**Targeted treatment of metastatic breast cancer by PLK1 siRNA delivered by an antioxidant nanoparticle platform**

Jingga Morry\*,1, Worapol Ngamcherdtrakul\*,1,2, Shenda Gu1, Moataz Reda1, David J. Castro1,2, Thanapon Sangvanich1, Joe W. Gray#,1, Wassana Yantasee#,1,2

1Department of Biomedical Engineering, Oregon Health and Science University

3303 SW Bond Ave, Portland, OR 97239, USA

2PDX Pharmaceuticals, LLC

3303 SW Bond Ave, Portland, OR 97239, USA

\*Authors with equal contribution

#Corresponding authors

**Supplementary Methods**

**Intracellular ROS assay.** To measure the impact of nanoparticles on cellular ROS induction, cells were pre-treated with siSCR-NP, siSCR-DharmaFECT (50 nM siRNA), 5 µM Diphenyleneiodonium chloride (DPI, Sigma Aldrich, St. Louis, MO), or 20 mM of N-acetylcysteine (NAC, Sigma Aldrich) antioxidant for 24 h. After 24 h, 100 µM of menadione was added into each well for 1 h to induce oxidative stress. At the end of the incubation period, cellular ROS was assayed using CellROX® deep red reagent (ThermoFisher Scientific) following the manufacturer’s protocol using flow cytometry.

**Cell viability assay.** The viability of treated cells was determined 72 h post-transfection using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) following the manufacturer’s protocol. The luminescent signal from CellTiter-Glo® assay was detected using Tecan Infinite M200 microplate reader and reported as fold change over the untreated control.

**Cell-cycle analysis.** After 24-h treatment with siSCR-NP, siPLK1-NP, or 10 nM BI2536 (Selleck Chemicals, Houston, TX), cells were washed once with PBS and stained with 10 µg/mL Hoechst 33342 (ThermoFisher Scientific) for 30 min at 37 oC. The cells were then trypsinized and spun down at 1000 rpm for 5 min. Cell pellets were washed twice in PBS and resuspended in 500 µL of FACS buffer (1X PBS (Ca/Mg2+ free), 1 mM EDTA, 25 mM HEPES pH 7.0, 1% FBS) and analyzed by flow cytometry (Guava Millipore Easycyte, EMD Millipore, Billerica, MA).

**In vitro scratch assay.** The in vitro scratchassay protocol was modified from Liang *et al.* (1). Briefly, 70-80% confluent LM2-4luc+/H2N cells in 12-well plate were treated with siSCR-NP, siPLK1-NP, siSCR-DharmaFECT, or siPLK1-DharmaFECT (all with 50 nm as siRNA dose). After 24 h treatment, a wound was created by scratching each well (~100% confluency) with a 200 µL pipette tip, washed three times with warm PBS, and replaced with serum-free medium. Each well was imaged at time 0 and 24 h after scratching with EVOS FL microscope (ThermoFisher Scientific). The migration of cells into the cell-free gap was quantified using ImageJ (NIH, Bethesda, MA) wound healing tool macros.

**Gelatin degradation assay.** This assay was performed following the established protocol (2) with slight modification. Briefly, Oregon-green 488 conjugate gelatin (ThermoFisher Scientific) was evenly coated (in 2% sucrose PBS) on the 12 mm glass coverslips for 30 min in the dark, fixed with 0.5% glutaraldehyde on ice for 15 min and quenched with 5 mg/mL sodium borohydride (Sigma Aldrich) for 3 min. Coverslips were then incubated in complete media overnight before use.

LM2-4luc+/H2N, pre-treated with siSCR-NP or 5 µM DPI, were seeded on the gelatin-coated coverslips for 24 h at 50,000 cells/well density before fixation and immunostaining. Cells were fixed with 4% PFA, permeabilized with 0.1% Triton-X for 30 min, and stained with Alexa-Fluor 568 Phalloidin (Thermo Fisher Scientific). Quantification of gelatin degradation activity was performed at 20X magnification with at least 8 randomly chosen fields per well, representing a minimum of 300 total cells scored per experimental point. The analysis of degradation area was performed with ImageJ software and calculated as the degraded area per field normalized to the number of cells in each field. Representative images were taken at 63X magnification with Zeiss Axioplan2 microscope equipped with Zeiss Axiocam HRm CCD camera using Zen software.

**Matrigel invasion assay.** LM2-4luc+/H2N cells (1 x 106 cells/dish) were seeded on a 10-mm petri dish and transfected with siSCR-NP overnight. Cells were then trypsinized and counted before being seeded onto the matrigel-coated invasion chambers in serum-free medium (125,000 cells/insert). DPI (5 µM) or NAC (2 – 30 mM) was added to the cells at the same time of cell plating on the upper chamber insert. After 48 h, the number of invaded cells were quantified according to the manufacturer’s instructions of Chemicon® QCM™ 24-well Fluorimetric Invasion assay kit (ECM554, Chemicon, Millipore).

**3D Matrigel culture and immunostaining.** 3D culture of LM2-4luc+/H2N cells were performed as described by Lee *et al.* (3) with slight modifications. Briefly, cells transfected overnight with DY677siSCR-NP were trypsinized and counted before being seeded onto matrigel-coated 4 well chamber slides (10,000 cells/well) and overlaid with complete medium containing 2% matrigel. Cells were grown on the slides for up to 5 days and imaged on alternate days starting from day 1 post seeding with the EVOS FL automated fluorescence microscope.

**Real time quantitative PCR (qPCR).** The RNA from the treated cells was isolated with the RNeasy Mini kit (Qiagen) following the manufacturer’s protocol. The amount of RNA was quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific). Purified RNAs (20 ng per reaction) were reverse transcribed with EXPRESS One-Step SuperScript qRT-PCR kit (ThermoFisher Scientific). Primer sequences chosen for qPCR (ThermoFisher Scientific) are: human PLK1 (Hs00983227\_m1), human NOX4 (Hs01558199\_m1), and human GAPDH (Hs02758991\_g1). qRT-PCR assays were performed in triplicate using an ABI 7500 Fast System (Applied Biosystems, Foster City, CA) under standard cycling conditions: 50 oC for 2 min, 95 oC for 10 min, 40 cycles of 95 oC for 15 s, and 60 oC for 1 min. Expression levels were analyzed using 2–ΔΔC(t) method using GAPDH as loading control.

**Western blot.** Cell lysate (30 µg protein/lane) were electrophoresed with denaturing sodium dodecyl sulfate (SDS)-polyacrylamide NuPage Novex 4% to 12% gels (Invitrogen) and blotted on a PVDF membrane (Millipore). Primary antibody incubation was carried out overnight at 4°C on a rocking platform (anti-PLK1, ab17056, Abcam; β-Actin (8H10D10), Cell Signaling Technology; anti-NOX4, ab109225, Abcam). Secondary incubation with IRDye 800CW and IRDye 680RD conjugated secondary antibodies (Licor Biosciences) was carried out at room temperature (2 h) the next day prior to imaging with the LI-COR infrared imaging system (LI-COR, Lincoln, NE).

**Histology.** All tissues were either fixed in 10% buffered formalin (Fisher Scientific) or snap-frozen in OCT compound (#4583, Tissue-Tek) prior to processing. For immunofluorescence staining, deparaffinized and rehydrated lung tissues were subjected to heat-mediated antigen retrieval in citrate buffer (10 mM, pH 6.0) for 30 min. The tissue sections were permeabilized with 0.25% Triton-X100 (PBS) for 30 min and blocked with 10% goat serum (Vector Laboratories Inc., Burlingame, CA) for 1 h at room temperature. Slides were then incubated with primary antibodies (5% goat-serum + 1% BSA in PBS) at 4 oC overnight (mouse monoclonal human vimentin, NCL-L-VIM-V9, Leica Biosystems; rabbit polyclonal Ki67, NCL-Ki67p, Leica Biosystems; rabbit polyclonal cleaved caspase-3, #9661, Cell Signaling Technology), washed, incubated with secondary antibodies (Alexa 647-conjugated anti-rabbit or anti-mouse Ab, Invitrogen) for 1 h at room temperature, and mounted with ProLong Gold Antifade reagent with DAPI (P-3691, Invitrogen). Images were taken with the EVOS FL fluorescence microscope at 20X magnification.

For quantification of Ki67 and cleaved-caspase 3, images from Ki67-immunostained lung sections were taken at 20X magnifications (five fields per section, 2 lesions per mouse). Percentage of Ki67-positive or cleaved-caspase3-positive cells within the lesion relative to total number of cells within the same region was quantified. Total of five mice per each group were analyzed.

**Dosing schedule optimization.** LM2-4luc+/H2N (high HER2, high luciferase) tumors were grown in MFP of SCID mice to 250 mm3 prior to treatment. When we i.v. injected 1.25 mg siLUC/kg on T-NP to the mice (n = 3 mice/group), which was followed by monitoring of luciferase levels of the tumors using an in vivo imaging system (IVIS). We found that the knock-down efficacy (vs. siSCR on T-NP) was 59% (p<0.0001) on day 1, 53% (p<0.05) on day 2, and 51% (p<0.01) on day 3, and was not significant at day 6 (p<0.2). Thus, we picked twice a week dosing schedule for this study.

**References**

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