Identification of a New Class of MDM2 Inhibitor That Inhibits Growth of Orthotopic Pancreatic Tumors in Mice

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Supplementary Methods

Materials and Methods

Chemicals, Plasmids, siRNA, and Other Reagents

The cell culture supplies and media, phosphate-buffered saline (PBS), sodium pyruvate, non-essential amino acids, and penicillin-streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was also obtained from Invitrogen (Carlsbad, CA). Anti-human Bax (N-20), Bcl-2 (100), Cyclin D1 (DCS-6), MDM2 (SMP14), PARP1 (H-250), and p21 (C19) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-human p53 (Ab-6) antibody was from EMD Chemicals, Inc. (Gibbstown, NJ). The anti-human caspase-3 (9662) antibody was from Cell Signaling Technology, Inc. (Danvers, MA). The anti-GST (GST01) antibody was from ThermoFisher Scientific (Pittsburgh, PA). The anti-ubiquitin (6C1.17) antibody was from BD Biosciences (San Jose, CA). Cycloheximide, MG132, glutathione-agarose beads, and the protease inhibitor were all purchased from Sigma (St. Louis, MO). The vectors for wild-type MDM2 and mutant MDM2 (C464A) without E3 ligase activity were kindly provided by Dr. J. Chen (Moffitt Cancer Center) and Dr. C.G. Maki (University of Chicago), respectively. The plasmids were transfected into the cells using Lipofectin (Invitrogen, Carlsbad, CA). MDM2 siRNA (h) (Santa Cruz Biotechnology, Inc. SantaCruz, CA) was

transiently transfected into pancreatic cancer cells using the DharmaFECT® system (ThermoFisher Scientific, Pittsburgh, PA).

Cell Lines and Culture

All cell culture media contained 10% FBS and 1% penicillin/streptomycin unless otherwise specified. HPAC cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate supplemented with 2 µg/mL insulin, 5 µg/mL transferrin, 40 ng/mL hydrocortisone, 10 ng/mL epidermal growth factor and 5% fetal bovine serum. Panc-1 cells were cultured with RPMI 1640 containing 1 mM HEPES buffer, 25 µg/mL gentamicin, 1.5 g/L sodium bicarbonate, and 0.25 µg/mL amphotericin B. Mia-Paca-2, Panc-1-Luc, AsPC-1 and IMR90 cells were all cultured in DMEM.

Assays for Cell Viability, Clonogenicity, Cell Proliferation, Apoptosis, and Cell Cycle Distribution

All *in vitro* assays were performed in triplicate, and all of the experiments were repeated at least three times, with similar results obtained for each replicate. ($^{\#}P < 0.05$, $*P < 0.01$).

Cell viability. The effects of the test compound on the cell viability were determined using the MTT assay. Briefly, cells were grown in 96-well plates at 3-4 × 10^3 cells per well and were exposed to SP141 (0 to 10 µM) for 72 h. After incubation with the compound, 10 µL of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg/mL; Sigma; St. Louis, MO) were added to each well. The plates were incubated for 2-4 hr at 37°C. The supernatant was then removed, and the formazan crystals were dissolved in 100 µL of DMSO.

The absorbance at 570 nm was recorded using a SYNERGY Mx microplate reader (BioTek, Winooski, VT). The cell survival percentages were calculated by dividing the mean OD of compound-containing wells by that of DMSO-treated control wells.

Colony formation assay. To assess the colony formation by the pancreatic cancer cells, the cells were seeded in six-well plates at 1,000 cells/well, allowed to attach overnight and treated with SP141 (0.25 or 0.5 mmol/L) for 24 h. The medium containing the drug was then replaced with fresh medium without SP141 and the cells were grown for another 14 days. The colonies in each well were fixed and stained with crystal violet.

Cell proliferation assay. The effects of SP-141 on cell proliferation were determined using the BrdUrd incorporation assay (Oncogene, La Jolla, CA). In brief, cells were seeded in 96-well plates (5-8× 10^3 cells/well) and incubated with various concentrations of SP-141 (0, 0.1 0.25, 0.5 and 1 μM) for 24 h. BrdUrd was added to the medium 10 hr before termination of the experiment. The BrdUrd incorporated into cells was determined using an anti-BrdUrd antibody, and the absorbance was measured at dual wavelengths of 450/540 nm using a SYNERGY Mx microplate reader (BioTek, Winooski, VT). The proliferation index was calculated in comparison to untreated cells.

Apoptosis assay. Cells in early and late stages of apoptosis were detected using an Annexin V-FITC apoptosis detection kit from BioVision (Mountain View, CA). In this procedure, 2-3 × 10^5 cells were exposed to SP141 (0, 0.2, 0.5 and 1 μM) and were incubated for 48 h prior to the analysis. The cells were collected and washed with serum-free media, then re-suspended in 500 μL of Annexin V binding buffer, followed by the addition of 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI). The samples were incubated in the dark for 5 min at room temperature and analyzed with a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA). Cells that

were positive for Annexin V-FITC alone (early apoptosis) and Annexin V-FITC and PI (late apoptosis) were counted. The apoptotic index was determined in comparison to the extent of apoptosis in untreated cells.

Cell cycle distribution analysis. To determine the effects of SP141 on the cell cycle, cells (2-3 × 10^5/well) were exposed to the test compound (0, 0.2, or 0.5 μM) and incubated for 24 h prior to analysis. The cells were then trypsinized, washed with PBS, and fixed in 1.5 mL of 95% ethanol and 0.5 ml 0.9% NaCl at 4°C overnight, followed by incubation with RNAse and staining with propidium iodide (Sigma, St. Louis, MO). The DNA content was determined by flow cytometry.

Immunoblotting and immunoprecipitation

The cells were transfected with the indicated plasmids in the presence of Lipofectin (Invitrogen, Grand Island, NY) or were treated with SP141 for various times and lysed in NP-40 lysis buffer containing a protease inhibitor mixture from Sigma (St Louis, MO). The cell lysates were fractionated with identical amounts of protein by SDS-PAGE, and were transferred to Bio-Rad trans-Blot nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The nitrocellulose membranes were incubated in blocking buffer (Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk) for 1 h at room temperature. Then, the membranes were incubated with the appropriate primary antibody overnight at 4°C with gentle shaking. The membranes were washed three times with rinsing buffer (Tris-buffered saline containing 0.1% Tween 20) for 15 min, and then incubated with goat anti-mouse/rabbit IgG-horseradish peroxidase-conjugated antibody (Bio-Rad Laboratories, Hercules, CA) for 1 h at room temperature. After repeating the washes in triplicate, the protein of interest was detected using

the enhanced chemiluminescence reagents from PerkinElmer LAS, Inc. (Boston, MA). Immunoprecipitation was performed using the indicated antibodies. The beads were washed, and the bound proteins were detected by immunoblotting. All experiments were repeated at least three times, and similar results were obtained each time.

Immunofluorescence

Human pancreatic cancer cells (HPAC and Panc-1) were seeded on coverslips in 12-well plates at a density of 10,000 cells/well, allowed to attach overnight, and were treated with SP141 (0 and 0.5 μM) for 24 h. The cells were fixed in a mixture of acetone and methanol (1:1), blocked in goat serum, and incubated with the primary antibody at 4^{o}C overnight. Then, the cells were washed with PBS and incubated with Alexa Fluor 594 (anti-rabbit) and Alexa Fluor 488 (anti-mouse) antibodies by gentle shaking for 1 h, followed by DAPI nuclear counterstaining. The coverslips were mounted on slides and photographed under a confocal microscope (Olympus Inc., Center Valley, PA). All assays were performed in triplicate, and all of the experiments were repeated at least three times, yielding similar results.

Development and Treatment of a Xenograft Model of Pancreatic Cancer

Female athymic pathogen-free nude mice (nu/nu, 4-6 weeks) were purchased from Charles River Laboratories International, Inc. (Wilmington, MA). To establish the Panc-1 human pancreatic cancer xenograft model, cells harvested from monolayer cultures were washed twice with serum-free medium, re-suspended in the medium, and then injected s.c. (5×10^6 cells, total volume 0.2 mL) into the left inguinal area of the mice. All animals were monitored for their activity, physical condition, body weight, and tumor growth. The animals bearing tumors were

randomly divided into a treatment group and a control group (10-15 mice/group). The control group received the vehicle only. SP141 was dissolved in PEG400:ethanol:saline (57.1:14.3:28.6, v/v/v), and was administered by intraperitoneal (i.p.) injection at a dose of 40 mg/kg/d, 5 d/wk for 18 days. At the different time points of the experiment, the xenograft tumors were removed and homogenized in NP40 buffer, and the resultant supernatants were used for a Western blot analysis.

Generation of an Orthotopic Model of Pancreatic Cancer and Treatment of Mice Bearing Orthotopic Tumors

The pancreatic cancer orthotopic model was developed by directly injecting a luminescent pancreatic cancer cell suspension (Panc-1-Luc and AsPC-1-Luc) into the pancreas of nude mice. The 4-6-week-old female athymic nude mice were anesthetized using isoflurane (Butler Animal Health Supply, Dublin, OH), then a 1-cm incision was made on the abdominal skin and muscle, slightly medial to the splenic silhouette. While gently retracting the pancreas laterally, a 30 Ga needle was inserted into the tail of the pancreas and passed into the pancreatic head region. 30 µL of Panc1-Luc and AsPC1 cell solution (1×10^6 cells in PBS) was slowly injected using a 1 cc syringe. To prevent leakage of the cells, a cotton wool tip was pressed onto the injection site for 30 seconds. To decrease post-surgical pain, mice were injected with 0.3 mg/kg buprenorphine hydrochloride immediately and with 0.1 mg/kg every 8 h for the initial two days. These mice were then administered luciferin salt (a luciferase substrate) by i.p. injection and were photographed using an IVIS Lumina XR *in vivo* imaging system (Caliper, Mountain View, CA). The treatment was carried out in the same way as described for the xenograft model. At the end of the experiment, the tumors were removed for immunofluorescence and Western blot analyses.