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Norstictic Acid Inhibits Breast Cancer Cell Proliferation, Migration, Invasion, and *In Vivo* Invasive Growth Through Targeting C-Met

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Abstract

Breast cancer is a major health problem affecting the female population worldwide. The triple-negative breast cancers (TNBCs) are characterized by malignant phenotypes, worse patient outcomes, poorest prognosis, and highest mortality rates. The proto-oncogenic receptor tyrosine kinase c-Met is usually dysregulated in TNBCs, contributing to their oncogenesis, tumor progression, and aggressive cellular invasiveness that is strongly linked to tumor metastasis. Therefore, c-Met is proposed as a promising candidate target for the control of TNBCs. Lichen-derived metabolites are characterized by their structural diversity, complexity, and novelty. The chemical space of lichen-derived metabolites has been extensively investigated, albeit their biological space is still not fully explored. The anticancer-guided fractionation of *Usnea strigosa* (Ach.) lichen extract led to the identification of the depsidone-derived norstictic acid as a novel bioactive hit against breast cancer cell lines. Norstictic acid significantly suppressed the TNBC MDA-MB-231 cell proliferation, migration, and invasion, with minimal toxicity to non-tumorigenic MCF-10A mammary epithelial cells. Molecular modeling, Z'-LYTE biochemical kinase assay and Western blot analysis identified c-Met as a potential macromolecular target. Norstictic acid treatment significantly suppressed MDA-MB-231/GFP tumor growth of a breast cancer xenograft model in athymic nude mice. Lichen-derived natural products are promising resources to discover novel c-Met inhibitors useful to control TNBCs.

Keywords

norstictic acid; lichen; c-Met; breast cancer; TNBC; *Usnea*

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SUPPORTING INFORMATION

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Conflict of Interest

Authors declare no conflicts of interest.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in women and the second leading cause of death among the female population (Siegel *et al.*, 2015). Over 234 000 women will be diagnosed with breast cancer and more than 40 290 will die from disease complications in 2015 in the U.S. according to recent statistics by the American Cancer Society.

Classically, breast cancer was classified based on clinical and histopathological criteria. In the past decade, microarray technology identified four main molecular subtypes of breast cancer; luminal, HER2, normal-like, and basal (Sørli *et al.*, 2003; Reis-Filho and Pusztai, 2011). In particular, most of the basal subtypes are negative for estrogen receptor (ER), progesterone receptor (PR), and HER2 and thus have been recognized as triple-negative breast cancers (TNBCs). TNBC is considered a heterogeneous group that differs in gene expression, response to chemotherapeutic drugs and biological therapies, and disease prognosis (Duffy *et al.*, 2012). Clinically, patients with TNBCs have worse outcomes and poor prognosis than those with the other forms of the disease, along with few available therapeutic options. Therefore, identification and validation of selective targeted therapies for TNBCs are important unmet medical need.

One of the promising candidate targets for the control of TNBCs is c-Met (mesenchymal epithelial transition factor). c-Met is a multifunctional membrane-bounded receptor tyrosine kinase that is normally expressed in epithelial cells of many organs (Comoglio *et al.*, 2008). Physiologically, c-Met and its high affinity ligand hepatocyte growth factor (HGF) act as a pleiotropic signaling axis promoting diverse phenotypes such as cell proliferation, survival, motility, invasion, differentiation, and morphogenesis (Organ and Tsao, 2011). Phenotypes result from c-Met activation rely on multiple stereotypical signaling pathways common to many RTKs (Trusolino *et al.*, 2010). For instance, c-Met activation positively regulates the critical PI3K/Akt/mTOR, Paxillin/Rac-1, and STATs signaling cascades (Paumelle *et al.*, 2002). HGF/c-Met axis has been found to be aberrantly dysregulated in TNBCs and associated with aggressive tumor growth, induction of angiogenesis, and metastasis (Danilkovitch-Miagkova and Zbar, 2002). Blockade of HGF/c-Met axis is documented to attenuate breast tumor growth and metastasis in animal models (Akl *et al.*, 2014). Recently, Chang and colleagues have summarized the current status of the c-Met inhibitors in preclinical and clinical studies (Chang *et al.* 2015). In particular, the first FDA approved c-Met inhibitors were crizotinib, a dual Met and ALK inhibitor approved for ALK-driven lung cancer and cabozantinib approved in 2012 for medullary thyroid cancer. Currently more than 240 entities are undergoing different clinical trial phases for the treatment of bladder, ovarian, prostate, brain, melanoma, breast, non-small cell lung, pancreatic, kidney and hepatocellular cancers. c-Met is a necessary oncogene for TNBCs growth and invasiveness; thus, c-Met targeted therapies would be effective front-line intervention to control TNBCs.

Lichens are unique symbiotic associations of fungi (mycobionts) and photosynthetic partners (photobionts) that are usually algae or cyanobacteria. Symbiosis allows lichens to grow under unusual environmental conditions, in which both partners could not grow alone (Nash, 2008). Thus, lichens biosynthesize diverse secondary metabolites to provide protection against negative physical and biological influences. Chemically, lichens' secondary metabolites comprise diverse classes, including mononuclear phenols, quinones,

dibenzofuranes, depsides, depsones, depsidones, γ -lactones, and xanthenes. Lichens had been commonly used for centuries in traditional herbal remedies to treat various human and animal disorders (Crawford, 2015). For instance, Iceland moss (*Cetraria islandica*) has been used for centuries to relieve chest ailments. Several species of the lichen *Usnea* and its major secondary metabolite usnic acid have been used in traditional Chinese medicine for thousands of years and as dietary supplements. To date, numerous pharmacological activities have been reported for lichen metabolites, including antioxidant, anti-inflammatory, antimicrobial, antiviral, and anticancer (Molnár and Farkas, 2010).

Norstictic acid (Fig. 1A) is a depsidone-derived metabolite common in several lichen species of the genera *Usnea*, *Ramalina*, and *Parmelia*. Various biological activities have been reported for norstictic acid including antioxidant, antimicrobial, and cytotoxic (White *et al.*, 2014). Interestingly, no previous data were reported addressing the possible macromolecular target(s) of norstictic acid in cancers.

The current study demonstrates the anticancer-guided fractionation of *Usnea strigosa* (Ach.) lichen extract to characterize the bioactive hit(s). Norstictic acid was identified as a novel bioactive metabolite against different human breast cancer cell lines. Norstictic acid was further appraised for its ability to inhibit *in vitro* migration and invasion of metastatic human breast cancer MDA-MB-231 cells. Molecular modeling, Z'-LYTE™ biochemical kinase assay and Western blot analysis confirmed c-Met as a possible molecular target. Norstictic acid attenuated the tumor growth of the TNBC MDA-MB-231/GFP cells in a xenograft breast cancer model in nude mice.

MATERIALS AND METHODS

Lichen collection, extraction, and bioassay-guided isolation of norstictic acid

The lichen *Usnea strigosa* (Ach.) family Parmeliaceae was collected April 2014 at the Russell Sage State Wildlife Management Area (Monroe, Louisiana) and identified by Dr. Joydeep Bhattacharjee (Department of Biology, School of Sciences, University of Louisiana at Monroe, Monroe, Louisiana). A voucher specimen (SE004B) was deposited at the Department of Basic Pharmaceutical Sciences, University of Louisiana at Monroe. Five hundred grams of dried *U. strigosa* were consecutively extracted with *n*-hexanes, EtOAc and EtOH (3X $\times$ 2L). Extracts were pooled, concentrated under vacuum, and kept at -20°C till used. Representative samples of each extract were prepared in DMSO (1mg/15 μL) and tested against the TNBC breast cancer cell line MDA-MB-231 and other breast cancer cell lines using MTT proliferation assay. EtOAc extract proved the most active and therefore was fractionated on C-18 RP-Si gel (40 μm , Sigma-Aldrich, St. Louis, MO) and eluted with mixtures of H_2O and CH_3CN . Similar fractions were pooled together and evaluated in MTT proliferation assay. Active fractions were further purified on C-18 RP-Si gel to afford the pure bioactive metabolite norstictic acid.

Cell lines and culture conditions

Human breast cancer cell lines; MDA-MB-231, MDA-MB-468, MCF-7, T-47D, BT-474, and SK-BR-3, and the human immortalized mammary epithelial MCF-10A cell line, were

obtained from the American Type Culture Collection (Manassas, VA). Breast cancer MDA-MB-231/Green Florescent Protein-tagged (MDA-MB-231/GFP) cell line was purchased from Cell Biolabs (San Diego, CA). Cells were cultured and maintained in RPMI-1640 medium (Corning, Manassas, VA) buffered with HEPES (5.95 g/L) and sodium bicarbonate (2.2 g/L) and supplemented L-glutamine (2.1mM), 10% (V/V) heat-inactivated fetal bovine serum (Valley Biomedical, Winchester, VA) and antibiotic/antimycotic solution (Corning, Manassas, VA); penicillin (100U/mL), streptomycin (100 µg/mL) and amphotricin B (0.25 µg/mL), in a 5% CO₂ humidified incubator at 37 °C. MCF-7 cells were cultured and maintained in DMEM (Gibco® by Life Technologies, Grand Island, NY) supplemented with 10% (V/V) hyclone FBS (GE Healthcare Life Sciences, Pittsburgh, PA). The immortalized mammary epithelial MCF-10A cells were maintained in DMEM/high glucose (Life Technologies, Grand Island, NY) supplemented with 10% (V/V) horse serum (Life Technologies, Grand Island, NY), EGF (Peprotech, Rocky Hill, NJ, 20 ng/mL), hydrocortisone (Corning, Manassas, VA, 0.5 mg/mL), cholera toxin (Sigma-Aldrich, St. Louis, MO, 100 ng/mL), bovine insulin (Sigma-Aldrich, St. Louis, MO, 10 µg/mL), and the same antibiotic/antimycotic solution used for other cell lines.

Cell proliferation assay

Breast cancer cells were trypsinized, harvested by centrifugation, and counted using trypan blue exclusion dye and a hemocytometer. Cells were seeded into 96-well plate at a density 5×10^3 cells/well in 100µL culture medium. Three wells containing only culture medium were used as blank for final absorbance readings. Cells were incubated overnight at 37 °C in a 5% CO₂ humidified incubator to recover and attach. Media were then carefully removed, and cells were washed with 50 µL PBS. Lichen extracts (1mg/15 µL) and purified norstictic acid (25 mM) were prepared as stock solutions in DMSO and immediately added to culture medium (supplemented with 100 ng/mL HGF, for c-Met-dependent cells) to prepare the final working concentrations. Treatment media (100 µL) were added, in triplicates, and cells were incubated for 72 h. Vehicle control wells were treated with culture media containing the maximum amount of DMSO added in treatment sets. At the end of incubation period, media were gently aspirated and cells were rinsed with 50µL PBS. One-hundred microliter fresh media and 50 µL of MTT in PBS were added to each well, and cells were then incubated for additional 4 h. After incubation, supernatants were carefully removed and formazan crystals were dissolved in 100µL DMSO. The 96-well plate was then incubated in dark for 5 min and the gently swirled before measuring the absorbance at 570 nm using Synergy 2 microplate reader (BioTek, Winooski, VT). Average values from triplicate readings were calculated and subtracted from the average value of blank. Cell numbers were deduced from a standard curve executed at the beginning of each experiment. IC₅₀ for each cell line was calculated using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA).

Cell migration assay

The TNBC MDA-MB-231 cells were seeded into a 24-well plate at a density 1×10^5 cells/well and then incubated overnight to recover and attach at 37 °C in a 5% CO₂ humidified incubator. Wounds were inflicted in confluent monolayers using sterile 200 µL pipette tips. Wells were then washed with PBS to remove cell debris and reincubated in serum-free

media for 5 h. After then, media were replaced by fresh ones supplemented with the scattering factor HGF (100 ng/mL) and containing different concentrations of norstictic acid or DMSO as vehicle control. The olive oil phenolic oleocanthal (5 μ M) was used as a positive standard antimigratory control (Akl *et al.*, 2014). Wounds were photographed at zero time using VistaVision Still camera (VWR, Radnor, PA) connected to an inverted VistaVision microscope (VWR, Radnor, PA). Wounds were monitored for closing up to about 24 h. When wound were about to close, media were aspirated and cells were washed by PBS and fixed with cold methanol for 15 min at 4 °C. Finally, wounds were photographed for treatments and vehicle control wells. Percentage cell migration were calculated using the following formula (Elsayed *et al.*, 2015):

$$\text{Percent cell migration} = \frac{[T_0 - T_t - T_{dms0}]}{[T_0 - T_{dms0}]} \times 100$$

where T_0 is wound thickness at zero time, T_{dms0} is wound thickness in DMSO-treated control wells, and T_t is wound thickness in treatment wells. IC₅₀ values were calculated using GraphPad Prism version 5.01 (GraphPad Software, CA).

Cell invasion assay

CultreCoat® 96-well BME invasion kit (Trevigen, Gaithersburg, MD) was utilized to assess the ability of norstictic to inhibit the *in vitro* invasion of the TNBC MDA-MB-231 cells through basement membrane extract (BME). The experiment was performed according to manufacturer procedures with optimization regarding the number of cells per well. The 96-well invasion chamber was kept at rt for 1 h to equilibrate prior use. Inserts were rehydrated by adding 25 μ L of warm RPMI-1640 media and incubated at 37 °C for 1 h. Cells in culture plates were serum-starved for 16 h prior to the assay. Cells were then harvested, resuspended and counted to prepare working concentration of 1×10^6 cell/mL. To the top hydrated inserts, 25 μ L of cell suspension (2.5×10^4 cells) were added. Meanwhile, 150 μ L of serum-free media were added to the bottom chamber, supplemented with 100ng/mL HGF and containing either norstictic acid or DMSO as a vehicle control. The olive oil phenolic oleocanthal (5 μ M) was used as a standard anti-invasive positive control (Akl *et al.*, 2014). Plates were then assembled and incubated at 37°C in a 5% CO₂ humidified incubator for 24 h. After incubation, top chamber was inverted and carefully shaken to remove the media, and then transferred to a black receiver plate provided with the kit. Wells of the top chamber were washed with 100 μ L warm washing buffer and placed back on the receiver plate. One-hundred microliters of cell dissociation solution/calcein AM were added to each well of the lower chamber, and plates were incubated for an additional 1h. Finally, the top chamber was removed and fluorescence in lower plate was measured at 485nm excitation, 520nm emission using Synergy 2 microplate reader (BioTek, Winooski, VT). Relative fluorescence units were used to calculate number of invaded cells in control and treatment wells, using a standard curve constructed prior start of the experiment. Mean percents invasion were calculated relative to the vehicle treated control wells. IC₅₀ value was calculated using GraphPad Prism version 5.01 (GraphPad Software, CA).

Lactate dehydrogenase (LDH) release assay

LDH cytotoxicity assay kit (Cayman, Ann Arbor, MI) was used to measure cell death in response to various concentrations of norstictic acid. The assay followed the manufacturer procedure after optimization regarding number of cells per well and serum concentration. Briefly, MDA-MB-231 cells were seeded into 96-well plate at a density 3×10^4 cells/well in 200 μ L culture media, while three wells were left without cells as background control. After cell recovery and attachment, media were removed and cells were treated with 200 μ L culture media supplemented with 5% FBS and containing norstictic acid at desired concentrations. Triton X-100 (20 μ L) was added to three wells containing the cells (as maximum release) and 20 μ L of assay buffer to other three cell-free wells (as spontaneous release). The plate was then incubated at 37 °C in a 5% CO₂ humidified incubator for 24 h. After incubation, plate was centrifuged at 400 $\times$ g for 5 min and 100 μ L of supernatant were then transferred to a new 96-well plate. Reaction buffer (100 μ L of NAD⁺, lactic acid, INT, reconstituted diaphorase) was added to each well and plate was incubated with gentle shaking on an orbital shaker for 30 min at rt. Finally, absorbance was measured at 490 nm using Synergy 2 microplate reader (BioTek, Winooski, VT). The percent cytotoxicity was calculated as following:

$$\% \text{Cytotoxicity} = \left[\frac{(\text{Experimental value A490}) - (\text{Spontaneous release A490})}{(\text{Maximum release A490}) - (\text{Spontaneous release A490})} \right] \times 100$$

MTT cytotoxicity assay

The human non-tumorigenic breast epithelial MCF-10A cells were used to assess selectivity of norstictic acid towards cancerous cells. In short, cells were seeded into 96-well plate at density 3×10^4 cells/well in 100 μ L culture media. Cells were incubated overnight at 37 °C in a 5% CO₂ humidified incubator to recover and attach. Media were then carefully removed, and cells were washed with 50 μ L PBS. Various concentrations of norstictic acid in culture media were added, in triplicates, while vehicle control wells were treated with media containing the maximum amount of DMSO added in treatment sets. Doxorubicin (10 μ M) was used as a cytotoxic standard positive control. Cells were then incubated for 24 h. At the end of incubation period, media were removed and cells were washed with PBS. One hundred microliters fresh culture media and 50 μ L of MTT in PBS were added to each well. Plate was then incubated to for 4 h. After crystals were fully grown, supernatants were removed and crystals were dissolved in 100 μ L DMSO. Plate was incubated in dark for 5 min and gently swirled before measuring the absorbance at 570 nm in Synergy 2 microplate reader (BioTek, Winooski, VT). Average values from triplicate readings were calculated and subtracted from the mean value of blank wells. Cell numbers were deduced from a standard curve executed at the beginning of the experiment. Percent cell viability was calculated by comparing number of cells in treatment wells and vehicle-treated control wells.

Biochemical c-Met kinase assay

The Invitrogen Z'-LYTE™ Kinase Assay-Tyr6 Peptide kit (Thermo Fisher Scientific, Madison, WI) was used to assess the ability of norstictic acid treatment to inhibit *in vitro* c-Met kinase activity. Briefly, 20 μ L/well reactions were set in 96-well plate containing kinase

buffer, 200 μ M ATP, 4 μ M Z'-LYTE™ Tyr6 Peptide substrate, 2500 ng/mL c-Met kinase domain, and various concentrations of norstictic acid. The olive oil phenolic oleocanthal (5 μ M) was used a standard c-Met inhibitor positive control (Akl *et al.*, 2014). Reaction plate was incubated for 1 h at rt, after which 10 μ L of the development solution containing site-specific protease were added to each well and plate was incubated for additional 1 h. The reaction was then stopped, and the fluorescence signal ratio of 445 nm (coumarin)/520 nm (fluorescein) was determined using FLx800™ plate reader (BioTek, Winooski, VT), which reflects the peptide substrate cleavage status and/or the kinase inhibition in the reaction. The IC₅₀ value was calculated by nonlinear regression of log concentration versus the % phosphorylation, implemented in GraphPad Prism version 5.01 (GraphPad Software, CA).

Protein extraction and Western blot analysis

The TNBC MDA-MB-231 cells were seeded at a density 5×10^5 /100 mm culture dish and incubated overnight to recover and attach at 37 °C in a 5% CO₂ humidified incubator. Cells were treated either with norstictic acid or DMSO as a vehicle control, in media supplemented with 100 ng/ml HGF for 48 h. Total protein content was obtained using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Madison, WI). Samples were diluted in Laemmli buffer (BIO-RAD, Hercules, CA) containing 5% β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO) prior loading on gels. Protein lysates (30 μ g) were electrophoresed on Mini-PROTEAN® TGX™ precast polyacrylamide gels (BIO-RAD, Hercules, CA) using Tris/Glycine/SDS running buffer and then transferred to Immobilon-P® PVDF membranes (BIO-RAD, Hercules, CA). Blotted membranes were subsequently blocked with 5% BSA (Cell Signaling Technology, Beverly, MA) in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) for 2 h with agitation at rt. Blots were incubated overnight at 4 °C with appropriate primary antibodies (Cell Signaling Technology, Beverly, MA). After incubation, membranes were washed with TBST and then incubated with HRP-labeled secondary antibodies (Cell Signaling Technology, Beverly, MA) for 1 h with agitation at rt. Chemiluminescence detection was performed using Supersignal West Pico kit (Thermo Fisher Scientific, Madison, WI) and G. BOX imaging system with high-resolution 100 m pixel camera (Syngene, Fredrick, MD).

Molecular modeling experiments

Molecular modeling—The *in-silico* experiments were carried out using a Schrödinger molecular modeling software package installed on an iMac 27-inch Z0PG workstation with a 3.5 GHz Quad-core Intel Core i7, Turbo Boost up to 3.9 GHz, processor and 16 GB RAM (Apple, Cupertino, CA).

Protein structure preparation—The experimental X-ray crystal structure of human HGF receptor (c-Met) kinase domain; PDB code: 4R1V (Dorsch *et al.*, 2015), was retrieved from the Protein Data Bank. The Protein Preparation Wizard of Schrödinger suite was implemented to prepare c-Met kinase domain by assigning bond orders, adding hydrogens, creating disulfide bonds and optimizing H-bonding networks using PROPKA (Jensen Research Group, Copenhagen, Denmark). Finally, energy minimization with a root mean square deviation value of 0.30 Å was applied using an Optimized Potentials for Liquid Simulation (OPLS_2005, Schrödinger, New York, NY) force field.

Ligand structure preparation—The 2D structure of norstictic acid was sketched in the Maestro 9.3 panel (Maestro, version 9.3, 2012, Schrödinger, New York, NY). The Lig Prep 2.3 module (Lig Prep, version 2.3, Schrödinger, New York, NY) of the Schrödinger suite was utilized to generate 3D structure and to search for different conformers. The Optimized Potentials for Liquid Simulation (OPLS_2005, Schrödinger, New York, NY) force field was applied to geometrically optimize the ligand and to compute partial atomic charges. Finally, at most, 32 poses per ligand were generated with different spatial features for the subsequent docking studies.

Molecular docking—The prepared X-ray crystal structure of c-Met catalytic domain was employed to generate receptor energy grids using the default value of the protein atomic scale (1.0 Å) within the cubic box centered on experimental cocrystallized ligand {3-[1-(3-{5-[(1-methylpiperidin-4-yl)methoxy]pyrimidin-2-yl}benzyl)-6-oxo-1,6-dihydropyridazin-3-yl]benzonitrile}. After receptor grid generation, norstictic acid was docked into the c-Met kinase domain using Glide 5.8 module (Glide, version 5.8, 2012, Schrödinger, New York, NY).

***In vivo* study of a breast cancer model in nude mice**

Animals—Female athymic nude mice (5–6 weeks old) were purchased from Harlan Laboratories (Cumberland, IN). Animals were housed at the Animal Facility (University of Louisiana at Monroe, School of Pharmacy, Monroe, LA) and maintained under clean conditions in sterile filter top cages, at temperature of 24 ± 2 °C, $50 \pm 10\%$ relative humidity and 12:12 h artificial light–dark cycle. Mice were received mouse chow and water *ad libitum*. All procedures were conducted in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. All *in vivo* studies were carried out under an approved protocol by University of Louisiana-Monroe Institutional Animal Care and Use Committee.

Xenograft breast cancer model—MDA-MB-231 Green Florescent Protein-tagged (MDA-MB-231/GFP) breast cancer cells were cultured and maintained in RPMI-1640 medium supplemented with 10% FBS. Cells were harvested, washed with PBS, and resuspended in RPMI-1640 medium. Cells (1×10^6 cells/25 μ L) were injected into the mammary fat pad of each nude mouse, using 29G hypodermic needle. Animals were observed daily for the growth of palpable tumor at the site of injection. Ten-day post-implantation, tumors became visible with average volume of 50 mm³. Mice were then randomized and allocated to control and treatment groups (4 mice/group). Norstictic acid was prepared as a stock solution in sterile DMSO (1mg/20 μ L). Prior treatment, the stock solution was diluted with PBS containing 0.1% Tween 80 and then injected intraperitoneal at a dose regimen of 15 mg/kg body weight, three times per week. Animals in the control group received the same volume of vehicle. Tumor dimensions were measured three times per week using a digital caliper (VWR, Radnor, PA). Animals were daily monitored for any signs of treatment/vehicle associated toxicity and weighed three times per week. Tumor volume was calculated using the well-established formula: tumor volume (mm³) = [(length $\times$ width²)/2]. Animals were sacrificed at the indicated times, unless they appeared to be moribund or tumor showed signs of necrosis. At termination, tumors were excised from the

connective tissues, weighed and then snap frozen. Visceral organs including spleen, liver, kidney, and lung were excised, weighed.

Statistical analyses

All *in vitro* experiments were performed in triplicates. Pooled data were subjected to statistical analyses using GraphPad Prism version 5.01 (GraphPad Software, CA). Differences between means from two different groups were subjected to Student's *t*-test, whereas one-way analysis of variance followed was used to analyze significant differences between three or more groups. The *in vivo* tumor growth data were subjected to two-tailed Student's *t*-test. Results were considered to be significantly different when *p* value < 0.05, indicated by * symbol.

RESULTS AND DISCUSSION

The hexane, EtOAc, and EtOH extracts of *U. strigosa* (Ach.) were screened against the TNBC cell line MDA-MB-231. The EtOAc extract showed a significant antiproliferative activity with IC₅₀ value of 3.7 µg/mL. The extract was then tested against the human breast cancer cell lines MDA-MB-468, BT-474, SK-BR-3, MCF-7, T-47D and showed IC₅₀ values of 4.5, 7.9, 7.5, 6.4, and 9.6 µg/mL, respectively (Fig. 1B). Further bioassay-guided fractionation identified the known lichen acid norstictic as a promising antiproliferative metabolite. The chemical identity of norstictic acid was confirmed by NMR spectroscopy (Figure S1, Supporting Information) and comparison with literature (Honda *et al.*, 2015). Norstictic acid was further evaluated against the six breast cancer cell lines in MTT proliferation assay. Data indicated that norstictic acid significantly inhibited the proliferation of breast cancer cell lines in a concentration-dependent manner (Fig. 1C). The best growth inhibition was observed for the c-Met overexpressing MDA-MB-231 and MDA-MB-468 cells, with corresponding IC₅₀=14.9 ± 1.4 and 17.3 ± 1.6 µM, respectively.

Cancer metastasis accounts for more than 90% of cancer-related mortality (Steeg, 2006 and Nguyen *et al.*, 2009). Therefore, the discovery of new antimigratory and anti-invasive hit entities would be important for the control of cancer metastasis. The wound healing assay is an adaptable procedure for monitoring two-dimensions cell motility *in vitro* in response to scatter factors that will mimic, at least in part, tumor cell motility during metastasis. Representative microscopic images of wounds at zero time and 21 h post-treatment for vehicle and norstictic acid are shown in Fig. 2A. Norstictic acid significantly reduced the MDA-MB-231 *in vitro* cell migration in a concentration dependent manner (Fig. 2B), with an IC₅₀ of 13.2 ± 1.9 µM.

CultreCoat® 96-well medium BME cell invasion kit was used to assess the ability of norstictic acid to inhibit MDA-MB-231 cell invasion through basement membranes, which considered as the major matrix barrier for metastatic tumor cells invasive cascade. Cells were induced to invade the BME by 100 ng/mL HGF for 24 h. Effective treatments resulted in high cell density at the upper-chamber invasion wells, while vehicle control and ineffective treatment wells displayed minimum cell density (higher cell invasion). Representative microscopic photographs of vehicle control and norstictic acid treated top wells are shown in Fig. 2C. Data indicated that norstictic acid treatment significantly

inhibited MDA-MB-231 cell invasion through BME in a dose dependent manner (Fig. 2C), with a corresponding IC₅₀ value of 18.2 ± 2.1 μM.

The tetrazolium-based anticancer screening assays have the limitation of being unable to differentiate between cell cycle inhibition and cellular death (Galluzzi *et al.*, 2009). Lactate dehydrogenase is a soluble cytosolic enzyme, which is released into surrounding culture medium upon cell damage or lysis during apoptosis and necrosis. Therefore, the detection of LDH in the culture medium can be used as a marker for cytotoxicity. To evaluate the effect of norstictic treatment on MDA-MB-231 cells' viability, LDH release was quantified. Data revealed that norstictic acid resulted in a minimal LDH release (Figure S2, Supporting Information). Therefore, norstictic acid treatment induced cell growth arrest rather than cellular death in MDA-MB-231 cells at achievable anticancer doses.

To evaluate the relative selectivity of norstictic acid against the breast cancer cells, the non-tumorigenic MCF-10A human mammary epithelial cells were treated with various concentrations of norstictic acid for 24 h. Results demonstrated that norstictic acid treatment induced a non-significant cytotoxicity at dose ranges achievable for antiproliferative, antimigratory and anti-invasive effects (Figure S3, Supporting Information). A 10 μM of the cytotoxic doxorubicin resulted in 57% reduction in the MCF-10A cell viability. A limited cytotoxicity was observed for norstictic acid only at relatively higher concentrations, 200 μM treatment induced 5.6 % cell death, which is more than tenfold its IC₅₀ values in different anticancer assays. Thus, norstictic acid exhibits preferential anticancer effects on breast cancer cells over the non-tumorigenic mammary epithelial cells. These findings further support the tolerability of norstictic acid administration in *in vivo* settings.

The proto-oncogenic receptor tyrosine kinase c-Met is found to be aberrantly dysregulated in TNBCs (Danilkovitch-Miagkova and Zbar, 2002). HGF/c-Met signaling hyperactivation contributes to oncogenesis, tumor progression and promotes aggressive cellular invasiveness and tumor metastasis (Cecchi *et al.*, 2010). Therefore, targeting intracellular c-Met kinase domain with small molecules would be an effective strategy to suppress the malignant phenotype of Met-dependent breast tumors. Previously, we reported the discovery of the olive oil phenolic oleocanthal (Akl *et al.*, 2014) and the marine-derived alkaloid araguspongine C (Akl *et al.*, 2015) as natural products-based c-Met inhibitors. The virtual binding characteristic of norstictic acid to the c-Met kinase domain was assessed by molecular modeling and docking studies. As depicted in Fig. 3A, norstictic acid adopted a shallow U-shaped conformation within the ATP binding cleft, characteristic for type-I Met inhibitors (Dussault and Bellon, 2008). Docking pose disclosed various interactions of norstictic acid with key amino acids in the kinase domain as shown in Fig. 3A. Norstictic acid's phenolic C-2'-OH accepted a hydrogen bond from the backbone amide hydrogen of the critical Met1160 in the hinge region and donated a hydrogen to interact with the amide carbonyl oxygen of the hinge region's critical Pro1158. Norstictic acid's ring A is anticipated to form triple molecular interactions with amino acids residue at different spots within the c-Met's catalytic domain. Initially, the phenyl moiety adopted an optimal orientation for π-π stacking with the aromatic side chain of the important Try1230 in the activation loop. Meanwhile, the phenolic C-4-OH donated a hydrogen bond to backbone amide carbonyl oxygen of Arg1208. Lastly, the aldehydic oxygen accepted a hydrogen bond

from the backbone amide of Arg1086 in the P-loop. Norstictic acid's ring D lactone was found to participate in GC-loop binding through hydrogen bonding interactions of its C-7'-OH with the backbone amide carbonyl of Ile1084.

To further disclose the location of norstictic binding spot within the c-Met catalytic pocket, an overlay of norstictic acid binding mode and the PDB 4R1V X-ray cocrystallized ligand {3-[1-(3-{5-[(1-methylpiperidin-4-yl)methoxy]pyrimidin-2-yl}benzyl)-6-oxo-1,6-dihydro pyridazin-3-yl]benzonitrile} was studied (Fig. 3B). The cocrystallized inhibitor and norstictic acid occupied almost the same binding region. In addition, ring A of norstictic acid was partially superposed with the dihydropyridazin ring of the cocrystallized ligand, while the lactone group was overlaid with the inhibitor methoxy linker. Norstictic acid's rings C and D were partially overlapped with the pyrimidin-2yl phenyl moiety of the cocrystallized inhibitor. Albeit, the ligand's piprazine ring provided an additional molecular extension to reach amino acids in the distant activation loop compared with the norstictic acid structure, justifying difference in potency. Therefore, further molecular extension of the norstictic acid structure towards the activation loop would probably enhance c-Met binding affinity and subsequent cellular potency.

To further validate c-Met molecular docking results, norstictic acid was tested to inhibit c-Met phosphoryl transferase activity in *Z'*-LYTE™ biochemical kinase assay. Cell-free biochemical assays have the advantages of excluding cellular barriers and metabolism in evaluating potential hits, where test compounds are brought to directly interact with macromolecules targets in a solution state. Biochemical results indicated that norstictic acid significantly inhibited the c-Met kinase catalytic activity in a concentration dependent manner, with corresponding IC₅₀ value of 6.5μM. The positive control c-Met inhibitor oleocanthal resulted in 53% reduction in the catalytic activity at 5μM, which is comparable to its reported activity (Fig. 3C).

To further confirm the inhibitory effect of norstictic acid on c-Met phosphorylation and dependent signaling pathways at cellular level, the TNBC and c-Met-dependent MDA-MB-231 cells were treated with various concentrations of norstictic acid followed by western blot analysis of its cell lysates. Results revealed that norstictic acid treatment induced a significant suppression of the HGF-induced c-Met phosphorylation (Fig. 3D). Norstictic acid-mediated p-c-Met inhibition was further associated with the inhibition of the downstream effectors mediating cell proliferation, survival, motility, and invasion. The antiapoptotic PI3K/AKT/mTOR pathway was significantly inhibited in a dose-dependent manner. Moreover, the phosphorylation level of transcription factor STAT3, mediating cell growth and differentiation, was also significantly suppressed, compared with the phosphorylated level in HGF-vehicle treated control cells. Furthermore, the phosphorylation of cell the motility-mediator paxillin/Rac-1 axis was also significantly suppressed in response to norstictic acid treatment in a concentration-dependent manner. Additionally, norstictic acid treatment resulted in a significant phosphorylation inhibition of the cell invasion-deriving non-receptor tyrosine kinase FAK (focal adhesion kinas). Collectively, these results confirm the inhibitory effects of norstictic acid on the c-Met phosphorylation and integrated downstream effectors in the TNBC MDA-MB-231 cells.

The *in vivo* effect of norstictic acid treatment on the growth of MDA-MB-231/GFP cells implanted into 5- to 6-week old athymic female nude mice has been evaluated and compared with vehicle-treated control animals (Fig. 4A). The mean $\pm$ SEM tumor volume in vehicle-treated control group was $1672.3 \pm 318.3 \text{ mm}^3$ at the experiment end. The norstictic acid-treated group showed a mean $\pm$ SEM tumor volume of $736.6 \pm 101.6 \text{ mm}^3$, suggesting significant growth inhibitory effects (Fig. 4B). The mean $\pm$ SEM tumor weight of norstictic acid treated animals was $0.89 \pm 0.13 \text{ g}$, compared with $2.09 \pm 0.52 \text{ g}$ mean $\pm$ SEM for vehicle-treated control group (Fig. 4C). Statistical analysis (Student's *t*-test) indicated that norstictic acid treated animals (15 mg/kg, i.p., 3 $\times$ weekly) had significantly smaller tumor size and less tumor weight, compared with the vehicle treated control animals. Western blot analysis of tissue lysates from tumor samples indicated a significant inhibition of c-Met phosphorylation in norstictic acid treated animal, when compared with vehicle control group (Fig. 4D).

Mice body weights were monitored and recorded throughout the course of study. Statistical analysis revealed that the mean body weight was not significantly different in norstictic acid treated group when compared with vehicle treated animals (Fig. 4E). Additionally, at the study termination, visceral organs (lung, liver, kidney, and spleen) were excised and weighed. No statistical difference was observed between mean weight of any visceral organ in control and norstictic acid treated groups at the end of the study (Fig. 4F). These results further suggest the tolerability of norstictic acid treatment in nude mice. Therefore, combined results strongly support the significant *in vivo* effect of norstictic acid treatment in attenuating MDA-MB-231/GFP tumor progression in treated nude mice and promote this natural product to a lead rank.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Chemical structure of norstictic acid (A) and effects of *Usnea strigosa* EtOAc extract (B) and norstictic acid (C) on the growth of the human breast cancer cell lines MDA-MB-231, MDA-MB-468, SK-BR-3, BT-474, MCF-7, and T-47D in MTT assay. Cells were seeded into a 96-well plate, incubated overnight to attach and then treated with either vehicle, extract or norstictic acid, in triplicates, at indicated concentrations for 72 h. Data represent mean ± SEM (% of control) of three independent experiments. * indicates a significant difference between the treatment and vehicle control wells ($P < 0.05$).

Figure 2. Effect of norstictic acid on the TNBC MDA-MB-231 cells migration and invasion. (A) Representative microscopic images of the wounds at zero time and 21 h post-treatment comparing vehicle control and treatment wells. (B) Concentration response diagram of norstictic acid treatment, in triplicates, and MDA-MB-231 percent cell migration. Data represent mean $\pm$ SEM of three independent experiments. (C) Representative microscopic images of vehicle control (high cell invasion and low cell density) and norstictic acid-treated (lower cell invasion and higher cell density) BME coated wells after 24 h. (D) Concentration response diagram of norstictic acid treatment, in triplicates, and MDA-MB-231 percent cell invasion. Data represent mean $\pm$ SEM of three independent experiments. * indicates significant difference between treatment and vehicle control groups ($P < 0.05$).

Figure 3.

In-silico interactions and *in vitro* effects of norstictic acid on c-Met. (A) Binding mode of norstictic acid in c-Met kinase domain (PDB 4R1V). Norstictic acid adopts a shallow U-shaped conformation to interact with the key amino acids (Ile1084, Arg1086, Pro1158, Met1160, Arg1208, and Tyr1230). (B) Overlay of norstictic acid and the X-ray cocrystallized inhibitor in the c-Met kinase domain showing good molecular overlapping. (C) Inhibitory effect of norstictic acid on the c-Met kinase activity in Z'-LYTE kinase assay. Data represent mean $\pm$ SEM of three independent experiments. (D) Effects of norstictic acid treatment on c-Met and downstream effectors in MDA-MB-231 cells using Western blot analysis. Immunoblots from 48 h-treated samples show p-Met, p-Akt, p-mTOR, p-Paxillin, p-Rac1, p-Stat3 and p-FAK downregulation. (E) Densitometric analysis of immunoblots. Data represent mean $\pm$ SEM of optical density normalized to β -tubulin, in three different experiments. * indicates a significant difference between the treatment and vehicle control groups ($P < 0.05$).

Figure 4.

Effect of norstictic acid on breast cancer xenograft model in nude mice. (A) Effect of norstictic acid treatment on *in vivo* growth of MDA-MB-231/GFP cells. Data represent mean $\pm$ SEM tumor volume at the start of treatment till the study end. (B) Representative photographs of mice from vehicle control and treatment groups after the termination of the study. (C) Tumor weight (mean $\pm$ SEM) of the animals in vehicle control and treatment group at the end of the study. (D) Effect of norstictic acid treatment on the c-Met phosphorylation in tumor detected by Western blot analysis. (E) Effect of norstictic acid treatment on the study animals' body weight. Data represent mean $\pm$ SEM body weight at indicated times. (F) Effect of norstictic acid treatment on the visceral organs weight. Data represent mean organ weight $\pm$ SEM after the study termination. * indicates a significant difference between the treatment and vehicle control groups ($P < 0.05$).