**A Highly Potent TACC3 Inhibitor as a Novel Anti-cancer Drug Candidate**

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**SUPPLEMENTARY MATERIALS and METHODS**

**Cell culture and reagents**

Human breast cancer cell lines MDA-MB-231, MDA-MB-436, MDA-MB-157, BT-474, MCF-7, ZR-75-1 and T-47D, mouse breast cancer cell line EMT6, human bladder cancer cell lines RT122 and RT4, mouse colorectal carcinoma cell line CT-26 and normal human breast epithelial cell line MCF-12A were purchased from ATCC (Manassas, VA, USA). JIMT-1, HCC1954, CAL51 and HCC1143 were kindly provided by Ali Osmay Güre from Bilkent University, Ankara, Turkey. Human colon carcinoma cell line, HCT-116, was a gift from Serkan Göktuna and mouse embryonic fibroblast cell line, MEF, was provided by Onur Cizmecioglu both from Bilkent University, Ankara, Turkey. Cells were cultured in Dulbecco’s modified Eagle’s medium (Lonza, NJ, USA), supplemented with 10% fetal bovine serum (FBS, Lonza), 1% non-essential amino acid (Gibco), 2 mM L-glutamine (Sigma Aldrich, MO, USA) and 50 U/ml penicillin/streptomycin (P/S). Additionally, BT-474 cells were grown in 0.1% insulin (Sigma Aldrich) containing medium. MCF-12A cells were also supplemented with 20 ng/ml epidermal growth factor (EGF) and 500 ng/ml hydrocortisone. T-47D and MCF-7 cells were cultured in phenol red–free DMEM (Gibco) with 10% FBS, 1% nonessential amino acids, 50 U/mL penicillin/streptomycin and 0.1% insulin. EMT6 and RT112 cells were maintained in RPMI-1640 (Biowest, Nuaille, France) while RT4 and HCT-116 cells were cultured in McCoy’s 5A (modified) (Gibco, Carlsbad, CA) medium supplemented with L-glutamine, FBS and P/S. The cell lines were authenticated and tested for mycoplasma contamination regularly using MycoAlert mycoplasma detection kit (Lonza, NJ, USA). The cumulative length of the cells between thawing and use in the experiments was less than 20 passages.

**Synthesis of BO-264**

* ***Ethyl 4-methoxybenzoate (1)***

Thionyl chloride (SOCl2) (34.4 mmol, 2.27 equiv) was added dropwise to a solution of 4-methoxybenzoic acid (15.15 mmol, 1 equiv) in absolute ethanol (25 ml) at room temperature (RT). The reaction was stirred at 80°C for 4 hours. After the reaction was complete, the resulting mixture was concentrated under reduced pressure to give colorless liquid (86% yield). HRMS (m/z): [M+H]+ calculated for C10H13O3: 181.0865; found:181.0858. CAS: 94-30-4

* ***3-(4-Methoxyphenyl)-3-oxopropanenitrile (2)***

Sodium hydride (NaH) (60% dispersion in mineral oil) (27.501 mmol, 3 equiv) and acetonitrile (MeCN) (27.501 mmol, 3 equiv) were added to a solution of **1** (9.167 mmol, 1 equiv) in dry toluene at RT and then refluxed under nitrogen atmosphere for 2 hours. The resulting mixture was cooled to RT. The salt obtained was filtered and washed with petroleum ether. The salt was dissolved in water and then acidified with concentrated hydrochloric acid (HCl). The resulting solid was filtered and triturated with a solution of NaHCO3, and then filtered to give light yellow solid. (77% yield). MP: 127.5-129.4oC (1) HRMS (m/z): [M-H]- calculated for C10H8NO2: 174.0561; found: 174.0564. CAS: 3672-47-7

* ***3-(4-Methoxyphenyl)isoxazol-5-amine (3)***

Compound **2** (2.8571 mmol, 1 equiv) and hydroxylamine hydrochloride (H2NOH.HCl) (2.8856 mmol, 1.05 equiv) were added to solution of sodium hydroxide (NaOH) (5.8570 mmol, 2.05 equiv) in water. The reaction mixture was refluxed for 4 hours. After the resulting mixture cooled, it was diluted with water and extracted with dichloromethane (DCM). The organic layer was dried, ﬁltered and evaporated to give the crude product which was then puriﬁed by automated-ﬂash chromatography on silica gel (24 g) eluting with a gradient of 0−60% ethyl acetate (EtOAc) in hexane (59% yield). MP: 135.4-137.2oC. (2) HRMS (m/z): [M+H]+ calculated for C10H11N2O2: 191.0821; found:191.0812 CAS: 86685-98-5

* ***N-(2-Chloropyrimidin-4-yl)-3-(4-methoxyphenyl)isoxazol-5-amine (4)***

Potassium tert-butoxide (t-BuOK) (2.4025 mmol, 2.5 equiv) was added to a solution of **3** (0.961 mmol, 1 equiv) in tert-butanol and the mixture was stirred for 1 hour at RT. 2,4-Dichloropyrimidine (1.441 mmol, 1.5 equiv) was added to the reaction mixture and it was stirred for 24 hours at RT. After the reaction was complete, the mixture was quenched by aqueous ammonium chloride (NH4Cl) solution and then extracted with EtOAc. The organic layer was dried, ﬁltered and evaporated to give the crude product which was puriﬁed by automated-ﬂash chromatography on silica gel (24 g), eluting with a gradient of 0−60% EtOAc in DCM (42% yield). MP: 197-198.8 oC HRMS (m/z): [M+H]+ calculated for C14H12N4O2Cl: 303.0649; found: 303.0645. 1H NMR (400 MHz, DMSO): δ 3.82 (3H, s), 6.68 (1H, s), 7.01 (1H, d, *J=*5.8 Hz), 7.07 (2H, d, *J=*8.6 Hz), 7.77 (2H, d, *J=*8.6 Hz ), 8.40 (1H, d, *J=*5.8 Hz), 11.73 (1H, s). 13C NMR (100 MHz, DMSO): δ 55.22, 85.54, 106.48, 114.42, 121.06, 127.86, 158.60, 158.72, 159.40, 160.69, 161.51, 162.26.

**3-(4-Methoxyphenyl)-N-(2-morpholinopyrimidin-4-yl)isoxazol-5-amine (5) (BO-264)**

Morpholine (1.0746 mmol, 3 equiv) was added to a solution of **4** (0.3582 mmol, 1 equiv) in n-butanol. The reaction mixture was refluxed under nitrogen atmosphere for 5 hours. After the reaction was complete, the mixture cooled down to RT, and then quenched by ice water to give light yellow solid. The resulting solid was filtered, dried to give crude product which was puriﬁed by automated-ﬂash chromatography on silica gel (24 g), eluting with a gradient of 0−60% EtOAc in DCM (70% yield). MP: 192.5-194.2oC HRMS (m/z): [M+H]+ calculated for C18H20N5O3: 354.156; found:354.1572. 1H NMR (400 MHz, DMSO): δ 3.68-3.70 (8H, m), 3.80 (3H, s), 6.24 (1H, d, *J*=5.6 Hz), 6.54 (1H, s), 7.04 (2H, d, *J*=9.0 Hz), 7.74 (2H, d, *J*=9.0 Hz), 8.11 (1H, d, *J*=5.6 Hz), 10.98 (1H, s). 13C NMR (100 MHz, DMSO): δ 44.10, 55.24, 65.99, 84.03, 97.00, 114.43, 121.43, 127.87, 157.38, 157.45, 160.61, 161.37, 162.13 162.61.

**Inhibitor treatments, cell viability assays and Annexin V/PI staining**

KHS101 (Sigma Aldrich) and SPL-B (Axon MedChem, VA, USA) were dissolved in 100% DMSO to yield a stock concentration of 50 mM. BO-264 was dissolved in 100% DMSO to yield a stock concentration of 10 mM. For cell viability assay, JIMT-1 (3x103 cells/well), MDA-MB-436 (4x103 cells/well), MDA-MB-157 (3x103 cells/well), RT112 (6x103 cells/well), RT4 (6x103 cells/well) and MCF-12A (5x103 cells/well) cells were seeded and 24 hours after cell seeding inhibitor treatments were performed at different concentrations. Cell viability was measured 72 hours after treatment with Sulforhodamine B (SRB) (Sigma Aldrich) as described previously (3). For western blotting, different concentrations of KHS101, SPL-B or BO-264 were given to JIMT-1 cells (1.5x105 cells/well) for 24 hours. HCT-116 (2x105 cells/well) and CT-26 (2x105 cells/well) cells were also treated with different doses of BO-264 for 24 hours. Annexin V/PI staining (Biolegend, USA) was performed according to manufacturer’s instructions using JIMT-1 cells treated with 500 nM BO-264 for 48 hours. For shRNA western blotting experiment, both control and TACC3 shRNA JIMT-1 cells were seeded (1.5x105 cells/well) and induced with 2 µg/ml doxycycline for 48 hours. Cells were then treated with BO-264 at different doses for 24 hours. For siRNA immunofluorescence experiment, JIMT-1 cells transfected with siRNAs targeting TACC3 were treated with BO-264 for 12 hours.

**Transient transfection with siRNAs and overexpression vectors**

For cell viability, JIMT-1 (3x103 cells/well), MDA-MB-436 (4x103 cells/well) and MDA-MB-157 (3x103 cells/well), cells were seeded into 96-well plates in P/S-free growth medium. 24 hours after seeding, cells were transfected with two different siRNAs targeting TACC3 (Dharmacon, CO, USA) at a final concentration of 20 nM (siTACC3#1: D-004155-03 and siTACC3#2: D-004155-02) using Lipofectamine 2000TM (Invitrogen, CA) transfection reagent as described previously (4). 72 hours following transfection, cell viability was measured with SRB assay. To assess the knockdown level with siRNAs, JIMT-1 (1.5x105 cells/well), MDA-MB-436 (1.5x105 cells/well) and MDA-MB-157 (1.5x105 cells/well) cells were transfected with both siTACC3s for 48 hours. Knockdown efficiency at TACC3 mRNA and protein level was analyzed by quantitative real-time PCR (qRT-PCR) and western blotting, respectively. For immunofluorescence experiments, JIMT-1 cells (3x105 cells/well) were seeded into 6-well plates, and next day transfected with two different siRNAs targeting TACC3 at a final concentration of 40 nM.

For transient TACC3 overexpression, MCF-12A cells were transfected with 250 ng of empty or TACC3 vector (OHu21751; Genscript, NJ, USA) for 48 hours. Overexpression efficiency at TACC3 protein level was assessed by western blotting.

**Generation of JIMT-1 cells stably expressing inducible shRNA**

pTRIPZ doxycycline-inducible lentiviral shRNA vectors (shTACC3: RHS4696-200764244 and non-silencing shControl: RHS4743) were obtained from Dharmacon (CO, USA). Packaging and infection of the lentiviral vector was carried out using trans-lentiviral packacing system (Dharmacon). Briefly, HEK293FT cells, acting as host, were transfected with shRNA vectors by calcium phosphate method. The supernatant containing virus particles were cleared through a 0.45-μm filter, collected and later used to infect JIMT-1 cells for 48 hours. shRNA expressing cells were selected with 2 μg/ml puromycin (Thermo Fisher Scientific, MA, USA) for at least two weeks.Resistant cells were induced with doxycycline (2 μg/ml) for 48 hours for TACC3 for both immunofluorescence and western blotting assays.

**Colony Formation Assay**

For monolayer culture, single-cell suspensions of JIMT-1 cells (3x103 cells/well) were plated in a 12-well plate. After 6 hours incubation, cells were treated with different doses of BO-264, SPL-B and KHS101. In order to test the colony formation capacity of MCF-12A cells during TACC3 overexpression, MCF-12A cells (2x105 cells/well) were seeded into 6-well plates, and transfection was performed next day. 48 hours following transfection, cells were counted and 1x103 cells/well were plated into 12-well plates. For both experimental setups, the media were refreshed every 4 days and cells were incubated for 12 days. Cells were then fixed with 2% paraformaldehyde for 15 min and stained with 1% crystal violet (Merck, Darmstadt, Germany) for 15 min at RT. Surviving colonies (composed of at least 50 cells) were counted with ImageJ software (NIH).

**Doubling time assessment**

To assess doubling time, normal breast epithelial cell line MCF-12A and breast cancer cell lines were plated (3x104 cells/well) in 6-well plates. Cells were collected by trypsinization and cell number was counted every 24 hours for one week. Growth curves for these cells were drawn as number of cells/cm2 versus days after seeding. The doubling time was calculated using the following formula;

Doubling time (DT) = ,

where t is the time spent in the logarithmic phase of cell growth, Xe and Xb are cell numbers at the end and beginning of the logarithmic phase, respectively.

**Western blotting**

Protein isolation and Western blotting were done as previously described (5). Primary and secondary antibodies were listed in Supplementary Table S1.

**Quantitative RT-PCR analysis**

To assess the efficiency of TACC3 knockdown, total RNA isolation, cDNA generation and quantitative RT-PCR analysis was done as previously described (4, 5). List of the sequences of qRT-PCR primers are provided in Supplementary Table S2. ΔΔCT method was used for the analysis of the data (6).

**Immunofluorescence**

Immunofluorescence staining of JIMT-1 cells were performed as previously described (7). Briefly, 1.5x105 JIMT-1 cells/well were seeded on glass coverslip in 6-well plates. Next day, the cells were treated with either vehicle or different doses of BO-264 for 12-24 hours. For the siRNA immunofluorescence experiments, 1.5x105 cells/well were seeded on glass coverslip in 6-well plates and transfected with two different siTACC3s. Cells were then treated with 0.2 µM BO-264 for 12 hours. After drug treatment, cells are fixed with ice-cold methanol for 10 min at -20oC. Cells were then blocked with 3% BSA in PBS solution for 1 hour at RT and incubated with primary and secondary antibodies for 1 hour at RT (for dilutions, see Supplementary Table S1). Cells were counter stained with DAPI for 5 min (0.01 μg/ml). Lastly, cover slides were mounted using ImmunoHistomounth (Santa Cruz). Images were taken with an upright fluorescent microscope equipped with DIC prisms (upright).

**Histological analysis**

Immunohistochemistry was performed on 5-μm-thick formalin-fixed paraffin-embedded (FFPE) tissue sections of major organs isolated from nude mice bearing JIMT-1 and Balb/c mice bearing EMT6 tumors. Tissue sections mounted on slides were deparaffinized at high temperature (60oC) overnight, and the paraffin was removed with xylene. Sections were stained with hematoxylin and eosin (H&E) staining as previously described (8).

***In vivo* studies**

Six-to-eight-week-old female athymic nude and Balb/c mice were housed with a temperature-controlled and 12-hour light/12-hour dark cycle environment. This study was carried out in accordance with Institutional Animal Care and Use Committee of Bilkent University and performed according to the institution's guidelines and animal research principles.

For *in vivo* breast tumor growth, 4x106 JIMT-1 cells were prepared in 150 μl of 1:1 DMEM and Matrigel (Corning, NY, USA), v/v) and injected into mammary fat pads (MFP) of female nude mice. Mouse weight and tumor volume were measured daily using calipers. Tumor volumes were calculated as length x width2 x 0.5. Once the tumor volume had reached about 90-100 mm3, xenografts were randomized into groups (4-5 mice per group). Animals were treated with vehicle, BO-264 or SPL-B (every other day 5 mg/kg oral gavage (po.)). The vehicle for these drugs includes 4% DMSO, 4% HCl in 0.9% NaCl. In a separate experiment, animals were also tested for a higher dose of BO-264 (25 mg/kg) dissolved in 0.05% (g/ml) HPMC (hydroxypropyl methylcellulose) and 2% Tween-80 in ddH2O. Mice were sacrificed 20-30 days after initiation of the treatment and the tumors were collected and stored for subsequent analyses. For the survival analysis, 1x106 EMT6 cells were prepared in PBS and injected into MFPs of immunocompetent female Balb/c mice. Similarly, once the tumor volume had reach to 90-100 mm3, mice were randomized into two groups and received either vehicle or 25 mg/kg BO-264 every day. Survival was calculated using a predefined tumor volume cut-off of 1500 mm3.

For the *in vivo* colon cancer studies, female nude and Balb/c mice were inoculated into the right flank with human colorectal carcinoma cell line, HCT-116, and murine colorectal carcinoma cell line, CT-26, respectively. 5x106 HCT-116 cells were prepared in 150 μl Matrigel:PBS (1:1) while 5x105 CT-26 cells were prepared in 100 μl PBS. Once tumor volumes reached 100 mm3, animals were given vehicle or 50 mg/kg BO-264 daily by oral gavage. Twenty days after the treatment, mice were sacrificed.

**Bioinformatics analyses**

TACC3 differential plots between different tumor and normal tissues was constructed using The Cancer Genome Atlas (TCGA) patients (9) and data was downloaded from <http://firebrowse.org/>. The microarray dataset GSE39582 including mRNA expression profiles of colorectal cancer and normal colorectal mucosa patients was used for TACC3 expression level analysis (10). For survival analysis and prognostic significance of TACC3, two independent publicly available cancer datasets were used. The first one is the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset (11). Here, TACC3 expression levels of METABRIC Discovery and Validation sets composed of 1992 tumors were used for tumor grade analysis. These data were accessed through European Genome-phenome Archive. TACC3 expression pattern on survival was also analyzed with Kaplan Meier plotter database which includes information on overall survival of 1402 breast cancer (12), 1926 lung cancer (13) and 876 gastric cancer (14) patients. Gene set enrichment analysis (GSEA) was performed using gene sets related to mitosis and DNA damage available at the Broad Institute website (http://software.broadinstitute.org/gsea/index.jsp). METABRIC patient gene expression profiles were used in GSEA analysis. Patients from 25th and 75th quartiles of TACC3 levels were used and defined as low and high TACC3, respectively. For the analysis of TACC3 dependency of NCI-60 cell lines, we used dependency data from combined RNAi screens of Broad Institute, Novartis and Marcotte et al. (15) which is available in <https://depmap.org/portal/>. Multivariate Cox regression analysis was done using METABRIC dataset in SPSS software. TACC3 level, tumor grade, tumor stage, ER, PR and HER2 status were selected as covariates. TACC3 expression was separated based on 25th percentiles.

**Statistical analyses**

Data were analyzed using GraphPad Prism software (GraphPad Software, Inc) and expressed as mean ± standard deviation from three independent experiments unless otherwise indicated. Statistical significance of two groups comparisons was determined by two-tailed Student’s t-test. To compare the doubling time curves of different cell lines one-way ANOVA was used. A multiple t-test was used to determine pair-wise significances between the treatment groups of tumor volumes of EMT6 tumor bearing mice. A *p* and an adjusted p (*q*) value of less than 0.05 were considered to be statistically significant. Kaplan-Meier survival curve analysis was performed using the Log-rank (Mantel-Cox) test.

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