Selective Inhibition of SIN3 Corepressor with Avermectins as a Novel Therapeutic Strategy in Triple-Negative Breast Cancer

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Abstract

Triple-negative breast cancers (TNBC) lacking estrogen, progesterone, and HER2 receptors account for 10% to 20% of breast cancer and are indicative of poor prognosis. The development of effective treatment strategies therefore represents a pressing unmet clinical need. We previously identified a molecularly targeted approach to target aberrant epigenetics of TNBC using a peptide corresponding to the SIN3 interaction domain (SID) of MAD. SID peptide selectively blocked binding of SID-containing proteins to the paired α-helix (PAH2) domain of SIN3, resulting in epigenetic and transcriptional modulation of genes associated with epithelial–mesenchymal transition (EMT). To find small molecule inhibitor (SMI) mimetics of SID peptide, we performed an in silico screen for PAH2 domain–binding compounds. This led to the identification of the avermectin macrocyclic lactone derivatives selamectin and ivermectin (Mectizan) as candidate compounds. Both selamectin and ivermectin phenocopied the effects of SID peptide to block SIN3–PAH2 interaction with MAD, induce expression of CDH1 and ESR1, and restore tamoxifen sensitivity in MDA-MB-231 human and MMTV-Myc mouse TNBC cells in vitro. Treatment with selamectin or ivermectin led to transcriptional modulation of genes associated with EMT and maintenance of a cancer stem cell phenotype in TNBC cells. This resulted in impairment of clonogenic self-renewal in vitro and inhibition of tumor growth and metastasis in vivo. Underlining the potential of avermectins in TNBC, pathway analysis revealed that selamectin also modulated the expression of therapeutically targetable genes. Consistent with this, an unbiased drug screen in TNBC cells identified selamectin-induced sensitization to a number of drugs, including those targeting modulated genes.

Introduction

Triple-negative breast cancer (TNBC), as defined by the absence of estrogen receptor (ER) and progesterone receptor (PR) and lack of overexpression of HER2, is an aggressive subtype comprising 10% to 20% of breast cancer incidences (1). TNBC patients have a shorter median survival time after relapse (~18 months) and more readily develop chemoresistant disease (2). In contrast to advances in the treatment of ER-positive and HER2-positive tumors, TNBC remains a disease with poor prognosis and limited treatment options (3). Molecular profiling suggests that while approximately 75% of TNBC tumors exhibit a basal-like phenotype (4) and commonly harbor BRCA1 and TP53 mutations (5), the subtype can, nevertheless, also be considered a heterogeneous entity (6).

In addition to underlying genetic factors, epigenetics are increasingly recognized as playing an important role in the phenotypic and molecular heterogeneity of TNBC, and aberrant DNA promoter methylation and histone modification can lead to the deregulated expression of key TNBC-associated genes (7, 8). Deregulated epigenetics have also been functionally linked to processes critical to breast cancer tumorigenesis such as epithelial–mesenchymal transition (EMT) that is necessary for the tumor invasion–metastasis cascade and acquisition and maintenance of a cancer stem cell phenotype (9). Epigenetic reconfiguration in cancer cells is brought about by aberrant recruitment of chromatin-modifying complexes that perform diverse activities. Important facilitators of epigenetic deregulation are the SIN3A, which has been implicated in breast cancer pathogenesis (10–12), and SIN3B multidomain adaptor proteins. SIN3A and SIN3B, which lack intrinsic DNA-binding activity, serve as molecular scaffolds that bridge interactions between chromatin regulators and sequence-specific DNA-binding transcription factors. The multi-protein repressor complexes that are generated control cell proliferation and differentiation (13–15). Both SIN3A and SIN3B...
are characterized by a unique arrangement of four paired amphipathic α-helix (PAH1–PAH4) motifs (Fig. 1). Despite sharing sequence homology, the different PAH domains mediate specific interactions with SIN3A and SIN3B, with the second PAH repeat (PAH2) reported to bind a functionally diverse group of proteins, including the Mad family of repressors, that contain a motif known as a SIN3 interaction domain (SID, Fig. 1; ref. 13).

We previously reported that inhibition of SIN3A to interact with partner proteins via its PAH2 domain induced differentiation and inhibited tumorigenicity in TNBC (10). This was achieved through the use of a peptide corresponding to the SID domain of MAD, which binds to SIN3-PAH2 and competes with PAH2 partner proteins. In this study, we sought to identify small molecule inhibitor (SMI) mimetics of SID. We describe an in silico screen of small molecule drugs, leading to the identification of two members of the avermectin family of macrocyclic lactones, selamectin and ivermectin, as clinical candidate compounds for the treatment of TNBC.

Materials and Methods

Cell lines and reagents

The metastatic mammary 4T1 tumor cell line (Cat# CRL-2539) and human MDA-MB-231 breast adenocarcinoma cell line (Cat# HTB-26) were purchased from the ATCC. The MDA-MB-231-Luc-D3H2LN Bioware (D3H2LN) cell line (16) was purchased from PerkinElmer (Cat# 119369). The mouse mammary tumor MMTV-Myc cell line has been previously reported (17, 18). Cell lines were authenticated by short tandem repeat (STR) profiling in accordance with the standard ASN-0002-2011 in April 2015 (DDC Medical). 4T1 cells were maintained in RPMI supplemented with 10% FBS and 1% antibiotic-antimycotic solution. Ivermectin was purchased from Sigma. Selamectin was synthesized in-house.

NMR spectroscopy

The PAH2 domain of mouse SIN3A was expressed in Escherichia coli BL21 (DE3) cells in the pET22b vector (Novagen) as previously described (19). Isotope-labeled protein was prepared from cells grown on a minimal medium containing 15NH4Cl with or without 13C-glucose in H2O. The protein was purified by nickel-IDA affinity chromatography, followed by thrombin cleavage to remove an N-terminal poly-His-tag. The protein solution for NMR study contained the SIN3A PAH2 domain at 0.1 mmol/L in 100 mmol/L phosphate buffer, pH 6.5, with 5 mmol/L perdeuterated DTT and 10% 2H2O. All NMR spectra were collected at 25 °C in a buffer consisting of 50 mmol/L Tris-HCl at pH 8.0, containing 150 mmol/L NaCl, 20% DMSO, and 10% 2H2O on NMR spectrometers of 800 or 600 MHz. The 1H, 13C, and 15N resonances of the protein were assigned by triple-resonance NMR spectra collected with a 13C/15N-labeled SIN3A PAH2 domain (20).

In silico chemical screening

Computational screening of chemical compounds was conducted using an ensemble of 20 NMR structures of the SIN3A PAH2 domain (19). For the virtual screening, three of these structures were selected: the one whose root-mean-square derivations (RMSD) with the rest are the smallest (i.e., the structure in the middle of the ensemble) and a pair of structures with RMSDs that were the largest among all pairs (i.e., the two extemes). The screening was performed on a collection of 2000 FDA-approved small molecule drugs (Microsource Discovery Systems). Two programs were used for virtual screening: Autodock-4, combined with AutoDockTools to set up the target structure file (21) and eHiTS (22). The screening and the analysis of the results were driven by the script set Full-screen and the program Dockxes (23). Docking was focused on the surface region of the PAH2 domain where the SID-containing protein binds, between the two longer helices near the end of the two shorter helices. The 20 top scoring ligands were tested for their binding to the SIN3A PAH2 domain experimentally by NMR spectroscopy.

GST pull-down assay

The experimental procedures were performed as reported (10). Briefly, GST and GST-SIN3A PAH2 were prepared in Escherichia coli according to the standard procedures. MAD was immunoprecipitated from MDA-MB-231 cell lysates using rabbit polyclonal anti-MAD (C19) antibody (Santa Cruz Biotechnology). Following vehicle, compound, or peptide treatment, bait protein (GST or GST-PAH2) and prey protein (immunoprecipitated MAD protein) were preincubated separately for 1 hour at 4 °C and subjected to GST pull-down assay procedures. The protein was visualized by immunoblotting using mouse monoclonal anti-MAD (F-1) antibody (Santa Cruz Biotechnology). Tat-SID peptide consists the amino acid sequence 5–24 of MAD protein and HIV type 1 Tat arginine-rich RNA-binding motif as a leader sequence that has been mutated (RRR–GGG) to improve nuclear entry (10).

Immunoblot analysis

Immunoblotting was performed according to standard procedures and visualized using ECL plus Western Blotting detection reagent (Life Technologies). Blots were probed with either anti-ERα antibody (Cell Signaling Technology, #8644) or E-cadherin antibody (Cell Signaling Technology, #3195). Anti-GAPDH antibody (Ambion, #4300) was used as a loading control.
Figure 2. Small molecule mimetics of MAD SID block interaction with SIN3 PAH2. A, mammalian two-hybrid analysis of 293T cells transfected with GAL4UASX5-Tk-Luc reporter (RE), GAL4DBD-SIN3B and VP16AD-MAD. Cells were treated for 24 hours with 15 μmol/L SID peptide or compounds (10 μmol/L) as indicated. Cells were also cotransfected with pSID or pSIDMUT expressing wild-type or mutated SID peptide, respectively, as indicated. Posttreatment cells were counted and measured. Shown is cell number ratio (treated/vehicle, blue) and luciferase activity relative to Renilla control (red). Cell number ratio and relative luciferase activity values were normalized to the value for untreated GAL4DBD-SIN3B and VP16AD-MAD. B, proximity ligation assay (PLA) analyzing SIN3A–MAD interactions following treatment for 24 hours with 15 μmol/L Tat-SID peptide or compounds (10 μmol/L) as indicated. Shown are numbers of signals (red dots) per cell relative to untreated control representing SIN3A-MAD interactions (see Supplementary Fig. S2A). Signal generation is dependent on close proximity (<40 nm) of antibody conjugated PLA probes that have been ligated, amplified, and detected with complementary fluorescent probes. (Continued on the following page.)
Mammalian two-hybrid assay

Experiments were performed as previously described (10). Cells were treated with 15 μM/L SID peptides or 10 μM/L compounds 24 hours after transfection. pSID and pSIDmut, GAL4, pVP16-MAD, pGALO- SIN3B, GAL4X5-Tk-luc have been previously described (10).

Duolink proximity ligation assay

MDA-MB-231 cells were plated onto coverslips in 12-well plates and treated with vehicle, Tat-SID or compound. Cells were stained with monoclonal SIN3A (sc-5299) 1:100 and polyclonal MAD (sc-222) 1:1,000 according to the manufacturer's instructions (Olink Bioscience) except utilizing 1% BSA in PBS as a blocking reagent and carrying out initial washes in PBS. Cells were counterstained in To-pro-3-iodide in PBS, 3 × 5 minute washes at room temperature, and mounted in Vectashield mounting medium (Vector Labs). Images were collected on a Zeiss LSM700 confocal microscope and Duolink software was utilized to quantitate signals.

Immunofluorescence

MDA-MB-231 cells were plated onto coverslips in 12-well plates and treated with vehicle or compound. Cells were stained with monoclonal E-cadherin (67A4) antibody (sc-21791) 1:50 overnight at 4°C according to standard protocols. Secondary antibodies (dilution 1:200 in 1% normal goat serum/PBS) were added for 1 hour and then washed. Cells were counterstained in To-pro-3-iodide in PBS, 3 × 5 minute washes at room temperature, and mounted in Vectashield mounting medium (Vector Labs). Images were collected on a Zeiss LSM700 confocal microscope.

Real-time quantitative PCR

RNA was prepared using RNeasy Plus Mini Kit (Qiagen). For real-time quantitative PCR, 1 μg RNA was reverse transcribed with SuperScript First-Strand Synthesis System (Invitrogen). Fifty to 250 ng of resulting cDNA were amplified and analyzed in real time with QuantiTect SYBR Green PCR Kit (Qiagen). Results are represented as fold changes according to the manufacturer’s instructions. Each comparison was made using triplicate reactions and in at least three experiments. Primer sequences are detailed in Supplementary Table S1.

Modified Boyden chamber invasion assay

A total of 5 × 10⁴ cells were seeded onto the top well of a 24-well Matrigel-coated filter insert (BD Biosciences) and 0.1% FBS was used as chemoattractant in the bottom chamber. The numbers of invaded cells were counted after 24 hours, stained with HEMA 3 stain set (Fisher Scientific), and the percentage of invasive cells calculated. A total 5 fields were counted per filter under 400× magnification using a Nikon Eclipse E600 microscope.

Estrogen receptor reporter luciferase assay

Luciferase reporter activities were measured using the Dual Luciferase reporter assay system (Promega) according to the manufacturer's instructions. To determine ERα transcriptional activity, cells grown in phenol-red free DMEM/F12 growth medium containing 5% charcoal-stripped FBS were pretreated with 1 μM/L selamectin (or ivermectin) for 4 days. Cells were split, replated, and cotransfected with 0.3 μg pGL3-ERE-responsive reporter plasmids and 0.1 μg pRL-Tk–expressing Renilla luciferase (24) prior to vehicle or drug treatment in phenol-red free DMEM/F12 containing 1% charcoal-stripped FBS for 48 hours. Firefly luciferase activity was normalized to Renilla luciferase activity and demonstrated as ERα reporter activity.

Tumorsphere formation assay

Cells were pretreated for 7 days in two-dimensional culture in growth medium. For tumorsphere cultures, cells (10,000 cells per well) were replated in ultra-low attachment plates (Corning Costar) for a further 7 days without treatment. Tumorsphere media contained DMEM/F12 supplemented with B27 growth supplement minus Vitamin A (Invitrogen), recombinant EGF (20 ng/mL, Invitrogen) and 1% antibiotic–antimycotic solution (Invitrogen). Cells were counted and spherical colonies with a diameter of greater than 50 μm considered tumospheres (25). Other cell aggregates were excluded.

Aldehyde dehydrogenase assay

D3H2LN cells were cultured and treated with vehicle or drugs as described for the tumorsphere formation assay. Tumospheres were dissociated using cell dissociation buffer (Invitrogen) and tested for ALDH activity (2 × 10⁵ cells/sample), using the Aldefluor assay (Aldegen) according to the manufacturer’s instructions.

In vivo animal studies

All procedures were approved by and performed according to IACUC procedures of the Icahn School of Medicine at Mount Sinai (New York, NY). For ex vivo treatments, MMTV-Myc cells were pretreated daily with selamectin (1 μM/L) in vitro for 7 days. After pretreatment, 200,000 cells were injected into inguinal mammary fat pads of 6- to eight-week-old female transgenic FVB/N mice (Jackson laboratory, n = 10). Tumor diameters were measured with a caliper every other day and volumes (mm³) were calculated according to the formula \( V = \frac{d^2 \times D}{2} \) (mm³), \( d = \) short diameter, \( D = \) long diameter. Mice were sacrificed 12 days after cell inoculation. For in vivo treatments, MMTV-Myc cells...
(50,000 cells per mouse) were injected into the left flank. From next day, mice were treated daily with 1.6 mg/kg/d selamectin by intraperitoneal injection for 15 days when tumor volume in the vehicle group reached 1,000 mm³. Mice were euthanized using CO₂. Tumor volume was calculated as indicated and dissected tumors were weighed. The lungs were removed from mice and fixed with Bouin fixative solution so that the number of lung metastases at the surface was counted.

**Lung metastasis dissemination studies**

BALB/c mice were inoculated subcutaneously with 1 × 10⁴ 4T1 cells (triple-negative breast cancer mouse model; ATCC) into the interscapular space. When the tumors reached approximately 300 mm³ in volume, established primary tumors were surgically resected under anesthesia/analgesia (ketamine/xylazine) following IACUC guidelines. The day after surgery the mice received vehicle (DMSO) or 3.2 mg/kg/d selamectin by intraperitoneal injection for 30 days. The mice were monitored for signs of cachexia (changes in weight, temperature, fur condition, activity, lethargy, respiratory distress). When signs of cachexia were detected, the mice were euthanized and the lungs were fixed in Bouin fixative solution and overt lung metastasis counted.

**Expression microarray analysis**

Subconfluent cultures of MDA-MB-231 cells were treated with vehicle or selamectin for 24 hours. Total RNA was isolated using the ZR RNA MiniPrep Kit (Zymo Research). The concentration and quality of the total RNA was assessed on an Agilent 2100 BioAnalyzer (Agilent Technologies). All samples were normalized to 200 ng and processed according to standard Affymetrix protocols using GeneChip WT Terminal Labeling and Controls Kit (Affymetrix) and WT Expression Kit (Ambion). The quality and quantity of labeled cRNA was checked and 750 ng of labeled cRNA were hybridized to a GeneChip Human Gene 1.0 ST Arrays using GeneChip Hybridization, Wash, and Stain Kit (Affymetrix). The arrays were scanned on a GeneChip Scanner 3000 7G. Array data were analyzed by Chipinspector 2.1 (Genomatrix) and transcripts were considered to be significantly regulated if at least 3 significant probes mapped to them and the log₂ fold change of the transcript calculated from these probes was above 1 or below −1. For all subsequent analyses, the median expression values of two independent biologic replicates were used. Replicates were combined exhaustively, i.e., mean fold changes were calculated by comparing each replicate from the treatment group with each replicate from the control group. Log₂ fold-change values for genes were calculated as the average of the log₂ fold-change values of the corresponding significantly regulated transcripts and a false discovery rate (FDR) was set as 5%. Ingenuity pathway analysis (IPA) software (Qiagen) was used to identify significantly overrepresent- ed pathways, cellular functions and upstream transcription factor analysis in the list of identified proteins. Vehicle versus selamectin treatment expression data were imported into IPA and core analysis was performed to identify the most significantly regulated proteins and associated cellular functions. Expression microarray analysis was performed according to Minimum Information About a Microarray Gene Experiment (MIAME) guidelines and data have been deposited on the Gene Expression Ontology (GEO) database with accession number GSE67438.

**Drug-sensitivity assay**

MDA-MB-231 cells (500 cells/well) were plated in 384-well plates and exposed to small molecule inhibitors selamectin (1 μmol/L) for 5 consecutive days after which cell viability in each well was estimated by the use of CellTiter Glo assay (Promega). Luminescence data were log₂ transformed, plate median centered, and then Z score standardized according to the library median effect and the library median absolute deviation (26). Drug sensitization Z-score values of <−1.5 were considered as representing selamectin sensitization effects.

**Statistical analyses**

Statistical analyses were performed with GraphPad Prism software (version 5.0). The experiments were conducted with at least three independent experiments unless otherwise mentioned. Where shown, P values were calculated using the unpaired Student t test, Mann–Whitney or one-way ANOVA as indicated.

**Results**

**Avermectins are small molecule mimetics of SID peptide**

We previously selectively targeted the PAH2 domain of SIN3 corepressor through introduction of a 20-mer peptide fragment comprising amino acids 5–24 of MAD SID. The peptide effectively bound the PAH2 domain and prevented interactions with SID-containing partner proteins, leading to epigenetic reprogramming, repression of COH1 and ESR1, and induction of differentiation in TNBC breast cancer cells (10). To identify small molecule inhibitor (SMI) mimetics of the SID motif, we selected 20 domain structures identified by nuclear magnetic resonance (NMR; ref. 19) and performed an in silico screen against the structures of 2,000 FDA-approved drugs. This structure-guided analysis identified 14 candidate compounds (Supplementary Fig. S1), which were screened using two complementary techniques to identify whether they could interfere with binding of MAD to SIN3 PAH2 (Fig. 2A and B). First, we used a mammalian two-hybrid assay as previously described (10) and consistent with previous data (10, 27), coexpression of GAL4DBD-Sin3B and VP16AD-MAD led a 3.7-fold decrease in luciferase activity compared with coexpression of GAL4DBD and VP16AD empty vectors on a reporter under the control of GAL4UAS elements (Fig. 2A). Treatment with Tat-SID peptide led to a 2-fold increase in luciferase activity compared with that found with GAL4DBD-Sin3B and VP16AD-MAD coexpression and the majority of candidate compounds were also found to increase luciferase activity, with the exception of compounds 9 and 10, which were cytotoxic.

We also used the proximity ligation assay (PLA; ref. 28) to confirm the ability of the candidate compounds to block SIN3A PAH2–SID interactions. Consistent with the mammalian two-hybrid results, the 12 compounds tested were able to interfere with SIN3A PAH2–SID interactions (Fig. 2B and Supplementary Fig. S2A). Compound 14 (selamectin) is a member of the avermectin family of macrocyclic lactones (29) and another member of this group, ivermectin, has been approved by FDA to treat human parasitic diseases, including scabies (30). Selamectin and ivermectin share the same macrocyclic ring and differ only in the number of sugar moieties (31). On the basis of the activity of selamectin in our SIN3A and SIN3B PAH2–SID interference assays and favorable drug-like qualities of avermectins, these
compounds were selected for further study. Consistent with the result from our initial focused screens, selamectin blocked interactions between SIN3A PAH2 and in vitro–translated MAD (Fig. 2C). Moreover, the degree of inhibition of interaction between SIN3A and MAD SID was dose-dependent (Fig. 2D and Supplementary Fig. S2B).

The NMR structure of SIN3A-PAH2 domain bound to MAD SID peptide (green) has previously been resolved (PDB: 1G1E; ref. 19; Fig. 2E, left) and we performed a detailed NMR titration to establish whether ivermectin interacts with PAH2 through residues critical for the PAH2–SID interaction. NMR titration of 15N-labeled PAH2 with ivermectin resulted in shift of signals for a
Ivermectin or selamectin treatment increases ESR1 and PGR expression, leading to restoration of tamoxifen and estrogen sensitivity in TNBC cells. A, quantitative PCR of ESR1 in MDA-MB-231 cells (SEL: ****, P < 0.0001; IVM: ****, P < 0.0001), D3H2LN (SEL: ****, P < 0.0001; IVM: ****, P < 0.0001), and MMTV-Myc (SEL: ***, P = 0.0001) following treatment with selamectin or ivermectin (5 days) as indicated. B, MDA-MB-231 cells were treated with selamectin (5 days) and subjected to immunoblot analysis to determine expression of ERα protein. Expression of ERα in T47D breast cancer cells is shown as a positive control. C, quantitative PCR of PGR in MDA-MB-231 cells (SEL: ****, P < 0.0001; IVM: ****, P < 0.0001) and D3H2LN (SEL: ****, P < 0.0001; IVM: ***, P = 0.0021) following treatment with selamectin or ivermectin (5 days) as indicated. D, MDA-MB-231 cells were pretreated with selamectin (1 μmol/L) for 4 days. Cells were transfected with a luciferase reporter under the control of an estrogen response element, and further treated with selamectin or selamectin plus tamoxifen (Tam) in the presence of 17β-estradiol (E2) for 48 hours (E2 vs. Tam: ***, P = 0.0056). Luciferase activity relative to Renilla control is shown. E, MDA-MB-231 or D3H2LN cells were pretreated with selamectin (1 μmol/L) for 4 days and then further treated with selamectin or selamectin plus tamoxifen in the presence of E2 for 48 hours. Cell viability was determined by MTS tetrazolium assay (E2 vs. Tam, MDA-MB-231: ****, P < 0.0001; D3H2LN: ****, P < 0.0001). Error bars, mean ± SD. P, unpaired t test.
Avermectins Disrupt SIN3 Corepressor Function

Ivermectin and selamectin upregulate CDH1 and ESR1, inhibit invasion, and confer tamoxifen sensitivity in TNBC

We tested the effect of selamectin and ivermectin on CDH1 and ESR1 expression in the wild-type MDA-MB-231 TNBC cell line as well as the mouse MMTV-Myc cell line (18). We also used the MDA-MB-231-derived reporter cell line (MDA-MB-231-luc-D3H2LN), which possesses greater metastatic potential compared with parental MDA-MB-231 cells (16). Consistent with our previous results with SID peptide (10), treatment with selamectin induced CDH1 mRNA expression by 2.5- to 12-fold in MDA-MB-231 and D3H2LN cell lines, respectively, and by 100-fold in MMTV-Myc cells (Fig. 3A). Upregulation of cytoplasmic and membrane-associated E-cadherin protein was also observed after selamectin or ivermectin treatment in MDA-MB-231 (Fig. 3B and C). We previously demonstrated that stable expression of SID inhibited the formation of large and invasive colonies in three-dimensional culture in Matrigel (10) and consistent with these results, both selamectin and ivermectin inhibited invasion in dose-dependent manner (Fig. 3D). Selamectin treatment also resulted in the downregulation in MDA-MB-231 cells of the MMP9 and MMT-MMP/MMPI4 genes that are associated with invasion and metastasis (refs. 32, 33; Fig. 3E). Treatment with selamectin also induced ESR1 mRNA expression by 4- to 11-fold, respectively, in MDA-MB-231 and D3H2LN cell lines, and by 1.5-fold in MMTV-Myc cells (Fig. 4A). Ivermectin was also found to increase ESR1 expression in MDA-MB-231 and D3H2LN cell lines by 3-fold and 6-fold, respectively. Consistent with these results, selamectin treatment also increased levels of ERα protein (Fig. 4B). Expression of progesterone receptor (PCR), a direct transcriptional target of ERα, was also increased by between 1.5- and 6-fold following treatment with selamectin or ivermectin (Fig. 4C). We next investigated the impact of reexpression of ESR1 on ERα function. Cotreatment with selamectin in the presence of 17β-estradiol (E2), led to a 2-fold increase in ERα activity in a reporter assay, whereas cotreatment with E2 antagonist tamoxifen led to a 2-fold reduction in ERα activity (Fig. 4D). Again, these results mirror those obtained with SID peptide (10). In addition, reexpression of ERα in response to selamectin treatment sensitized MDA-MB-231 and D3H2LN cells to the growth promoting or inhibiting effects of E2 or tamoxifen, respectively (Fig. 4E).

Ivermectin and selamectin target TNBC clonogenic self-renewal to inhibit growth and metastasis

In embryonic stem (ES) cells, SIN3A is a part of a complex that positively regulates NANOG and SOX2, expression of which are hallmarks of stem cell pluripotency and self-renewal (34). Furthermore, both NANOG and SOX2 interact with SIN3A-containing complexes to repress gene targets in ES cells (35). Consistent with this, treatment of D3H2LN cells with 0.5 μmol/L of selamectin or ivermectin reduced NANOG (Fig. 5A) and SOX2 (Fig. 5B) gene expression by 50% to 80%. Furthermore, treatment with selamectin or ivermectin diminished by 90% to 100% clonogenic, self-renewal–dependent (32, 33), tumorsphere growth (Fig. 5C). Basal-B subtype cell lines such as MDA-MB-231 have increased ALDH activity, which is another marker of normal and malignant breast stem cells (36). Compared with vehicle-treated cells (18.6 ± 104 ALDH+ cells), reduced ALDH activity was observed after treatment with selamectin (13 ± 105 ALDH+ cells) or ivermectin (7.8 ± 104 ALDH+ cells, P = 0.0149; Fig. 5D). Our in vitro results could also be recapitulated in vivo. Both in vitro pretreatment of

Table 1. Genes involved in EMT that are downregulated in MDA-MB-231 cells treated with selamectin

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<th>Gene</th>
<th>Fold change</th>
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MMTV-Myc cells with selamectin (Fig. 6A) and in vivo administration of the drug (Fig. 6B and C) led to a significant reduction in volume and mass of orthotopically implanted tumors. Selamectin-treated tumors were also observed to be locally less invasive (not shown). In vivo treatment with selamectin also lead to a dramatic reduction in the number of lung metastases observed (Fig. 6D). Finally, the effect of selamectin on tumor cell dissemination and metastasis was examined following surgery to remove established tumors arising from implantation of 4T1 metastatic mammary tumor cells and was found to greatly reduce the number of lung metastases under these conditions (Fig. 6E–G).

Selamectin treatment alters the expression of genes involved in clinically targetable TNBC pathways, leading to drug sensitization

To further investigate the mechanism of action of selamectin in TNBC, we performed expression microarray analysis at 24 hours to identify genes and pathways upstream of E-cadherin and ERα.

Figure 6. Treatment with selamectin inhibits TNBC growth and metastasis in vivo. A, MMTV-Myc mouse mammary tumor cells were pretreated in vitro with vehicle or selamectin for 7 days. A total of 200,000 cells were inoculated into the inguinal mammary fat pads of FVB/N mice (n = 10 per arm). Tumor volume (mm³) was calculated on the days indicated (∗, P = 0.0017). B–D, 50,000 MMTV-Myc cells were inoculated into the flanks of FVB/N mice on day 0 and treated with selamectin (1.6 mg/kg/d) for 15 days (n = 10 mice per arm). Tumor volume (B, ∗∗∗∗, P < 0.0001), tumor mass (C, ∗, P = 0.0161), and number of lung metastases (D, ∗∗, P = 0.0224) in each group were measured. E–G, 4T1 cells (10,000 per mouse) were inoculated into the flanks of BALB/c mice. Tumors were allowed to grow for 10 days before surgical removal. Treatment was then initiated with selamectin (3.2 mg/kg/d for 30 days, n = 4) or vehicle (n = 5). Animals were sacrificed after 30 days and the total number of lung metastases were measured (E and F, ∗, P = 0.0017). Also measured were number of metastases according to size in each group (G; <1 mm: ∗, P = 0.0198; 1–2 mm: ∗, P = 0.0255; >2 mm: ∗∗, P = 0.0447). Error bars, mean ± SD. P, unpaired t test.
Table 2. Pathway analysis for genes differentially expressed in MDA-MB-231 cells treated with selamectin

<table>
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<th>Canonical pathway</th>
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<td>Estrogen-dependent breast cancer signaling</td>
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<td>1.06E−02</td>
</tr>
<tr>
<td>HGF signaling</td>
<td>−0.707</td>
<td>2.25E−02</td>
</tr>
<tr>
<td>FGF signaling</td>
<td>−1334</td>
<td>4.98E−02</td>
</tr>
</tbody>
</table>

Pathway analysis identified downregulation of important markers of EMT: FGFR2—4, SMAD2, ID2, PIK3CA, and WNT5A (Table 1 and Supplementary Table S2; refs. 37–40). Genes involved in estrogen-dependent signaling were modulated (Table 2 and Supplementary Table S3) and upstream regulator analysis also identified activation of ER receptor signaling (Z score, 0.433; P = 1.36E−05) and the β-estradiol pathway (Z score, 0.577; P = 3.77E−01). Other associated pathways were significantly negatively regulated included FGF, integrin-linked kinase (ILK) signaling (which plays an important role in silencing of E-cadherin; ref. 41), growth hormone, and HGF cytokine signaling (Table 2 and Supplementary Table S3). Analysis of the genes differentially expressed in the pathways modulated by selamectin treatment revealed four additional genes that have been identified as playing critical roles in TNBC (IGFIR, −1.58 fold; IRS1, −1.52 fold; ITGB3, −1.48 fold; see Supplementary Table S2). Importantly, together with those identified as markers of EMT, these genes encode factors that either sit at the top of clinically relevant receptor tyrosine kinase (RTK) pathways or, as is the case with PIK3CA (encoding PI3K subunit p100), occupy a central node of control. PI3K is a key player in TNBC pathogenesis and represents a major therapeutic target, and a number of the receptor tyrosine kinases downregulated by selamectin, including IGF1R (42) and FGF (40), signal through PI3K via RAS (43). The finding that selamectin also downregulates ITGB3 is also significant given the role of integrin α,β1 in EMT, maintaining a cancer stem cell phenotype and conferring resistance to RTK inhibitors (44, 45).

In parallel, we carried out a sensitivity screen in MDA-MB-231 cells to identify the compound to which selamectin treatment conferred sensitization, using a focused library of 80 drugs that are either currently used in the treatment of cancer or are in late-stage clinical development. We measured viability in cells exposed to compound alone versus compound in addition to selamectin. Drug sensitization Z scores of less than −1.5 were considered significant and 23 compounds passed this threshold (Table 3). Consistent with our results, and validating this approach, selamectin caused sensitization to 4-hydroxytamoxifen (4-OHT), an active metabolite of tamoxifen (Z score: −2.93 at 50 nmol/L/4-OHT). In the context of TNBC therapy, other drugs with significant Z-scores included the FGFR inhibitor AZD4547 (Z score: −1.65), RTK inhibitor sunitinib (Z score: −2.76), PI3K/mTOR inhibitor PF-04691502 (Z score: −2.78), and the IGF1R inhibitor GSK1904529A (Z score: −2.18). It is notable that many of the drugs to which selamectin conferred sensitivity target genes and pathways modulated by selamectin treatment.

Discussion

In this study, we have identified the macrocyclic lactones selamectin and ivermectin as small molecule mimetics of a therapeutic peptide corresponding to the MAD SID motif. We demonstrated that the effects of these drugs on TNBC phenocopy SID peptide and that selamectin treatment as a single agent exhibits inhibitory activity against TNBC tumor growth and metastasis. Work to identify the molecular mechanisms underlying the activity of selamectin or ivermectin in TNBC is ongoing but candidate SIN3A/SIN3B-PAH2–interacting proteins include PF1 (PHF12) and TIEG1 (KLF10). PF1 recruits a complex containing two known breast cancer factors, EMSY and JARID1B (46–48). JARID1B is a H3K4 demethylase and inhibition of this complex...
could explain, at least in part, the epigenetic remodeling we have observed with SID peptide treatment (10). PF1 also interacts with SIN3B to modify chromatin downstream of transcriptional start sites and mitigate RNA polymerase II progression within transcribed loci (49), although it is unclear whether this plays a role in aberrant gene expression in TNBC. As its name implies, TIEG1 (TGFβ-inducible early gene), plays a role in the TGFβ/SMAD signal transduction pathway where it drives expression and activation of SMAD2 (50, 51). A direct link between TIEG1 and TGFβ pathway-associated EMT in TNBC remains to be established but it is notable that expression of SMAD2 is downregulated following selamectin treatment. The identities of DNA- or chromatin-binding factors that recruit SIN3-containing repressive complexes also remain to be established but candidates include ZEB1 (via CTRP and/or SMARCA4) (32) and components of the DNA methylation machinery such as MeCP2, MBD2, or DNMT3B (53–55).

While up to 33% of TNBC patients achieve a pathologic complete response following neoadjuvant chemotherapy and have a good prognosis, overall survival for those TNBC patients with residual disease is dramatically worse compared with non-TNBC patients with residual disease (56, 57). A major reason for this discrepancy lies with the inability of TNBC patients to respond to conventional hormone or HER2-directed therapies that improve overall survival in other breast cancer subtypes. Thus, our results demonstrating that selamectin and ivermectin treatment leads to reexpression of ERα and induction of tamoxifen responsiveness have clinical significance. These findings are consistent with HDAC and DNMT inhibition where it has been reported that tamoxifen-assoicated reactivated ERα recruited the Mi2/NuRD corepressor complex but not the SIN3A corepressor complex to ERα-responsive genes (58). The inactivation of specific SIN3A repressor functions is therefore not predicted to interfere with tamoxifen-induced downstream activities of ERα. Furthermore, given that TNBC patients with metastatic disease relapse very quickly when on chemotherapy (59), the ability of selamectin to inhibit metastasis is particularly exciting. With conventional treatment options delivering poor results in TNBC, there has been much focus in recent years on the development of molecularly targeted strategies to combat this disease. However thus far, with a few notable exceptions, molecularly targeted drugs have not been curative or even effective in cancer in general and rational combinatorial approaches are needed to improve outcome (60, 61). Thus, our results showing that selamectin modulates expression of therapeutically relevant genes to confer sensitization to molecularly targeted drugs in clinical development warrant further investigation.

The results of this study are in line with recent research demonstrating that avermectin treatment at concentrations comparable with those used here inhibited the WNT/TCF pathway, leading to inhibition of colon and lung tumor growth in vivo and in vitro without nonspecific cell toxicity (62). Oral ivermectin is already extensively used to treat humans including river blindness (63) with few side effects observed at clinical doses (64, 65). Selamectin and ivermectin do not readily cross the blood–brain barrier (66) and thus far only dogs with mutated ABCB1 (MDR1) and knockout mice for this gene have exhibited neurotoxicity (67) with these drugs. In summary, our results strongly suggest that selective inhibition of SIN3 function using selamectin or ivermectin should be investigated clinically in combinatorial adjuvant and neoadjuvant therapy, both with existing agents such as tamoxifen as well as molecularly targeted drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.-J. Kwon, K. Petrie, B.A. Leibovitch, L. Zeng, M. Mezei, L. Howell, R. Christova, S. Yang, E.V. Ariztia, C.J. Lord, A. Zelent, E. Farias, M.-M. Zhou, S. Waxman

Writing, review, and/or revision of the manuscript: Y.-J. Kwon, K. Petrie, B.A. Leibovitch, A. Zelent, E. Farias, M.-M. Zhou, S. Waxman

Study supervision: Y.-J. Kwon, B.A. Leibovitch, A. Zelent, E. Farias, M.-M. Zhou, S. Waxman

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Conflict of Interest

Authors declare no potential conflicts of interest.

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Molecular Cancer Therapeutics

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Yeon-Jin Kwon, Kevin Petrie, Boris A. Leibovitch, et al.


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