Supporting Information

Discovery of AMG 232, a Potent, Selective, and Orally Bioavailable MDM2-p53 Inhibitor in Clinical Development

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(i) In vitro biological assays

Biochemical (HTRF) assay. ***Materials:*** Human MDM2 (GST-thrombin-hMDM2(1-188)) was produced in house. It was expressed in E coli and purified by Glutathione Sepharose 4B, Q-HP and Superdex 200 column. Human p53 (Avi-TrxA-6His-Thrombin-S-tag-EK-p53(1-83)) was expressed in E. coli and passed though Ni-NTA, Hydroxyapatite, Superdex 75 columns to reach purity over 80%. Human serum was from Bioreclamation (HMSRM, non-filtered). Monoclonal anti GST antibody labeled with europium cryptate (Eu-anti-GST, 61GSTLB) and SA-Xlent (611SAXLB) were from Cisbio. DTT, BSA, KH_2PO_4, Na_2HPO_4, DMSO, NaCl and KF were all from Sigma. The serum-free reaction buffer is composed of 1.06 mM KH_2PO_4, 2.96 mM Na_2HPO_4, 0.155 M NaCl, 0.1% BSA and 1 mM DTT. HTRF assay in serum used reaction buffer added with 15% human serum. The assay plate for HTRF was White 384 Opti plates from Perkin Elmer (6007299). The Envison (Perkin Elmer) was set at excitation 320 nm. Emissions were measured at 665 and 615 nm and the ratio of Em665/Em615 represented the interaction of MDM2-p53. Time-resolved fluorescence was measured 50 flashes for both detectors with 60 μs delay after each excitation. The reading time was 300 μs. Vprep was a product from Velocity11. Wellmate microplate dispenser was from Thermo Scientific. Serial Killer was made in house. ***Methods (Determination of inhibitor potency in HTRF assay):*** 20 μL 1.5 mM inhibitor was serial diluted to 20 μL DMSO for 22 points by Serial Killer. 1 μL of such diluted inhibitor was transferred to reaction plate with 9 μL reaction buffer by Vprep. 10 μL 1 nM of MDM2 was dispensed to reaction plate and incubated with inhibitor for 20 minutes before 20 μL 1.25 nM of p53 was added. After 60 minutes, the detection mixture (10 μL, 1 nM SA-Xlent, 3 nM Eu-anti-GST and 0.5 M KF) was dispensed to the reaction mixture and the plate was read on Envision after 18 hrs incubation. Total reaction volume is 50 μL. MDM2, p53 and detection were delivered to assay plates by Wellmate. IC_{50} was determined from duplicate data. ***For HTRF assay in 15% serum:*** the sequence of addition was the same as serum-free assay. 10 μL of 12.5 nM MDM2 diluted in reaction buffer containing 30% human serum was added to 10 μL of buffer and compound mixture to generate serum at 15%. 20 nM p53 was diluted in buffer with 15% human serum. The detection buffer was in 15% serum with 10 nM SA-Xlent, 3 nM Eu-anti-GST and 0.5 M KF.

Surface Plasmon Resonace (SPR) spectroscopy binding assay. ***Materials*****:** Biacore T100 instrument (GE Healthcare), CM5 sensor chip (BR-1000-12), amine couple kit (BR-1000-50) including 1-ethyl-3-(3-dimethlamino-propyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS)

and ethanolamine-HCl, GST capture kit (BR-1002-23), HBS-N buffer (BR-1006-70) were from GE Healthcare. All buffers were filtered through 0.2 μM nylon membrane (VWR 87006-076). ***Kinetics study of inhibitors on Biacore:*** CM5 chip was preconditioned with 10 mM NaOH, 10 mM HCl and 0.1% SDS each for 10s injection twice at 100 μL/min in HBS-N buffer. Immobilization and capture steps were performed as described: 1. Equal volume of 0.4 M EDC and 0.1 M NHS was mixed and injected over the chip surface to activate it for 10 min at 10 μL/min; 2. Anti-GST antibody (30 μg/ml in 10 mM sodium acetate, pH5) was immobilized to the chip by being injected to the surface for 15 min at 8μL/min; 3. To deactivate excess reactive groups and wash off unbound anti-GST antibody, 1 M ethanolamine-HCl pH 8.5 was pumped across the surface for 10 min at 8μL/min. The resulting antibody immobilization level was about 18000 RU; 4. 10μg/ml GST in HBS-N buffer was injected over one flow cell for 15 min at 5μl/min to create control surface of 1500 RU; 5. Sample surface was generated by injecting 20μg/ml MDM2 (1-188) over another flow cell for 15min twice at 5μl/min to reach capture level about 2000 RU. The kinetic binding study of inhibitors (molecular weight ranging from 450-600) was performed at 25 °C at a constant flow of 90 μL/min. The sample analysis buffer consisted of 25 mM Tris, pH 7.5, 150 mM NaCl, 0.005% (v/v) Tween 20, 0.2 mM TCEP plus 2.5% (v/v) DMSO. The inhibitors were diluted from 1 mM stock (in 100% DMSO) in sample analysis buffer to 12 nM. The inhibitors were then serially diluted 2 times to generate 8 points. Diluted inhibitors were injected over the control and sample surface for 1-2 minutes and dissociation observed for 3-4 minutes. Buffer containing 2.3- 3.5% DMSO were injected to construct a DMSO correction curve. All the sensorgrams were processed using Biacore Evaluation software (GE Healthcare) with double reference procedure and DMSO calibration. K_D values were established using a 1:1 binding model including a mass transfer limitation term.

Functional p21 induction assay (SJSA-1 p21 TaqMan® assay). In order to assess the potency of MDM2 inhibitors, quantitative reverse transcription polymerase chain reaction (qRT-PCR or TaqMan®) was used to measure the levels of p21 transcript in compound-treated cells relative to dimethyl sulfoxide (DMSO)-treated control cells. SJSA-1 cells were plated at a density of 2×10^4 cells/well in 96-well cell culture plates in 100 μl of growth medium (RPMI 1640, 10 mM HEPES, 1 mM sodium pyruvate, 1X Penicillin-Streptomycin-Glutamine (PSQ), and 10% fetal bovine serum (all components from Invitrogen). The cells were cultured overnight at 37 °C and 5% CO_2. The following day, MDM2 inhibitors were serially diluted in DMSO (Sigma #D2650), then diluted again in assay medium (RPMI 1640, 10 mM HEPES, 1 mM sodium pyruvate, and 1X PSQ) containing either no serum or 10% human serum (Bioreclamation #HMSRM) with a final DMSO concentration of 1%. The cells were incubated in the presence of inhibitor at 37 °C and 5% CO_2 for 7 hours. Total RNA was

purified from the inhibitor- and DMSO-treated SJSA-1 cells using the Qiagen BioRobot Universal workstation following the RNeasy 96 BioRobot 8000 kit (Qiagen #967152) protocol from the manufacturer, with the following exceptions: the protocol began with RLT lysis buffer addition, omitted DNase treatment, omitted addition of Top Elute fluid, and changed the final elution volume to 120 µl. To measure the levels of p21 transcript present, qRT-PCR was used. The levels of both p21 and the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were assayed from total RNA from each inhibitor- or DMSO-treated well using the TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Invitrogen #4309169); p21 20X TaqMan® Gene Expression Assay (Invitrogen #Hs00355782_m1) or GAPDH 20X TaqMan® Gene Expression Assay (Invitrogen #Hs99999905_m1). The qRT-PCR reactions were assayed on the Applied Biosystems Prism 7900HT instrument, using the relative quantification (ΔΔCt) method. The data were analyzed with Applied Biosystems SDS2.2 software, using GAPDH as the endogenous control and DMSO-treated samples as the calibrator. The SDS2.2 software calculated relative quantification (RQ) or fold increase of p21 levels relative to DMSO control for each treated sample. Dose-response curves were generated using XLFit software to calculate IC_{50} values for each inhibitor tested.

SJSA-1 cell proliferation assay (Click-iT EdU HCS assay). SJSA-1 cells were plated at a density of 2.8×10^3 cells/well in 384-well cell culture plates (Perkin Elmer, #6007460) in 40 µl of growth medium (RPMI 1640 supplemented with 10 mM HEPES, 1 mM sodium pyruvate , 1X Penicillin-Streptomycin, 2 mM Glutamine, and 10% fetal bovine serum). The cells were cultured for 24 hours at 37 °C and 5% CO_2. The next day, cells were treated with MDM2 inhibitors for 16 hours in the presence of 10% human serum. On day 3, Click-iT EdU assay procedures were performed according to the manufacturer's instructions with assay volumes reduced to 25 µL to adjust for 384 well formats (Invitrogen, #C10357). In short, EdU (5-ethynyl-2´-deoxyuridine) was added to cells to a final concentration of 10 µM and incubated for 1 hour. After labeling, cells were fixed with 4% formaldehyde and permeabilized with 0.1% Triton-X 100. After washing, cells were incubated with Click-iT reaction buffer and then with nuclear stain. Cells were then washed and imaged using Opera High Content Screening System (Perkin Elmer). Percentage of EdU incorporation was calculated and used for IC_{50} calculations. IC_{50} values were determined using a four-parameter logistical (4PL) Hill model.

BrdU proliferation assay (HCT116 specificity assay). The potency of MDM2 inhibitors was also determined by assaying the effect on cell proliferation by quantifying the amount of 5-bromo-2-deoxyuridine (BrdU) incorporation in compound-treated cells vs. DMSO-treated control cells. HCT116 $p53^{WT}$ or $p53^{-/-}$ cells were plated at a density of 6×10^3 cells/well in 96-well cell culture plates in 100

μl of growth medium (McCoy's 5A, 1X PSQ, and 10% fetal bovine serum (all components from Invitrogen)). The cells were initially cultured for 24 hours at 37°C and 5% CO_2 before addition of compound. The MDM2 inhibitors were serially diluted in DMSO (Sigma #D2650), then diluted again in assay medium (McCoy's 5A, 1X PSQ, 10% human serum (Bioreclamation #HMSRM)) with a final DMSO concentration of 1%. The cells were incubated in the presence of inhibitor at 37°C and 5% CO_2 for 16 hours and then pulsed with diluted BrdU labeling reagent (1:100 final dilution, Invitrogen #00-0103) for 1 hour at 37°C and 5% CO_2. Following the BrdU pulse, the medium was removed and the cells were fixed and stained for BrdU incorporation. The amount of BrdU incorporation was assayed using either flow cytometry or the Cellomics Array Scan Vti plate reader with the Target Activation bioapplication. The percentage of BrdU-positive cells in the DMSO-treated control wells was used to normalize the signal and calculate percent inhibition for each of the compound-treated wells. Dose-response curves were generated using XLFit software to calculate IC_{50} values for each inhibitor tested.

(ii) In vivo study protocols

All animal experimental procedures were conducted in accordance with the guidelines of the Amgen Animal Care and Use Committee and the Association for Assessment and Accreditation of Laboratory Animal Care standards. All studies utilized 4-6 week old female athymic nude mice (Harlan Laboratories, Hsd:Athymic Nude-*Foxn1*^{nu}). The mice were housed five per filter-capped cage in sterile housing in an environmentally controlled room (temperature 23 ± 2°C, relative humidity 50 ± 20%) on a 12-hour light/dark cycle. The mice were fed commercial rodent chow (Harlan Laboratories, #2020SX) and received filter-purified tap water *ad libitum*. The mice were individually identified by microchips (Bio Medic Data Systems) which were implanted subcutaneously at least two days prior to the study.

SJSA-1 Pharmacodynamic assay. In order to evaluate the pharmacodynamics (PD) of MDM2 inhibitors in vivo, the levels of p21 transcript were examined in SJSA-1 tumor xenografts, a human osteosarcoma model. On Day 0, 5×10^6 SJSA-1 cells in 0.2 ml of Matrigel mixture (1 part Matrigel™ (BD Biosciences #354234) and 2 parts RPMI 1640 (Invitrogen #11875)) were implanted subcutaneously in the right flank . Approximately 12 days post-implantation, when the average tumor volume reached ~150 mm^3, the tumor-bearing mice were randomized into various treatment groups (n = 5/group). Each group was dosed QD with the inhibitor or vehicle control *per os* (p.o.) of varying concentrations. At selected time points following the initial dose, the mice were sacrificed, and the tumor and the plasma were harvested for PD (p21 mRNA levels) and pharmacokinetic (PK) analysis, respectively. In order to perform qRT-PCR analysis of p21 transcript levels, total RNA was purified from each of the snap-frozen tumors. Tumors were placed into microcentrifuge tubes each containing a stainless steel bead (Qiagen #69989) and Buffer RLT containing β-mercaptoethanol (β-ME, 1:100

dilution, Sigma #M7522) and lysed using the Qiagen TissueLyser for 3 minutes at a frequency of 30/second. The lysates were centrifuged at 13,000 rpm for 5 minutes in a microcentrifuge, and a portion of the supernatant was used for the total RNA extraction procedure on the Qiagen BioRobot Universal workstation following the RNeasy 96 BioRobot 8000 kit (Qiagen #967152) protocol from the manufacturer, with the following exceptions: excluded DNase treatment, omitted the Top Elute Fluid addition, and changed the final elution volume to 100 µl. To measure the levels of p21 transcript present in each of the tumor samples, qRT-PCR was used. The levels of both p21 and the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were assayed from total RNA from each sample using the TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Invitrogen #4309169); p21 20X TaqMan® Gene Expression Assay (Invitrogen #Hs00355782_m1) or GAPDH 20X TaqMan® Gene Expression Assay (Invitrogen #Hs99999905_m1). A known amount of plasmid containing p21 or GAPDH cDNA was used to generate a standard curve. The qRT-PCR reactions were assayed on the Applied Biosystems Prism 7900HT instrument, using the absolute quantification method. The data were analyzed with Applied Biosystems SDS2.2 software. The SDS2.2 software calculated the p21 and GAPDH copy number in each of the tumor samples. The copy number of p21 was normalized to the copy number of GAPDH, and the fold increase of normalized p21 levels were calculated relative to vehicle control for each sample. Statistical significance was determined by ANOVA (Kruskal-Wallis) followed by Dunnett's post-hoc analysis using JMP software v8.

SJSA-1 Tumor xenograft study. SJSA-1 cells (5×10^6) were implanted subcutaneously into female athymic nude mice. Treatment with vehicle or inhibitor at the designated doses by oral gavage began on day 11 when tumors had reached ~200 mm^3 (n = 12/group). Tumor sizes and body weights were measured twice per week. Statistical significance was evaluated by Repeated Measures ANOVA followed by Dunnett's post hoc test using JMP software v8.

(iii) Determination of co-crystal structures of 25 with MDM2

Human MDM2 (17-111) with a cleavable N-terminal GST tag was expressed in *E. Coli* and purified using glutathione affinity chromatography. The N-terminal GST tag was then cleaved by thrombin and the untagged MDM2 was further purified by cation exchange chromatography. Crystals of MDM2 with **25** were obtained at 4 ^{0}C in hanging drops with 100 mM citrate pH 5.0, 2.0–2.7 M $(NH_4)_2SO_4$. These crystals belong to the spacegroup $C2_12_12_1$ with unit cell parameters of a=56.55, b=99.04, c=107.08 Å. Paratone-N mineral oil was used as cryo protectant. Diffraction data for all crystals in this work were collected on beamline 21-ID-F at the Advanced Photon Source (APS) and processed and scaled with HKL 2000. The co-crystal structures were solved by molecular replacement with AMoRe using PDB

entry code 1YCR as the template. Model building was carried out with QUANTA and refinement was done using CNS.