MOLECULAR CANCER THERAPEUTICS | SMALL MOLECULE THERAPEUTICS

An Orally Available Tubulin Inhibitor, VERU-111, Suppresses Triple-Negative Breast Cancer Tumor Growth and Metastasis and Bypasses Taxane Resistance

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ABSTRACT

Triple-negative breast cancer (TNBC) accounts for approximately 15% of breast cancer cases in the United States. TNBC has poorer overall prognosis relative to other molecular subtypes due to rapid onset of drug resistance to conventional chemotherapies and increased risk of visceral metastases. Taxanes like paclitaxel are standard chemotherapies that stabilize microtubules, but their clinical efficacy is often limited by drug resistance and neurotoxicities. We evaluated the preclinical efficacy of a novel, potent, and orally bioavailable tubulin inhibitor, VERU-111, in TNBC models. VERU-111 showed potent cytotoxicity against TNBC cell lines, inducing apoptosis and cell-cycle arrest in a concentration-dependent manner. VERU-111 also efficiently inhibited colony formation, cell migration, and invasion. Orally administered VERU-111 inhibited MDA-MB-231 xenograft growth in a dose-dependent manner, with similar efficacies to paclitaxel, but without acute toxicity. VERU-111 significantly reduced metastases originating from the mammary fat pad into lung, liver, and kidney metastasis in an experimental metastasis model. Moreover, VERU-111, but not paclitaxel, suppressed growth of luciferase-labeled, taxane-resistant, patient-derived metastatic TNBC tumors. In this model, VERU-111 repressed growth of preestablished axillary lymph node metastases and lung, bone, and liver metastases at study endpoint, whereas paclitaxel enhanced liver metastases relative to vehicle controls. Collectively, these studies strongly suggest that VERU-111 is not only a potent inhibitor of aggressive TNBC phenotypes, but it is also efficacious in a taxane-resistant model of metastatic TNBC. Thus, VERU-111 is a promising new generation of tubulin inhibitor for the treatment of TNBC and may be effective in patients who progress on taxanes.

Results presented in this study demonstrate the efficacy of VERU-111 in vivo and provide strong rationale for future development of VERU-111 as an effective treatment for metastatic breast cancer.

Introduction

Breast cancer is the second leading cause of cancer-related deaths in women in the United States, leading to approximately 40,920 deaths in 2017 (1). Approximately 15% of all breast cancer cases are classified as the triple-negative breast cancer (TNBC) subtype, characterized by the absence of estrogen receptor, progesterone receptor, and HER2 (2). Conventional endocrine therapy or HER2-targeted therapy is effective for the hormone receptor–positive or HER2-positive breast cancers, but not for TNBC due to the lack of targets (3, 4). Thus, cytotoxic chemotherapy is still a primary treatment regimen for patients with TNBC. TNBC is highly aggressive and is characterized by poor prognosis due to relatively quick relapse after chemotherapy (5, 6), with enhanced rates of visceral metastasis. Therefore, there is an urgent need to develop novel effective therapeutics for the treatment of TNBC, particularly for chemorefractory disease.

Microtubules, composed of α- and β-tubulin heterodimers, are important for cell structure maintenance, cell mitosis, and intracellular transport (7). Interference with microtubule assembly arrests cell-cycle progression, and ultimately induction of cell death, making microtubules an attractive target in anticancer drug discovery (8, 9). At least four major binding sites on the microtubules for antimitotic agents have been discovered: taxol-binding site, vincristine-binding site, colchicine-binding site, and laulimalide-binding site (10). Existing tubulin inhibitors, such as paclitaxel, are highly successful in breast cancer treatment; however, due to poor aqueous solubility, they must be intravenously infused. In addition, their clinical use is often limited by drug resistance mediated by ATP-binding cassette (ABC) transporters and neurotoxicities (11–13). Extensive preclinical studies have suggested that tubulin inhibitors targeting the colchicine-binding site are significantly less vulnerable to transporter-mediated drug resistance, and several of these agents have been tested in clinical trials (10, 14). However, up to this point, none of these colchicine-binding site inhibitors (CBSIs) have gained FDA approval, mainly due to toxicities and their lack of clinical benefit. Although targeting tubulin polymerization may involve inherent toxicity, for many of these CBSIs, the major toxicities that led to clinical failures are related to their specific chemical structures (15, 16). Developing a new generation of CBSI with a unique chemical structure that possesses a good therapeutic index could lead to a new anticancer agent for safe and effective chemotherapy, particularly for metastatic disease.

A series of novel compounds that target the colchicine-binding site, termed ABI-III chemotypes, were designed and synthesized on the basis of our previously reported 2-aryl-4-benzoyl-imidazole...
analogue (17). These ABI-III analogues possessed nanomolar cytotoxicity against a panel of melanoma and prostate cancer cell lines and they overcame multidrug resistance mediated by the ABC transporter P-glycoprotein (P-gp; ref. 17). Among these, VERU-111, also named ABI-231, showed the most potent antiproliferative activity against five melanoma and prostate cancer cell lines and is the clinical candidate for trials for advanced prostate cancer (NCT 03752099). Oral activity and efficacy of VERU-111 were previously evaluated in paclitaxel- or docetaxel-resistant tumor models; VERU-111 has favorable pharmacokinetic properties with acceptable oral bioavailability, ranging from 21% to 50%, in mice, rats and dogs (18). Therefore, we investigated the antitumor activity of VERU-111 specifically for the treatment of metastatic TNBC. The in vitro efficacy of VERU-111 was assayed using two conventional TNBC cell lines (MDA-MB-231 and MDA-MB-468) and two luciferase-labeled TNBC primary cell lines derived from metastatic patient-derived xenograft (PDX) models created at the Huntsman Cancer Institute (HCI; ref. 19), HCI-2-Luc2 (treatment-naïve) and HCI-10-Luc2 (taxane refractory). VERU-111 had potent antiproliferative activity against all models tested, including the taxane-resistant HCI-10 model (low nanomolar range). Furthermore, VERU-111 inhibited cancer cell colony formation, cell migration, and invasion, likely through the antiproliferative related mechanisms regulating microtubule assembly, G2-M cell-cycle arrest, and induction of apoptosis. In a MDA-MB-231 xenograft model, orally administered VERU-111 inhibited TNBC xenograft tumor growth in a dose-dependent manner with antitumor potency similar to paclitaxel and repressed visceral metastasis in both an orthotopic setting and in an experimental metastasis model. VERU-111, but not paclitaxel, significantly repressed primary tumor growth, growth of preestablished axillary lymph node (AxLN) metastases, and repressed endpoint metastasis in mice bearing HCl-10-Luc2 xenografts derived from the PDX model (20). Collectively, these data position VERU-111 as a promising drug candidate for the more effective treatment of metastatic TNBC, potentially including patients who progress on taxanes.

Materials and Methods

Chemical compounds and cell lines

Colchicine was purchased from Sigma-Aldrich. Paclitaxel was purchased from LC Laboratories. VERU-111 was synthesized by a reported method (21); purity (≥98%) and identity were verified by HPLC, HR-MS (Waters), and proton nuclear magnetic resonance (Bruker). MDA-MB-231 and MDA-MB-468 were purchased from ATCC and authenticated prior to use and then authenticated every year at the University of Arizona Genetics Core. Cells were cultured in DMEM-Hi (Mediatech, Inc.) supplemented with 10% FBS (Atlanta Biologicals) and 1% antibiotic–antimycotic solution (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO2. Spent media were routinely tested for Mycoplasma using the MycoAlert Kit (Lonza). The parental HCl-2 and HCl-10 PDX breast tumor lines (TNBC: ER-/PR-/HER2+) were originally provided by Dr. Alania Weil and the HCl tissue resource and application core (19). HCl-2-Luc2 and HCl-10-Luc2 patient-derived tumor xenografts were developed by transient cell culture of parent primary tumor duct cell tumors in stem cell conditions with a lentivirus expressing luciferase-2 and puromycin, followed by transplant into and exclusive passage in immunocompromised recipient mice (20). Primary cell lines generated from the Luc2-labeled tumor xenografts were grown in adherent conventional cell culture conditions and were maintained in M87 growth medium (19, 20). Luciferase-labeled HCl PDX-derived primary cell lines and subsequent tumor xenograft material were authenticated by matching to the original deidentified patient sample by whole-genome expression profiling at the HCI.

Cell growth inhibition assay

A MTS assay was used to score for cell growth inhibition effects of an increasing concentration range of colchicine, paclitaxel, and VERU-111 in human melanoma (A375 and M14), human HER2-positive breast (SKBR3) and TNBC (MDA-MB-231, MDA-MB-453, and MDA-MB-468) cell lines for 72 hours as described previously (21). HCl-2- or HCI-10-Luc2 patient-derived primary cell lines were seeded at 20,000 cells/well and grown in the presence of drugs for 6 days. Drug response was normalized to untreated and vehicle-only controls, plotted on log scale, and analyzed using nonlinear regression best fit analysis modules in Prism 7.0. For all cell models, at least three biological replicate experiments were averaged to calculate the grand mean IC50 ± SEM.

Colony formation assay

MDA-MB-231 or MDA-MB-468 cells were seeded into 12-well plates (250 or 500 cells/well, respectively) and then treated 24 hours later with increasing concentrations of colchicine, paclitaxel, and VERU (8, 16, or 32 nmol/L). Medium was replaced with fresh drug once per week. Cells were fixed with methanol and stained with 0.5% crystal violet, and colony morphology was visualized by a microscope and colonies quantified using ImageJ software (NIH, Bethesda, MD).

Immunofluorescence staining

MDA-MB-231 (1 × 105) or MDA-MB-468 (2 × 105) cells were seeded in 6-well plates onto sterile coverslips for 24 hours prior to treatment with 16 nmol/L of colchicine, paclitaxel, or VERU-111 for 18 hours. For tubulin visualization, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 minutes, and permeabilized with 0.2% Triton X-100/PBS for 15 minutes. Following blocking in 1.5% BSA, 0.1% Tween 20 in PBS for 1 hour, coverslips were incubated with anti-α-tubulin antibody (Thermo Fisher Scientific) in 1% BSA/0.1% Tween 20/PBS overnight at 4°C. Cells were then washed and incubated with Alexa Fluor 647 goat anti-mouse IgG (Molecular Probes) for 1 hour at room temperature, mounted with Prolong Diamond Antifade containing DAPI (Invitrogen), and images captured with a Keyence BZ-X700 microscope.

Cell migration and invasion assay

Cell migration and invasion were measured, respectively, using transwell 24-well plates with a noncoated membrane (pore size 8 µm) or a Matrigel-coated insert (BD Biosciences). MDA-MB-231 and MDA-MB-468 cells were starved in serum-free medium for 24 hours, followed by suspension of the cells (MDA-MB-231: 4 × 104 and MDA-MB-468: 105) in serum-free medium containing 8 nmol/L of VERU-111 and then plating in the top chamber of the inserts. Medium containing 10% FBS was added to the lower chamber as a chemoattractant and cells were incubated for 24 hours (MDA-MB-231) or 48 hours (MDA-MB-468). The chambers were fixed in cold methanol, stained with 0.5% crystal violet, and imaged. The number of migrating or invading cells was counted using ImageJ software and normalized to the control group for each cell line (100%). Random cell migration was then analyzed by a manual scratch wound healing assay. MDA-MB-231 cells (105 cells/well) and MDA-MB-468 (2 × 105 cells/well) cells were seeded in 12-well plates and allowed to adhere overnight. The following day, a scratch was made in the confluent cell monolayer using a sterile 200 µL pipette tip. The cell culture medium was replaced by medium containing vehicle (DMSO), colchicine, paclitaxel, or
VERU-111 (16 nmol/L). At 12, 24, and 48 hours, wound width was imaged with an Evos Fl Imaging System (Life Technologies) and closure expressed relative to the size of the original scratch width at each measured time point in the drug-treated wells relative to the vehicle control.

Cell-cycle analysis
After treatment with each drug, cells were harvested by trypsinization, fixed, permeabilized, stained with anti-pherin-histone H3 (Ser 10)-Alexa Fluor 488 antibody on ice for 1 hour, and then incubated with freshly prepared propidium iodide/RNase solution for 30 minutes at room temperature per the manufacturer’s protocol (#FCCH025103, EMD Millipore Corporation). Stained cells were analyzed using a Bio-Rad ZE5 instrument (Bio-Rad) and data were analyzed using FlowJo version 10 (FlowJo, LLC) in the University of Tennessee Health Science Center (UTHSC) Flow Cytometry and Cell Sorting core. First, total cells were gated, followed by gating out cell doublets and debris. Cells in sub-G1, G1, S, and G2 phases were then gated according to DNA content, as determined from histogram plots of PI staining-Area. Cell populations were de
gated, followed by gating out cell doublets and debris. Cells in the M-phase were identi
d by comparing the compensation control sample with the pherin-histone H3-Alexa Fluor 488-stained samples and gated only for pherin-histone H3-stained cells.

Detection of apoptosis
MDA-MB-231 and MDA-MB-468 cells were seeded in 6-well plates (2 × 10^4/well). The next day, cells were treated with 100 nmol/L of VERU-111 for 24, 48, or 72 hours. Alternatively, cells were treated with increasing concentrations of VERU-111 for 24 hours. At harvest, 10^5 cells were suspended in 185 L of Annexin-V-FITC binding buffer (eBioscience) and 5 L of Annexin-V-FTC (eBioscience) and 10 L of propidium iodide were added, followed by incubation for 10 minutes at room temperature, and analysis in the UTHSC Flow Cytometry and Cell Sorting core.

Caspase-3/7 activity assay
Apoptosis induced by VERU-111 was measured using the Caspase Glo 3/7 assay system (Promega; ref. 22). Cells (5,000 cells/well) were seeded into a 96-well plate and treated with either colchicine, paclitaxel, or VERU-111 (20 nmol/L) for 24 hours, and caspase-3/7 activity was normalized by total protein content.

Western blotting
Cells grown to 70% confluence were incubated with increasing concentrations of VERU-111 or 100 nmol/L colchicine or paclitaxel for 24 hours. For time-dependent studies, cells were exposed to 100 nmol/L VERU-111 or 100 nmol/L colchicine or paclitaxel for 24 hours. For P-gp detection, PDX xenograft cohorts were dosed orally five times per week (Monday–Friday), and animals treated with paclitaxel were dosed every other day (intraperitoneally). Primary tumor size was monitored twice a week and the body weight of the mice was recorded daily. Tumor volume was calculated by the formula volume = (width^2 × length)/2. All animals were euthanized when tumor volume in the vehicle control group exceeded 1,000 mm^3 (after 18 days on therapy). Excised tumors were photographed, and final tumor volume was calculated from ex vivo caliper measurements. Tumors were then divided in half and either flash frozen or fixed overnight at room temperature in buffered formalin. Lungs were harvested after inflation with saline and postfixed in formalin overnight prior to downstream histologic analysis using the UTHSC Research Histology Core.

Experimental lung metastasis model
NSG female mice (7–8 weeks old) were injected with 2 × 10^3 MDA-MB-231 cells in 100 L of HBSS via tail vein. Twenty-four hours later, mice were treated with either vehicle, 10 mg/kg of VERU-111, or 10 mg/kg paclitaxel (n = 7 mice/group) using the same dosing regimen as the orthotopic xenograft model. Body weight was monitored weekly. After 23 days, mice were sacrificed, and lungs, livers, kidneys, and spleens were photographed and then fixed in formalin overnight.

Histology and IHC analysis
Fixed tumors and organs were embedded in paraffin and unstained slides were prepared for immunostaining or stained with H&E. IHC staining was performed as described previously (21). Primary antibodies included rabbit anti-Ki67 (1:400), rabbit anti-CD31 (1:100; #9027, #77699, Cell Signaling Technology), rabbit anti-cleaved PARP (1:500), rabbit anti-cleaved caspase-3 (1:1,000), rabbit anti-CD31 (1:100), CD95 (1:50), rabbit anti-cleaved PARP (1:500), and rabbit anti-CD3 (1:1,000). Detection of PARP and caspase-3 was performed by a biotinylated horse anti-rabbit IgG antibody (BA-1100, Vector Laboratories Inc.) second antibody. Anti–human-specific mitochondria IHC staining (ABcam, cat# ab92824) was performed at a 1:1,000 dilution to visualize metastases. All slides were developed with DAB Impact (Vector Labs), counterstained in Gill’s hematoxylin (Vector Labs), and mounted with Cytoseal XYL mounting media. Tissue images were captured using a Leica DM 2500 microscope (Leica Microsystems) and analyzed using ImageJ (National Institutes of Health) software.

In vivo orthotopic MDA-MB-231 xenograft model
All protocols and methods were approved by the UTHSC Animal Care and Use Committee (ACUC), consistent with the Guide for the Care and Use of Laboratory Animals, 8th edition (protocols #17-080 and #17-056). Female NSG mice at 5 to 6 weeks of age were housed with a 12:12 hour light/dark cycle and fed a breeder chow diet. MDA-MB-231 cells suspended in 10 mL of HBSS (2.5 × 10^6) were surgically inoculated into the left and the right inguinal mammary gland fat pads of deeply anesthetized NSG mice using a Hamilton syringe mounted with a 26G PT2 point needle (23). Mice were palpated and measured with digital calipers weekly until the average tumor size per mouse was 100 mm^3. Mice were then randomly divided into 5 groups such that the mean tumor volumes were equivalent across cohorts (Cohort 1: vehicle, 1:1 ratio of PEG 300:water, orally, n = 14 mice; Cohort 2: 5 mg/kg VERU-111, orally, n = 8 mice; Cohort 3: 10 mg/kg VERU-111, orally, n = 8 mice; Cohort 4: 12.5 mg/kg VERU-111, orally, n = 8 mice; Cohort 5: 12.5 mg/kg paclitaxel, intraperitoneally, n = 8 mice). Animals in the vehicle and VERU-111 MDA-MB-231 xenograft cohorts were dosed orally five times per week (Monday–Friday), and animals treated with paclitaxel were dosed every other day (intraperitoneally). Primary tumor size was monitored twice a week and the body weight of the mice was recorded daily. Tumor volume was calculated from ex vivo caliper measurements. Tumors were then divided in half and either flash frozen or fixed overnight at room temperature in buffered formalin. Lungs were harvested after inflation with saline and postfixied in formalin overnight prior to downstream histologic analysis using the UTHSC Research Histology Core.

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acquired with a Keyence BZ-X700 microscope. Quantification of metastatic burden was performed by digital scanning of whole stained slides using a Panoramic FLASH III system (3D Histech), followed by manual counting of metastatic lesions present in the tissue section. Quantification of Ki67-, CD31-, cleaved PARP-, and cleaved caspase-3-positive tumor cells was performed by calculating the area of positive cells in 4 to 5 representative fields per section using the Keyence Hybrid Cell Count module.

**Patient-derived xenograft models**

The parental HCI-10 PDX model was originally derived from a pleural effusion of a heavily treated patient diagnosed with metastatic breast cancer (19). To facilitate whole-body bioimaging, this model was previously labeled with firefly luciferase as described (20). After transient cell culture in stem cell culture conditions to achieve viral transduction and reporter labeling, HCI-10-Luc2 cells were implanted into a donor female NSG mouse and then serially retransplanted and/or cryopreserved to maintain the line. Tumor fragments from a donor tumor-bearing mouse were bilaterally implanted into the cleared inguinal mammary fat pads of recipient NSG female mice (4–6 weeks of age). Primary tumor growth and metastasis were monitored biweekly by caliper measurement and bioimaging (PerkinElmer XMRS; once/week) beginning two weeks post-surgery. Body weights were recorded every other day. As soon as one tumor per mouse grew to approximately 100 mm³, mice were randomized such that mean tumor volumes were equivalent in each cohort: Cohort 1: vehicle (orally, five consecutive days/week, n = 12 mice), Cohort 2: 10 mg/kg paclitaxel (intravenously, once/week, n = 13 mice) and Cohort 3: 10 mg/kg VERU-111 (orally, 5 consecutive days/week, n = 13 mice). Mice were then bio-imaged the day before treatment to screen for preexisting AxLN metastases. All bio-imaging data are shown as total flux (photons/s), calculated using Living Image software. After 8 weeks on treatment, when mean tumor volume in the vehicle control group was approximately 500 mm³, all animals were euthanized. All organs were collected and bioimaged ex vivo to confirm location of metastatic signals observed in intact mice and to quantitate metastatic burden specific to that organ. Collected tumors and organs were processed for histology as described above.

**Statistical analysis**

All data were analyzed using GraphPad Prism 7. In vitro experiments were repeated using at least three technical replicates per group and each assay performed over three biological replicates. One-way or two-way ANOVA tests were first employed for experiments comparing more than two groups/conditions, followed by pairwise, two-tailed Student t tests or a Dunnett multiple comparison test. Student t tests were used to compare treatment group with the control group for the in vivo migration and invasion assays. One-way ANOVA analyses followed by the Dunnett test was used to evaluate differences between treatments and controls for the in vivo colony formation, scratch, and cell apoptosis detection assays, as well as all in vivo xenograft studies. Significance levels are defined as *P < 0.05; **P < 0.01; ***P < 0.001; ****, P < 0.0001.

**Results**

**VERU-111 attenuates proliferation of breast cancer cells and impairs microtubule assembly and mitotic spindle organization**

VERU-111 (Fig. 1A) has an average IC₅₀ of 4 nmol/L in melanoma cells (17). We evaluated whether VERU-111 could inhibit the growth of HER2⁺ (SKBR3) and TNBC breast cancer cells (MDA-MB-231, 468, and 453 cells). VERU-111 had potent antiproliferative effect in all cells, with IC₅₀ values in the low nanomolar range: SKBR3 (14 nmol/L) and 8 to 14 nmol/L in TNBC cells (Supplementary Table S1). The tubulin-stabilizing agent colchicine and the tubulin-stabilizing agent paclitaxel were then compared with VERU-111 using two TNBC cell lines (Fig. 1B). All three inhibitors inhibited cell proliferation. Mean IC₅₀ values of colchicine, paclitaxel, and VERU-111 ranged from 9.8 to 17.5 nmol/L, 3.1 to 4.6 nmol/L, and 8.2 to 9.6 nmol/L, respectively, depending on the model. VERU-111 reduced colony formation of TNBC cells in a concentration-dependent manner (Fig. 1C). In MDA-MB-231 cells, paclitaxel completely repressed colony formation at a concentration as low as 8 nmol/L. In contrast, colony formation was reduced, but not eliminated, at an equivalent concentration of either colchicine or VERU-111, although 16 nmol/L of VERU-111 was more effective than 16 nmol/L of colchicine. Similar results were observed in MDA-MB-468 cells.

VERU-111 demonstrates robust antitumor activity by destabilizing tubulin polymerization through targeting the colchicine-binding site (17). Thus, we performed immunofluorescence staining to visualize the microtubule network after treatment with colchicine, VERU-111, and paclitaxel. Control cells showed intact microtubule fibers and typical microtubule organization (Fig. 1D). As expected, treatment with paclitaxel produced aggregation and condensation of microtubules due to increased tubulin polymerization. Similar to colchicine, cells treated with VERU-111 were smaller and the cell shape changed from spindle-like to round and irregular, and the microtubules were depolymerized, confirming that VERU-111 disrupts tubulin polymerization in TNBC cells.

**VERU-111 inhibits TNBC cell migration and invasion**

VERU-111 (8 nmol/L) inhibited cell migration by 44% in MDA-MB-231 cells and by 37% in MDA-MB-468 cells relative to the control (set to 100%; Fig. 2A). Likewise, VERU-111 reduced invasion through Matrigel membrane by 50% in MDA-MB-231 and by 45% in MDA-MB-468 cells (Fig. 2B). Similar results on invasion potential were obtained when cells were exposed to 16 nmol/L of VERU-111 (Supplementary Fig. S1). VERU-111, colchicine, and paclitaxel showed effective inhibition of wound healing in a scratch wound assay at 16 nmol/L (Fig. 2C). After 24 hours, the average rates of migration in colchicine, paclitaxel, and VERU-111–treated MDA-MB-231 cells were 67.3%, 43.3%, and 44.9%, respectively, relative to vehicle control (DMSO). Similarly, after 48-hour treatment, colchicine, paclitaxel, and VERU-111 reduced the average migration of MDA-MB-468 cells by 15.8%, 14.5%, and 17.9%, respectively. Images of wounds are shown in Supplementary Fig. S2. We also verified that lowering the concentration of VERU-111 to 8 nmol/L also inhibited wound healing in TNBC cells (Supplementary Fig. S3A and S3B). Finally, the ability of VERU-111 to inhibit wound healing in a concentration-dependent manner over time was shown using MDA-MB-231 cells grown in the IncuCyte S3 live cell imaging system (Supplementary Fig. S3C). In summary, although VERU-111 impaired cell migration, invasion, and wound healing, it is not possible to rule out the impact of VERU-111–dependent effects on growth inhibition and/or cytotoxicity on these assays.

**VERU-111 blocks TNBC cells at G₂–M checkpoint and induces cell apoptosis**

Microtubule disruption typically leads to the mitotic arrest of growing cells in metaphase, ultimately causing cell death (24, 25). To examine whether VERU-111 induces cell-cycle arrest, cells treated with 100 nmol/L of colchicine or paclitaxel and increasing
Concentrations of VERU-111 for 24 hours were profiled by flow cytometry. Each compound showed divergent effects on cell-cycle progression in each cell line. In MDA-MB-231 cells, VERU-111 treatment induced a G2–M block in a concentration-dependent manner, with a concomitant reduction in cells in the G1 and S phases. Colchicine and paclitaxel, also arrested MDA-MB-231 cells in the G2 and M phases, as expected (Fig. 3A; Supplementary Fig. S4A). Likewise, VERU-111 induced a G2 phase arrest in MDA-MB-468 cells, reducing the population of cells G1, with little effect on the S-phase. In contrast to VERU-111, paclitaxel arrested MDA-MB-468 cells in the G2 and M phases (Fig. 3A). VERU-111 treatment caused a significant G2–M accumulation at a concentration of 20 nmol/L in MDA-MB-231 cells, and at 50 nmol/L in MDA-MB-468 cells, with maximum accumulation observed in response to a concentration of 100 nmol/L.

The potential of VERU-111 to induce apoptosis in TNBC cells was analyzed using Annexin-V-FITC/PI costaining. VERU-111 initiated apoptotic cell death in a concentration-dependent manner, as indicated by the appearance of Annexin-V−/PI− cells, Annexin-V−/PI+ cells, and Annexin-V+/PI+ cells (Fig. 3B; Supplementary Fig. S4B). The potency of VERU-111 to induce apoptosis was identical to colchicine but required a higher concentration for paclitaxel. MDA-MB-231 and MDA-MB-468 cells treated with 100 nmol/L VERU-111
VERU-111 represses TNBC cell migration and invasion. Directed migration (A) or invasion (B) of MDA-MB-231 (top) and MDA-MB-468 (bottom) cells was determined using a transwell 24-well plate assay following treatment with VERU-111 (8 nmol/L) for 24 and 48 hours, respectively. C, The effect of VERU-111 on MDA-MB-231 (left) and MDA-MB-468 (right) on wound healing was determined by the scratch assay after treatment for 24 and 48 hours, respectively. Migration rates of cells treated with VERU-111, colchicine, and paclitaxel (each 16 nmol/L) were determined by the percentage of wound closure relative to cells in the control group. All data (A–C) are presented as the grand mean ± SEM of three independent experiments, with P values determined relative to the control group (normalized to 100%). All images are shown with a scale bar of 200 μm.

Figure 2

VERU-111 represses TNBC cell migration and invasion. Directed migration (A) or invasion (B) of MDA-MB-231 (top) and MDA-MB-468 (bottom) cells was determined using a transwell 24-well plate assay following treatment with VERU-111 (8 nmol/L) for 24 and 48 hours, respectively. C, The effect of VERU-111 on MDA-MB-231 (left) and MDA-MB-468 (right) on wound healing was determined by the scratch assay after treatment for 24 and 48 hours, respectively. Migration rates of cells treated with VERU-111, colchicine, and paclitaxel (each 16 nmol/L) were determined by the percentage of wound closure relative to cells in the control group. All data (A–C) are presented as the grand mean ± SEM of three independent experiments, with P values determined relative to the control group (normalized to 100%). All images are shown with a scale bar of 200 μm.
Figure 3.
VERU-111 induces G2-M cell-cycle arrest and apoptosis in TNBC cells. A, Cell-cycle distribution was determined by flow cytometry after staining of MDA-MB-231 cells (left) and MDA-MB-468 cells (right) with phospho-histone H3 (Ser10) and propidium iodide posttreatment with 100 nmol/L colchicine or 100 nmol/L paclitaxel as controls, or VERU-111 at concentrations of 10, 20, 50, and 100 nmol/L for 24 hours. The percentage of cells in each phase of the cell cycle is shown as the grand mean ± SEM calculated from three independent experiments. B, Apoptosis was compared after treatment with the same drugs by Annexin-V/PI costaining and flow cytometry analysis expressed as the grand mean of the apoptotic cells (%) ± SEM calculated from three independent experiments, as compared with control. C, Flow cytometry analysis of apoptotic cells detected by Annexin-V/PI staining in MDA-MB-231 (left) and MDA-MB-468 cells (right) treated with vehicle, or VERU-111 at 100 nmol/L for 24, 48, or 72 hours, expressed as the grand mean of the apoptotic cells (%) ± SEM of three independent experiments, as compared with control. D, Cleaved caspase-3 and PARP cleavage were determined by Western blotting after treatments as in A. GAPDH was used as a loading control. Signal intensity was evaluated by ImageJ densitometry, with 100 nmol/L VERU-111 treatment set to 1.00. E, Cleaved caspase-3 and PARP cleavage by Western blotting following VERU-111 treatment (100 nmol/L) for 24, 48, and 72 hours. Signal intensity was evaluated by ImageJ densitometry, with 100 nmol/L VERU-111 treatment at 72 hours set to 1.00. F, Caspase-3/7 activity following treatment with 20 nmol/L of each drug for 24 hours in MDA-MB-231 (left) and MDA-MB-468 (right) cells. Bar graphs represent the grand mean of the fold change of caspase-3/7 activity ± SEM of three independent experiments as compared with control.
for 24, 48, and 72 hours underwent apoptosis in a time-dependent manner as shown in Fig. 3C and Supplementary Fig. S4C. Caspases and PARP play an important role in the initiation and execution of programmed cell death (26, 27). To determine whether VERU-111 triggers apoptotic cell death through these pathways, expression of cleaved caspase-3 and cleaved PARP in TNBC cells treated by VERU-111 was analyzed by Western blotting (Fig. 3D and E), revealing increased expression of cleaved caspase-3 and cleaved PARP in a concentration-dependent manner after 24-hour treatment, although cleavage was overall lower than in cells treated with paclitaxel. Colchicine also induced the upregulation of cleaved caspase-3 and cleaved PARP in MDA-MB-468 cells after 24-hour treatment (Fig. 3D). There was also a clear, time-dependent increase in cleaved caspase-3 and cleaved PARP expression after VERU-111 treatment (Fig. 3E). Caspase-3/7 activity was next evaluated in each cell line using the Caspase Glo 3/7 assay, including colchicine and paclitaxel as positive controls (Fig. 3F). VERU-111, colchicine, and paclitaxel displayed up to 4-fold higher caspase-3/7 activity levels relative to the control, consistent with induction of apoptosis effectors shown in Fig. 3B and C.

VERU-111 inhibits TNBC primary tumor growth

We next tested the antitumor activity of VERU-111 in an orthotopic metastatic TNBC mouse model (MDA-MB-231). Because paclitaxel is one of the most widely used chemotherapeutics for stage IV breast cancer, we incorporated paclitaxel as the comparison treatment. NSG mice bearing MDA-MB-231 xenografts were treated with either vehicle, 5-, 10-, or 12.5 mg/kg of VERU-111 (orally) or 12.5 mg/kg paclitaxel (intraperitoneally) relative to vehicle-treated mice. mean tumor size was significantly decreased in the 10 and 12.5 mg/kg VERU-111 cohorts and in the paclitaxel-treated groups, whereas VERU-111 given at 5 mg/kg showed less potent inhibition (Fig. 4A). No significant loss of body weight was observed in the VERU-111–treated groups, although 12.5 mg/kg dosing of VERU-111 did appear to inhibit the weight gain observed in the other treatment groups over the course of the treatment. In contrast, the body weight of mice in the paclitaxel treatment group was significantly decreased (~10%), suggesting an accumulated toxicity (Fig. 4B). Relative to controls, each dose of VERU-111 significantly reduced mean tumor volume and mean weight with the largest effect observed at 12.5 mg/kg (Fig. 4C and D). Moreover, VERU-111 had comparable efficacy with paclitaxel because there were no significant differences between the means of the two groups at the same dose (276.61 mm³ vs. 254.32 mm³; Fig. 4C). Similar changes were observed when the mean final tumor weights were compared (Fig. 4D). Both VERU-111 and paclitaxel treatment significantly reduced overall tumor size as compared with the vehicle control (Fig. 4E).

VERU-111 induces tumor necrosis, antiangiogenesis, and apoptosis in vivo

Paraffin-embedded tumor sections were then stained with H&E and with Ki67, CD31, cleaved PARP, and cleaved caspase-3 by IHC (Fig. 5A). As necrotic areas would be expected in the tumor centers due to hypoxia, all H&E and IHC pictures were imaged near the tumor margins. Both VERU-111 and paclitaxel treatment increased the number of necrotic tumor cells with pyknosis, indicated by the nuclear shrinkage (Fig. 5A). The percentage of tumor area occupied by necrotic cells was not statistically significant between vehicle and the 5 mg/kg dose of VERU-111 (Fig. 5B); however, necrosis significantly increased in response to 10 or 12.5 mg/kg of VERU-111. Approximately half of tumor area in the 12.5 mg/kg VERU-111 treatment group was necrotic, which was comparable with the necrotic area in response to paclitaxel (41.5%). All doses of VERU-111 treatment significantly decreased, in a dose-dependent manner, the number of KI67-positive cells, and CD31-positive cells relative to the vehicle-treated tumors. Paclitaxel has been previously shown to inhibit breast tumor angiogenesis (28). Therefore, in vivo, like paclitaxel, VERU-111 inhibited the proliferation of TNBC cells and disrupted the tumor vasculature (Fig. 5C and D). A dose-dependent increase in the percentage of cells expressing both cleaved PARP and cleaved caspase-3 was also observed, confirming that VERU-111 efficiently induces apoptosis in vivo (Fig. 5E and F). As expected, tumor growth suppression, tumor vasculature disruption, and apoptotic cell death induction were also evident for the paclitaxel treatment group (Fig. 5C–F). Overall, we conclude that the antitumor activity of VERU-111 is equivalent to paclitaxel in vivo, but without evidence of toxicity.

VERU-111 inhibits metastasis either from the mammary fat pad or in an experimental metastasis model

The lungs were harvested from the same cohort of MDA-MB-231 mammary tumor–bearing mice shown in Fig. 4 for each dose of VERU-111 and for paclitaxel. Lung macrometastases visible to the naked eye after H&E staining were only detected in the vehicle group (Fig. 6A). In the 5 mg/kg VERU-111 group, 2 of 8 treated mice did not develop any visible lung metastases, increasing to 4 of 8 mice in the 10 mg/kg VERU-111 treatment group and to 7 of 8 mice in the 12.5 mg/kg VERU-111 group. In comparison, no lung metastases were observed by either H&E staining or IHC in the 12.5 mg/kg paclitaxel-treated group (0 of 8 mice developed metastases). To test whether VERU-111 represses the conversion of seeded tumor cells into lethal lung macrometastases, an experimental metastasis (tail vein) model was used. Because mice subjected to experimental metastases typically lose weight as metastases develop, the dose of VERU-111 and paclitaxel was lowered to 10 mg/kg. As expected, metastases were readily detectable in all lungs from the vehicle group, whereas the lungs in VERU-111- and paclitaxel-treated groups exhibited considerably fewer metastases, which were also smaller in area (Fig. 6B). Both VERU-111 and paclitaxel were also highly effective in repressing metastasis to the kidney, where no metastases were detected in any mice. In contrast, VERU-111 appeared to be more effective than paclitaxel to repress liver metastases, as fewer lesions were observed (Fig. 6B). Importantly, the body weight (Supplementary Fig. S5A) and the physical activity of mice were normal in VERU-111–treated group. In fact, only the VERU-111–treated mice gained weight over the course of the experiment. As expected, mice in the vehicle treatment group lost weight over time. In the paclitaxel-treated group, mice lost approximately 10% of their initial body weight, demonstrating the relative toxicity of paclitaxel versus VERU-111. H&E staining also showed that multiple metastases with varying sizes were observed in the lung, liver, and kidney of vehicle-treated mice, whereas the metastases in the VERU-111 and paclitaxel-treated mice were overall less frequently observed and were smaller (Supplementary Fig. S5B). Collectively, these results support our conclusions that VERU-111 shows comparable efficacy with paclitaxel in suppressing metastasis of TNBC cells at equivalent doses, but without significant toxicity to mice, and with the distinct advantage of being orally bioavailable.

VERU-111 suppresses the TNBC growth and metastases in a taxane-resistant metastatic mouse model

To determine whether VERU-111 is effective in a taxane-resistant TNBC model, we first utilized two primary cell lines derived from well-characterized TNBC PDX models to compare...
colchicine, paclitaxel, and VERU-111 efficacy in vitro, a treatment-naive TNBC model (HCI-2-Luc2), and a taxane-resistant TNBC model (HCI-10-Luc2). Because both primary cell lines double slowly, approximately every 3 days (20), the duration of drug treatment was six days. As expected, the HCI-10 cells were more resistant to paclitaxel treatment than HCI-2 cells (grand mean IC\textsubscript{50} 39.27 ± 5.4 nmol/L vs. 6.20 ± 1.70 nmol/L; determined from n = 4 biological replicate experiments; Fig. 7A; Supplementary Table S2). However, for VERU-111, the IC\textsubscript{50} concentration in each cell model was nearly equivalent (mean IC\textsubscript{50} 11.89 ± 2.76 nmol/L vs. 10.78 ± 1.20 nmol/L), suggesting that VERU-111 maintained its efficacy to suppress growth of a taxane-resistant TNBC model. Colchicine is included as an assay control, although it is not clinically approved for breast cancer chemotherapy due to high toxicity. The HCI-10 cell line appeared to be refractory to colchicine compared with the HCI-2 cell line (46.08 ± 5.53 nmol/L vs. 36.93 ± 10.57 nmol/L), which is likely because relative to HCI-2 cells, HCI-10 cells over-expresses P-gp, which effluxes colchicine (Supplementary Fig. S6A; Figure 4. Antitumor effect of VERU-111 in orthotopic MDA-MB-231 xenografts. For all comparisons, one-way ANOVA was performed followed by Dunnett multiple comparisons testing and all P values are relative to the vehicle control group at experimental endpoint. A, The mean percent increase ± SEM in tumor volume relative to initial tumor volume when drug treatment began (tumors ~100 mm\textsuperscript{3}) following dosing with 5, 10, or 12.5 mg/kg VERU-111 (5 times/week, orally) or 12.5 mg/kg paclitaxel (every other day, i.p.). B, Mean percent change in mouse body weight ± SEM relative to body weight at the time of initiating drug treatment. Dashed lines indicate weight relative to the baseline (y-axis = 0) and 20% weight gain. A and B, Time is expressed as days postinjection of MDA-MB-231 cells to the mammary fat pad. C and D, Scatter plots of final tumor volume ± SEM of excised tumors measured ex vivo with calipers (C) and final tumor weight wet (D); the mean is indicated above each treatment group. E, Images representative of tumors for each cohort’s mean final tumor volume and wet weight.
Figure 5.
VERU-111 therapy induces tumor necrosis, represses proliferation and angiogenesis, and induces apoptosis in vivo. 

A, Representative images of H&E-stained whole tumor sections bisected at the widest width and representative stained slides following immunostaining with Ki67, CD31, cleaved PARP, and cleaved caspase-3. Images were captured at either ×20 or ×40 magnification; scale bar, 50 μm. Inserts show low-power images.

B, Quantification of the mean percentage of necrotic area ± SEM of H&E sections as scored by an experienced pathologist, expressed relative to the vehicle control.

C–F, Quantification of the mean percentage ± SEM of Ki67 (C), CD31 (D), cleaved PARP (E), and cleaved caspase-3 (F)–positive cells relative to the vehicle control group (100%); the mean value is indicated above each group. All P values are determined by one-way ANOVA followed by a multiple comparisons test.
Figure 6. VERU-111 treatment suppresses metastasis formation in an orthotopic xenograft model and in an experimental lung metastasis model using MDA-MB-231 cells. A, Representative H&E-stained sections of lung metastases derived from orthotopically implanted MDA-MB-231 cells. Lung metastases are indicated by yellow arrows in the H&E-stained slides; ×4 magnification; scale bar, 100 μm. Lung metastatic burden of each section from each mouse was quantified after scanning whole slides. B, Anti-human specific mitochondria IHC staining to detect metastases in lung, liver, and kidney sections; scale bar is 200 μm for primary figures and 50 μm for inserts. Scatter plots of mean ± SEM show the quantification of metastases present in the lung, liver, and kidney (A and B). The number above each treatment group correspond to the mean.
Antitumor efficacy of VERU-111 in the taxane-resistant HCI-10-Luc2 TNBC xenograft model. A, Antiproliferative effect of colchicine, paclitaxel, and VERU-111 was measured in vitro. The bar graphs represent the grand mean ± SEM of the calculated IC50 values from independent drug dosing experiments (n = 4) as calculated by the MTS method and validated by IncuCyte S3 live cell imaging (Supplementary Fig. S6A). B, The mean ± SEM tumor volume based on weeks on therapy in response to vehicle, 10 mg/kg VERU-111, orally (3 times/week) or 10 mg/kg paclitaxel, i.p. (once/week). At all time points beginning at week 1.5 of therapy, the differences between vehicle or paclitaxel versus VERU-111 are statistically significant; two-way ANOVA followed by multiple comparisons test. C, Mean percent change in mouse body weight ± SEM during therapy relative to the starting body weight at week 0. Dashed lines indicate weight decreases below baseline (y-axis = 0) and ±10% increase in body weight. D–K, For all experiments, statistically significant differences were determined by one-way ANOVA followed by Dunnett multiple comparison test at experimental endpoint; only those pairwise analyses that are significant (P < 0.05) are shown. The cohort mean is shown above each treatment group. For panels G–H, data are plotted on a log-scale along the y-axis, and for I–K, log10-transformed data are plotted. D, Scatter plot of ex vivo final tumor volume ± SEM. E, Scatter plot of final tumor wet weight ± SEM. F, Images of tumors representative of each group’s final tumor volume and wet weight. G, Mean ± SEM of total photon flux of AxLN metastases present in intact mice 1 day prior to administration of drug therapy, after assignment to a cohort; data are significant at experimental endpoint between either the vehicle control or the paclitaxel-treated group compared with VERU-111 (solid bracket). The colored dashed brackets compare the baseline AxLN signal of each group from week 0 to week 8. H, Mean ± SEM of total photon flux of AxLN metastases present at experiment endpoint. I–K, Mean ± SEM of log10-transformed total photon flux of lung (I), liver (J), and leg bone (K) metastases as measured ex vivo at experiment endpoint. J, Of note, whereas no signal for liver metastases was detectable in the VERU-111 treatment group, paclitaxel treatment significantly enhanced liver metastasis relative to the vehicle control.
A major clinical challenge is to treat established metastases of the stage IV patient. The HCl-10-Luc2 model metastasizes during early tumor outgrowth to the AxLNs (20). Therefore, by bio-imaging mice the day prior to drug treatment, after cohorts were randomized, we were able to identify a subset of mice in each cohort with pre-established AxLN mets prior to drug treatment and then to follow metastatic growth during treatment. There was exponential increase in AxLN metastatic signal measured by total photon flux comparing week 0 on therapy to week 8 on therapy at endpoint between the vehicle and paclitaxel cohorts (100- and 65-fold, respectively; Fig. 7B). The use of PDX xenograft models derived from patients and primarily passaged in mice are preferred xenograft models due to their conserved similarities to original patient material (19, 30, 31). We engrafted HCI-10-Luc2 patient-derived tissue fragments into female NSG mice and each recipient entered a treatment cohort when the tumor volume was approximately 100 mm^3. VERU-111 (10 mg/kg, orally) significantly suppressed xenograft growth over the entire course of the study, whereas there were no significant changes in tumor volume between the vehicle-treated or paclitaxel-treated (10 mg/kg, once/week, i.v.) cohorts at any time point (Fig. 7B). It should be noted that the dosing regimen for paclitaxel was changed for the HCl-10 xenograft model because the tumor fragments grow slower in vivo than MDA-MB-231 cells. Paclitaxel was provided 10 mg/kg i.v. once/week instead of 12.5 mg/kg i.p. every other day as in Fig. 4 to reduce potential cumulative toxicity due to the extended treatment regimen (~5 weeks longer duration of treatment than used for MDA-MB-231 xenografts; ref. 32). As observed for MDA-MB-231 xenograft model, there was no significant body weight loss during VERU-111 treatment (Fig. 7C). All animals were euthanized after 8 weeks of drug treatment. As expected, tumors in VERU-111-treated mice were significantly smaller than vehicle-treated mice, with reduced mean tumor volume and tumor weight by 73.7% and 72.0%, respectively (Fig. 7D and E). In contrast, paclitaxel treatment slightly increased mean tumor volume and tumor weight relative to the vehicle controls (Fig. 7D and E). Representative tumors of the mean tumor volume from vehicle-treated, paclitaxel-treated, and VERU-111-treated mice are shown in Fig. 7F.

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Discussion

The 5-year survival rate of patients diagnosed with metastatic TNBC is less than 30% (33–35). Challenges for patients with TNBC are a lack of effective targeted therapies and a more rapid onset of chemoresistance to conventional cytotoxic therapies. Chemotherapy is the primary systemic treatment for patients with TNBC, with the goal of inhibiting cancer cell growth and dissemination (36). Dynamic microtubule organization is one of the most successful targets due to the critical roles of the microtubule network in cell proliferation, particularly cell division (37). FDA-approved drugs, such as paclitaxel, are effective in treating neoadjuvant, adjuvant, or metastatic TNBC, but intrinsic or acquired resistance to taxanes is commonly observed. Moreover, paclitaxel needs to be formulated as an albumin-bound formulation or with a vehicle containing Cremophor EL due to poor aqueous solubility and then delivered intravenously by slow infusion (6, 38). There are also neurotoxicities associated with taxane agent use, including persistent peripheral neuropathies, which significantly impact the patient’s quality of life. As alternatives to paclitaxel, microtubule-targeted agents that bind to the colchicine-binding site of tubulin have attracted recent attention. Several colchicine-binding site inhibitors, including VERU-111, are currently undergoing clinical trials. In contrast, the CBSI combretastatin A-4 phosphate (CA-4) has been evaluated in clinical trials for over a decade and is not yet FDA approved (14, 39).

We previously reported a series of novel 2-aryl-4-benzoyl-imidazoles and derivatives that target the colchicine-binding site with potent cytotoxicity, have improved aqueous solubility and overcome multidrug resistance, including P-gp overexpression (17, 40, 41). We evaluated VERU-111, the most potent 2-aryl-4-benzoyl-imidazole analogue, in the treatment of TNBC, as its antiproliferative efficacy was not previously defined, using colchicine and paclitaxel as positive controls. We assessed the efficacy of VERU-111 against MDA-MB-231 and MDA-MB-468 TNBC cell lines. VERU-111 showed strong anti-proliferative activity in all these models with an IC50 value in the low nanomolar range. In a colony formation assay, VERU-111 reduced the clonogenic survival of TNBC cells. VERU-111 efficacy was superior to colchicine, although less potent than paclitaxel at the equivalent concentration to repress clonogenic survival.

Most of the microtubule-stabilizing agents enhance microtubule polymerization by targeting taxane-binding site located at the interior surface of the microtubules, while microtubule depolymerizing agents inhibit microtubule polymerization by binding in one of two domains on tubulin (7). To confirm that VERU-111 depolymerizes microtubules, we stained for α-tubulin in TNBC cells treated with VERU-111, which showed that VERU-111–treated cells displayed aberrant microtubule network arrangement and organization, similar to results obtained with colchicine-treated TNBC cells. Therefore, VERU-111 is a novel microtubule-depolymerizing agent. Because of the pivotal role of asymmetric regulation of microtubule dynamics and stability played in cell migration and invasion, we then investigated the effect of VERU-111 using cell migration and invasion assays (42, 43). Our results showed that VERU-111 inhibited TNBC cell motility and invasion potently.

Several antimotic agents have been reported to target microtubules, disrupt microtubule dynamics, induce cell-cycle arrest, and cause eventual cell death (44, 45). We found that VERU-111 arrested MDA-MB-231 cells at the G2–M checkpoint in a concentration-dependent manner. The effects of colchicine, paclitaxel, and VERU-111 were similar in MDA-MB-231 cells, with accumulated cells at the G2–M checkpoint; VERU-111 also induced cells into the M-phase in a concentration-dependent manner. However, VERU-111 or colchicine primarily induced a G2 phase arrest in MDA-MB-468 cells, whereas paclitaxel arrested cells in both the G2 and M phase. One reason for this result may be that MDA-MB-231 cells are a “type A” cell line that...
displays prominent mitotic arrest, whereas the MDA-MB-468 cell line is a "type B" cell line, displaying less prominent mitotic arrest, but possessing significant mitotic arrest at higher concentrations of microtubule-targeting agents (46). It is widely believed that antimitotic agents cause apoptosis along with cell-cycle arrest (47, 48). Cleaved caspase-3 is an effector caspase involved in both intrinsic and extrinsic apoptosis pathways; it can cleave its downstream target PARP, which displays prominent mitotic arrest, whereas the MDA-MB-468 cell line is a "type B" cell line, displaying less prominent mitotic arrest, but possessing significant mitotic arrest at higher concentrations of microtubule-targeting agents (46). It is widely believed that antimitotic agents cause apoptosis along with cell-cycle arrest (47, 48). Cleaved caspase-3 is an effector caspase involved in both intrinsic and extrinsic apoptosis pathways; it can cleave its downstream target PARP, which

data suggests that VERU-111 is efficacious at killing TNBC cells in a time- and concentration-dependent manner, and it promoted the cleavage of caspase-3 and PARP in MDA-MB-231 and MDA-MB-468 cells. Together, these results indicate that VERU-111 induces cell-cycle arrest and ultimately apoptosis in TNBC cells.

The chemotherapeutic strategies for treating TNBC primarily include DNA-damaging agents, antimitotic agents, and targeted antiangiogenic therapy. For example, the combination use of carboplatin, paclitaxel, and bevacizumab, respectively, is recommended as a first-line or second-line therapy for TNBC treatment (51–53). However, novel chemotherapies are required to improve the survival rates for TNBC due to taxane-induced dose-limiting neurotoxicities or cardiotoxicities and acquired resistance to taxanes (54). We evaluated the antitumor effect of orally delivered VERU-111 versus paclitaxel (provided intraperitoneally or intravenously) in two orthotopic, highly metastatic TNBC models to determine whether VERU-111 is an efficacious novel anti-tubulin inhibitor to treat TNBC. For the MDA-MB-231 xenograft model, for which cells were not previously exposed to taxanes, VERU-111 inhibited TNBC tumor growth in a dose-dependent manner without reducing body weight, and with equivalent efficacy to paclitaxel to reduce tumor volume/wet weight. Both drugs were observed to increase tumor necrosis, to decrease cancer cell proliferation, to reduce angiogenesis and to cause apoptotic cell death via increased the expression of cleaved caspase-3 and cleaved PARP.

TNBC frequently progresses to stage IV, metastatic disease, the leading cause of breast cancer–associated mortality (55). In the MDA-MB-231 model, either paclitaxel or VERU-111 inhibited the formation of lung metastasis from the mammary pad, reducing the total number and size of metastases that had completed the entire metastatic cascade. One limitation in this experiment is that the tumors were not equal sizes at the time of euthanasia and lung harvest (both paclitaxel- and VERU-111–treated tumors were smaller than vehicle-treated tumors). Therefore, to bypass primary tumor growth, we supplemented these results by a tail vein assay in which mice were treated with either VERU-111 or paclitaxel 24 hours after tumor cell injection. Fewer metastases were observed in lungs, livers, and kidneys by H&E or IHC following VERU-111 or paclitaxel treatment. Although both drugs appeared equally effective to repress lung and kidney metastasis, VERU-111 appeared to be more effective than paclitaxel in reducing liver metastasis.

Finally, we have shown in vivo that VERU-111 is efficacious at similar nanomolar concentrations in vitro for both a taxane-resistant patient-derived primary cell line (HCl-10-Luc2) and a treatment-naïve patient-derived primary cell line (HCl-2-Luc2). In contrast, the IC_{50} concentration of paclitaxel required to inhibit growth of HCl-10 cells was between 6.33- and 8.5-fold higher than for HCl-2 cells, depending on the method used to calculate IC_{50} values. These data provide rationale that VERU-111 likely bypasses taxane resistance in breast cancer, as we have previously shown for VERU-111 in prostate cancer models (18). VERU-111 efficacy was then tested in vivo using the luciferase-labeled HCl-10 taxane-resistant model. VERU-111 therapy was highly effective in blocking primary tumor growth, whereas paclitaxel treatment was completely ineffective relative to vehicle-treated controls at all time points. In addition, VERU-111 treatment significantly reduced outgrowth of preestablished AxLN metastases over the course of drug treatment and the endpoint metastatic burden for AxLNs, lung, and bone while blocking liver metastasis. Collectively, these data strongly suggest that VERU-111 may also be an effective second-line therapy for patients with TNBC who progress on taxanes (i.e., develop taxane resistance).

In summary, we have shown in vivo in two aggressive TNBC xenograft models that a novel microtubule-targeting agent to the colchicine-binding site, VERU-111, which is orally bioavailable and well-tolerated, displays potent anti-breast cancer activity, including antimitastatic potential. VERU-111 has been previously reported to inhibit tubulin polymerization and to effectively overcome P-gp–mediated multidrug resistance, which frequently develops in patients treated with conventional taxanes (17). The distinct advantages of VERU-111 therapy include equivalent efficacy as paclitaxel in vivo and in treatment-naïve TNBC cell line models, and the ability to effectively treat taxane-resistant disease in vivo. Moreover, compared with taxanes, VERU-111 analogues do not induce peripheral neuropathy in mice and dogs (56), addressing a currently unmet clinical challenge impacting patient quality of life. A phase I/II clinical trial of VERU-111 has begun for patients with advanced prostate cancer, including patients who have already failed taxane-based therapies. Our results suggest that clinical trials for patients with stage IV metastatic breast cancer, including patients diagnosed with TNBC, are also supported by strong preclinical data.

Disclosure of Potential Conflicts of Interest

W. Li is a scientific consultant at and reports receiving a commercial research grant from Veru, Inc. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: S. Deng, Z. Lin, T.N. Seagroves, W. Li
Development of methodology: S. Deng, W. Li
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Deng, R.I. Krutliina, Q. Wang, Z. Lin, D.N. Parke, H.C. Playa, T.N. Seagroves
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Deng, Z. Lin, H.C. Playa, H. Chen, T.N. Seagroves, W. Li
Writing, review, and/or revision of the manuscript: S. Deng, Q. Wang, Z. Lin, D.D. Miller, T.N. Seagroves, W. Li
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.D. Miller, T.N. Seagroves, W. Li
Study supervision: T.N. Seagroves, W. Li

Acknowledgments

We thank Dr. Louisa Balazs and the UTHSC Research Histology Core (RHC) for assistance in quantifying the necrotic percentage of representative tumor tissues and Drs. Desidee Daria and Tony Marion of the UTHSC Flow Cytometry and Cell Sorting (FCCS) core for assistance with cell-cycle analysis using FlowJo. We thank Dr. Radika Sekhi and the Department of Pathology at UTHSC for generously scanning all stained slides. We thank Dr. Alana Wehn at the Huntsman Cancer Institute (HCI) for providing the original PDX models and for performing whole-genome expression analysis of the Lu2-labeled PDX derivatives to verify their origin. This work was supported by the NIH grants R01CA193609 (to W. Li), R01CA148706 (to W. Li and D. D. Miller), and R01CA138488 (to T. Seagroves), and the 2018 West Cancer Center Research Awards (to W. Li and to T. Seagroves). The contents of the article are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

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Received May 21, 2019; revised August 27, 2019; accepted October 15, 2019, published first October 23, 2019.
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Published OnlineFirst October 23, 2019; DOI: 10.1158/1535-7163.MCT-19-0536
Molecular Cancer Therapeutics

An Orally Available Tubulin Inhibitor, VERU-111, Suppresses Triple-Negative Breast Cancer Tumor Growth and Metastasis and Bypasses Taxane Resistance

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Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-19-0536

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