Ibulocydine inhibits migration and invasion of TNBC cells via MMP-9 regulation

Mi Ri Kwon
University of Ulsan College of Medicine

Ji Soo Park
Seoul National University

Eun Jung Ko
ASAN Medical Center

Jin Park
ASAN Medical Center

Eun Jin Ju
ASAN Medical Center

Seol Hwa Shin
ASAN Medical Center

Ga Won Son
ASAN Medical Center

Hye Won Lee
University of Ulsan College of Medicine

Hee Hyun Park
ASAN Medical Center

Yun-Yong Park
Chung-Ang University

Myoung-Hee Kang
ASAN Medical Center

Yeon Joo Kim
ASAN Medical Center

Byeong Moon Kim
Seoul National University

Hee Jin Lee
ASAN Medical Center

Si Yeol Song
ASAN Medical Center

Seok Soon Park (pssooni@amc.seoul.kr)
ASAN Medical Center

Seong-Yun Jeong
Abstract

Background

Triple-negative breast cancer (TNBC) accounts for approximately 15–20% of all breast cancer types, indicating poor survival prognosis with more aggressive biology of rapidly progressive growth, metastasis to the lung, and short response duration to available therapies. TNBC is characterized by the negative expression of three hormone receptors. Therefore, compared to other breast cancers, TNBC is difficult to treat using hormone inhibitors and is resistant to chemotherapy. Additionally, the lack of effective targets limits the development of therapeutics. Ibulocydine (IB) is a novel (cyclin-dependent kinase) CDK7/9 inhibitor prodrug displaying potent anti-cancer effects against various cancer cell types. We performed the following experiments to determine whether IB inhibits metastasis and eventually overcomes the poor drug response in TNBC.

Methods

Colony-forming, cell counting kit-8 (CCK-8), wound healing, trans-well assays, and western blotting were performed in vitro. An experimental metastasis model was developed via intravenous injection of MDA-MB-231-Luc cells in vivo, and tumor growth was monitored using an In Vivo Imaging System (IVIS) spectrum.

Results

The result showed that IB reduced the viability of various TNBC cell lines in a dose-dependent manner. Pretreatment with z-VAD effectively blocked IB-induced cell death and cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP) in TNBC cells. A reduction in the migration and invasion abilities of TNBC cell lines was observed following IB treatment in migration and invasion assays. We determined the expression levels of metastasis-related markers using western blotting and found that the expression of matrix metalloproteinase-9 (MMP-9) decreased in an IB dose-dependent manner. In addition, IB-induced inhibition of migration and invasion was blocked in MMP9-overexpressing MDA-MB-231-Luc cells. Results of in vivo experiments using the metastasis model showed that metastasis of MDA-MB-231-Luc cells to the lung was inhibited by IB.

Conclusions

Collectively, these results showed that IB inhibited the growth of TNBC cells by inducing caspase-mediated apoptosis and blocking metastasis by reducing MMP-9 expression, suggesting a novel therapeutic agent for metastatic TNBC.
Background

Triple-negative breast cancer (TNBC) refers to breast cancer that does not express the genes for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [1]. It is highly aggressive, with metastasis, resistance to various treatments, and a poor overall survival rate compared to other subtypes of breast cancer cells [2, 3]. Despite various studies, the lack of clear molecular targets for TNBC has limited the development of therapies [1, 3–5]. Therefore, more effective treatment strategies are required.

In TNBC, Cyclin-dependent kinase 7 (CDK7) expression is associated with poor prognosis [6, 7]. CDK7 plays two primary roles in regulating the cell cycle and transcription factors [8]. During cell cycle progression, CDK7 activates CDK1 and CDK2 during the S/G2 phases and CDK4/6 during the G1 phase via phosphorylation [9]. During transcription, CDK7 phosphorylates serine 5 (Ser5) and Ser7 of the RNA polymerase (Pol II)-C-terminal domain (CTD) and CDK9, which induces Ser2 phosphorylation of the Pol CTD [8]. CDK7 is a good anticancer target because of its dual role in regulating cell cycle and transcription.

For various cancers, the development of therapies targeting CDK7 is ongoing [8, 10]. Early CDK7 inhibitors were not initially CDK7-specific but multi-CDK inhibitors. Albocidib (flavopiridol), an inhibitor of CDK1, 2, 4, 6, 7, and 9, was evaluated in phase I clinical trials for numerous cancer types [11] but showed a limited clinical response [12, 13]. Seliciclib (roscovitine), another inhibitor of CDK1, 2, 5, 7, and 9, was assessed in clinical trials in various tumor types; however, this had limited clinical activity [14, 15]. SNS-032, which inhibits CDK2, 7, and 9, has been evaluated for advanced solid tumors; however, the drug has not progressed further than phase I [16, 17]. Recently, various CDK7-specific inhibitors have been developed, such as BS-181 [18], ICEC0942 (CT7001; samuraciclib) [19–21], LY3405105 [22], LDC4297 [23], SY-1365 [24] (phase 1), THZ1 (SY-079) [25], THZ2 [6], YKL-5-124 [26], QS11189 [27], and SY-5609 [28]. Among them, SY-5609, THZ1, and THZ2 were tested preclinically against TNBC [6, 28]. ICEC0942 is currently in phase I clinical trials for patients with breast (TNBC, HR+/HER- breast type) or prostate cancers (clinical trial ID: NCT03363893) [29]. LY3405105 is also in phase I for advanced or metastatic solid tumors (clinical trial ID: NCT03770494). SY-1365 is currently in phase I trials for advanced solid tumors, ovarian cancer, and HR+ metastatic breast cancer (clinical trial ID: NCT03134638). In addition, SY-5609 is entering phase I in select advanced solid tumors (clinical trial ID: NCT04247126). Thus far, four CDK7 inhibitors have been in clinical trials.

Ibulocydine (IB) is a novel CDK7/9 inhibitor prodrug with anticancer effects against human hepatoma cells (HCC) [30] and sensitizes TRAIL-induced apoptosis in HCC cells [31]. Previously, we reported that IB sensitizes radiotherapy (RT) in lung and colon cancer cells [32]. CDK7/9 is highly expressed in TNBC [6, 7, 33–36]. For a follow-up study of IB, we evaluated whether IB has anticancer efficacy and the ability to inhibit metastasis in TNBC.

Materials and methods
Cell culture

The human TNBC cell lines (Hs578T and MDA-MB-435S) were purchased from the American Tissue Culture Collection (ATCC, VA, USA). The human TNBC cell line (MDA-MB-231-Luc) was purchased from Caliper Life Sciences (MA, USA). Hs578T cells were cultured in DMEM (4.5 g/L D-glucose) (Gibco, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (Gibco). MDA-MB-231-Luc cells were maintained in MEM (Gibco) supplemented with 10% FBS and 1% P/S. MDA-MB-435S cells were cultured in DMEM (1 g/L D-glucose) (Gibco) supplemented with 10% FBS and 1% P/S. All cells were incubated in 5% CO₂ at 37°C. Cell lines were tested using the MycoAlert PLUS Mycoplasma Detection Kit (LT07-710, Lonza, MD, USA). To establish stable cell lines, the MMP-9-FLAG plasmid (RC202872, OriGene, MD, USA) was transfected with the FuGENE HD Transfection Reagent (E2312, Promega, WI, USA). Transfected cells were selected using 500 µg/mL neomycin (108321-42-2, Corning, NY, USA).

Reagents

Ibulocydine (IB) was obtained from Seoul National University and dissolved in dimethyl sulfoxide (DMSO) to prepare 10 mM stock solutions for the in vitro experiments. DMSO was purchased from Sigma-Aldrich (MA, USA), and z-VAD-fmk from R&D Systems (MN, USA). The primary antibodies against α-tubulin (2125), cleaved caspase 3 (9661), MMP-2 (4022) were purchased from Cell Signaling Technology (MA, USA), β-actin (A5441) from Sigma-Aldrich (MA, USA), MMP-9 (PA5-13199) from Invitrogen (MA, USA), Bcl-xL (ADI-AAM-080-E) and Mcl-1 (ADI-AAP-240) from Enzo Life Sciences (NY, USA), cleaved PARP (ab32561), SNAIL 1/2 (ab135708), and TWIST (ab175430) from Abcam (Cambridge, UK), Survivin (NB500-201) from Novus Biologicals (CO, USA), XIAP (610762) from BD Biosciences (NJ, USA), and ZEB1 (polyclonal goat anti-human; sc-10572) from Santa Cruz Biotechnology (TX, USA).

Cell viability and clonogenic assays

For cell viability assays, cells were seeded in 24-well plates and treated as indicated in Fig. 1B. Cytotoxicity was assayed using Cell Counting Kit-8 (CCK-8) (Cat.no. CK04, Dojindo, Tabaru, Japan) according to the manufacturer’s protocol. For clonogenic assays, cells were seeded in 6-well plates and exposed to different doses of IB (1 or 3 µM) for 12 h. The cells were incubated for nine days to allow colony formation and stained with 0.5% crystal violet solution in 10% methanol. Colonies with > 50 cells were counted.

Western blotting analysis

Cells were washed in PBS and lysed in 2× sodium dodecyl sulfate (SDS) sample buffer (ELPIS-BIOTECH, Daejeon, South Korea). The lysates were boiled for 5 min, separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to an Immobilon membrane. After blocking nonspecific binding sites for 30 min using 5% skim milk, membranes were incubated overnight at 4°C or for 2 h at room temperature with specific antibodies. Subsequently, membranes were washed
three times with TBST and further incubated for 1 h at room temperature with peroxidase-conjugated
donkey anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch Laboratories, PA, USA). Protein
bands were visualized using ECL (Amersham Life Science, Bucks, UK) and ImageQuant LAS-4000 (GE
Healthcare Life Sciences, MA, USA).

Cell migration and invasion assays

Polycarbonate membrane Transwell with a pore size of 5 µm (Cat no. 3421, Corning) and Matrigel (Cat
no. 356237, Corning) was used for the invasion assays. Upper chambers were coated with Matrigel (150
µg/100 µL) and incubated at 37 °C for 30 min before cell seeding. Following Matrigel suction, Hs578T
and MDA-MB-231-Luc (3 × 10^4 cells/100 µL) and MDA-MB-435S (4 × 10^4 cells/100 µL) cells were seeded
into the upper chamber in DMEM (4.5 g or 1 g/L D-glucose). MEM with 10% FBS and 600 µL media was
added to the lower chamber. After 24 h at 37 °C with 5% CO2 in an incubator, the upper chamber was
washed with PBS, Hs578T and MDA-MB-231-Luc (3 × 10^4 cells/100 µL) and MDA-MB-435S (4 × 10^4 cells/100 µL) cells were seeded
into the upper chamber in DMEM (4.5 g or 1 g/L D-glucose). MEM with 10% FBS and 600 µL media was
added to the lower chamber. After 24 h at 37 °C with 5% CO2 in an incubator, the upper chamber was
treated with IB (0.5 and 1 µM) and 0.1% FBS ‐ media for 24 h. The media in the bottom well was replaced
with fresh media containing 10% FBS used as a chemoattractant. The non‐invasive cells were removed
with a cotton swab. After washing with PBS, the upper chamber was fixed, stained with crystal violet
(20% methanol + 0.5% crystal violet) for 30 min, and washed. The invasive cells were observed using a
microscope (BX53, OLYMPUS, Tokyo, Japan) in five independent fields of view per sample at ×4 and ×10
magnifications. To examine cell migration, wound healing assays were performed. Cells were seeded (1–
3 × 10^4 cells/70 µL/insert) on Culture-Insert 2 Well in µ-Dish (Cat no. 81176, ibidi, Gräfelfing, Germany).
After 24 h, inserts were removed and scratched. Media with or without IB (1 and 3 µM) was added and the
cells were then incubated for 24 h. Cell migration was monitored using a phase-contrast microscope
(IX71, Olympus, Tokyo, Japan) at ×10 magnification. ImageJ software was used to evaluate the images.

Gene expression omnibus (GEO) analysis

To complement our experimental results, additional analyses for the association between CDK7/9 gene
expression and patient survival were performed using the publicly available GEO database (GSE16446),
The Cancer Genome Atlas (TCGA), and the Netherlands Cancer Institute (NKI). Survival estimates were
calculated using the Kaplan–Meier method and compared using log-rank tests.

Animal models

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Asan
Institute for Life Science (2021-12-080).

BALB/c nude mice (6 weeks old, female, SLC, Shizuoka, Japan) were used to generate mouse models. An
experimental metastasis model was developed via intravenous injection of MDA-MB-231-Luc cells (1 ×
10^6 cells/100 µL). MDA-MB-231-Luc cells were treated with 3 µM IB 6 h before injection. Tumor growth
was monitored through the IVIS spectrum (Perkin Elmer, MA, USA).

Statistical analysis
Results

Ibulocydine has anticancer effects on TNBC cells

IB is an isobutyrate ester prodrug of a novel synthetic CDK inhibitor that has activity against CDK7 and CDK9 [30]. To confirm the association between CDK7/9 expression and TNBC, we determined survival rates and CDK7/9 levels in patients with TNBC. The relationship between overall survival (OS) and patients with high expression of both CDK7 and CDK9 was significant (Fig. 1A).

To examine the cytotoxic effects of IB, the viability of TNBC cells after IB treatment was evaluated using a CCK-8 assay. The results showed that IB decreased cell viability in a dose-dependent manner for various TNBC cells (Fig. 1B). In addition, IB inhibited the long-term survival of TNBC cells in colony-forming assays (Fig. 1C). Collectively, these results suggest that IB exerts anticancer effects on TNBC cells.

Ibulocydine induces apoptotic death of TNBC cells

To explore the mechanism of IB-induced cell death, we examined whether IB affects the expression of apoptosis-related proteins. The results showed that cleaved caspase-3 and cleaved PARP levels effectively increased in TNBC cells after 24 h of IB treatment (Fig. 2A). In contrast, anti-apoptotic protein levels decreased after 24 h of IB treatment (Fig. 2B). To confirm whether IB-induced cell death was apoptosis, we pretreated cells with z-VAD-fmk, a pan-caspase inhibitor. Consequently, IB-induced cell death in TNBC cells was almost blocked by z-VAD-fmk pretreatment (Fig. 2C). Additionally, IB-induced cleavage of caspase-3 and PARP was blocked by z-VAD-fmk pretreatment (Fig. 2D). Collectively, these results demonstrate that IB induces cell death in TNBC cells via induction of caspase-mediated apoptosis.

MMP-9 plays a crucial role in Ibulocydine-induced inhibition of metastasis in TNBC cells

We evaluated whether IB inhibits the metastasis of TNBC cells because TNBC is aggressively metastatic [37]. In wound healing assays, IB effectively blocked the migration of TNBC cells into the scratch area (Fig. 3A). The invasion assays also demonstrated that the number of invading TNBC cells was significantly reduced compared with that in the control cells (Fig. 3B). Therefore, these results showed that IB inhibited migration and invasion of TNBC cells.
Epithelial-mesenchymal transition (EMT) is part of the metastatic process, where cancer cells lose their epithelial characteristics and gain mesenchymal characteristics [38]. To investigate the underlying mechanism of the metastasis inhibition by IB, changes in the levels of various metastasis-regulating proteins were determined using western blotting. The protein levels of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), which are involved in the EMT and metastasis [39], decreased after IB treatment of TNBC cells (Fig. 3C). Moreover, the levels of mesenchymal marker proteins, including Snail 1/2, TWIST, and ZEB1 [40], decreased after IB treatment (Fig. 3D). Because overexpression of MMP-9 is associated with poor prognosis in patients with TNBC [41], we examined whether MMP-9 is important in the inhibition of IB-induced metastasis. For this study, we established stable MDA-MB-231-Luc cell lines with MMP-9 overexpression. MMP-9 was successfully overexpressed by the MMP-9-FLAG plasmid in MDA-MB-231-Luc cells, and its expression was confirmed using western blotting (Fig. S1A). Results of wound healing assays using MMP-9 overexpressing stable cell lines showed that the inhibition of migration by IB was reduced (Fig. 3E). Likewise, the inhibition of invasion by IB was blocked in MMP-9 overexpressing cells (Fig. 3F). Collectively, these results indicate that IB inhibited TNBC metastasis by downregulating MMP-9.

Ibulocydine inhibits metastasis to the lungs in an animal model

TNBC frequently metastasizes to the lungs [37, 42, 43]. Therefore, we constructed an experimental lung metastasis model using MDA-MB-231-Luc cells and evaluated the inhibition of metastasis by IB. MDA-MB-231-Luc cells were pretreated with 3 µM IB in the lung metastasis animal model. After 6 h, treated cells were injected intravenously in the tail (Fig. 4A). No difference in the growth of tumor cells was observed between untreated cells and those treated with IB for 6 h in colony-forming assays (Fig. S1B). When the growth of metastatic tumors was monitored using the in vivo imaging system (IVIS) spectrum, tumors rarely formed in the lungs of the IB-treated group (Fig. 4B). After 56 days, all mice were sacrificed and dissected to identify metastatic tumors in the lung. IB-treated MDA-MB-231-Luc cells showed a reduction in tumor burden on bioluminescence imaging (Fig. 4C). These in vivo data demonstrated that IB inhibits lung metastasis in the mimic metastatic environment.

Discussion

Recently, there has been increased interest in the application of CDK inhibitors in patients with TNBC in preclinical and clinical trials [8, 44–46]. Samuraciclib (CT7001), a CDK7 inhibitor, has completed phase 1 clinical trials against TNBC (NCT03363893) [29]. Most known CDK7 inhibitors inhibit CDK7 and other CDKS (for example, CDK1, CDK2, CDK4/6, CDK9, and CDK12) [8, 47, 48]. Herein, we used IB, a CDK7/9-specific inhibitor, which is expected to be a new CDK inhibitor in TNBC treatment.

First, we investigated the relationship between the OS of patients with TNBC and CDK7/9 expression. The data revealed that patients with high CDK7/9 expression had poor survival rates (Fig. 1A). To confirm the anti-cancer effects of CDK7 inhibitor on TNBC cells, IB, a novel CDK7/9 inhibitor, was used.
Unsurprisingly, IB showed strong anticancer effects on TNBC cells (Figs. 1B and 1C). Additionally, we demonstrated that IB-induced cell death was dependent on caspases and that IB decreased the expression levels of anti-apoptotic proteins (Fig. 2). Cho et al. [30] reported that IB downregulates anti-apoptosis proteins (Mcl-1, survivin, and XIAP) by inhibiting RNA polymerase II phosphorylation in HCC cells. Based on these reports, we hypothesized that IB may disrupt anti-apoptotic gene transcription regulation, leading to apoptosis in TNBC cells. We further verified the ability of IB to inhibit metastasis of TNBC cells. IB blocked migration and invasion in TNBC cells (Figs. 3A and 3B). However, unlike Hs578T and MDA-MB-231-Luc cells, MDA-MB-435S cells showed less metastatic characteristics. Recently, it has been reported that MDA-MB-435S is closer to the melanoma type than breast cancer [49, 50]. This could explain the reduced metastatic properties of MDA-MB-435S cells. We evaluated protein level changes of EMT markers to investigate the mechanism underlying the suppression of metastasis by IB. In TNBC cells, MMP-9, MMP-2, and mesenchymal markers (Snail 1/2, TWIST, and ZEB1) were dose-dependently downregulated (Figs. 3C and 3D). Matrix metalloproteinases (MMPs) dissolve the extracellular matrix (ECM) and the basement membrane (BM) to facilitate the invasion of cancer cells [39]. MMP-2 and MMP-9 play crucial roles in the cell migration involved in wound healing [51]. Because MMP-9 expression in TNBC is highly associated with metastasis [52], we hypothesized that MMP-9 is important for the suppression of metastasis by IB. Migration and invasion assays were performed in MMP-9 overexpressing MDA-MB-231-Luc cells. Therefore, the IB-induced inhibition of metastasis was significantly prevented by MMP-9 overexpression (Figs. 3E and 3F). Collectively, these results indicate that MMP-9 plays a crucial role in IB-induced inhibition of metastasis in TNBC cells. Furthermore, we evaluated whether IB inhibits lung metastasis in vivo. We designed an experiment in which mice were intravenously injected with MDA-MB-231-Luc cells pretreated for 6 h with IB to test whether it could prevent metastasis rather than have an anticancer effect on an already metastasized cancer. Using colony-forming assays, we confirmed that tumorigenesis did not differ between untreated and IB-treated cells (Fig. S1B). Mice of the IB-treated group exhibited successfully inhibited tumor growth on bioluminescence imaging (Figs. 4B and 4C). Taken together, these results suggest that the ability of IB to suppress metastasis and induce cytotoxicity may represent an effective therapeutic strategy against TNBC.

**Conclusion**

This study demonstrated that IB can induce caspase-dependent cell death and suppress metastasis by regulating MMP-9 in TNBC. This preclinical evidence may provide an effective therapeutic strategy against TNBC and a promising clinical candidate as a novel CDK7/9-specific inhibitor.

**Abbreviations**

Triple-negative breast cancer (TNBC)

Cyclin-dependent kinase 7 (CDK7)
Cyclin-dependent kinase 9 (CDK9)

Ibulocydine (IB)

Metalloproteinase-2 (MMP-2)

Matrix metalloproteinase-9 (MMP-9)

_in vivo_ imaging system (IVIS)

Intravenous (_i.v._)

---

**Declarations**

**Ethics approval and consent to participate**

The animal experiments were performed following the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the Asan Institute for Life Science (2021-12-080).

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data involved in this study are available in the main text or the supplementary materials. Materials can be available through an agreement with the corresponding authors.

**Competing interests**

Not applicable.

**Funding**

This study was supported by the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) funded by the Ministry of Health & Welfare, Republic of Korea (HI20C1586 (EKC)), the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (2018R1D1A1B0704997013 (YJK), 2020R1F1A1073962 (SSP), 2020R1A2C2101994 (SYJ), (Bio&Medical Technology Development Program, RS-2023-00227084 (SYJ)).

**Authors’ contributions**

MRK wrote—original draft & editing, methodology, investigation, and validation. JSP and MBK provided IB. JP, EJJ, EJK, SHS, GWS, HWL, and HHP designed in vivo experiments, edited figures, and reviewed and
corrected the manuscript. YYP and MHK analyzed genomic data. YJK acquired funding. HJL and SYS gave clinical advice. SSP advised conceptualization, methodology and investigated research data and gave funding acquisition. SYJ advised conceptualization, edited manuscript, and gave funding acquisition. EKC supervised and gave funding acquisition.

References


5. Knowlson C, Haddock P, Bingham V, McQuaid S, Mullan PB, Buckley NE: **Pin1 plays a key role in the response to treatment and clinical outcome in triple negative breast cancer.** *Ther Adv Med Oncol* 2020, **12**:1758835920906047.


49. MDA-MB-435, and its derivation MDA-N, are Melanoma cell lines, not breast cancer cell lines. [https://dtp.cancer.gov/discovery_development/nci-60/mdi-mb-435.htm](https://dtp.cancer.gov/discovery_development/nci-60/mdi-mb-435.htm)


**Figures**
Figure 1

Ibulocydine induces cell death in various TNBC cells. A Patients with TNBC were divided by relatively high or low CDK7/CDK9 expression, and a Kaplan–Meier plot was generated. The differences between these groups were statistically significant in terms of overall survival (OS), metastasis, and distant metastasis-free survival (DMFS). B Cell viability was measured using the CCK-8 assay after treatment with the indicated concentrations of IB for 24 h. The percentage of live cells was normalized to that of untreated
control cells (100%). Data represent the mean ± SD. **Cells were treated with two different doses of IB and incubated for 10 days for colony-forming assays. Representative graphs are shown with the number of colonies. Data are presented as the mean ± SD. * p < 0.05, ** p < 0.01 vs. untreated control.
Ibulocydine induces apoptotic cell death. **A** TNBC cells were treated with the indicated concentrations of IB for 24 h. Cell extracts were prepared from the treated cells, and western blotting was performed using anti-cleaved caspase 3 and anti-cleaved PARP antibody. α-Tubulin was used as a loading control. **B** Cell extracts were prepared from the cells treated with the indicated concentrations of IB for 24 h. Western blotting of the anti-apoptotic family proteins was performed. α-Tubulin was used as a loading control. **C** Various TNBC cells were pretreated with 50 µM z-VAD-fmk for 30 min and further treated with 3 µM IB for 24 h. Cell viability was assessed using the CCK-8 assay. Data represent the mean ± SD. *** p < 0.001 vs. untreated control. ## p < 0.01, ### p < 0.001 vs. IB treatment. **D** Cells were untreated or pretreated with 50 µM z-VAD-fmk and further treated with 3 µM IB for 24 h. Western blotting of the indicated proteins was performed. α-Tubulin was used as a loading control.
MMP-9 downregulation plays a critical role in IB-induced metastasis inhibition in human TNBC cells. 

**A** After treatment with 1 or 3 µM IB, wound healing scratch assays were performed with TNBC cells. Cell migration was monitored under a phase-contrast microscope for 24 h. Bar, 50 µm. Data are shown as mean ± SD. * $p < 0.05$, *** $p < 0.001$ vs. 24 h untreated control. 

**B** Invasion assays of TNBC cells treated with 0.5 or 1 µM IB for 24 h were performed. Invading cells were stained with crystal violet and observed.
using a fluorescence microscope (4). Data are presented as the mean ± SD. *** p < 0.001 vs. untreated control. C Cell extracts were prepared from cells treated with the indicated concentrations of IB for 24 h. MMP-9 and MMP-2 levels were detected using western blotting. α-Tubulin was used as a loading control. D TNBC cells were treated with the indicated concentrations of IB for 24 h. Cell extracts were prepared for western blotting of mesenchymal markers. E MMP-9 overexpressing stable cells were scratched in wound healing assays. Cell migration was monitored for 24 h and observed under a phase-contrast microscope. Bar, 50 μm. The graphs quantitatively show the area of wound recovery. Data are presented as the mean ± SD. *** p < 0.0001 vs. IB-treated vector cells. F Invasion assays of MMP-9 overexpressing stable cell lines treated with 0.5 or 1 μM IB for 24 h were performed. Invading cells were stained with crystal violet and observed using a fluorescence microscope (4). Data are shown as the mean ± SD. * p < 0.05, *** p < 0.001 vs. IB-treated vector cells.
Figure 4

Inhibition of metastasis by IB in animal models of metastasis. A Schematic diagram of the establishment of the experimental metastasis animal model and experimental schedules. B BALB/c nude mice were intravenously injected with MDA-MB-231-Luc cells treated with 3 μM IB for 6 h. For 56 days, lung metastasis was monitored by IVIS spectrum. Quantitative graphs of luciferase total flux on days 14, 28, 42, and 56 are shown. Data are presented as the mean ± SD. *** p < 0.001 vs. Control. C Mice with lung
metastasis were sacrificed at the endpoint of 56 days. Images of lung metastasis were obtained with the IVIS spectrum. Quantitative graphs show the luciferase total flux. Data are shown as the mean ± SD. ** $p < 0.01$ vs. Control.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryfigure.docx