Supplementary Materials for

NL-103, a novel dual-targeted inhibitor of histone deacetylases and hedgehog pathway, effectively overcomes vismodegib resistance conferred by Smo mutations

Jie Zhao, Haitian Quan, Chengying Xie, Liguang Lou*

Authors' affiliation: *Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China*

***Corresponding author**: Prof. Liguang Lou, 555 Zuchongzhi Road, Shanghai 201203, China. Phone: 86-21-5080 6056; Fax: 86-21-5080 7088. E-mail: **lglou@mail.shcnc.ac.cn**

Running Title: NL-103 overcomes vismodegib resistance conferred by Smo mutations

Supplementary Materials and Methods

Antibodies

Mouse anti-acetylated-α-tubulin (Catalog Number: T7451) and mouse anti-β-tubulin (Catalog Number: T4026) antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA); rabbit anti-acetylated-lysine (Catalog Number: 9441) antibody from Cell Signaling Technology (Beverly, MA, USA); rabbit anti-detyrosinated-tubulin (Catalog Number: AB3201) antibody, horseradish peroxidase-conjugated goat anti-mouse (Catalog Number: 401215) and horseradish peroxidase-conjugated goat anti-rabbit (Catalog Number: 401315) secondary antibodies from Merck Millipore (Darmstadt, Germany); Alexa Fluor® 488-conjugated goat anti-rabbit (Catalog Number: A-11008) and Alexa Fluor® 633-conjugated goat anti-mouse (Catalog Number: A21052) secondary antibodies from Life Technologies (Carlsbad, CA, USA).

Genes and plasmids

Human Smo-WT and Smo-M2 genes were generously provided by Genentech (South San Francisco, CA, USA). Both genes were separately amplified by high-fidelity PCRs and the PCR products were separately cloned into the pcDNA3.1-Myc-His (Life technologies, Carlsbad, CA, USA) and pEYFP-N1 (Clontech, Palo Alto, CA, USA) vectors for different purposes. Specific primers were deliberately designed so that Smo genes cloned into pEYFP-N1 were in frame with the coding sequence of enhanced yellow fluorescence protein (EYFP), with no

intervening in-frame stop codons. Smo-WT-EYFP and Smo-M2-EYFP fusion genes were amplified by PCRs and the products were separately cloned into the pBabe-puro vector for other purposes. The Hh pathway reporter was constructed by ligating 12 tandemly-repeated Gli binding sites into the multiple cloning site of the pGL4.26 vector (Promega, Madison, WI, USA). Certain point mutations were generated following the protocol 3 of mutagenesis in Molecular Cloning.

Cell culture and transfection

HEK293, HEK293T (HEK293 and HEK293T were obtained from the cell bank of Shanghai Institute for Biological Sciences, Chinese Academy of Science, Shanghai, China), NIH3T3, and cell lines derived from NIH3T3 were all cultured in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA), 100 units/ml of penicillin, and 100 μg/ml of streptomycin in a 37°C incubator (Thermo Scientific, Waltham, MA, USA) containing a humidified atmosphere of 5% CO_2. Before transfection, seeded cells were stabilized for 24 h in an incubator and then were transfected with constructs using Lipofectamine® 2000 Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Retroviral preparation and transfection were performed according to the protocol and guidelines provided by the Nolan Lab at Stanford University. Retroviral supernatants were obtained 48 hours after transfection of plasmids encoding Smo-EYFP variants into phoenix-eco packaging cell line with Lipofectamine® 2000.

Immunoblotting

After drug treatment, cells were washed twice with ice-cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) and lysed in sodium dodecyl sulfate (SDS) sample buffer. Equal amounts of whole cell lysates were separated by SDS-polyacrylamide gel electrophoresis, and electroblotted onto Immobilon PVDF membranes (Merck Millipore, Darmstadt, Germany). After incubation in the blocking buffer (tris-buffered saline containing 5% nonfat dry milk and 0.1% Tween-20, pH 7.6), membranes were incubated with the appropriate primary antibodies at 4°C overnight and then switched to Horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence system (Pierce Chemical, Rockford, IL, USA). Primary and secondary antibodies for western blotting were used at 1/1000 and 1/5000 final dilutions, respectively.

Construction of Hh pathway responsive reporter cell line

NIH3T3 cells were transfected with the Hh pathway reporter construct using Lipofectamine® 2000. 48 hours later, cells were incubated in their regular growth medium containing 100 μg/ml hygromycin B to select for stable transfectants. Foci of hygromycin-resistant stable transfectants were individualized after 10 days incubation. These transfectants were diluted appropriately and seeded into 96-well plates ensuring that most wells contained only one cell. 10 days later, monoclonal colonies were picked and expanded.

BODIPY-Cyclopamine/Smo Binding Assay

HEK293T cells were transfected with the Smo expression vector pcDNA3.1-Smo-WT. 24 hours after transfection, the cells were washed with PBS, trypsinized, and fixed with 4% (wt/vol) paraformaldehyde for 10 min at room temperature. The cells were washed, resuspended in PBS containing 1% BSA (wt/vol), and incubated with 10 nM BODIPY-cyclopamine and the indicated competitors for 1 hour at room temperature. The treated cells then were collected by centrifugation and analyzed on a FACSCalibur^{TM} flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

Immunofluorescence protocol

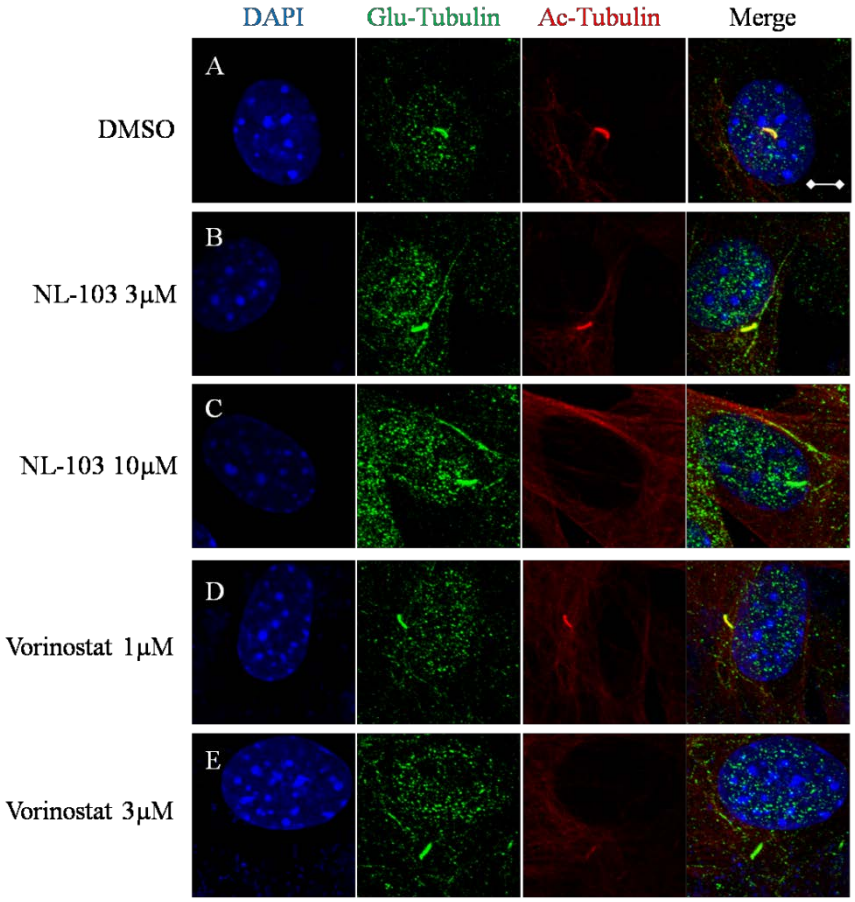
Monoclonal cells were separately seeded into 6 well plates containing polyD-lysine-coated 20-mm glass coverslips. The cells were grown to 90% confluence, and then switched to low serum medium with indicated treatments. After 18hours, the cells were fixed in 4% paraformaldehyde for 10 min at room temperature, washed5 min with PBS three times, permeabilized and blocked simultaneously with PBS containing 0.1% Triton X-100 and 5% bovine serum albumin (BSA) for 30 min. The coverslips coated with cells were then treated with mouse anti-acetylated-α-tubulin (1/1000 in blocking buffer) for 2 h at room temperature and washed 5 min with PBS three times. The coverslips were incubated next with Alexa Fluor 633-conjugated goat anti-mouse IgG antibody (1/500 in blocking buffer) for 1 h at room temperature. After washes with PBS and 5-min incubation with DAPI, the

samples were mounted using fluorescence anti-fade mounting medium, and imaged with an inverted Fluoview laser scaning confocal microscope (Olympus, Japan).

Quantitative real-time PCR Primers

Quantitative real-time PCR was performed using primers against mouse Ptch-1 (forward: 5′-ATGGCCGCATTGATCCCTATCC-3′ and reverse: 5′-TATGGGTTTC GTGGGCGAAGCT-3′), Gli-1 (forward: 5′-GTTGCAGCCAGGAGTTCGATTC-3′ and reverse: 5′-CTTCCGACAGCCTTCAAACGTG-3′), Gli-2 (forward: 5′-TCAGCC TTTGGACACACACCAC-3′ and reverse: 5′-AGCTGACTCGCTGTTCTGCT TG-3′), Gli-3 (forward: 5′-TTTCCCTGCCTTCCATCCTCCT-3′ and reverse: 5′-CTGCTGTGCAATGAGCGGATGT-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5′-GAAGGTCGGTGTGAACGGATTTG-3′ and reverse: 5′-AGACTCCACGACATACTCAGCAC-3′).

Supplementary Figure



Supplementary Figure 1. NL-103 and vorinostat downregulates the acetylation but not the detyrosination levels of cilia. NIH3T3-12Gli cells were treated with indicated compounds or conditions. Primary cilia were marked with detyrosinated-tubulin antibody (also known as glu-tubulin) (green) and acetylated-α-tubulin antibody (red) simultaneously by immunofluorescence. Nuclei (blue) were visualized by DAPI staining. Representative images are provided (Scale bars: 5 μm).