

SUPPLEMENTAL MATERIALS AND METHODS

HTRF assay for inhibitors of p53-MDM2 binding

Homogeneous time-resolved fluorescence (HTRF) assay measures the signal generated by two components when they are in close proximity. The p53-MDM2 binding assay uses a biotinylated peptide derived from the MDM2-binding domain of p53 and a truncated N-terminal portion of recombinant human GST-tagged MDM2 protein containing the p53 binding domain. Binding is detected by anti-GST antibody conjugated with APC and streptavidin-linked Eu. Upon binding of biotinylated peptide and GST-MDM2, Eu and APC are brought in close proximity resulting in energy transfer between the two fluorophors and a long-lived signal at 665 nm, which represents the bound fraction.

The p53-MDM2 HTRF assay was performed in buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM DTT, 0.2 mg/ml BSA. RG7112 was stored in aliquots as 10 mM stock solution in DMSO at 4° C in 96-deep-well plate. It was thawed and mixed immediately prior to testing. The compound was incubated with GST-MDM2 and biotinylated peptide (RO0274812-001-003) for one hour at 37°C. Phycolink goat anti-GST (Type 1)-allophycocyanin and Eu-8044-streptavidin were then added and followed by one hour incubation at room temperature. Plates were read using the Envision fluorescence reader. IC₅₀ values were determined from inter-plate duplicate or triplicate sets of data. Data were analyzed by XLfit4 (Microsoft) using a 4 Parameter Logistic Model (Sigmoidal Dose-Response Model) and the equation $Y = (A + ((B - A) / (1 + ((C/x)^D))))$, where A and B are enzyme activity in the absence or presence of infinite inhibitor compound, respectively, C is the IC₅₀ and D is the Hill coefficient.

Biacore studies

Human recombinant MDM2 (aa 25-108) L33E was expressed in E. coli and extracted from inclusion bodies by classical methods. The protein was refolded and purified to homogeneity by conventional purification including a last step size exclusion chromatography to confirm its monomeric nature. MDM2 protein was minimally biotinylated using Sulfo-NHS-LC-biotin (Pierce Inc) at a reagent to protein molar ratio of 1:3. The reaction mixture was incubated at 4°C for 20 hours and then exhaustively dialyzed into a TBS buffer at pH 7.5 including 1mM DTT to remove free biotin before capture. The biotinylated protein was captured on a Sensor Chip SA (streptavidin)

(Biacore Inc.) at a density conducive to kinetic analysis (theoretical response maximum of ~40 units). Compounds were serially diluted 1:1 and run in a concentration series from 100 nM to 0.78 nM. Association time was 60 seconds and dissociation time was 240 seconds. The TBS running buffer contained 0.005% P20 detergent (Biacore Inc.) and a final DMSO concentration of 5%. Experimental data was exported to Scrubber II, external software, for kinetic evaluation. The biosensor data were fit to a rate equation describing a Langmuir 1:1 binding model (Scrubber, BioLogic Software). The fit yields the association rate (k_a), the dissociation rate (k_d) and the dissociation constant (K_D). The dissociation constant is defined by the ratio of k_d/k_a .

Crystal structure studies

Proteins were expressed in *E. coli* strain BL21 using the helper plasmid pUBS 520 coding for the lacI^q repressor and the rare tRNA^{Arg} [AGA/AGG]. Cells were grown in 10 L fermenters at 37°C and expression was induced by 1 mM IPTG at $\text{OD}_{600\text{nm}}=10$. Over-expressed protein was found in inclusion bodies, exclusively. Cells were centrifuged and resuspended in 0.1 M Tris/HCl pH 7.0 by adding lysozyme and DNase. After cell disruption by french press, inclusion bodies were separated by centrifugation and washed with a) 30 g/l Brij 35, 0.75 M NaCl, 30 mM EDTA, pH 7.0 and b) 0.1 M Tris/HCl, 20 mM EDTA, pH 6.5. For further use the material was stored at -80 °C. Inclusion bodies were solubilized in 6 M GdnHCl, 100 mM Tris/HCl, 5 mM EDTA, 10 mM DTT, pH 7.5 and the proteins refolded by slowly diluting (hours) the samples 100-fold into refolding buffer (50 mM MES, 50 mM NaCl, 1 mM EDTA, 5 mM DTT, pH 7.0) at room temperature.

After removal of insoluble material ammonium sulfate was added to a final concentration of 1.5 M. Protein was adsorbed on Butyl-Sepharose 4 fast flow (Pharmacia, Sweden), washed with high-salt buffer (25 mM MES, 1.5 M Ammonium sulfate, 1 mM EDTA, pH 7.0) and eluted with 25 mM MES, 1 mM EDTA, pH 7.0. After adjusting the pH to 6.5 (HCl) further purification was performed immediately by cation exchange chromatography on SP-Sepharose (Pharmacia) at 4°C applying a gradient from 0-500 mM NaCl. Final purification was achieved by gel filtration on Superdex 75 (Pharmacia) using 10 mM MES, 100 mM NaCl, 1 mM EDTA, pH 7.0. Proteins were concentrated to 1.5-2.0 mg/ml and stored at -80°C.

For crystallization, the frozen protein was thawed and concentrated to 9.8 mg/ml using a Centricon concentrator (3000 MW cutoff). The complex was then formed by

combining the protein with a slight molar excess of the inhibitor (stock solution is 100 mM in DMSO) and this solution was allowed to sit for four hours at 4°C. Crystals were grown using the hanging drop vapor diffusion method. The reservoir contained 50% saturated ammonium sulfate, 100 mM Bis-Tris, pH 6.5, 5% (v/v) PEG 200, and 5 mM DTT. Drops were formed by combining equal volumes (2 µL) of protein/inhibitor solution with the reservoir solution. Crystals could be flash frozen in liquid nitrogen by first passing them through a cryo-preservation solution consisting of equal parts crystal growth reservoir and 4M malonate.

Cryo-preserved crystals were used to collect diffraction data on beamline X8C at the National Synchrotron Light Source at Brookhaven National Laboratory in Upton, NY. Data were collected at 100°K with the Quantum 4 detector (ADSC) to 1.55Å resolution and processed with HKL2000. The crystal used for structure solution was in space group C222(1) with cell dimensions $a = 43.08 \text{ \AA}$, $b = 67.49 \text{ \AA}$, and $c = 66.72 \text{ \AA}$. To 1.55 Å resolution, the dataset was 82.6% complete, had an overall redundancy of 3.6 and an excellent merging R-factor ($R_{\text{merge}} = 0.037$). Structure solution was achieved by applying program MOLREP from the CCP4 suite and a search model based on the published XDM2 structure (PDB code 1YCQ) of Kussie et al. (1). There is one molecule of the protein/inhibitor complex in the asymmetric unit.

Due to low completeness of the data in the higher resolution ranges, refinement was restricted to 1.70 Å resolution. At this resolution, the dataset is 96.9% complete. Refinement was performed with CNS and the R-factor for the final model is 0.208 (R_{free} based on 5% of the data not used for refinement is 0.229). The final structure is of high quality with rmsd for bond lengths equal to 0.004 Å and for bond angles equal to 1.38°. Sequence of humanized Xenopus MDM2 protein used for crystallography (hXDM2 21-105 (I50L, P92H, L95I) including N-terminal Met: MEK-LVQ-PTP-LLL-SLL-KSA-GAQ-KET-FTM-KEV-LYH-LGQ-YIM-AKQ-LYD-EKQ-QHI-VHC-SND-PLG-ELF-GVQ-EFS-VKE-HRR-IYA-MIS-RNL-VS

Cell proliferation/viability assays

Cell proliferation/viability was evaluated by the tetrazolium dye (MTT) assay. Each cell line in its optimal medium was plated at the appropriate seeding density to give logarithmic growth over the course of the assay in a 96-well tissue culture plate as previously described (2). Cell growth kinetics were measured using the IncuCyte™ live cell imaging system (Essen BioScience, Inc.). SJSA1 cells were seeded in 6-well plates

(BD Biosciences) at 2.5×10^5 cells per well and 24 hours later incubated with indicated concentrations of RG7112. Data was acquired every hour (9 points per well) for a total of 68 hours per treatment condition and growth curves were generated using IncuCyte software.

Cell cycle and apoptosis analysis

Cells were cultured in T75 flask with appropriate growth medium (10^6 cells/condition in 10 ml) and incubated overnight at 37°C . They were incubated with test compounds and processed as previously described (2). Apoptosis was determined using the Annexin V assay. Cells were seeded in six-well tissue culture plates (4×10^5 cells/well) 24 h prior to incubation with RG7112 for the indicated times. No treatment controls were established in parallel for each cell line. Culture media was collected and attached cells were trypsinized. Cells were combined with corresponding media and collected by centrifugation at 1600 rpm for 10 minutes at 4°C . Annexin V-positive cells were tagged using the GuavaNexin TM apoptosis detection kit (Guava Technologies, Hayward, CA) and percent apoptosis determined by using a Guava Personal Cell Analyzer following the manufacturer's protocol.

Animal studies

All animals were allowed to acclimate and recover from any shipping-related stress for a minimum of 72 hours prior to experimental use. Autoclaved water and irradiated food (5058-ms Pico Chow; Purina, Richmond, IN) were provided *ad libitum*, and the animals were maintained on a 12-hour light and dark cycle. Cages, bedding and water bottles were autoclaved before use and were changed weekly. The health of all animals was monitored daily by gross observation and analyses of blood samples of sentinel animals. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee.

For SJSA-1, SJSA-1luc2, and MHM xenograft studies, female Balb/c nude (CAnN.Cg-Foxn1nu/Crl, Charles River Laboratories, Wilmington, DE) mice were implanted sc in the right flank with 5×10^6 cells suspended in a 0.2 ml volume of a 1:1 mixture of matrigel:PBS. For studies with hormone-dependent LNCaP xenografts, castrated male Balb/c nude (CAnN.Cg-Foxn1nu/Crl, Charles River Laboratories, Wilmington, DE) were implanted with 12.5 mg sustained-release testosterone pellets (Innovative Research of America, Sarasota, FL) 5 days prior to sc inoculation with 1 x

10^7 cells suspended in 0.2 ml of matrigel:PBS. Mice were randomized into treatment groups ($n = 10$ per group) when mean tumor volume reached approximately 150 to 400 mm^3 . In all studies, mice received either vehicle (1% Klucel LF/ 0.1% Tween 80) or RG7112, administered as an oral suspension (*po*) at the dose indicated (25-200 mg/kg). For assessment of androgen ablation treatment in combination with RG7112 in LNCaP xenograft-bearing mice, testosterone pellets were removed under ketamine/xylazine anesthesia. Tumor volume was monitored by caliper measurement, and body weights were recorded 2-3 times weekly. Tumor volume (in cubic millimeters) was calculated as described previously (3). Efficacy data were graphically represented as the group mean tumor volume \pm SEM. Statistical analysis was determined by two-way ANOVA and Bonferroni post-test or (GraphPad Prism version 5.03). Differences between groups were considered significant when the probability (*p*) value was ≤ 0.05 .

Following cessation of RG7112 treatment, tumor regrowth was evaluated in mice bearing LNCaP xenografts. Median survival was determined utilizing the Kaplan-Meier survival analysis method, with tumor regrowth to a volume of 1000 mm^3 utilized as a surrogate endpoint for death. A log rank test was performed to evaluate statistical significance (GraphPad Prism version 5.03), and results were plotted as the percentage survival vs. days post- tumor implant. Percentage ILS was calculated as $100 \times ((\text{median survival of treated group} - \text{median survival of control group}) / \text{median survival of control group})$.

For Western blot analysis, mice bearing established SJSA-1 *sc* xenografts received a single *po* dose of vehicle or 50, 100, or 200 mg/kg RG7112, and tumors were harvested at 4, 8, and 24 h post-dosing. Antibodies against p53 (sc-263) and MDM2 (sc-965) were purchased from Santa Cruz Biotechnology and respectively. Anti-p21^{WAF1} (OP64) was purchased from Calbiochem. Anti- β -actin antibody was purchased from Sigma Aldrich. Protein was extracted from tumor tissue with 1X RIPA buffer (Sigma Aldrich) containing protease inhibitors (Roche Diagnostics) by homogenization. Equal amounts of total protein were resolved on 4-12% NuPAGE gradient gel (Invitrogen) and blotted with antibody dilutions as recommended by manufacturer. The chemiluminescent signal was generated with enhanced chemiluminescence Plus (GE Healthcare) and detected with Fujifilm LAS-3000 imager. The densitometric quantitation of specific bands was determined using Multi Gauge Software (Fujifilm).

For bromodeoxyuridine (BrdU) incorporation, 1 ml of a 1 mg/ml solution was administered *ip* to mice 2 hr prior to sample collection. Xenografts were formalin-fixed

overnight, processed and paraffin-embedded. Immunohistochemical analysis was performed using the Ventana Discovery XT platform (Ventana Medical System, Tucson, AZ). Briefly, 5-micron sections were stained with anti-BrdU (Abcam, Cambridge, MA) or anti-cleaved PARP1 (Epitomics, Burlingame, CA) antibodies, using heat-induced epitope retrieval and a 3-step biotin-streptavidin-HRP detection method, followed by DAB chromagen (Ventana Medical System, Tucson, AZ). Tissue sections were counterstained with hematoxylin, dehydrated and mounted with Permount mounting media. Glass slides were scanned at 20X magnification using a Zeiss-Mirax digital slide scanner (Carl Zeiss Imaging, Thornwood, NY), and Definiens Tissue Studio software (Definiens, Munich, Germany) was used to generate image analysis solutions. Custom-made image analysis algorithms were applied to the digital slides, to automatically detect and quantify viable xenograft areas, and areas of DAB deposition (positive staining within viable regions). Numerical results were expressed as percentage of positively-labeled area (area of DAB labeling/xenograft viable area). Statistical analysis was determined by two-way ANOVA and Bonferroni post-test (GraphPad Prism version 5.03). Differences between groups were considered significant when the probability (p) value was ≤ 0.05 .

For bioluminescence imaging, SJSA-1 cells were transfected with a pGL4.51 vector (Promega) using FuGene reagent (Roche Applied Sciences) and selected in medium containing 0.5 $\mu\text{g}/\text{ml}$ Geneticin (Gibco Life Technologies) to create stable luciferase- expressing cells. A clone with optimal luciferase expression was selected by limited dilution (SJSA-1luc2). In order to monitor RG7112-mediated apoptosis *in vivo*, imaging of SJSA-1luc2-bearing mice was performed at 24 hrs post-dosing with vehicle or 200 mg/kg of RG7112. Z-DEVD-aminoluciferin (VivoGloTM Caspase-3/7 Substrate, Promega) was administered *ip* at a dose of 50 mg/kg. Mice were anesthetized with 2-4% isoflurane/oxygen and images were acquired every 5 minutes for up to 25 minutes using an IVIS Spectrum (Caliper Life Sciences). Luciferase signal intensity was quantified using Living Image software (Version 4.2, Caliper Life Sciences). Statistical analysis was determined by two-way ANOVA and Bonferroni post-test (GraphPad Prism version 5.03). Differences between groups were considered significant when the probability (p) value was ≤ 0.05 .

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2. Tovar C, Rosinski J, Filipovic Z, Higgins B, Kolinsky K, Hilton H, et al. Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. *Proc Natl Acad Sci U S A*. 2006;103:1888-93.
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