Inhibition of FoxM1-Mediated DNA Repair by Imipramine Blue Suppresses Breast Cancer Growth and Metastasis

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Abstract

Purpose: The approaches aimed at inhibiting the ability of cancer cells to repair DNA strand breaks have emerged as promising targets for treating cancers. Here, we assessed the potential of imipramine blue (IB), a novel analogue of antidepressant imipramine, to suppress breast cancer growth and metastasis by inhibiting the ability of breast cancer cells to repair DNA strand breaks by homologous recombination (HR).

Experimental Design: The effect of IB on breast cancer growth and metastasis was assessed in vitro as well as in preclinical mouse models. Besides, the therapeutic efficacy and safety of IB was determined in ex vivo explants from breast cancer patients. The mechanism of action of IB was evaluated by performing gene-expression, drug–protein interaction, cell-cycle, and DNA repair studies.

Results: We show that the systemic delivery of IB using nanoparticle-based delivery approach suppressed breast cancer growth and metastasis without inducing toxicity in preclinical mouse models. Using ex vivo explants from breast cancer patients, we demonstrated that IB inhibited breast cancer growth without affecting normal mammary epithelial cells. Furthermore, our mechanistic studies revealed that IB may interact and inhibit the activity of proto-oncogene FoxM1 and associated signaling that play critical roles in HR-mediated DNA repair.

Conclusions: These findings highlight the potential of IB to be applied as a safe regimen for treating breast cancer patients. Given that FoxM1 is an established therapeutic target for several cancers, the identification of a compound that inhibits FoxM1- and FoxM1-mediated DNA repair has immense translational potential for treating many aggressive cancers.

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Introduction

Mammalian cells have evolved robust DNA damage surveillance mechanisms to maintain genome integrity. The defect in DNA damage response serves as a major factor predisposing normal cells to acquire oncogenic mutations (1). However, after tumor develops, malignant cells adapt various mechanisms such as repairing DNA damage resulting from unchecked DNA replication to manage their survival (2). Moreover, the ability of cancer cells to repair radiation or chemotherapy drug-induced DNA damage also serves as one of the mechanisms for therapy resistance. Consistent with that, several DNA repair factors are reported to be overexpressed in cancer patients (1, 3, 4). On the basis of these premises, the inhibition of DNA repair mechanisms has lately been considered to be a promising therapeutic target for treating cancers.

In this study, we report that a novel compound "Imipramine blue (IB)" may serve as a potent therapeutic agent as it induces DNA damage and inhibits the ability of breast cancer cells to repair DNA. IB is derived from imipramine, which is an FDA-approved drug for treating depression. IB was synthesized by a single-step process in which an electrophilic addition occurs at the ortho or para position to hydroxy or amine groups in the phenol or aniline compounds, or meta to nitro or carboxyl groups (5). IB was previously reported to inhibit invasion of glioma and head and neck squamous cell carcinoma (5, 6). However, IB's safety and viability to be used as a novel drug and its clinical relevance as well as utility in the treatment of breast cancers in particular is lacking. Most importantly, the mechanisms by which IB may inhibit breast cancer growth in particular are virtually unknown. Here, for the first time, using a clinically relevant ex vivo model of tumor explants from breast cancer patients, we show that IB inhibited breast cancer growth without any effect on
Inhibition of FOXM1-Mediated DNA Repair by Imipramine Blue

Translational Relevance
Pharmacologic inhibition of factors that contribute to the ability of cancer cells to repair DNA damage from unchecked DNA replication holds great promise for cancer treatment. Here, we demonstrate that imipramine blue (IB), a novel analogue of antidepressant imipramine, suppresses breast cancer growth and metastasis by inhibiting FoxM1- and FoxM1-associated signaling known to play critical roles in DNA repair. Our studies showing inhibition of breast cancer growth by IB in preclinical mouse models and in clinically relevant explants from breast cancer patients without inducing any toxicity suggest that IB may serve as a novel therapeutic lead compound for treating breast cancer patients. Because IB is derived from a FDA approved drug, it bears the potential to be quickly translated to the clinic.

normal mammary epithelial cells. In addition, the systemic delivery of IB suppressed breast cancer growth and metastasis in preclinical orthotopic mouse models without inducing any toxicity. Importantly, we report that IB inhibits breast cancer growth and metastasis by inhibiting homologous recombination (HR)-mediated DNA repair. Our results reveal that IB inhibits the levels and activity of DNA repair gene Forkhead Box M1 (FoxM1; ref. 7) and subsequently its transcriptional targets including S-phase kinase-associated protein 2 (Skp2; refs. 8, 9) and Exonuclease 1 (Exo1; ref. 10). Our interaction studies suggest that IB may affect the stability and transactivation function of FOXM1. Collectively, these findings indicate that IB may serve as a novel therapeutic lead compound with negligible toxicity for treating breast cancer patients. Furthermore, establishing the therapeutic potential of a compound that inhibits FoxM1, which is highly expressed and induces growth and progression of several cancers (11, 12), should exert much broader impact.

Materials and Methods
Human breast cancer cell lines and culture conditions
Breast cancer cells lines MDA-MB-231, MDA-MB-468, BT-549, MCF-7, and SKBR3 were purchased from the ATCC and cultured according to their guidelines. The cell lines were authenticated annually by using PCR for short tandem repeats.

Breast cancer tissues
For expression analysis and ex vivo explants, breast cancer tissues along with normal matched tissues were collected from Breast Cancer Clinic at UT Health Science Center San Antonio, TX (UTHSCSA) after obtaining approval (IRB #HSC20120041H).

Plasmid and cloning
FoxM1 cloning vector (pDNR-dual-FoxM1) was purchased from DNA repository at Arizona State University (DNASu, Arizona State University). FoxM1 insert was digested from pDNR-dual-FoxM1 vector and cloned in pCMV6 at EcoRI and HindIII sites.

Cell proliferation assay
Breast cancer cells were seeded in 96-well plates at a density of 5 × 10³ cells per well and after 20 to 24 hours of incubation, cells were treated either with DMSO alone (0.02%, vehicle control) or with varying concentrations of IB (0.5–5 μmol/L) in DMSO for additional 24, 48, and 72 hours in CO₂ incubator at 37°C. Cell viability was assessed by using CellTiter-Glo (Promega Inc.) assay.

Colony formation assay
200,000 cells per well were plated in 6-well plates and after 20 to 24 hours of incubation, cells were treated either with DMSO alone or with varying concentrations of IB (1–5 μmol/L) in DMSO for another 24 hours. Next, 1,000 cells per well were re-seeded in 6-well plates for additional 7 days until colonies were clearly visible. Colonies were fixed with 4% paraformaldehyde and visualized by staining with 1% crystal violet and wells were scanned using scanner. Visible colonies were counted using image analysis software.

Invasion and migration assays
Breast cancer cells were pre-treated with IB at different concentrations for 24 hours and subjected to invasion and migration assays as described previously (13, 14). For rescue experiments, breast cancer cells were pre-treated with IB for 3 hours followed by FoxM1 expression for 72 hours and then were subjected to migration and invasion assays.

Animal studies
Orthotopic xenograft study. All animal experiments were performed after obtaining UTHSCSA-IACUC approval and the animals were housed in accordance with the UTHSCSA's protocol for animal experiments. For experimental metastasis, model, MDA-MB-231-GFP-Luc cells (2 × 10⁶) were injected into the tail vein of athymic nude mice. Starting from 8 days after tumor cell injection, animals were randomized into two groups. Group 1 animals received DMSO and group 2 animals received IB weekly once for 3 weeks via tail vein. Group 2 animals initially received 1.5 mg/kg body weight of IB in the first week and 3 mg/kg body weight of IB for the remaining treatment period. The Xenogen Small-Animal Imaging System was used for subcellular imaging in live mice once a week. After 36 days, animals were sacrificed and lungs were fixed and analyzed for metastatic foci. Liver, spleen, and kidney tissues were also fixed and analyzed for toxicity studies.

Orthotopic xenograft model. For orthotopic tumor xenograft assays, 2 × 10⁶ MDA-MB-231 cells were mixed with an equal volume of matrigel and implanted in a mammary fat pad of 6-week-old female athymic nude mice as previously described (15, 16). Once tumors reached measurable size, mice were divided into control and treatment groups. The control group received vehicle (DMSO), and the treatment groups received IB conjugated with liposome-nanoparticle (3 mg/kg body weight in 20% intralipid) intravenously twice a week for 3 weeks. Tumor volumes and body weight were measured twice a week. After the 30th day, the mice were euthanized, and the tumors were isolated and processed for molecular and immunohistologic studies. Tumor volume was calculated by using the formula 0.5236L₁L₂², where L₁ is long axis and L₂ is the short axis of the tumor. At
the end of the experiment, mice were sacrificed, and tumors were excised, weighed, and fixed in buffered formalin for further analysis.

Ex vivo explants
Excised breast tumor and normal matched tissues were provided by a pathologist in accordance with UTHSCSA Institutional Review Board (IRB) approval (Control# HSC20120041H). Culture media consisted of RPMI-1640 supplemented with 10% FBS, 1% antibiotic/antimycotic solution, 0.01 mg/ml hydrocortisone, and 0.01 mg/ml insulin. A core piece of 8-mm tissue was dissected into 1 mm³ pieces and cultured on pre-soaked gelatin sponges (Johnson and Johnson, New Research) in 500 μl RPMI containing vehicle or nanoparticle conjugated-IB for 72 hours in 24-well plates. All explant tissues were cultured at 37°C in a 5% CO₂ incubator for 72 hours and were subsequently either formalin-fixed and paraffin-embedded or preserved for RNA isolation.

Ki-67 and TUNEL analysis
For IHC analysis, explants and xenograft tumor tissues were processed, paraffin-embedded and incubated with antibody against Ki-67 (#NB500, Novus) or subjected to apoptosis assay using the TUNEL Assay Kit (#G3250, Promega Inc.) as described previously (13). Ki-67- and TUNEL-positive cells were counted at ten arbitrarily selected fields at ×40 magnification. The proliferation/apoptotic index (per ×40 microscopic field) was determined as (number of Ki-67/TUNEL-positive cells × 100)/total number of cells.

Gene-expression profiling
Total RNA was isolated from MDA-MB-231 cells following treatment with vehicle and IB for 24 hours, respectively. RNA samples were further processed at the UTHSCSA Genomics Core for gene-expression profiling using Illumina Human HT-12 v4 Expression BeadChip following the manufacturer’s standard protocol (Illumina). Gene-expression data were quantified and normalized (quantile normalization) using the BeadStudio software (Illumina). Gene-expression data are publicly available at NCBI/GEO (GSE72363).

RNA and protein analyses
Total RNA extracted from tumors, normal tissues, cell lines, xenograft tumors, and explants were subjected to qRT-PCR analysis and Western blot analysis as described previously (13). Supplementary Table S1 lists primer sequences for all genes studied in the present study. Antibodies against β-actin (A3854) and Aurora kinase A (T6199) were purchased from Sigma-Aldrich. Antibodies against FOXM1 (sc-502), XRCC3 (sc-271714), EXO-1 (sc-19941), and GADD45-A (sc-797) were purchased from Santa Cruz Biotechnology. Antibody against PLK (#627701) was purchased from BioLegend and against NOX4 (NB110-58849) was purchased from Novus. Calnexin antibody was kindly provided by Dr. Hima Bansal, UTHSCSA (San Antonio, TX). Other antibodies, including SKP2 (#4358), cyclin D1 (#2978), E2 (#4132), CDK2 (#2546), CDK4 (#12790), p-JNK (#9251), total JNK (#9252), and NFκB p65 (#8242), were purchased from Cell Signaling Technology. RAD51 (#70005) antibody was purchased from BioAcademia.

Immunofluorescence
To determine DNA damage, immunofluorescence was performed with vehicle- and IB-treated cells using 53BP1 rabbit antibody (#A300-272AT; Bethyl Laboratories) as previously described (17).

Homologous recombination assay
The ISce1-based DR-GFP reporter assay was performed in cells following IB treatment (1, 2.5 or 5 μmol/L for 3, 12, or 24 hours) to evaluate frequency of double strand break repair by HR as described previously (18–20). For rescue experiments, cells were pre-treated with IB or vehicle control for 3 hours followed by transfection with control, FoxM1 expression plasmid and ISCe1 expression vector, respectively, for 48 hours.

Cell-cycle distribution and apoptosis assay by flow cytometry
Both cell-cycle distribution and AnnexinV/PI-positive cells were analyzed using flow cytometry as described previously (21).

Statistical analysis
All values and error bars in graphs are means ± SEM; respective n values are indicated in figure legends; P values are determined by two-tailed Student t tests.

Results
IB inhibits breast cancer cell viability
To address the effect of IB on breast cancer growth and progression, we first examined short- and long-term survival of IB-treated breast cancer cells. MDA-MB-231, MDA-MB-468, BT-549, SKBR3, and MCF-7 cells were treated with varying concentrations of IB (0–5 μmol/L) and cell proliferation was measured using CellTiter-Glo assay. IB treatment resulted in dramatically decreased breast cancer cell proliferation in a time and dose-dependent manner (Fig. 1A and Supplementary Fig. S1A). Interestingly, IB selectively targeted cancer cells without inducing any significant killing in normal human mammary epithelial cells (HMEC) that proliferate normally and maintain 24 hours doubling time (Fig. 1B). Next, we evaluated the long-term survival of IB-treated breast cancer cells. Indeed, pre-treatment with IB for 24 hours inhibited colony forming ability of breast cancer cells (Fig. 1C and Supplementary Fig. S1B). In addition to cell viability, IB treatment drastically reduced the migratory and invasive capabilities of the breast cancer cells (Fig. 1D and E and Supplementary Fig. S2A). To ensure that reduced migration and invasion of IB-treated breast cancer cells is not due to compromised viability, breast cancer cells were treated for a shorter duration (3 hours) and with lower concentration of IB that has minimum effect on cell viability followed by 12 hours of migration and invasion assays (Supplementary Fig. S2B). To further substantiate these findings, we performed Matrigel/3D invasion assay and showed that IB dramatically reduced the invasion of breast cancer cells (Supplementary Fig. S2C).

Therapeutic potential of IB and feasibility of systemic delivery
To test IB’s tumor suppressor and metastasis suppressor-like activity in vivo and to address whether IB can be systemically delivered, we used two tumor xenograft models. First, we tested the efficacy of IB in an orthotopic xenograft model. MDA-MB-231 cells were implanted into the mammary fat pad of mice followed by treatment with specified concentrations of IB or vehicle control.
after a week when tumors reached measurable size. For systemic delivery of IB, we conjugated IB with liposome-nanoparticle (Nano-IB; 3 mg/kg body weight) and injected (intravenous) twice a week for 3 weeks. Interestingly, the systemic delivery of Nano-IB caused marked inhibition of mammary tumor growth when compared with vehicle control (Fig. 2A). To investigate whether IB can also inhibit tumor progression in vivo, we used a breast cancer experimental lung metastasis model, which recapitulates extravasation, seeding, and growth of metastatic process. MDA-MB-231 cells stably expressing GFP-luciferase reporters were injected through tail vein and mice were treated either with IB or vehicle control after 8 days when metastasis foci were first detected in the lungs. Intravenous (tail vein) injection of Nano-IB (1.5 mg/kg body weight of IB in the first week and to 3 mg/kg body weight of IB for the remaining treatment period for 4 weeks) into tumor-bearing mice resulted in the inhibition of metastasis, whereas vehicle-injected mice showed increased metastasis (Fig. 2B–D and Supplementary Fig. S3A).
Although vehicle-treated mice showed aggressive multifocal metastasis outside of the vessel in the lung and with metastatic tissues attached to the diaphragm, IB-treated mice showed contrast effect as evident from the significant reduction or elimination of lung metastases (Fig. 2D). Importantly, analysis of tissues from IB-treated mice from both tumor models revealed that IB had no obvious toxicity because mice did not exhibit any weight loss and liver, kidney, spleen, or lung sections from the IB-treated group showed no evidence of hepatotoxicity and kidney toxicity in both experimental metastasis and orthotopic transplant models.

Figure 2.
IB treatment inhibits breast cancer growth and metastasis in vivo. A, bar graph showing mean tumor volume for vehicle or IB-conjugated (n = 10) nanoparticle-treated mice. MDA-MB-231 cells were subcutaneously implanted into mammary fat pad of athymic nude mice. After tumors reach approximately 100 mm³, mice were treated with either vehicle- or IB (3 mg/kg body weight)-conjugated with nanoparticle every 5 days for 25 days. B, live bioluminescence images of mice treated with vehicle- or IB-conjugated with nanoparticle using the Xenogen In Vivo Imaging System (IVIS; Xenogen). C, tumor metastasis volume (mean ± SEM; n = 5) was assessed starting from day 8 until animals were sacrificed at day 36. Using ROI analysis, tumor light intensity was calculated in photon/s, which corresponds with the number of live cells in vivo. D, representative hematoxylin and eosin (H&E)-stained lung sections showing metastatic foci from each group. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus control group, t test. E, H&E-stained sections of livers and kidneys from nano-IB-treated mice show no signs of hepatotoxicity and kidney toxicity in both experimental metastasis and orthotopic transplant models.
Figure 3.
IB is safe and effective against human breast cancers. A, left, schematic of the tumor tissues from breast cancer patients cultured as explants on gelatin sponge and growth media as described in Materials and Methods section; right, IHC using antibody against Ki-67 on freshly frozen breast cancer tissue (specimen) and explant cultured for 72 hours showing comparable tissue architecture. B and C, IHC using antibody against Ki-67 (B) and TUNEL assay (C) on breast cancer and normal adjacent tissue explants treated with either nano-vehicle or nano-IB for 72 hours. Representative photographs are presented at ×40. Bar graphs show average number of Ki-67- and TUNEL-positive cells derived from randomly selected 10 microscopic fields from each of three different explants from each of four breast cancer patients. Ki-67 score was defined as the percentage of positively stained cells among the total number of malignant cells scored. Scoring was done in whole tumor section and not limiting to most obvious positive cells; *, P < 0.01; **, P < 0.001; ***, P < 0.0001 versus control group, t test.
Figure 4.
IB inhibits expression of cell cycle- and DNA damage repair-associated genes. A, heat map representing microarray analysis on total RNA isolated from vehicle- and IB-treated MDA-MB-231 cells. The light color indicates decrease and the dark color indicates increase in mRNA levels. B, real-time qPCR validation of highly altered genes (obtained from microarray analysis) in vehicle control- and IB-treated MDA-MB-231 cells using gene-specific primers. The relative expression of each gene was quantified by measuring Ct values and normalized with GAPDH. C, Western blot analysis of MDA-MB-231 and MDA-MB-468 cells treated with either vehicle or IB (5 μmol/L) for 24 hours using antibodies against indicated proteins. Membranes were reprobed with different antibodies and with β-actin, which served as a loading control. D and E, real-time qPCR analysis of selected IB target genes on total RNA isolated from vehicle control- or IB-treated orthotopic xenograft tumor tissues (n = 3; D) and tumor explants from breast cancer patients (n = 4; E) using gene-specific primers. F, histogram showing cell-cycle distribution of vehicle- and IB-treated MDA-MB-231 and MDA-MB-468 cells. Breast cancer cells were treated with vehicle or IB for 72 hours, stained with PI and analyzed for cell-cycle distribution by flow cytometry. The data shown are mean ± SEM of three samples for each treatment and represent three independent experiments. G, Western blot analysis of MDA-231 and MDA-468 cells treated with vehicle or IB (5 μmol/L) using antibodies against cyclin D1, cyclin E2, CDK2, CDK4, and GADD45A. Membranes were reprobed with β-actin for loading control. H, Western blot analysis of vehicle or IB-treated MDA-MB-231 and MDA-MB-468 cells using antibodies for p-JNK and total JNK. β-actin was used as a loading control. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001 versus control group, t test.
suggest that IB may serve as a potent therapeutic for treating both primary and metastatic breast cancers with negligible toxicity.

IB is safe and effective against human breast cancers

Having established the therapeutic efficacy of IB in mouse models, we asked whether IB is indeed a safe and viable therapeutic alternative for treating breast cancer patients. To address this, we tested the efficacy of IB in ex vivo explants using tumor tissues collected from breast cancer patients. Our results show that ex vivo explants recapitulate the structural complexity and individual heterogeneity of human breast cancers, indicating that explants can be used to assess the therapeutic response of cancer treatment drugs (Fig. 3A). Interestingly, Nano-IB reduced the growth of tumor cells as revealed by dramatically reduced Ki-67 staining and increased apoptosis in explants from breast cancer patients (Fig. 3B and C and Supplementary Fig. S4). Importantly, IB did not affect the growth of the normal cells as revealed by comparable Ki-67 levels and absence of apoptotic cells in untreated and Nano-IB–treated explants from normal adjacent mammary tissues (Fig. 3B and C). These findings are highly significant as they indicate that explants recapitulate the microenvironment of human tumors (22), and therefore, can serve as a bona fide clinical model to determine therapy response in patients. Furthermore, it underscores the therapeutic potential of IB for treating breast cancers in most physiologically relevant milieu.

IB targets genes associated with cell-cycle progression and DNA damage surveillance pathway

To understand the mechanism by which IB may inhibit breast cancer growth and progression, we performed gene-expression analyses on breast cancer cells treated with or without IB. Interestingly, gene ontology enrichment analysis using DAVID of differentially expressed genes revealed that genes involved in the cell-cycle and DNA repair pathways were highly enriched (Supplementary Table S2). Examples of those genes included FoxM1, cell division cycle 25 (Cdc25), stathmin1 (Stmn1), aurora kinase (Aurak), polo-like kinase 1 (Plk1), CyclinD1, Skp2, Eso1, and Xrc3 that are known to play important role in cell-cycle progression and DNA damage surveillance or repair (7–10, 23–26; Fig. 4A and Supplementary Fig. S5A). To confirm our microarray results, we performed gene-expression analyses on breast cancer cells treated with or without IB. Interestingly, gene ontology enrichment analysis using DAVID of differentially expressed genes revealed that genes involved in the cell-cycle and DNA repair pathways were highly enriched (Supplementary Table S2). Examples of those genes included FoxM1, cell division cycle 25 (Cdc25), stathmin1 (Stmn1), aurora kinase (Aurak), polo-like kinase 1 (Plk1), CyclinD1, Skp2, Eso1, and Xrc3 that are known to play important role in cell-cycle progression and DNA damage surveillance or repair (7–10, 23–26; Fig. 4A and Supplementary Fig. S5A). To confirm our microarray results, we...
determined the target gene expression at the RNA and protein levels. IB treatment resulted in significant inhibition of target genes both at the RNA and protein levels (Fig. 4B and C). To further substantiate these findings, we determined the levels of IB-targeted genes in our tumor xenograft tissues and ex vivo explants from breast cancer patients. As expected, the levels of target genes were significantly reduced in IB-treated tumors and explants when compared with vehicle-treated tumors (Fig. 4D and E). We also determined the levels of these genes in breast cancer patients. Our analysis revealed that these genes are overexpressed in tumor tissues from breast cancer patients when compared with normal control tissues (Supplementary Fig. S5B).

Previous reports have shown that IB inhibits invasion of glioma and head and neck squamous cell carcinoma by reducing the levels of reactive oxygen species (ROS), NADPH oxidase 4 (NOX4), and NF-κB in breast cancer cells. Interestingly, we did not observe any significant difference in NF-κB levels in IB-treated breast cancer cells when compared with vehicle-treated cells (Supplementary Fig. S5C). Although IB treatment resulted in reduced ROS and NOX4 levels in some breast cancer cells, the effect was not consistently observed in all breast cancer cell lines (Supplementary Figs. S5D and S5E). Our findings suggest that NOX4-ROS-NF-κB signaling cascade may not be the predominant mechanism by which IB affects breast cancer growth.

Because several cell cycle–associated proteins were altered in IB-treated cells, we reasoned that IB may affect a specific phase of cell cycle progression. Interestingly, IB treatment resulted in S-phase arrest when compared with control (Fig. 4F). The observed accumulation of cells in S-phase was accompanied by a concomitant decrease in G2–M phase cell population in IB-treated breast cancer cells (Fig. 4F). Consistent with this, FoxM1, which is known to regulate both G1–S and G2–M progression via several proteins, including cyclin D1, CDC25, AURAK, STM1, and PLK1 (27), showed significantly reduced level in IB-treated breast cancer cells (Fig. 4C and G). To further substantiate these findings, we determined the levels of cyclin-dependent kinases (CDK) and other cyclins, which are the key regulators of cell-cycle progression. By qRT-PCR and Western blot analyses, we observed that IB treatment resulted in the inhibition of cyclin E2 as well as CDK2 and CDK4 (Fig. 4G and Supplementary Fig. S6A). In addition to CDKs and cyclins, we tested the levels of growth arrest and DNA damage-induced protein GADD45A, which is required for proper S-phase control and checkpoints (28). IB-treated breast cancer cells showed increased levels of GADD45A when compared with vehicle-treated cells (Fig. 4G). Because GADD45 is known to activate c-Jun N-terminal kinase (JNK)–dependent programmed cell death (29), we examined the effect of IB on JNK and apoptosis. Breast cancer cells treated with IB showed increased levels of JNK and apoptosis when compared with vehicle-treated cells as revealed by Western blot analysis and annexin V staining followed by FACS analysis, respectively (Fig. 4H and Supplementary Fig. S6B).

**IB inhibits breast cancer cell's ability to repair DNA by HR**

Given that GADD45a is known to be activated in response to damage, we hypothesized that IB may be inducing DNA damage, either directly or indirectly. To address this, we assessed the amount of DNA strand breaks induced by IB by determining the levels of p53-binding protein (53BP1) nuclear foci (30). Immunofluorescence analysis showed significantly increased number of 53BP1 foci in IB-treated breast cancer cells when compared with vehicle-treated breast cancer cells (Fig. 5A). Because IB can induce apoptosis, we reasoned that IB-induced DNA strand break may not be properly repaired. Considering the accumulation of cells in S-phase, we examined HR repair activity, a key DNA strand break repair pathway during S- and G2-phases of the cell cycle (10). To address this, first, we assessed the kinetics of repair by examining the levels of RAD51, which is a FoxM1 transcriptional target (31) and is known to play critical role in HR (32). Western blot analysis showed significantly reduced levels of RAD51 in breast cancer cells treated with IB (Fig. 5B and Supplementary Fig. S6C) supporting the notion that IB may inhibit DNA repair by HR. To confirm these findings, we performed a functional assay to monitor HR events. We used IScel-based DR-GFP reporter assay that measures the frequency of double strand break repair by HR (18–20). FACS analysis showed significantly reduced number of GFP-positive cells in IB-treated cells stably expressing DR-GFP reporter (Fig. 5C). Interestingly, we observed a time- and dose-dependent inhibition of GFP in IB-treated cells (Fig. 5C). These findings suggest that inhibition of FoxM1/FoxM1-associated signaling and HR-mediated DNA repair may be one the specific mechanisms by which IB affects growth and progression of breast cancers in particular and FoxM1-overexpressing cancers in general. Consistent with this, IB treatment resulted in reduced levels of FoxM1 and RAD51 and subsequent inhibition of long-term growth of lung, cervical, and prostate cancer cells that express high levels of FoxM1 (Supplementary Fig. S7).

**Mechanism of regulation of IB targets**

To begin to address the mechanism by which IB may regulate its targets, we focused on FoxM1 because several IB target genes, including Skp2 (33), Etx1 (31), Rad51 (31), Smn1 (34), and Aurora (27), are reported to be transactivated by FoxM1. The dramatically reduced expression of FoxM1 in IB-treated breast cancer cells prompted us to test whether IB may directly interact with FoxM1. We explored the surface of FoxM1 using SiteMap tool from Schrodinger suite to identify potential landing spots for IB. Interestingly, we found at least one potential binding site for IB. Our results reveal that residues Met296-Tyr317, which form a β-hairpin, are major contributors to the cavity, whereas residues Arg254-Arg256 complement its structure. The docking of IB showed considerable complementarity between shape of the cavity and topology of the IB (Fig. 6A). Our interaction model suggests that dibenzazepine part of the IB binds to the carboxylic group of Glu298, whereas the die moieties lands on side chains of Val296, Glu298, His311, Pro312, and the cα-helix H3 that binds to the DNA groove (Fig. 6A). Because FoxM1 has flexible, it is likely that specific binding of a small molecule (like IB) will constrain this flexibility. Given that interdomain interactions (DNA-binding forhead domain, N-terminal auto-inhibitory domain, and cα-terminus transcriptional transactivation domain) are critical for FoxM1 stabilization and activity. Moreover, FoxM1–DNA interaction is reported to be weak and any minor adjustment to FoxM1 structure (such as
IB inhibits breast cancer growth and progression by directly interacting and inhibiting FoxM1 activity. A, a model of IB–FoxM1 complex, left, FoxM1 shown as solvent accessible surface to highlight complementarity of shapes of the binding site and IB (ball and sticks). DNA shown in cartoons; right, same as left with protein shown in ribbons. FoxM1 residues that participate in interaction are shown in sticks. B, FoxM1 rescues IB-mediated inhibition of HR. Flow-cytometry analysis showing level of GFP-positive cells reflecting HR events in cells treated with vehicle, IB or IB-pretreated and FoxM1-transfected cells (IB + FoxM1). DAPI-based homologous assay was done as described in Fig. 5. C, photomicrographs of migrated (top) and invaded (bottom) MDA-MB-231 cells treated with vehicle or IB (1 μmol/L) or pre-treated with IB (1 μmol/L) and transfected with FoxM1. D, bar graphs show number of migrated and invaded cells counted microscopically in 10 different fields per filter. The data shown are mean ± SEM of three independent experiments; **, P < 0.01; ***, P < 0.001 versus control group, t test. E, a model showing IB mode of action. FoxM1 is a proto-oncogene that is known to play a critical role in cell cycle as well as DNA repair by HR. We posit that IB inhibits breast cancer growth and progression by directly interacting and inhibiting FoxM1 and subsequently its transcriptional targets, including Skp2, Exo1, CyclinD1, Plk1, and Aurak, that are known to play important role in cell-cycle progression and DNA repair. Because DNA strand breaks often occur during S-phase as a result of replication fork collapse, which should be repaired before mitosis by HR during S- and G2-phases, we propose that reduced FoxM1 expression resulting in reduced RAD51 and EXO1 levels as well as S-phase arrest is causal in IB-induced inhibition of DNA repair process.
interference with β-hairpin flexibility by IB) may affect both FoxM1 transactivation function as well its stability. There are two lines of evidence that further support the notion that interaction of IB with FoxM1 will result in FoxM1 transcriptional and translational instability. First, FoxM1 is reported to induce cyclin D1 transcription (27), whereas cyclin D1–CDK4 is reported to phosphorylate and stabilize FoxM1 protein (35). Therefore, it is likely that inhibition of FoxM1 by IB results in reduced cyclinD1–CDK4 levels (Fig. 4), which in turn affect FoxM1 levels. Second, FoxM1 transcriptional target Skp2 is reported to activate ATM via Mre11/Rad50/Nbs1 (MRN) complex and ATM is known to transcriptionally activate FoxM1 (8, 9). Therefore, IB–FoxM1 interaction resulting in FoxM1 inhibition may result in Skp2 and subsequently ATM downregulation, which in turn may affect FoxM1 transcription. Indeed our expression and Western blot analyses revealed that Skp2, Nbs1, and ATM levels were significantly reduced in IB-treated cells (Fig. 4 and Supplementary Figs. SSA and S6D). To further substantiate these findings, we performed rescue experiments. Transfection of FoxM1 expression vector rescued the IB’s inhibitory effect on HR-mediated DNA repair as revealed by IScel-based DR-GFP reporter assay (Fig. 6B). In addition, FoxM1 expression rescued the migration and invasion of breast cancer cells pre-treated with IB (Fig. 6C and D). Though these results indicate a possible FoxM1–IB interaction, future in-depth structural and biophysical studies will be required to unambiguously establish IB binding to FoxM1 protein.

Discussion

Our results reveal that IB may serve as a novel cancer treatment agent with negligible toxicity as it selectively inhibited breast cancer growth and metastasis without targeting normal mammary epithelial cells. We demonstrate that IB inhibits breast cancer growth and progression by regulating genes involved in DNA damage repair.

The presence of DNA damage from dysregulated growth control and upregulated DNA damage response are two common features that characterize cancer development as well as progression (2). Therefore, overexpression of factors that are capable of inducing both DNA damage and DNA repair responses may promote cancer cell survival and successful targeting of these factors will yield to promising therapeutic outcomes. One such factor is FoxM1, which is highly expressed in breast cancer patients (12) and is reported to regulate DNA damage response to support the uncontrolled proliferation associated with cancer (7, 36, 37). The advantage conferred by overexpression of FoxM1 by increasing DNA repair capacity is highlighted by the finding that cells depleted of FoxM1 have increased DNA strand breaks (31). Because FoxM1 is a transcriptional activator, it is likely that FoxM1 regulates a network of genes to mediate DNA damage sensing and repair in breast cancer cells (33). An example of such a gene includes Skp2, which is a transcriptional target of FoxM1 and is significantly downregulated in IB-treated cells. Skp2 plays a critical role in the cellular response to DNA damage and the maintenance of chromosome integrity by activating ATM via Mre11/Rad50/Nbs1 (MRN) complex that results in the recruitment of ATM to the DNA damage foci to initiate HR-mediated repair (8, 9). Another factor that may play an equally important role in IB-mediated and FoxM1-dependent HR event is cyclin D1 (26), which is highly overexpressed in many human cancers, including breast cancers (26). Consistent with this, loss of cyclin D1 expression in cancer cells has been shown to inhibit the recruitment of RAD51 to damaged DNA and consequently inhibit the DNA repair by HR (38). Because Rad51 is a direct transcriptional target of FoxM1 (31), it is functionally linked with Skp2 (39) and physically and functionally interacts with cyclin D1 (26), it is likely that the FoxM1–cyclin D1–Skp2–RAD51 signaling cascade is a central component mediating IB’s effect on HR (Fig. 6E). In addition to FoxM1–RAD51/Skp2 signaling cascade, Xrcc3 is another gene that may mediate DNA repair events in IB-treated breast cancer cells as Xrcc3 is known to participate in HR to maintain chromosome stability and repair DNA damage (23). Importantly, increased Xrcc3 levels are associated with increased HR (25) and variants of Xrcc3 were recently shown to be associated with increased risk for breast cancer patients (40).

DNA repair and replication are closely linked processes (10); therefore, we wondered whether increased DNA strand breaks in response to IB may be due to aberrant replication and increased replication stress. Supporting this, our results revealed that Exo1, which is a FoxM1 transcriptional target (31), is significantly downregulated in IB-treated breast cancer cells. In addition to being involved in HR, EXO1 processes single-stranded DNA arising due to stalled and collapsed replication forks (10). Additional evidence for the involvement of replication errors following IB treatment comes from our observation that level of RAD51, which prevents replication error-associated DNA strand breaks and has been shown to promote restart/repair of stalled/collapsed fork (41), is significantly reduced in IB-treated breast cancer cells. Because DNA strand breaks often occur during S-phase as a result of replication fork collapse (10), it is likely that reduced FoxM1 expression resulting in reduced RAD51 levels and S-phase arrest is causal in IB-induced inhibition of DNA repair process. In summary, IB mediates its antitumor growth and metastasis functions by inhibiting critical DNA repair genes such as FoxM1 and subsequently HR-mediated DNA repair (Fig. 6E). FoxM1 is highly expressed in breast cancers (12) and are known to play a causal role in promoting breast cancer growth and metastasis. In addition, FoxM1 is known to transcriptionally regulate the expression of Vegf, which is an important angiogenic factor that promotes growth, invasiveness, and metastasis of several tumors (42–46). Therefore, pharmacologic inhibition of FoxM1 expression and its associated signaling is of significant clinical interest and holds great promise for treating cancer. Collectively, this study provides a strong rationale for developing IB as a novel therapeutic for treating cancer in general and breast cancers in particular and establishes several aspects that are needed for future drug development.

Disclosure of Potential Conflicts of Interest

J.L. Arbiser holds ownership interest (including patents) in ABBY. No potential conflicts of interest were disclosed by the other authors.

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