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Research Methods Used to Study Pharmacological Intervention of Nicotine-Induced Breast Cancer Formation and Metastasis

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RESEARCH METHODS USED TO STUDY PHARMACOLOGICAL
INTERVENTION OF NICOTINE-INDUCED BREAST CANCER FORMATION AND
METASTASIS

EASTERN KENTUCKY UNIVERSITY

Research Methods Used to Study Pharmacological Intervention of Nicotine-

Induced Breast Cancer Formation and Metastasis

(Adopted from “Bromo-enol Lactone Attenuates Nicotine-Induced Breast
Cancer Cell Proliferation and Migration”)

Honors Thesis

Fall 2016

By

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RESEARCH METHODS USED TO STUDY PHARMACOLOGICAL INTERVENTION OF NICOTINE-INDUCED BREAST CANCER FORMATION AND METASTASIS

Abstract

The effects of iPLA₂β (Ca²⁺-independent Phospholipase A₂β) and MMP-9 (Matrix Metalloproteinase-9) have been a recent focus of research in the progression of cancer cell proliferation and migration. However, their roles are not entirely known, especially concerning nicotine-induced breast cancer cell advancement. The purpose of this study was to determine the involvement of iPLA₂β in nicotine-induced breast cancer cell proliferation. BEL (bromo-enol lactone), a suicide inhibitor of iPLA₂β, was hypothesized to attenuate nicotine-induced breast cancer cell proliferation and tumor growth. Following the BEL treatment, the *in-vitro* results exhibited a significant decrease in 4T1 cell proliferation for both basal and nicotine-induced, as shown by MTT proliferation assays. Scratch and transwell assays revealed migration was also significantly attenuated. In addition, the expression of MMP-9 was discovered to have expressed in an iPLA₂β-dependent manner, which further supports the idea that iPLA₂β plays a significant role, concerning the mediation of basal and nicotine-induced cancer cell progression. Our *in-vivo* results also supported this conclusion. In mice, basal and nicotine-induced tumor growth was significantly decreased, in comparison with the control group. The presence of nicotine-induced tumors in lung tissue was reduced significantly. *In-vitro* and *in-vivo* results suggest that iPLA₂β plays a critical role in the regulation of breast cancer cell proliferation and migration and is found upstream of MMP-9. In addition, nicotine-induced breast cancer is promoted through iPLA₂β expression. Because BEL was found to reduce the effects of this, it may be a possible chemotherapeutic drug to clinically develop. *Keywords:* iPLA₂β, MMP-9, BEL, proliferation, migration, metastasis

RESEARCH METHODS USED TO STUDY PHARMACOLOGICAL
INTERVENTION OF NICOTINE-INDUCED BREAST CANCER FORMATION AND
METASTASIS

Table of Contents

Abstract.....	ii
List of Figures.....	iv
List of Tables.....	v
Acknowledgements.....	vi
Introduction.....	1
Methods.....	3
Results.....	7
Discussion.....	10
Figures.....	11
References.....	23

RESEARCH METHODS USED TO STUDY PHARMACOLOGICAL
INTERVENTION OF NICOTINE-INDUCED BREAST CANCER FORMATION AND
METASTASIS

List of Figures

Figure 1 Nicotine-induced Cell Proliferation is attenuated by BEL.....11

Figure 2 Nicotine-induced Cell Migration is decreased by BEL.....12

Figure 3 BEL decreases Nicotine-induced MMP-9 Expression and Secretion.....13

Figure 4 iPLA2 β and MMP-9 Expression is upregulated in 4T1 tumor tissue.....14

Figure 5 Nicotine-induced Tumor Growth is decreased by BEL.....15

Figure 6 Nicotine-induced iPLA2 β and MMP-9 Expression in Tumor Tissue is decreased
by BEL.....16

Figure 7 Nicotine-induced CD31 is decreased by BEL.....17

Figure 8 Nicotine-induced breast cancer metastasis in lung tissue was attenuated by
BEL.....18

Figure 9 Nicotine-induced breast cancer metastasis in lung tissue was attenuated by
BEL.....19

RESEARCH METHODS USED TO STUDY PHARMACOLOGICAL
INTERVENTION OF NICOTINE-INDUCED BREAST CANCER FORMATION AND
METASTASIS

List of Tables

Figure 1A Nicotine-induced cell proliferation (Cell Count analysis).....	20
Figure 1B BEL attenuates nicotine-induced cell proliferation (MTT assay).....	20
Figure 2A Nicotine-induced cell migration is attenuated by BEL (Scratch assay).....	20
Figure 2B Nicotine-induced cell migration is attenuated by BEL (Transwell Assay).....	20
Figure 3C Nicotine-induced MMP-9 secretion is attenuate by BEL (Gel Zymography)..	21
Figure 5A Tumor Volume to Body Weight.....	21
Figure 5B Liver Weight to Body Weight.....	22
Figure 8B Nicotine-induced lung tumor area μm^2	22

RESEARCH METHODS USED TO STUDY PHARMACOLOGICAL
INTERVENTION OF NICOTINE-INDUCED BREAST CANCER FORMATION AND
METASTASIS

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RESEARCH METHODS USED TO STUDY PHARMACOLOGICAL INTERVENTION OF NICOTINE-INDUCED BREAST CANCER FORMATION AND METASTASIS

Introduction

According to the American Cancer Society (2016), there is an estimated 246,660 new cases of breast cancer for 2016, with 40,450 deaths in the United States. At an occurrence of 29% out of all cancers, breast cancer is most common type of cancer found in women. Stage IV breast cancer is the most invasive. At this stage, metastasis has occurred, which means that tumors have spread to other parts of the body (American Cancer Society, 2016). There is an increased risk of mortality as cancer spreads. For breast cancer, there is a 99% survival rate for local breast cancer compared to a 26% survival rate for distant, which means metastasis has occurred (American Cancer Society, 2016).

It has been found that cigarette smoke increases the risk of getting breast cancer. In a cohort study, it was found that the rate of new cases of breast cancer was higher in smokers than in non-smokers (Simon, 2013). Cigarette smoke has been linked to an increase in the overall invasiveness of breast cancer, including an increase in anchorage-independent growth, increased motility, and fibroblastoid morphology. Therefore, cigarette smoke contributes to the metastatic ability of breast cancer cells (Simon, 2013). The FDA (2012) has identified many HPHC's (Harmful and Potentially Harmful Constituents) in cigarette smoke. These include carcinogens, respiratory toxicants, cardiovascular toxicants, reproductive or developmental toxicants, and addictive components, which add up to 93 identified harmful components, 78 of which are carcinogens.

Nicotine, in particular, has been shown to stimulate apoptosis resistance of breast cancer cells, which would enhance proliferation, leading to tumor growth (Guha, et al., 2014). In addition, nicotine is very nonpolar, which allows for its ability to diffuse through the lipid bilayer of cells, without the use of a carrier protein. This gives nicotine the potential to enter all cells. Though nicotine has been linked with increased progression of breast cancer, the pathways involved nicotine-induced breast cancer cell proliferation and migration are still unknown.

The enzyme iPLA₂ β has been identified as a contributor to different cancer types, including prostate and ovarian cancers. It is involved in the proliferation and migration of ovarian cancer cells (Li et al., 2014) and the survival of prostate cancer cells (Nicotera et al., 2009). It is found in the cytosol and membranes and is a member of the iPLA₂ family of enzymes. One of its major functions is to maintain membrane homeostasis by degrading phospholipids. The enzyme hydrolyzes at the *sn*-2 position, releasing arachidonic acid, which leads to a variety of outcomes, including inflammation (Akiba & Sato, 2004). iPLA₂ β is associated with cancer, among other disease states. Upregulation of iPLA₂ β has been connected with hypertension (Calderon et al., 2012) and it is hypothesized that the regulation of iPLA₂ β may be effective against β -cell dysfunction related to diabetes (Lei, Zhang, Bohrer, Barbour, & Ramanadham, 2012). In addition, iPLA₂ β is linked with alzheimer's (Talib, Hototian, Joaquim, Forlenza, & Gattaz, 2015).

MMP-9 (Matrix Metalloproteinase 9) contributes to tumor vascularization and growth and is known to be involved in the metastasis of breast cancer. MMP's break down basement membranes, which allows cancerous cells to get into the blood stream

(Mehner, et al., 2014). Both iPLA₂β and MMP-9 play roles in the progression of breast cancer, and may be in the same cell signaling cascade.

In this study, Bromoenol Lactone (BEL) was examined to be a chemotherapeutic agent. BEL is an irreversible inhibitor, specific to iPLA₂β (Jenkins, Han, Mancuso, & Gross, 2002) and has been used in order to stimulate apoptosis (Fuentes, Perez, Nieto, Balsinde, & Balboa, 2003). In this study, the effects of nicotine on iPLA₂β were observed, as well as the link between iPLA₂β and MMP-9, as well as the results of BEL administration. The cell signaling cascade related to iPLA₂β may be effective in providing a new proposal for an effective chemotherapeutic treatment.

Methods

(Referring to our paper “Bromoenol Lactone Attenuates Nicotine-Induced Breast Cancer Cell Proliferation and Migration”)

Cell Culture

A GFP (Green Fluorescent Protein) labeled 4T1 mouse mammary tumor cell line was used, which was a donation from Dr. Shu Liu and Dr. Kai Su; this was originally from ATCC. Dulbecco’s Modified Eagle Medium (DMEM) was used to culture the cell line, with the addition of 100U/ml penicillin, 100µg/ml streptomycin, and 10% (vol/vol) Fetal Bovine Serum. These cultures remained incubated at 37° C, with 95% air and 5% CO₂.

Animals

Ten-week-old female BALB/c mice were obtained from Jackson Laboratory (BarHarbor, ME). The mice were on exposed to 12 hour light and dark periods and fed

standard rodent chow (Prolab ISOPRO RMH 3000Irradiated LabDiet; Purina Mills International). They were given one week to habituate. The committee on animal research care and use at Eastern Kentucky University approved the protocols used.

***In-Vivo* Xenograft Model of Breast Cancer and Drug Delivery**

DMEM (100 μ l) without FBS was used to suspend 1×10^5 tumor cells, which was injected into the right mammary fat pad of the mice. Following anesthetization with gaseous isoflurane mixed with O₂ (3–5% isoflurane/97% O₂), mini-osmotic pumps (Alzet model 2004, 28-day release, Alza Co., Palo Alto, CA) were inserted subcutaneously via a scapular region incision onto the right flank. The pumps contained either Nicotine (Sigma-Aldrich [5 mg/kg/day]) or Saline (50% DMSO). Throughout the procedure, the mice were kept under using isoflurane mixed with O₂ (1–2% isoflurane/97% O₂). A Drager 19.1 model isoflurane machine (Highland Medical Equipment) was used for anesthetization. Intraperitoneal injections of either BEL (Bromo-enol Lactone), [Cayman Chemical Company, lot 70700; (10 μ g/g/day)] or saline was given daily. Tumor volumes (mm³) were found using the equation $(\text{width})^2 \times \text{length}/2$, in order to measure the tumor growth; width was the smaller measurement. The mice were put down after 2 weeks, and tumor volume and weight were recorded. Tissues were isolated and mounted or formalin fixed. Mounting was accomplished using Optimal Cutting Temperature compound (OCT; Tissue Tek, Torrance, CA).

Histological and Immunohistochemical Staining

Paraffin-embedded tissues were cut 5 μ m thick and were deparaffinized with xylene. They were then rehydrated, followed by immunohistochemistry procedures as

previously described (Liu et al., 2013). Slides were incubated at 4°C with primary antibodies. These antibodies included iPLA₂β (1:100, donation from Dr. Guo University of Kentucky), MMP-9 (1:500, Cell Signaling), MMP-2 (1:500, IHC World), and GFP (1:500, Cell Signaling). Counterstaining was done using hematoxylin and mounted on slides using paramount; Hematoxylin and Eosin (Surgipath) were used to stain lung and liver stains. An Olympus IX70 microscope equipped with Olympus DP70 digital camera was used to magnify the images 20 to 40x. An Olympus digital camera with Olympus MicroSuit-B3 Software was used to analyze large tumor areas.

Cell Proliferation

A cell count assay was performed, which aided in predicting the lowest nicotine concentration that would produce cell proliferation. Cells (1×10^4) were spread on 6 well plates, and media was added, which contained 10% FBS. Either DMSO (control) or Nicotine (1μM, 5μM, or 10μM) was added to the plates. All *in-vitro* experiments utilized S-BEL (Cayman Chemical Company, 10006801). The cells were left to grow for 24, 48, and 72 hours. This was followed by trypsinization and suspension with typhan blue. The cells were then counted using a hemocytometer, which was useful for analyzing the degree of proliferation.

An MTT assay was used to determine the potentiality of BEL reducing the nicotine-induced cell proliferation. Cells (500 cells/μl) were spread in a 96-well plate, followed by the addition of DMSO (control), 10 μm Nicotine, 3 μm BEL, or both BEL and Nicotine, which was repeated after 24 hours. After 48 hours, MTT solution (10 μl) from Vbrant MTT Cell proliferation assay kit (Life Technologies) was used for the incubation of the cells (37°C for 4 hours). Cells were solubilized, mixed with SDS

(sodium dodecyl sulfate), and a Phenix Genios Tecon 96 well plate reader was used to read the absorbance at 595 nm.

Cell Migration

A scratch/wound healing assay and transwell assay were performed. A 6-well plate was used for cell culturing for the scratch assay. Culturing continued until a confluent monolayer arose. The wells were scratched using a 20 μ l pipet tip. PBS was used to rinse the wells in a sequential fashion, and the wells were cultured in DMEM with 10% FBS. DMSO (control), 10 μ mNicotine, 3 μ m BEL or both BEL and Nicotine were added to the wells. At 0, 6, and 24 hours, four images (10x) were taken. Three leading edge measurements were used to find the gap width.

Three μ m BEL (30 minutes pretreatment), 10 μ m Nicotine, both BEL and Nicotine, or DMSO (control) were used to treat cells (1×10^5) for the transwell assay as well. Using DMEM with 0.1% FBS, cells were spread in the upper insert (8- μ m pore; corning), and 10% FBS was in the fitted culture dishes; the cells were left for 4 hours. The chambers and plates were separated, the chambers were fixed, and the cells that migrated were stained with DAPI. Four images were taken of each well (20x).

Gel Zymography

Parallel gel zymography experiments were used in order to determine which MMP (matrix metalloproteinase) is the most prominently secreted due to nicotine treatment. Serum was not added to the culture medium during these experiments. The experiments were performed following 24 hours of cell starvation. No MMPs were found in the culture medium. Ten μ l of culture medium was tested after the previously described treatment for 24 hours of 1×10^6 cells. Samples were stained with GelCode Blue

Phalloidin (Life Technologies, Grand Island, NY) following the addition of native Tris-Glycine with 0.15% gelatin and incubation with reaction buffer. ImageJ software was used to analyze the sample band densities.

***In situ* Zymography**

The MMP-9 secreted due to nicotine treatment was analyzed. The 4T1 cells (1×10^4) were placed on cover slides, starved for 24 hours, and treated as previously described for 24 hours. The procedure was performed as described previously (Liu et al., 2013), and the cells were counterstained with DAPI following incubation with MMP-9 (1:500, Cell Signaling).

Statistics

Data was pooled and described as mean \pm SEM. GraphPad, Prism 6 (San Diego, CA) was used for statistical analyses, and T-tests with one- and two-way ANOVAs were utilized.

Results

(Referring to our paper “Bromoenol Lactone Attenuates Nicotine-Induced Breast Cancer Cell Proliferation and Migration”)

Nicotine-induced cell proliferation and migration is attenuated by BEL

Different concentrations of nicotine were administered to the breast cancer cells. At 10 μ M, which was the highest concentration, the cell proliferation increased significantly (Figure 1A). The MMT assay exhibited that—in the presence of BEL—the cell proliferation was diminished, both with nicotine and without nicotine treatment (Figure 1B).

Not only did BEL reduce breast cancer cell proliferation, but it was also found to be effective in reducing the migration of breast cancer cells—even the cells that were treated with nicotine. This was shown in the scratch assays (Figure 2A-B). A transwell assay was conducted in order to confirm the ability of BEL to reduce breast cancer cell migration. Again, BEL was found to reduce breast cancer cell migration—both basal and nicotine-induced (Figure 2C-D). Due to the role of BEL—as a suicide inhibitor of $iPLA_2\beta$ —the results are indicative of $iPLA_2\beta$ being a contributor to breast cancer cell proliferation and migration, both basal and nicotine-induced.

MMP-9 expression and secretion is decreased by BEL

In order to examine the effects of $iPLA_2\beta$ on metastasis, gel zymography experiments were performed to find the type of MMP produced as a consequence of nicotine treatment. The particular MMP found to be increased was MMP-9, and its expression was reduced by BEL (Figures 3A-C).

The amount of $iPLA_2\beta$ exhibited in breast cancer tumor tissue and normal mammary tissue was examined; there was an upregulation in the tumor tissue (Figure 4). An upregulation of MMP-9 in tumor tissue was also discovered through GFP staining. This association points to a link between $iPLA_2\beta$ and MMP-9—it appears that $iPLA_2\beta$ results in MMP-9 production in tumor tissue. This would mean that $iPLA_2\beta$ may be a promotor of metastasis in stage IV breast cancer.

Nicotine-induced breast cancer tumor growth is attenuated by BEL

The 4T1 breast cancer cells produced tumors, which were grown in the mice—some with nicotine and some without. BEL injections were given daily, and tumor size was continually monitored. The nicotine-treated tumor growth and basal tumor growth

were both attenuated by BEL (Figure 5A). The liver was also examined, specifically weight and cytotoxicity, in order to see if there were any major physiological side effects due to BEL treatment; no differences in liver tissue were observed (Figure 5B-C).

Nicotine-induced metastasis is decreased by BEL

The effects of nicotine on breast cancer metastasis was examined through tissue immunohistochemistry. The tumor tissue exhibited an upregulation of iPLA₂β in the presence of nicotine, and BEL reduced this upregulation, for both basal and nicotine-induced (Figure 6). In addition, there was also an upregulation of MMP-9 in tumor tissue; this was also reduced by BEL—both basal and nicotine-induced (Figure 6). The results point to a link between nicotine and the upregulation of iPLA₂β and MMP-9, which leads to an increase in metastasis.

Nicotine was found to upregulate HIF-1 alpha (Figure 6) and CD31 (Figure 7) in tumor tissue, which was decreased through BEL treatment. In hypoxic tumor tissue, angiolytic factor production and blood vessel formation are increased. The upregulation of HIF-1 expression suggests that nicotine increases tumor hypoxia conditions (Figure 6). The increase in endothelial cells in the tissue is suggestive of angiogenesis. These results support the idea that nicotine can promote metastasis by giving breast cancer cells access to

The data indicated that nicotine promotes metastasis. The lung tissue was analyzed, and this conclusion was further supported. There was a significant increase in nicotine-induced breast cancer tumor colonization, compared to basal tumor colonization (Figure 8). BEL not only attenuated nicotine-induced tumor formation, but basal as well. This was shown through HE staining and GFP cells (Figure 8). The area analysis of the

lung sections further verified the attenuation of nicotine-induced tumor colonization by BEL (Figure 9).

Discussion

Cigarette smoke has been shown to contribute to the progression of breast cancer through mediating cell proliferation and metastasis. However, the pathways related to this enhancement are unknown. Research has found iPLA₂β to play a role in cancer development. In this study, iPLA₂β was shown to be linked to nicotine-induced breast cancer progression. The cell proliferation and migration, enhanced by nicotine, occurred in an iPLA₂β dependent manner. Our data exhibited that blocking iPLA₂β can be effective against stage IV breast cancer, as well as stage IV nicotine-induced breast cancer. Bromoenol lactone was able to reduce tumor growth in both control and nicotine-induced breast cancer. In addition, BEL treatment kept metastasis from occurring in the lung tissue. There was not only a reduction of iPLA₂β with BEL treatment, but MMP-9—a contributor to metastasis—as well. Because BEL is an inhibitor specific to iPLA₂β, this suggests that these two enzymes are linked in a cell signaling cascade that promotes the progression of breast cancer—control and nicotine-induced. The effectiveness against stage IV breast cancer progression—including proliferation, migration, and metastasis—suggests that BEL would also be effective against early stages of breast cancer. Furthermore, there was no cytotoxicity found in the liver, which means there are no apparent severe side effects. The data shows that iPLA₂β mediates the production of MMP-9, both of which are enhanced with nicotine. In conclusion, bromoenol lactone was able to greatly reduce the production of these enzymes, leading to decreased tumor size and metastasis, with no toxicity.

Figures

(Obtained from “Bromo-enol Lactone Attenuates Nicotine-Induced Breast Cancer Cell Proliferation and Migration”)

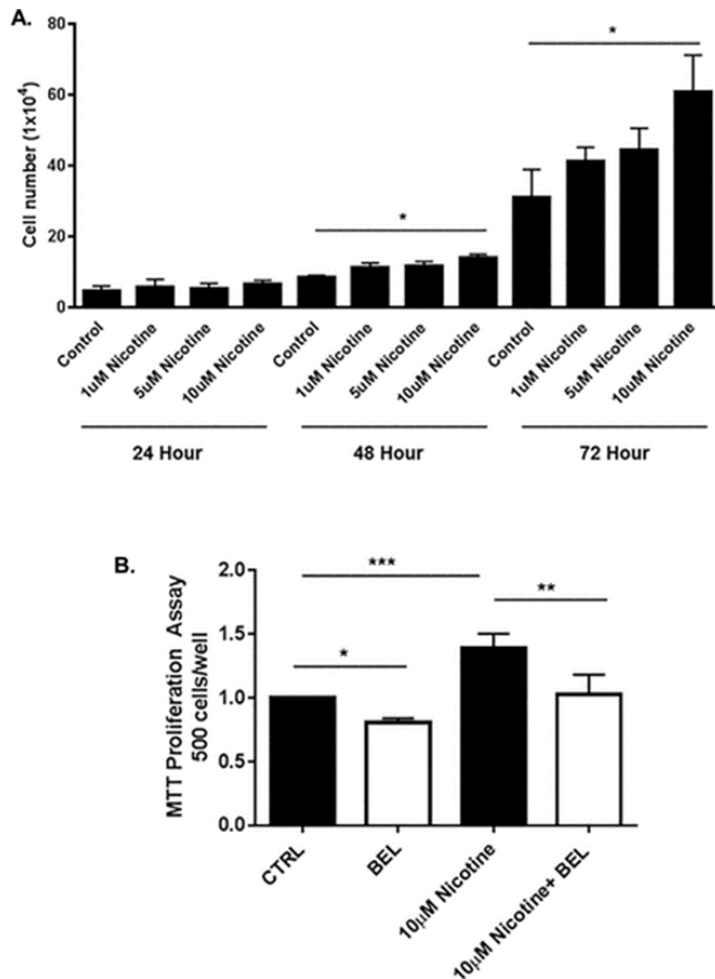


Fig 1. Nicotine-induced Cell Proliferation is attenuated by BEL.

Promotion of 4T1 cell proliferation by nicotine dose dependently (1, 5, 10µM) was analyzed by cell count analysis for time intervals indicated above. Control wells were treated with DMSO (A). Cells were treated with nicotine (10µM) in the presence or absence of BEL (3µM) and proliferation measured by MTT assay (B). (n = 6–7). *, p<0.05 **; p<0.01 ***; p<0.00; two and one-way ANOVA.

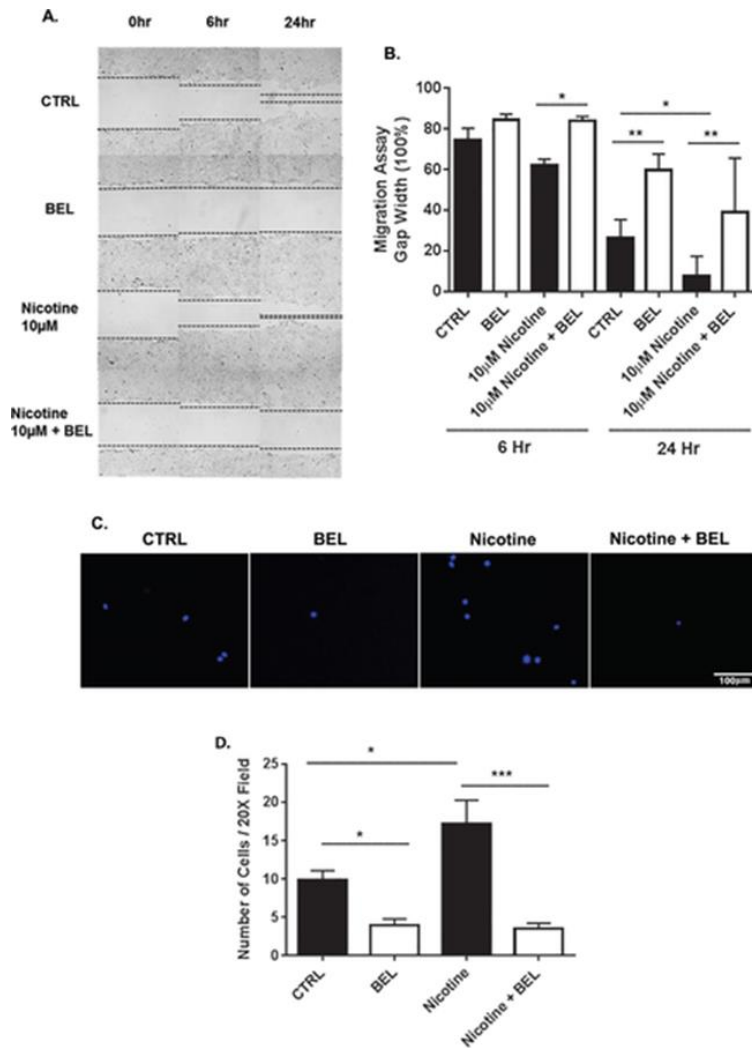


Fig 2. Nicotine-induced Cell Migration is decreased by BEL.

Cultured 4T1 cells were scratched under normal conditions (DMSO) and nicotine (10µM) in the presence or absence of BEL (3µM). Representative 10x images of scratch migration assay, indicating BEL treatment attenuated nicotine induced migration (A). Cell migration was quantitatively evaluated by measuring the distance between the scratch edges after 6, 24, and 48 hr (B). Representative images of Boyden chamber migration assay, cells were treated with nicotine (10M) in the presence or absence of BEL (3µM, 30 minute pretreatment) for 4 hr (C). Cell migration was quantitatively evaluated by counting the number of cells migrated (D). (n = 3–5). *, p<0.05 ** ,p<0.01; one-way ANOVA.

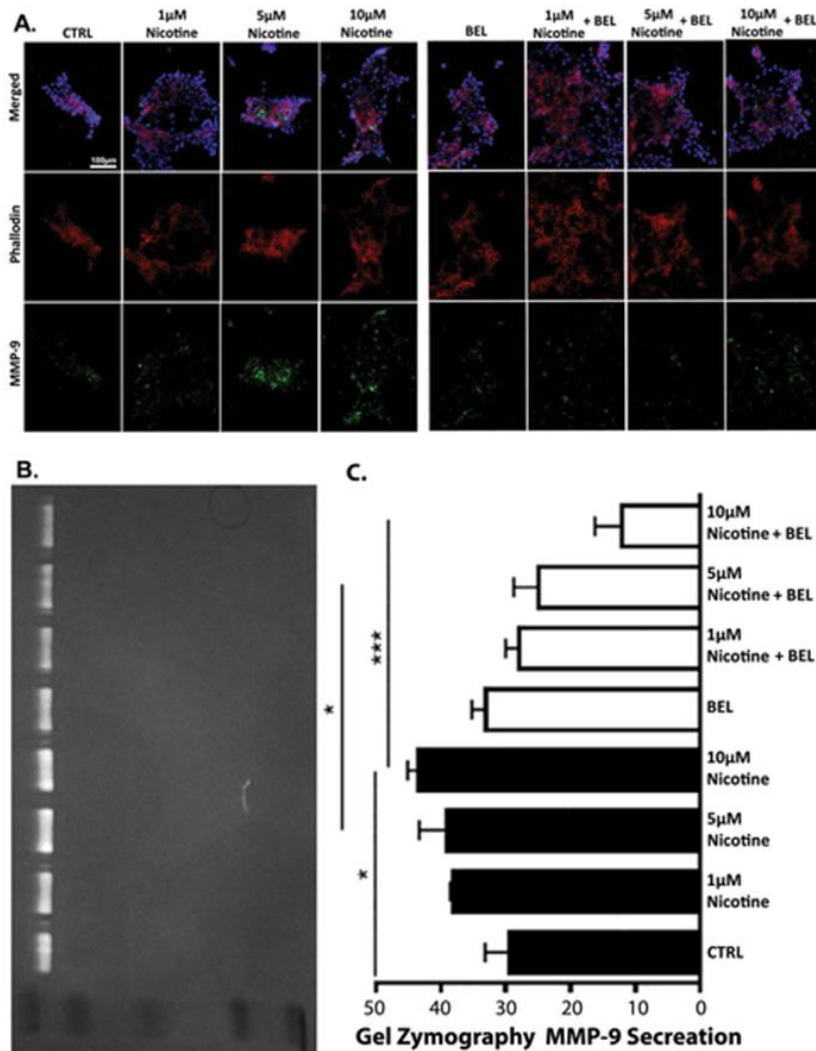


Fig 3. BEL decreases Nicotine-induced MMP-9 Expression and Secretion.

An In-situ zymography was conducted with cells treated dose dependently with nicotine (1, 5, 10 μ M) in the presence or absence of BEL (3 μ M) for 24 hr. Cells were stained with MMP-9, phalloidin, and DAPI (A). Cell culture media was isolated from cells treated as previously described and a gel zymography was performed; the gel molecular weight standard from top to bottom is 103kDa, 77kDa, 49kDa, 34kDa, and 28kDa. A representative image of gel zymography shows a band corresponding to the molecular weight of proMMP-9 (92kDa) (B) and the area of gelatin dissolved was quantified (C). n = 3, *, p<0.05. ***, p<0.001; one-way ANOVA.

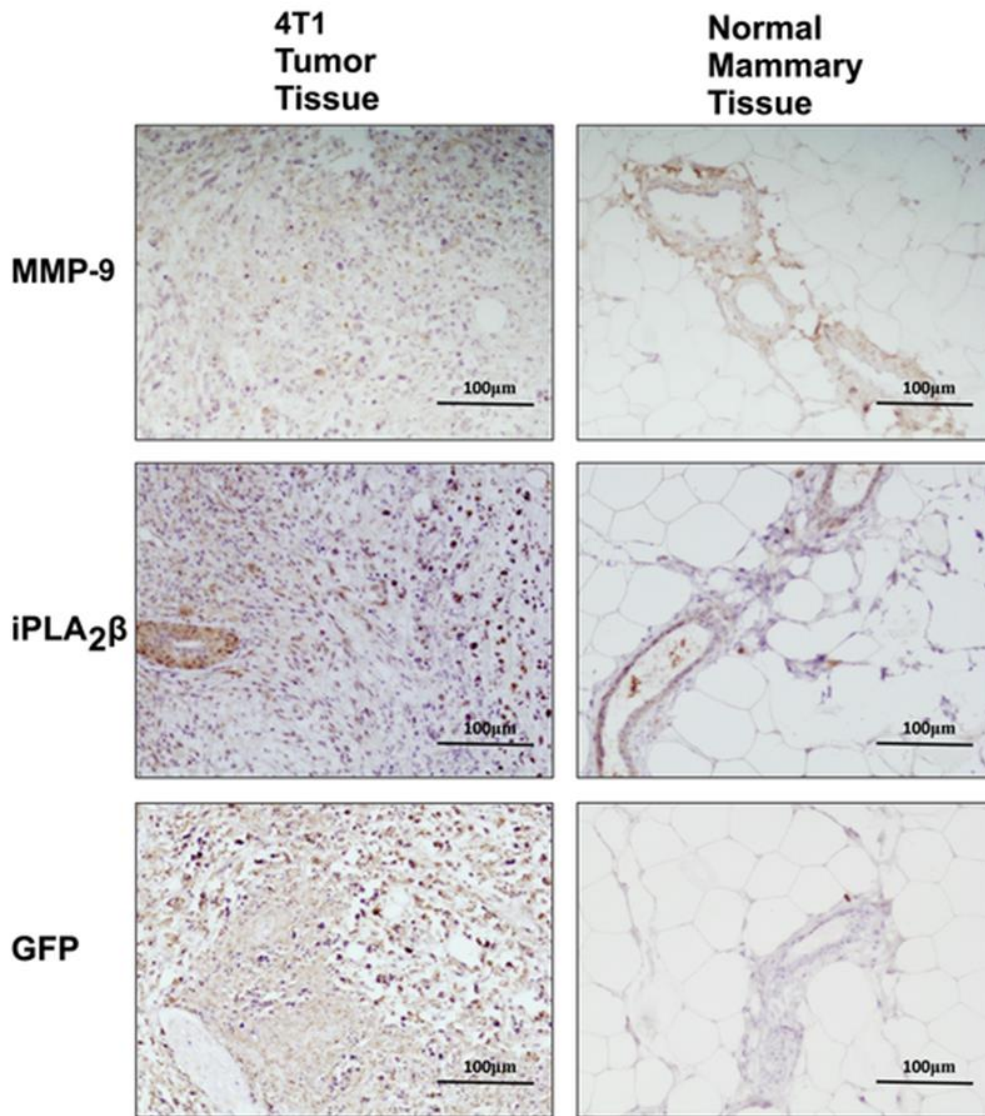


Fig 4. iPLA₂β and MMP-9 Expression is upregulated in 4T1 tumor tissue.

Normal mammary or 4T1 tumor tissue grown for 2wks in 12wk old Female BALB/c mice were isolated and embedded in paraffin. Tissue sections were stained with anti-GFP (green-fluorescent protein), iPLA₂β, or MMP-9 antibodies. Positive staining is indicated by dark brown coloration and representative images shown.

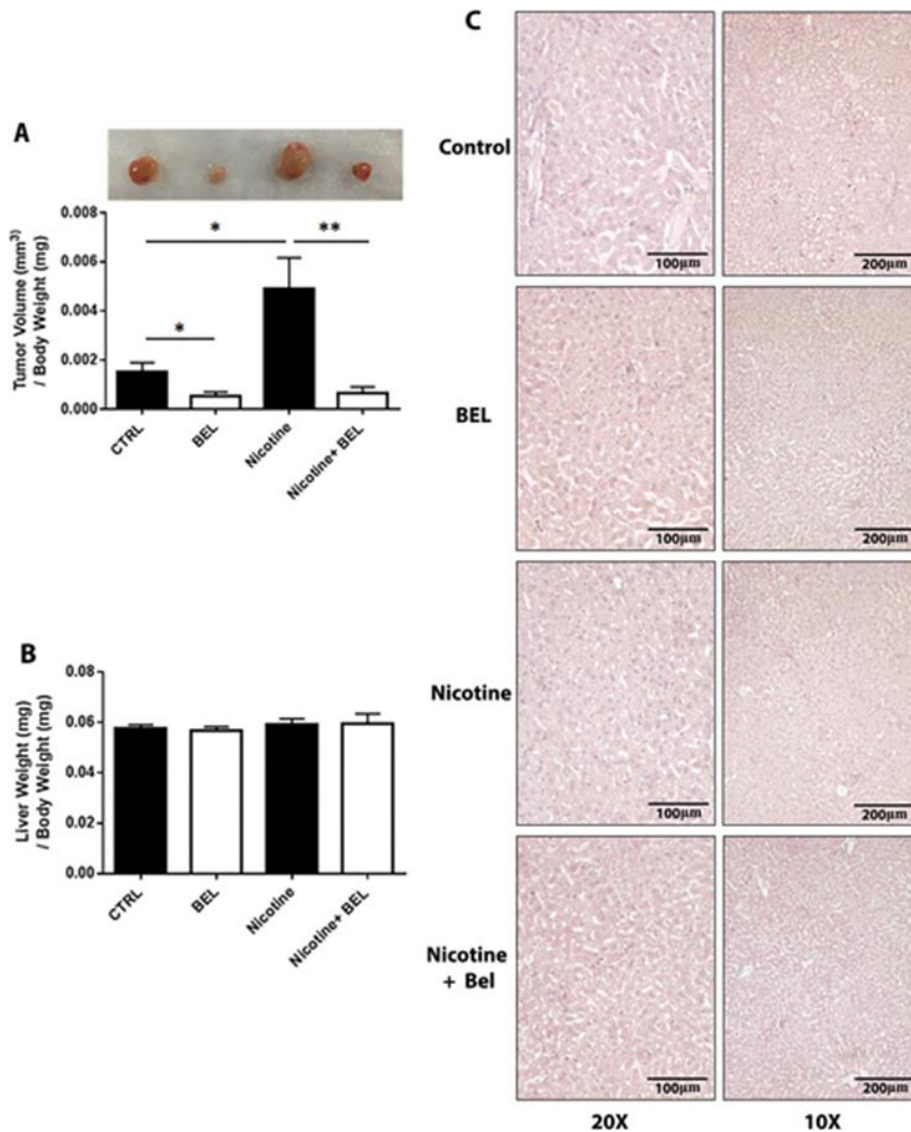


Fig 5. Nicotine-induced Tumor Growth is decreased by BEL.

Female BALB/c mice were implanted with 4T1 cells (1X10⁵) in the right second mammary fat pad. Tumors were grown for 2wks in mice infused either with nicotine (5mg/kg/day) or saline (50% DMSO) and in the presence or absence of BEL (10ug/g/day). Tumors were isolated and final tumor volume (A) and liver weight (B) measured. Liver cytotoxicity was analyzed by HE staining with representative 10x and 20x images shown (C) (n = 8). *, p<0.05 ***, p<0.01; unpaired t-test.

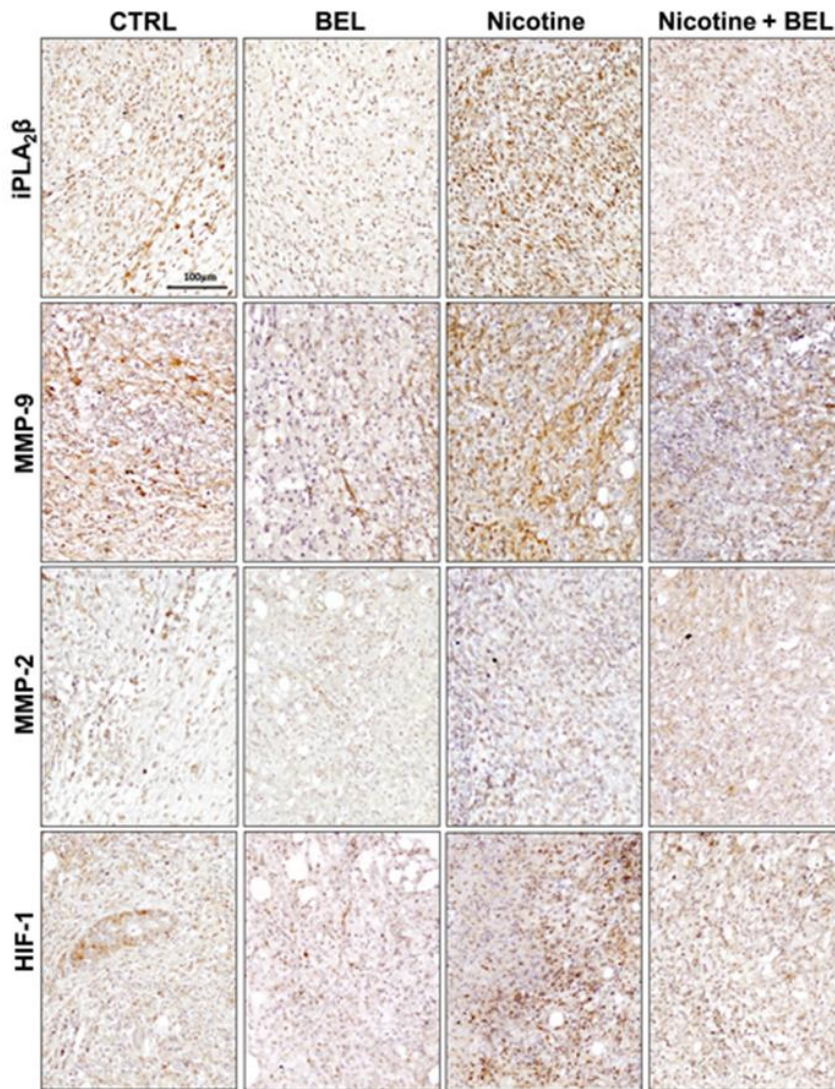


Fig 6. Nicotine-induced iPLA₂β and MMP-9 Expression in Tumor Tissue is decreased by BEL.

Control versus BEL and Nicotine versus Nicotine plus BE (n = 4): 4T1 tumor tissue grown for 2wks in 12wk old Female BALB/c mice were isolated and embedded in paraffin. Tissue sections were stained with anti-iPLA₂β, MMP-9, MMP-2, and HIF-1α antibodies. Positive staining is indicated by dark brown coloration and representative images shown.

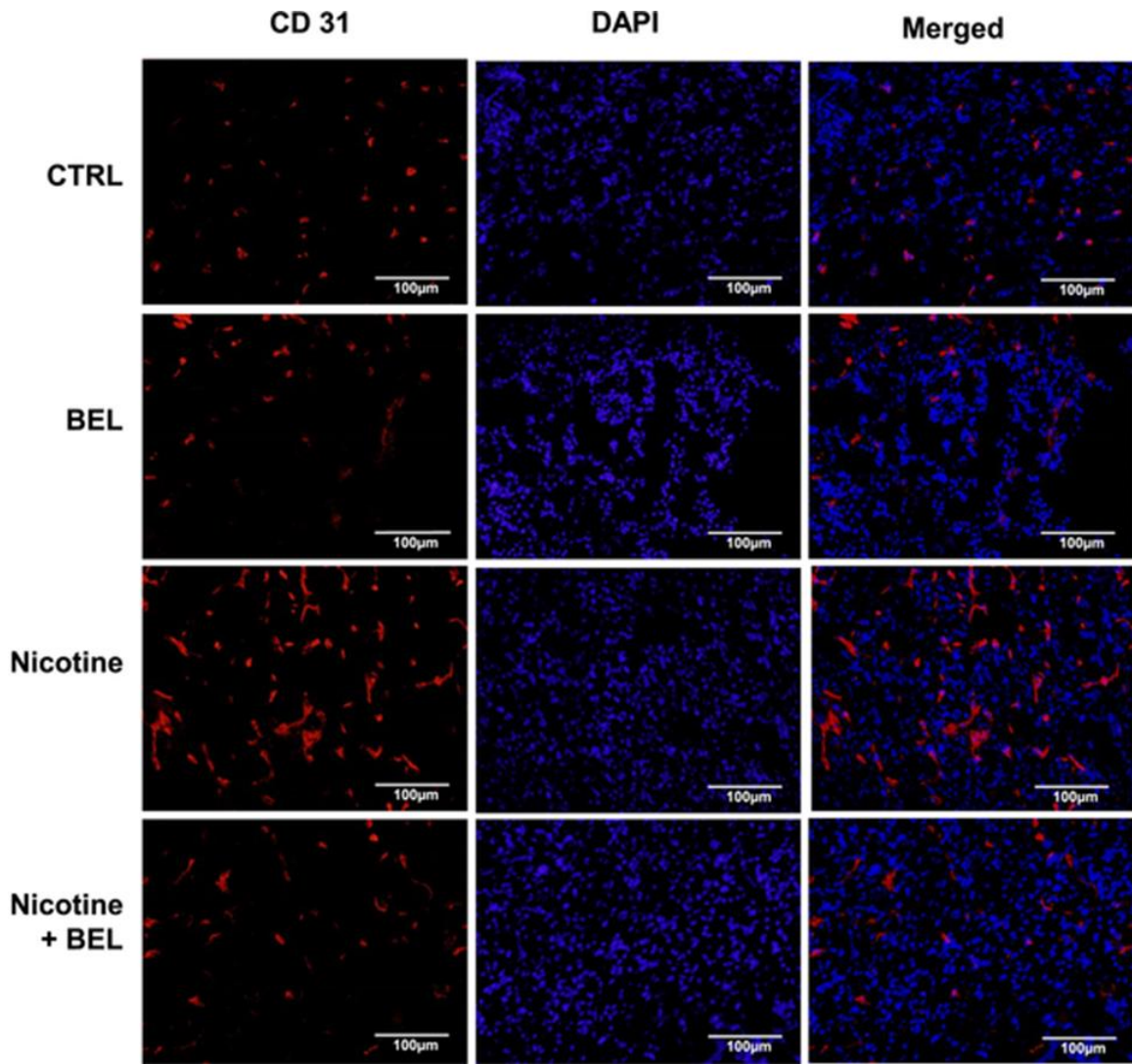


Fig 7. Nicotine-induced CD31 is decreased by BEL.

Isolated tumor tissue was embedded in OCT and stained with CD31 and counterstained with DAPI. Representative images are shown. n = 4.

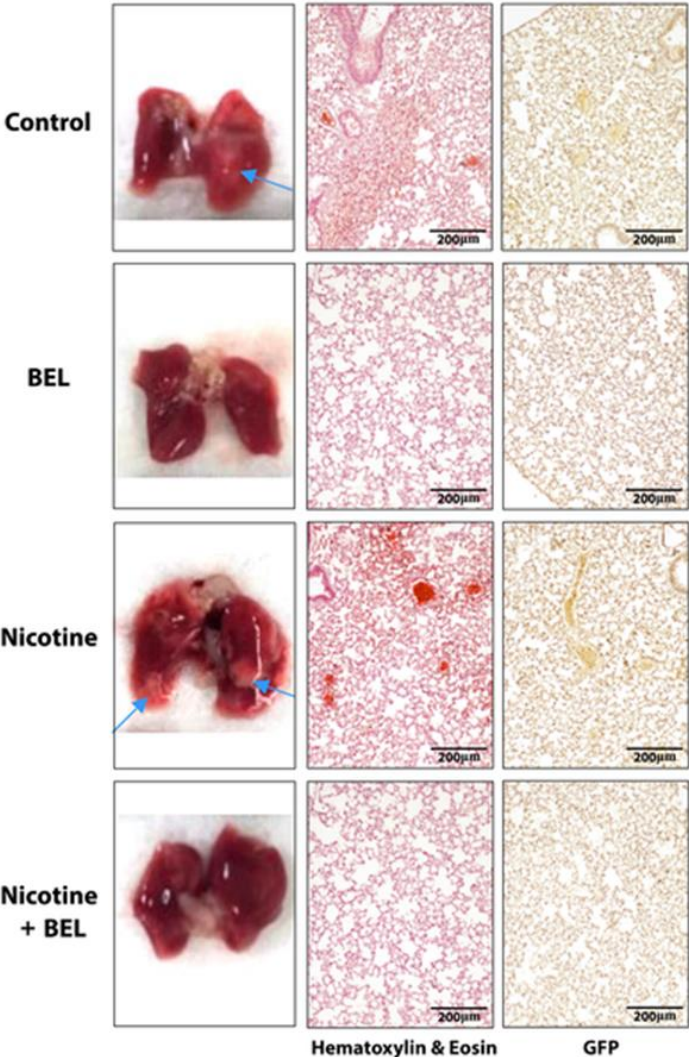


Fig 8. Nicotine-induced breast cancer metastasis in lung tissue was attenuated by BEL.

Lungs were isolated and representative 10x images of tumor colonization shown by HE and GFP staining.

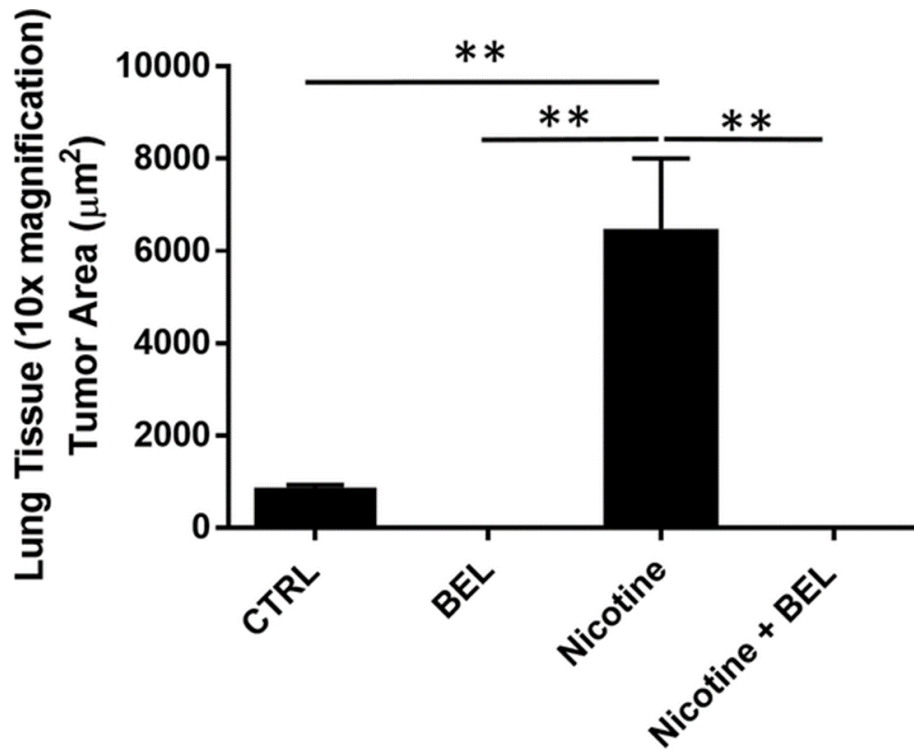


Fig 9. Nicotine-induced breast cancer metastasis in lung tissue was attenuated by BEL.

Tumor area (μm²) was quantified in the lung sections stained with HE. n = 4, **, p<0.01; one-way ANOVA.

Figure 1A: Nicotine-induced cell proliferation (Cell Count analysis)

Trials	24 Hours				48 Hours				72 Hours			
	Control	1uM Nicotine	5uM Nicotine	10uM Nicotine	Control	1uM Nicotine	5uM Nicotine	10uM Nicotine	Control	1uM Nicotine	5uM Nicotine	10uM Nicotine
1	7.67	11.5	9	9.25	9.8	12.7	13.5	16.9	46.89	51.89	62	86
2	3.6	4.75	5.6	5.5	8	11.6	10.4	14	33.2	41.25	39.2	52.3
3	5.75	5.5	4.5	7.25	8.3	13	14	12.2	34.8	38.75	42.5	67.33
4	2	1.3	2.5	4.25	8.25	8	9.2	12.7	9.78	33	34.5	38

Figure 1A: Nicotine-induced cell proliferation (Cell Count analysis)

Column1	Control	BEL	Nicotine	Nicotine + BEL
Trial 1	7.3	4.6	17.3	2.5
Trial 2	10	3.75	11.6	4.5
Trial 3	16.25	6.5	23.75	4
Trial 4	9.75	6.75	24.25	5.25
Trial 5	11	3.5	9.3	1.5
Trial 6	7.4	1.3		
Trial 7	8	1.25		

Figure 1B: BEL attenuates nicotine-induced cell proliferation (MTT assay)

Column1	Control	10mM Nic	10mM Nic + BEL	BEL	CTR L	10mM Nic2	10mM Nic + BEL3	BEL4
Trial 1	74.3	50	75.5	74.9	39.4	20	59.14	52.3
Trial 2	72	67.9	84.8	84.09	0.045	0.04	45	45.7
Trial 3	73.8	70	91.65	94.2	28.8	10.7	51.81	59
Trial 4	66.2	65.1	82.13	90	37	0.116	0.0007	82
Trial 5	77.9	61.8	83.4	79.4				
Trial 6	83.2	58.15	86.39	84.3				

Figure 2A: Nicotine-induced cell migration is attenuated by BEL (Scratch assay)

Column1	Control	BEL	Nicotine	Nicotine + BEL
Trial 1	10	4.6	17.3	2.5
Trial 2	7.3	3.75	11.6	4.5
Trial 3	16.25	6.5	23.75	4
Trial 4	9.75	6.75	24.25	5.25

Trial 5	11	3.5	9.3	1.5
Trial 6	7.4	1.3		
Trial 7	8	1.25		

Figure 2B: Nicotine-induced cell migration is attenuated by BEL (Transwell Assay)

Column1	Trial 1	Trial 2	Trial 3
Control	32.75	21.81	33.58
1µM Nicotine	38.18	38.88	37.08
5µM Nicotine	46.21	31.81	39.59
10µM Nicotine	46.33	43.52	40.39
BEL	35.53	34.92	28.66
1µM Nicotine + BEL	26.49	32.03	25.46
5µM Nicotine + BEL	20.51	32.5	21.87
10µM Nicotine + BEL	13.03	4.49	18.84

Figure 3C: Nicotine-induced MMP-9 secretion is attenuate by BEL (Gel Zymography)

Control	BEL	Nicotine	Nicotine + BEL
0.00116	0.00102	0.00119	0.00049
0.00298	0.0012	0.0065	0.00018
0.000745	0.00027	0.0026	0.00201
0.0023	0.000078	0.00616	0.00034
0.00088	0.00096	0.00214	0.000155
0.00063	0.00013	0.00265	0.00027
0.0035	0.000545	0.0123	0.0011
0.0013	0.00008	0.00557	
0.00016			

Figure 5A: Tumor Volume to Body Weight

Control	BEL	Nicotine	Nicotine + BEL
0.064	0.051	0.071	0.056
0.056	0.055	0.055	0.057

0.0544	0.058	0.064	0.0821
0.0633	0.058	0.059	0.055
0.056	0.053	0.053	0.052
0.06	0.063	0.053	0.06
0.054	0.059	0.057	0.055
0.0522		0.0619	

Figure 5B: Liver Weight to Body Weight

Control	BEL	Nicotine	Nicotine + BEL
1400.83	0	8597.29	0
868.65	0	9472.54	0
397.99	0	6351.36	0
693.6	0	2814.02	0
394.69		1667.93	
946.26		5649.5	
873.6		4150.01	
		19440.5	
		7167.16	
		1193.98	
		4772.6	

Figure 8B: Nicotine-induced lung tumor area μm^2

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