches for breast cancer. Although few studies have linked Rho GTPases and the MAPK pathway (46,47), the exact mechanism involved and the crosstalk between these requires further investigation. Future studies should investigate the effectiveness of LeTx treatment on the inhibition of metastasis *in vivo*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DEC and MAH performed the experiments and analyzed the data. RAH and MES are the principal investigators on the project who designed and supervised the project, wrote the manuscript and provided the resources. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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