

Implementation of a 220,000-Compound HCS Campaign to Identify Disruptors of the Interaction between p53 and hDM2 and Characterization of the Confirmed Hits

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In recent years, advances in structure-based drug design and the development of an impressive variety of high-throughput screening (HTS) assay formats have yielded an expanding list of protein-protein interaction inhibitors. Despite these advances, protein-protein interaction targets are still widely considered difficult to disrupt with small molecules. The authors present here the results from screening 220,017 compounds from the National Institute of Health's small-molecule library in a novel p53-hDM2 protein-protein interaction biosensor (PPIB) assay. The p53-hDM2 positional biosensor performed robustly and reproducibly throughout the high-content screening (HCS) campaign, and analysis of the multiparameter data from images of the 3 fluorescent channels enabled the authors to identify and eliminate compounds that were cytotoxic or fluorescent artifacts. The HCS campaign yielded 3 structurally related methylbenzo-naphthyridin-5-amine (MBNA) hits with IC_{50} s between 30 and 50 μ M in the p53-hDM2 PPIB. In HCT116 cells with wild-type (WT) p53, the MBNAs enhanced p53 protein levels, increased the expression of p53 target genes, caused a cell cycle arrest in G1, induced apoptosis, and inhibited cell proliferation with an IC_{50} $\sim$ 4 μ M. The prototype disruptor of p53-hDM2 interactions Nutlin-3 was more potent than the MBNAs in the p53-hDM2 PPIB assay but produced equivalent biological results in HCT116 cells WT for p53. Unlike Nutlin-3, however, MBNAs also increased the percentage of apoptosis in p53 null cells and exhibited similar potencies for growth inhibition in isogenic cell lines null for p53 or p21. Neither the MBNAs nor Nutin-3 caused cell cycle arrest in p53 null HCT116 cells. Despite the relatively modest size of the screening library, the combination of a novel p53-hDM2 PPIB assay together with an automated imaging HCS platform and image analysis methods enabled the discovery of a novel chemotype series that disrupts p53-hDM2 interactions in cells. (*Journal of Biomolecular Screening* 2010:766-782)

Key words: high-content screening, protein-protein interaction, biosensor, p53, hDM2, cell-based assays, imaging, anticancer drugs

INTRODUCTION

THE TRANSCRIPTIONAL ACTIVATOR P53 FUNCTIONS as a tumor suppressor by regulating the expression of genes that contribute to multiple processes that serve to limit the initiation, progression, or survival of tumor cells; cell cycle arrest; DNA damage repair; apoptosis; senescence; metastasis; and angiogenesis.¹⁻⁸ For

example, in response to DNA damage, p53 activation switches on target genes that promote cell cycle arrest (p21/WAF, Reprimo, and 14-3-3 σ) or that facilitate DNA repair through transcription-dependent and transcription-independent mechanisms.^{3,5,8} p53 also upregulates the expression of proapoptotic genes (*BAX*, *NOXA*, *PUMA*, *BID*, *CD95*, *APAF-1*, *DR5*, and *p53AIP1*) so that if the DNA repair process is unsuccessful, these proteins initiate apoptosis to kill the cell and prevent genomic damage and instability from accumulating in daughter cells.^{1,2,8} Due to the importance and complexities of p53 signaling cascades in this process, it is perhaps not surprising that numerous mutations have been identified that affect various steps in this pathway. Indeed, p53 is inactivated by single-point missense mutations in the DNA binding domain in around 50% of human cancers, resulting in deficient regulation of p53 target genes. In tumors where p53 is wild type and functional, other disruptions to the regulatory framework prevent normal p53 function. One prominent p53-deactivating mechanism is the overexpression of hDM2, common in acute lymphoblastic leukemia^{9,10} and a number of other cancers.¹¹ The

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binding site for hDM2 overlaps with the N-terminal transactivation domain of p53, and through its E3-ubiquitin-ligase activity, hDM2 targets p53 for degradation.^{12–17} hDM2 is a target gene for p53-mediated transactivation, and the 2 proteins participate in a tight autoregulatory feedback loop.^{12–14,16,17} Consequently, it has been proposed that the disruption of p53 binding and/or ubiquitination by hDM2 should produce increased p53 protein levels and reactivation of p53 tumor suppressor activity.^{7,18–23}

The structural interactions between p53 and hDM2 have been well characterized.^{24,25} The N-terminus of p53 (Phe19, Trp23, and Leu26) binds to a small hydrophobic pocket on the surface of hDM2. Nutlin-3s, a class of *cis*-imidazoline analogs, were the first potent small-molecule inhibitors of the p53-hDM2 protein-protein interaction to demonstrate in vivo stabilization of p53 and suppression of tumor growth in xenograft models.²¹ Since then, other small molecules have been identified that disrupt the p53-hDM2 interaction.²⁶ Compounds that inhibit the E3-ubiquitin ligase activity of hDM2 directly are also under investigation as a means to stabilize p53 protein levels and suppress tumor progression.^{27,28} Another popular strategy to reactivate p53 is to pharmacologically assist mutant (or in some cases wild-type) p53 to fold into an active state and to restore transactivation activity. CP-31398,¹⁹ PRIMA,¹⁸ RITA,²⁹ MIRA,³⁰ and MITA²⁰ all stabilize mutant p53 and permit transactivational activity. However, the mechanism(s) by which these compounds reactivate p53 remain unclear. Despite a significant drug discovery effort culminating in the identification of several lead compounds that stabilize p53 protein levels and reactivate p53 functions, none of these compounds has yet to be approved for clinical use in cancer therapy.

In this report, we present the results from the implementation of a high-content screening (HCS) campaign to identify disruptors of the interaction between p53 and hDM2 using a novel p53-hDM2 protein-protein interaction biosensor (PPIB) to screen a 220,017-compound library. We document the performance of the p53-hDM2 PPIB in HCS and illustrate the use of the multi-parameter image analysis data extracted from the 3 fluorescence channels of the PPIB to identify cytotoxic compounds and fluorescent outlier artifacts. We present data on the confirmation of primary HCS actives in concentration response assays together with the chemical structures of the hit compounds. We present data on the characterization of the methylbenzonaphthylidene hit series for stabilization of p53, transcriptional activation of hDM2 and p21 in Western blotting assays, effects on growth inhibition, cell cycle arrest, and the induction of apoptosis in a panel of isogenic HCT116 cell lines that are wild type (WT) for p53 or that are p53 or p21 null.

MATERIALS AND METHODS

Reagents

Nutlin-3, nocodazole, camptothecin, formaldehyde, and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis,

MO). DMSO (99.9% high-performance liquid chromatography grade, under argon) was from Alfa Aesar (Ward Hill, MA).

Cell culture

HCT116 and U-2 OS cell lines were acquired from American Type Culture Collection (Manassas, VA). The HCT116-p53 null and HCT116-p21 null cell lines were a gift from Dr. Bert Vogelstein (Johns Hopkins University). All cell lines were maintained in McCoy's 5A medium with 2 mM L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) and 100 U/mL penicillin and streptomycin (Invitrogen) in a humidified incubator at 37°C with 5% CO₂.

Compound library

The 220,017 compounds and cherry-picked actives from the primary p53-hDM2 HCS assay were supplied to the Pittsburgh Molecular Library Screening Center (PMLSC) by the National Institutes of Health (NIH) small-molecule repository (SMR; Biofocus-DPI, a Galapagos company, San Francisco, CA) as part of the Molecular Library Screening Center Network (MLSCN) pilot phase as described previously.^{31–33} The NIH library was supplied at 10 mM concentration in DMSO arrayed into 384-well microtiter master plates. NIH compounds were identified by their PubChem substance identity numbers (SIDs). Daughter plates containing 2 µL of 10 mM compounds in DMSO were prepared and replicated from the MLSCN master plates using the VPrep (Velocity11, Menlo Park, CA) outfitted with a 384-well transfer head. Aluminum adhesive plate seals were applied with an Abgene Seal-IT 100 (Rochester, NY) plate sealer, and plates were stored at –20°C in a Matrical MatriMinistore™ (Spokane, WA) automated compound storage and retrieval system. For the primary screen, immediately prior to use, daughter plates were withdrawn from –20°C storage, thawed at ambient temperature, and centrifuged 1 to 2 min at 50 g, and the plate seals were removed prior to the transfer of 78 µL of McCoy's 5A media into wells using the FlexDrop liquid handler (PerkinElmer, Waltham, MA) to generate a 200-µM intermediate stock concentration of library compounds (in 2.5% DMSO). The diluted compounds were mixed by repeated aspiration and dispensation using a 384-well P30 dispensing head on the Evolution-P3 (EP3) liquid handling platform (PerkinElmer), and then 5 µL of diluted compounds was transferred to the wells of assay plates. In the primary screen, compounds were individually screened at a final concentration of 25 µM (0.25% DMSO). For the determination of the 50% inhibition concentrations (IC₅₀), 10-point 2-fold serial dilutions of test compounds in McCoy's 5A medium-DMSO were performed using a 384-well P30 dispensing head on the EP3 liquid handling platform. The diluted compounds were mixed by repeated aspiration and dispensation using a 384-well P30 dispensing head on the EP3, and then 5 µL of diluted compounds

was transferred to the wells of assay plates to provide a final concentration response ranging from 0.195 to 50 μ M.

p53-hDM2 protein-protein interaction biosensor HCS assay

The components of the p53-hDM2 protein-protein interaction biosensor have been described previously.³⁴ N-terminal residues 1-131 of p53 encompass the binding site for hDM2 that overlaps with the p53 transactivating domain. The p53-monomeric TagGFP (Evrogen, Inc., Moscow, Russia) fusion protein is targeted and anchored in the nucleolus by the inclusion of a nuclear localization (NLS) sequence. N-terminal residues 1-118 of hDM2 encompass the domain for binding to the N-terminal transactivating domain of p53. The hDM2-monomeric Tag RFP (Evrogen, Inc.) cytoplasm-nucleus shuttling protein interaction partner includes both an NLS and a nuclear export sequence (NES). When U-2 OS cells are coinfecting with both adenovirus constructs, the hDM2 component binds to the p53 component, and both proteins become localized to the nucleolus (**Fig. 1A**). Upon disruption of the p53-hDM2 protein-protein interaction, the p53-green fluorescent protein (GFP) interaction partner remains nucleolar, but the hDM2-RFP interaction partner component redistributes into the cytoplasm (**Fig. 1A**). The disruption of the p53-hDM2 interaction biosensor is measured by determining the difference of the average fluorescence intensity associated with the hDM2-RFP shuttling component of the biosensor in the cytoplasm and nuclear compartments.

Image acquisition and analysis

Images of 3 fluorescent channels (Hoechst, GFP, and red fluorescent protein [RFP]) were sequentially acquired on the ArrayScan V^{TI} platform using a 10 $\times$ 0.3 NA objective with the XF93 excitation and emission filter set (Hoechst, FITC, and TRITC), and the images were analyzed using the molecular translocation image analysis algorithm. The ArrayScan V^{TI} platform was set up to acquire 250 selected objects (nuclei) or 2 fields of view, whichever came first. The nucleic acid dye Hoechst 33342 was used to stain and identify the nucleus, and this fluorescent signal was used to focus the instrument and define a nuclear mask. Objects in channel 1 that exhibited the appropriate fluorescent intensities above background and size (width, length, and area) characteristics were identified and classified by the image segmentation as nuclei. The nuclear mask was eroded to reduce cytoplasmic contamination within the nuclear area, and the reduced mask was used to quantify the amount of target channel, p53-GFP (channel 2) or hDM2-RFP (channel 3), fluorescence within the nucleus. The nuclear mask was then dilated to cover as much of the cytoplasmic region as possible without going outside the cell boundary. Removal of the original nuclear region from this dilated mask created a ring mask that covered the cytoplasmic region outside the nuclear envelope. The image analysis algorithm outputs quantitative data such as the total and average

fluorescent intensities of the Hoechst-stained objects (channel 1), the selected object or cell count from channel 1, the total and average fluorescent intensities of the p53-GFP (channel 2), and the hDM2-RFP (channel 3) signals in the nucleus (Circ) or cytoplasm (Ring) regions as an overall well average value or on an individual cell basis. To quantify the translocation of the hDM2-RFP from the nucleus to the cytoplasm induced by disruptors of the p53-hDM2 protein-protein interaction, the image analysis algorithm calculates a mean average intensity difference by subtracting the average hDM2-RFP intensity in the Ring (Cytoplasm) region from the average hDM2-RFP intensity in the Circ (Nuclear) region of channel 3: mean Circ-Ring average intensity difference channel 3 (MCRAID-Ch3).

p53-hDM2 automated HCS protocol

For a typical 50-plate day of HCS operations, 1×10^7 U-2 OS cells were coinfecting with the p53-GFP and hDM2-RFP adenovirus expression constructs by incubating cells with the manufacturer's (Cellumen, Inc., Pittsburgh, PA) recommended volume of virus in 1.5-mL culture medium for 1 h at 37°C, 5% CO₂ in a humidified incubator. Typically, $\geq 95\%$ of cells were coinfecting with both adenoviruses under these conditions. Coinfecting cells were then diluted to 5.6×10^4 cells/mL, and 45 μ L (2500 cells/well) were seeded in each well of a 384-well collagen-coated barcoded microplate (#781986, Greiner Bio-One, Monroe, NC) using a Zoom liquid handler (Titertek, Huntsville, AL). Plates were incubated overnight at 37°C, 5% CO₂ in a humidified incubator. Compounds or plate controls (5 μ L) were added to appropriate wells using a VPrep (Velocity11) or an Evolution P3 (PerkinElmer) for a final screening concentration of 25 μ M. Plates were incubated at 37°C for 90 min. Samples were fixed by the addition of 50 μ L of prewarmed (37°C) 7.4% formaldehyde and 2 μ g/mL Hoechst 33342 in phosphate-buffered saline (PBS) using a BioTek ELx405 (BioTek, Winooski, VT) or a MapC liquid handler (Titertek) and incubated at room temperature for 30 min. Liquid was then aspirated and plates were then washed twice with 85 μ L PBS using the BioTek or MapC liquid handler and sealed with adhesive aluminum plate seals (Abgene) with the last 85- μ L wash of PBS in place. Fluorescent images were then acquired on an ArrayScanV^{TI} automated imaging platform (Thermo Fisher Scientific, Waltham, MA), and the images were analyzed using the molecular translocation image analysis algorithm as described above.

HCS data analysis, visualization, statistical analysis, and curve fitting

Data analysis for the primary HCS assay and IC₅₀ determinations were performed using ActivityBaseTM (IDBS, Guildford, UK) and CytoMiner (University of Pittsburgh Drug Discovery Institute), and processed data and HCS multiparameter features were visualized using SpotfireTM DecisionSite[®] (Somerville,

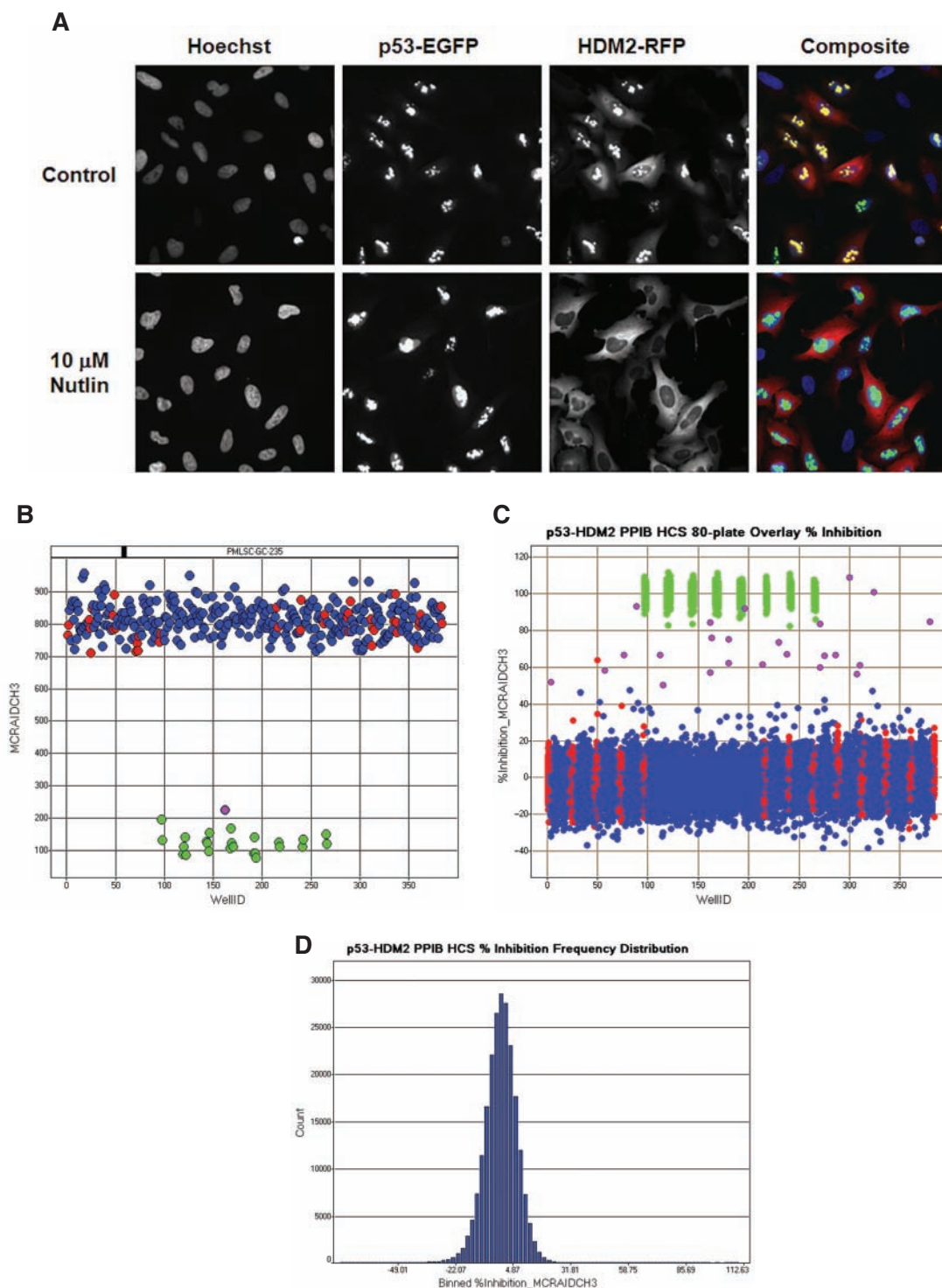


FIG. 1. High-content screening (HCS) assay performance. (A) Representative images of the p53-hDM2 biosensor acquired on the ArrayScan V^{TI} using a 20× 0.4 NA objective and the XF93 filter set. Cells were infected with the adenovirus protein-protein interaction biosensor (PPIB) expression constructs and plated as described in Materials and Methods. Samples were treated with 0.25% DMSO (top row) or 10 μ M Nutlin-3 in 0.25% DMSO (bottom row), and images were acquired in each of the 3 fluorescent channels. Composite color images of the overlays of the 3 channels are also presented. (B) Raw screening data (MCRAIDCh3) SpotfireTM scatter plot from a single plate. Positive controls (10 μ M Nutlin-3 in 0.25% DMSO, green, $n = 32$) are well separated from the negative controls (0.25% DMSO, red, $n = 32$) and the majority of compounds (25 μ M in 0.25% DMSO, blue, $n = 320$). A single hit (>50% inhibition) can be seen (magenta). (C) Overlay SpotfireTM scatter plot of a single 80-plate assay run plotted as normalized percent inhibition versus well number. Color coding is identical to B. (D) Binned histogram of the normalized percent inhibition for the entire screening campaign.

MA) software. An ActivityBase primary HTS template was created that automatically calculated percent inhibition together with plate control signal-to-background (S/B) ratios and Z' factors. For the HCS campaign, we used the mean MCRAID-Ch3 value of the DMSO minimum plate control wells ($n = 32$) and the mean MCRAID-Ch3 value of the 10 μ M Nutlin-3 maximum plate control wells ($n = 24$) to normalize the MCRAID-Ch3 compound data and to represent 0% and 100% disruption/inhibition of the p53-hDM2 interactions, respectively. The PMLSC also constructed an ActivityBase concentration-response template to calculate percent inhibition together with plate control S/B ratios and Z' factors for quality control purposes. IC_{50} values were calculated using an XLfit 4-parameter logistic model, also called the sigmoidal dose-response model: $y = (A + ((B - A)/(1 + ((C/x)^D))))$, where y was the percent activation and x was the corresponding compound concentration. The fitted C parameter was the IC_{50} and defined as the concentration giving a response halfway between the fitted top (B) and bottom (A) of the curve. The A and B parameters were locked as 0 and 100, respectively. We also used GraphPad Prism 5 software to plot and fit data to curves using the following sigmoidal dose-response variable slope equation: $Y = \text{Bottom} + [\text{Top} - \text{Bottom}]/[1 + 10^{(\text{LogEC}_{50} - X) * \text{Hill slope}}]$.

Z-score statistical methods for HCS outlier analysis and hit criterion

We used the Z-score plate-based statistical scoring method to estimate the compounds that behaved as outliers compared with the other substances ($n = 320$, no controls) tested on an assay plate for selected HCS multiparameter measurements output by the image analysis module: $Z\text{-score} = (x_i - X)/\sigma$, where x_i was the raw measurement on the i th compound, and X and σ were the mean and standard deviation of all the sample measurements on a plate. We used the selected object counts per valid field of view (SCCPVF) parameter derived from the Hoechst DNA staining in channel 1 to identify compounds that were cytotoxic. A deviation of 4σ below the sample average X selected object counts per field of view on the plate (SCCPVF Z-scores < -4) was designated as the threshold or cutoff for cytotoxic compounds. We also calculated Z-scores for 2 fluorescent intensity parameters from each of the 3 fluorescent channels: the mean nuclear total intensity (MNTI-Ch1) and the mean nuclear average intensity from the Hoechst channel (MNAI-Ch1), the mean Circ (nuclear) average intensity (MCAI-Ch2) and the mean Ring (cytoplasm) average intensity (MRAI-Ch2) from the GFP channel, and the mean Circ (nuclear) average intensity (MCAI-Ch3) and the mean Ring (cytoplasm) average intensity (MRAI-Ch3) from the RFP channel. Compounds that exhibited a deviation of 4σ above the sample average X (Z-scores > 4) in either one or both of the intensity parameters from each channel were considered fluorescent outliers.

Compounds that met all of the following criteria were designated active in the p53-hDM2 PPIB primary HCS: $\geq 50\%$ inhibition of the MCRAID-Ch3, a SCCPVF Z-score > -4 , an MNTI-Ch1 Z-score < 4 , an MNAI-Ch1 Z-score < 4 , an MCAI-Ch2 Z-score < 4 , an MRAI-Ch2 Z-score < 4 , an MCAI-Ch3 Z-score < 4 , and an MRAI-Ch3 Z-score < 4 .

Compound structure clustering analysis

Disruptors of the p53-hDM2 interaction that were identified in the HCS and confirmed in 10-point concentration-response IC_{50} assays were analyzed using the Leadscope Enterprise 2.4.6-1 software. The confirmed hit compounds were subjected to structure-based clustering and classification techniques based on recursive partitioning as described previously.^{31-33,35}

Cell proliferation assay

Cell proliferation was measured using a previously described high-content image analysis method.³⁶ HCT116 WT, p53 $-/-$, p21 $-/-$, and U-2 OS WT cell lines were seeded at 1000 cells/well using a Zoom liquid handler in collagen-coated 384-well black-walled clear-bottom plates (#781986, Greiner Bio-One) and cultured for 2 to 72 h at 37°C in a 5% CO_2 atmosphere and 95% humidity. After a 2-h cell attachment period, 1 plate of cells was fixed, stained, and analyzed as described below to establish the cell density at the time of treatment; the other plates were treated with the indicated compound concentrations by transfer of stocks to the 384-well plate by the Evolution P3 liquid handling platform using a 384-well P30 dispensing head and cultured for an additional 22 to 70 h. At the indicated time points, plates were fixed with 3.7% formaldehyde containing 1 μ g/mL Hoechst 33342 at 37°C for 30 min, the fixed cells were then washed twice with PBS, and plates were sealed with adhesive aluminum plate seals (Abgene). Images using a 10 $\times$ objective of the Hoechst-stained nuclei from 9 fields of view covered the entire well and provided the total cell count per well. The extent of growth inhibition observed in compound-treated wells is expressed as a percentage of the total cell counts in these wells relative to the total cell counts in DMSO control wells: % growth inhibition = $([\text{mean total cell count in DMSO control wells} - \text{total cell count in treated wells}] \times 100)/\text{mean total cell count in DMSO control wells}$.

Immunoblotting

Cells were harvested in a modified immunoprecipitation buffer as described previously³⁷ and incubated on ice for 30 min with frequent, vigorous vortexing. Lysates were cleared by 13,000 g centrifugation for 15 min at 4°C. Protein concentration was determined by the Bradford method. Total cell lysates (10-20 μ g protein) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 16% Tris-glycine gels and

transferred to nitrocellulose membranes using the i-Blot semi-dry transfer system (Invitrogen) for Western blotting. Antibodies against p53, phosphorylated p53 (Ser15), and γ H2A.X were from Cell Signaling Technologies (Danvers, MA). The hDM2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), the β -tubulin antibody was obtained from Cederlane Labs (Burlington, NC), and the anti-p21 antibody was received from Merck KGaA (Darmstadt, Germany). Bound primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and proteins were visualized using Pierce Enhanced Chemi-luminescence Western Blotting Substrate (Pierce, Rockford, IL). Chemiluminescence was detected using an LS3000 image analyzer and quantitated using Multigauge software (Fujifilm, Woodbridge, CT).

Flow cytometry

M phase cells were identified by simultaneous staining with propidium iodide (PI) and phospho-histone H3 using an anti-phospho-histone H3 antibody (Upstate, Lake Placid, NY) as previously described³⁸ using the manufacturer's recommended protocol. Briefly, cells were washed twice with cold PBS and detached from dishes using 0.05% trypsin/EDTA. Culture medium, PBS washes, and detached cell suspension were pelleted by a 5-min centrifugation at 300 g and washed twice with cold PBS to remove trypsin. Cells ($\sim 1 \times 10^6$) were fixed overnight in 70% ethanol and permeabilized with 0.25% Triton X-100 in PBS for 8 min on ice. After washing with 1% bovine serum albumin (BSA) in PBS, cells were incubated with 1 μ g anti-phospho-histone H3 antibody for 2 h. Cells were subsequently washed with PBS containing 1% BSA before incubation with 1 μ g anti-rabbit IgG-FITC for 40 min in the dark. Finally, cells were resuspended in 500 μ L PI/RNase staining buffer (BD Pharmingen, San Diego, CA) for 15 min, and 20,000 cells were analyzed by fluorescence-activated cell sorting (FACS) using an EasyCyte Plus flow cytometer (Guava Technologies, Hayward, CA).

S phase cells were identified using a BrdU flow kit (BD Pharmingen). Cells were treated and stained per the manufacturer's recommendations. Briefly, cells were stained with 10 μ M bromodeoxyuridine (BrdU) 30 min prior to harvesting. Cells were washed twice with cold PBS and detached from dishes using 0.05% trypsin/EDTA. Culture medium, PBS washes, and detached cell suspension were pelleted by a 5-min centrifugation at 300 g and washed twice with cold PBS to remove trypsin. Cells were then permeabilized with saponin and fixed with 4% paraformaldehyde in PBS. Cells were then treated with DNase to expose BrdU epitopes. Immunofluorescence staining was achieved using a FITC-conjugated anti-BrdU antibody followed by 7-amino-actinomycin-D (7-AAD) for staining of total DNA content. Cells were then resuspended in PBS and analyzed by FACS as described above.

Apoptotic cells were identified using the Annexin V-FITC Apoptosis Detection Kit I from BD Pharmingen. Briefly, cells

were washed twice with cold PBS and gently detached from dishes using 0.05% trypsin/EDTA. Culture medium, PBS washes, and detached cell suspension were pelleted by a 5-min centrifugation at 300 g and washed twice with cold PBS to remove trypsin. Cells were resuspended in 100 μ L binding buffer plus FITC-Annexin V and PI and incubated at room temperature in the dark for 15 min. Samples were diluted with 400 μ L binding buffer and acquired by FACS as above. WinMDI software (TSRI, La Jolla, CA) was used to determine phospho-histone-positive, BrdU-positive, and Annexin V-positive/PI negative cells in their respective experiments.

RESULTS

p53-hDM2 PPIB HCS assay performance

An initial characterization of the components of the p53-hDM2 PPIB HCS assay has been described previously.³⁴ Briefly, U-2 OS cells were coinfecting with 2 recombinant adenovirus expression constructs: a nucleolar-localized p53-GFP fusion component and a cytoplasmic-localized hDM2-RFP fusion component. When U-2 OS cells were coinfecting with both adenovirus constructs, the shuttling hDM2 interaction partner bound to the anchored p53 interaction partner, and both proteins became localized to the nucleolus (**Fig. 1A**). However, when the PPI was disrupted by a small molecule (e.g., Nutlin-3), the p53-GFP interaction partner remained anchored in the nucleolus while the shuttling hDM2-RFP interaction partner was exported to the cytoplasm (**Fig. 1A**). The relative distribution of the hDM2-RFP shuttling protein in the cytoplasmic and nuclear regions of compound-treated cells was used to measure the disruption of the p53-hDM2 interaction and could be quantified by analyzing the digital images acquired on automated imaging platforms. The primary output for the interaction of p53 with hDM2 from images acquired on the ArrayScanV^{TI} and analyzed with the molecular translocation algorithm was the Mean Circ-Ring Average Intensity Difference in Channel 3 (MCRAID-Ch3; **Fig. 1B**). The raw MCRAID-Ch3 data from a single 384-well screening plate are presented in **Figure 1B**. In the DMSO minimum plate control wells ($n = 32$), the interaction between p53 and hDM2 was intact; hDM2-RFP was primarily localized within the nucleolus, and the corresponding MCRAID-Ch3 value was large (~ 800 ; **Fig. 1B**). In the 10- μ M Nutlin-3 maximum plate control wells ($n = 24$), however, the interaction between p53 and hDM2 was disrupted; hDM2-RFP was predominantly localized in the cytoplasm, and the corresponding MCRAID-Ch3 value was relatively smaller (~ 100 ; **Fig. 1B**). The DMSO plate control wells (red) clustered with the overwhelming majority of the inactive compound-treated wells (blue)—well separated from the cluster of Nutlin-3-treated plate controls (green) (**Fig. 1B**). A single active compound on the plate was readily identified (magenta) with a low MCRAID-Ch3 value approaching those of the Nutlin-3 controls.

Over the course of a 6-week period, in 13 separate 2-day HCS operational runs, the UPDDI screened 220,017 compounds from the NIH compound library at 25 μ M in the p53-hDM2 PPIB HCS assay. For the HCS campaign, we used the mean MCRAID-Ch3 value of the DMSO minimum plate control wells ($n = 32$) and the mean MCRAID-Ch3 value of the 10 μ M Nutlin-3 maximum plate control wells ($n = 24$) to normalize the MCRAID-Ch3 compound data and to represent 0% and 100% disruption/inhibition of the p53-hDM2 interactions, respectively. **Figure 1C** shows an overlay of the normalized percent inhibition data from a single 80 (384-well) plate operational run. The primary HCS active criterion was set at $>50\%$ inhibition MCRAID-Ch3, and primary HCS actives identified in the 25,600 compounds screened in this 80-plate run are indicated (magenta). The p53-hDM2 PPIB HCS assay exhibited a robust and reproducible assay signal window between the Nutlin-3 and DMSO-treated plate controls and performed very consistently from plate to plate (**Fig. 1C**). For the 697 384-well plates screened during the p53-hDM2 campaign, the average Z' factor was 0.64 ± 0.13 , and the average S/B ratio was 31.0 ± 9.8 -fold. Indeed, the p53-hDM2 PPIB HCS assay performed very consistently throughout the HCS campaign, as indicated by the normal percent inhibition results frequency distribution for the 220,017 compounds in which the majority of compounds were inactive centered at 0% inhibition (**Fig. 1D**).

Outlier analysis and confirmation of hits

We used a Z-score plate-based statistical scoring method to estimate the compounds that behaved as outliers compared with the other substances ($n = 320$) tested on an assay plate for selected HCS multiparameter measurements extracted from the digital images by the molecular translocation image analysis algorithm: the SCCPVF, the mean nuclear total intensity (MNTI-Ch1) and the mean nuclear average intensity from the Hoechst channel (MNAI-Ch1), the mean Circ (nuclear) average intensity (MCAI-Ch2) and the mean Ring (cytoplasm) average intensity (MRAI-Ch2) from the GFP channel, and the mean Circ (nuclear) average intensity (MCAI-Ch3) and the mean Ring (cytoplasm) average intensity (MRAI-Ch3) from the RFP channel. A SCCPVF Z-score <-4 was designated as the threshold or cutoff for cytotoxic compounds. Compounds that exhibited Z-scores >4 in either one or both of the intensity parameters from any of the 3 channels were considered fluorescent outliers. Compounds that met all of the following criteria were designated active in the p53-hDM2 PPIB primary HCS: $\geq 50\%$ inhibition of MCRAID-Ch3, a SCCPVF Z-score >-4 , an MNTI-Ch1 Z-score <4 , an MNAI-Ch1 Z-score <4 , an MCAI-Ch2 Z-score <4 , an MRAI-Ch2 Z-score <4 , an MCAI-Ch3 Z-score <4 , and an MRAI-Ch3 Z-score <4 . After excluding 133 cytotoxic compounds (0.06% of the library) with cell counts below the threshold and 3037 compounds that were fluorescent outliers (1.38% of the library) in 1 or more of the intensity parameters from the 3 channels, only

312 compounds (0.14% of the library) exhibited $\geq 50\%$ disruption of the p53-hDM2 interaction (Suppl. Table S1).

Of the 312 primary HCS actives, only 284 compounds were available for cherry picking by the NIH SMR. To confirm their initial activity in the p53-hDM2 PPIB HCS assay, we conducted duplicate 10-point concentration-response assays starting at a maximum of 50 μ M to determine the reproducibility of the initial hit characterization and an IC_{50} for the compounds. The images and multiparameter HCS data from these studies were rigorously investigated for the presence of outliers and artifacts (**Fig. 2** and Suppl. Table S1).

Figure 2A shows a scatter plot of SCCPVF versus the MCRAID-Ch3 activity data. The maximally disruptive Nutlin-3 controls (green) cluster well to the right of the DMSO controls (red) and the majority of inactive compounds (blue). The SCCPVF value for the majority of compound wells is within the 100 to 400 range. In contrast, for the 3 highest concentrations of SID 26725305 (50, 25, and 12.5 μ M), the SCCPVF values were below the cytotoxic threshold of 100 (magenta), and the corresponding 3-color composite images from these wells and a nontoxic concentration (6.25 μ M) are presented. Only 3 (1%) of the compounds tested in concentration-response assays were identified as cytotoxic with a SCCPVF Z-score <-4 (Suppl. Table S1).

Despite the filtering of the primary HCS actives for fluorescence outliers at 25 μ M (Suppl. Table S1), the concentration-response assays starting at a maximum of 50 μ M yielded compounds that were fluorescent outliers in 1 or more of the intensity parameters from the 3 channels (**Fig. 2B-D** and Suppl. Table S1). A scatter plot of the MNTI-Ch1 and the MNAI-Ch1 data from the concentration-response assays revealed 2 distinct populations of fluorescent outliers in the Hoechst channel (**Fig. 2B**). Some of the compounds with SCCPVF values below 100 (**Fig. 2A**) also exhibited very low fluorescent intensity values in the Hoechst channel (**Fig. 2B**; circled in black, images labeled “low”), whereas other compounds exhibited very high MNTI-Ch1 and/or MNAI-Ch1 fluorescent intensity values (**Fig. 2B**; magenta, images labeled “high”). Insoluble compounds that form fluorescent precipitates in the Hoechst channel contribute to some of the high-intensity outliers in channel 1 (**Fig. 2B**; images labeled “high”). For other compounds, the Hoechst channel fluorescence was not restricted to the nuclear region but rather was throughout the entire cell (**Fig. 2B**; images labeled “high”), and the image analysis algorithm created a whole cell mask instead of a nuclear mask and invalidated the analysis. Forty-two (14.8%) of the 284 compounds tested in concentration-response assays were identified as fluorescent outliers (Z-scores >4) in channel 1 (Suppl. Table S1). A scatter plot of the MCAI-Ch2 and the MRAI-Ch2 data from the concentration-response assays revealed a number of compounds that were fluorescent outliers in the GFP channel (**Fig. 2C**; magenta and images). Two types of green fluorescent artifacts were apparent from the images; some compounds were

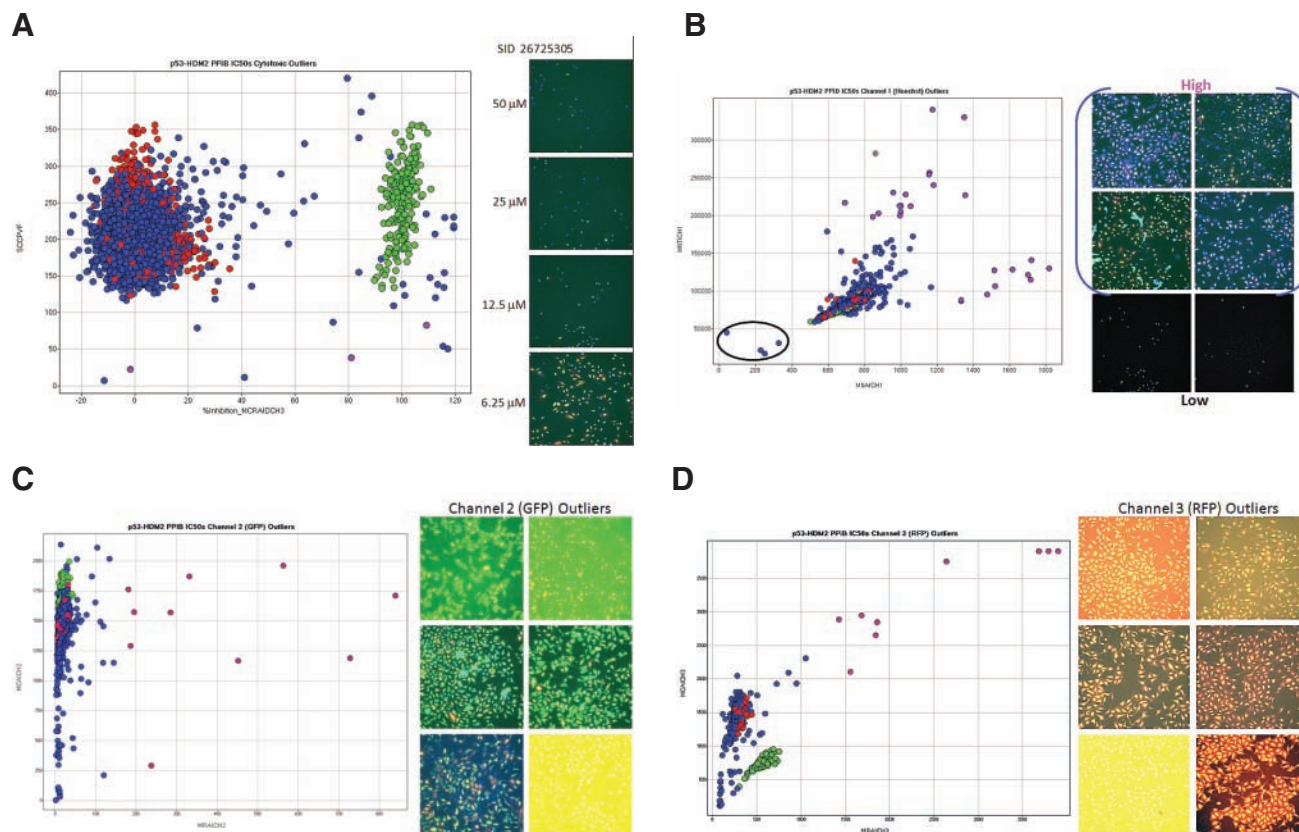


FIG. 2. Hit confirmation data outlier analysis. (A) Spotfire™ scatter plot of SCCPVF versus percent inhibition. The positive control (10 μM Nutlin-3 in 0.25% DMSO; green) is a distinct cluster from the negative control (0.25% DMSO; red) and majority of compounds in 0.25% DMSO (blue). Any compound with a selected object counts per valid field of view (SCCPVF) >4 σ from the controls (100 was used as the cutoff value because 4 σ from the mean was less than 100) was tagged as a cytotoxic outlier. Nuclear images of a selected outlier compound (magenta), as well as the first noncytotoxic concentration for that compound, are presented to the right of the plot. (B) Spotfire™ scatter plot of MNTI-Ch1 versus MNAI-Ch1. Controls are colored as in A, with nuclear outliers defined as >4 σ (magenta) for MNTI-Ch1 or MNAI-Ch1. Selected images of nuclear outliers (labeled “high”) are shown to the right of the plot. Cytotoxic compounds can also be identified from this plot (circled in black). Selected images of cytotoxic compounds (labeled “low”) are presented to the right of the plot. (C) Spotfire™ scatter plot of MCAI-Ch2 versus MRAI-Ch2 colored as in A. Outliers (magenta) are defined as above except that the MCAI-Ch2 and MRAI-Ch2 parameters were analyzed. Selected images from outlier wells are presented to the right of the plot. (D) Scatter plot of MCAI-Ch3 versus MRAI-Ch3 colored as in A. Outliers (magenta) are defined as above except that the MCAI-Ch3 and MRAI-Ch3 intensity parameter were analyzed. Representative images are presented to the right of the plot.

strongly fluorescent in solution throughout the whole well, whereas others were strongly fluorescent within the cytoplasm and/or nucleus of the cell (**Fig. 2C**; images). Twenty-three (8.1%) of the 284 compounds tested in concentration-response assays were identified as fluorescent outliers (Z-scores >4) in channel 2 (Suppl. Table S1). Some of the compounds were so intensely fluorescent in the green channel that they bled through the filter set into the red channel (**Fig. 2C**; composite images—yellow) and produced false-positive MCAID-Ch3 values in the hDM2-RFP channel. The scatter plot of the MCAI-Ch3 and MRAI-Ch3 data from the concentration-response assays revealed a number of compounds that were fluorescent outliers in the RFP channel (**Fig. 2D**;

magenta and images). Similar to the GFP channel, 2 types of red fluorescent artifacts were apparent from the images: some compounds were strongly fluorescent in solution throughout the whole well, whereas others were strongly fluorescent within the cytoplasm and/or nucleus of the cell (**Fig. 2D**; images). Once again, some of the compounds were so intensely fluorescent in the red channel that they produced false-positive MCAID-Ch3 values for the subcellular distribution of hDM2-RFP and/or bled through into the GFP channel (**Fig. 2D**; composite images-yellow).

Of the compounds tested in concentration-response assays, 213 (75%) failed to confirm the activity observed in the p53-hDM2 PPIB primary HCS assay. After excluding 3 cytotoxic compounds

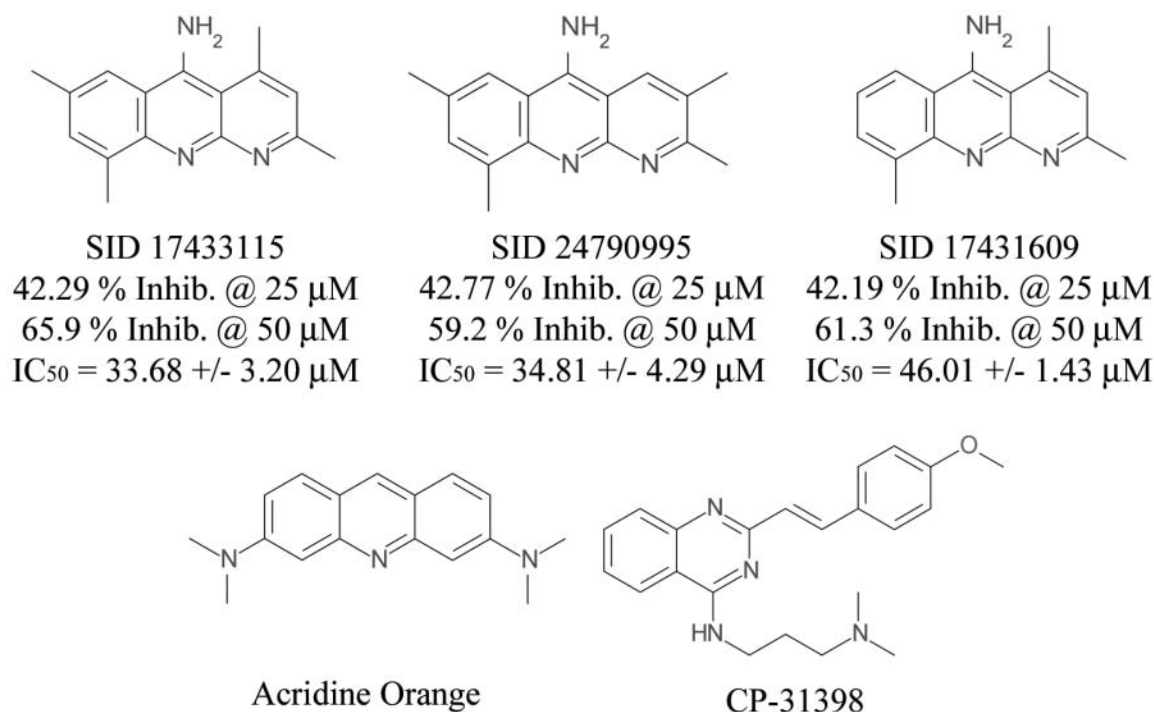


FIG. 3. Compound structures of p53-hDM2 biosensor hits. The chemical structures of the methylbenzonaphthyridin-5-amines (MBNAs) (PubChem SIDs 17433115, 24790995, and 17431609), together with their corresponding percent inhibition from the primary high-content screening and the mean IC₅₀s from the 10-point concentration confirmation assays, are presented. For comparison, the structures of acridine orange and CP-31398 are shown.

(1%) with cell counts below the cutoff threshold and 66 compounds that were fluorescent outliers (23.2%) in 1 or more of the intensity parameters from the 3 channels, only 3 of the compounds (1.06%) exhibited reproducible concentration-dependent disruption of the p53-hDM2 interaction (Suppl. Table S1). The chemical structures of the 3 confirmed hits with their average IC₅₀s and percent inhibition at 50 μ M are presented in **Figure 3**. A single cluster of 3 structurally related methylbenzonaphthyridin-5-amine (MBNA) compounds reproducibly exhibited IC₅₀s in the 30- to 50- μ M range: SID 17433115 (2,4,7,9-tetramethylbenzo[b][1,8]naphthyridin-5-amine), SID 17431609 (2,4,9-trimethylbenzo[b][1,8]naphthyridin-5-amine), and SID 24790995 (2,3,7,9-tetramethylbenzo[b][1,8]naphthyridin-5-amine). Images from the hDM2-RFP channel of the p53-hDM2 PPIB in wells treated with 50 μ M of the 3 MBNA hits were compared with those from the DMSO and Nutlin-3 (10 μ M) controls (Suppl. Fig. S3). Just as with Nutlin-3, the images from treated wells reveal that the short-term 90-min exposure of the U-2 OS cells to the highest concentrations of the MBNAs tested (50 μ M) did not significantly reduce the SCCPVF counts (Suppl. Fig. S3). A cross-target query of the PubChem database revealed that although each of the

MBNA compounds had been screened in ≥ 210 bioassays, there were very few active flags recorded in the database. Furthermore, the concentration dependency of these compounds had been confirmed in only a very few (≤ 4) assays (Suppl. Table S2). For comparison, the chemical structures of 2 compounds, the styrylquinazoline CP-31398 and acridine orange, that have been shown to reactivate mutant p53 and/or activate wild-type p53 are shown.^{19,39,40} However, because acridine orange analogs are fluorescent when bound to DNA/RNA, we measured the absorbance and emission spectra for 50 μ M SID 17433115 and SID 24790995 in 0.5% DMSO in PBS. Both MBNA compounds exhibited an absorbance peak at 400 nm and an emission peak at 465 nm. However, consistent with our outlier analysis and using the image acquisition conditions optimized for the p53-hDM2 PPIB HCS, U-2 OS cells that were exposed to 50 μ M of any of the MBNA compounds for 90 min exhibited slightly more background fluorescence in images of the Hoechst channel and no signal in either the GFP or RFP channels (data not shown). The higher background fluorescence in the Hoechst channel did not interfere with either the ability of the ArrayScan V^{TI} to focus in the well or the ability of the image analysis algorithm to segment these images, identify

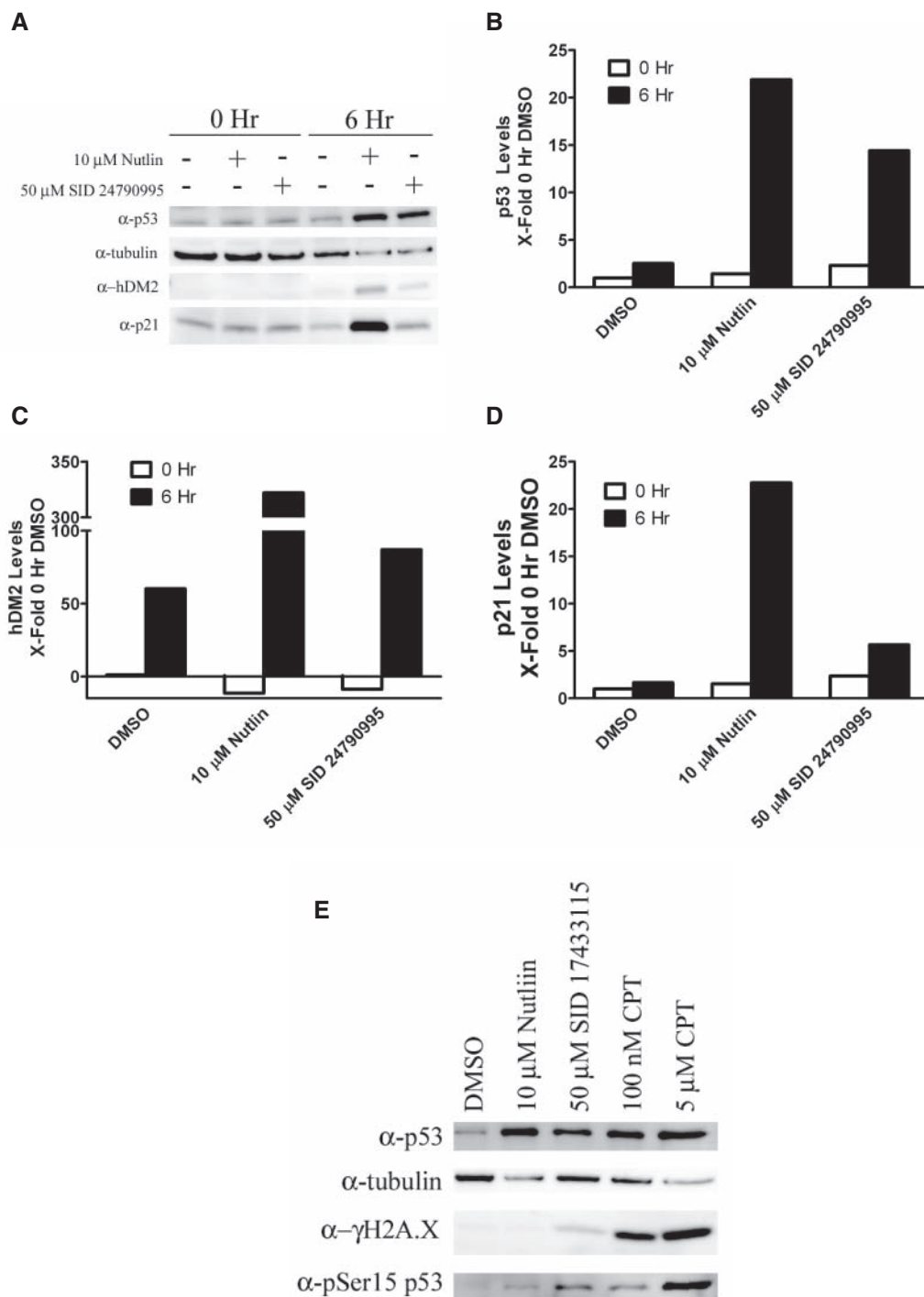


FIG. 4. Methylbenzo-naphthyridin-5-amines (MBNAs) increased p53 activity through a DNA damage-independent pathway. HCT116-WT cells were treated with 0.25% DMSO control, 10 μ M Nutlin-3 in 0.25% DMSO, or 50 μ M SID 24790995 in 0.25% DMSO for 0 or 6 h. (A) Cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane, and selected antibodies were used to probe for the indicated proteins. (B) Quantification of p53 protein levels from A. Scanning densitometry values were normalized to the 0-h DMSO control. (C) As in B, quantification of hDM2 protein levels from A. (D) As in B, quantification of p21 protein levels from A. (E) Similar to A, cells were treated with 0.25% DMSO, 10 μ M Nutlin-3, 50 μ M SID 17433115, 100 nM camptothecin (CPT), or 5 μ M CPT for 24 h, and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and probed for the indicated proteins.

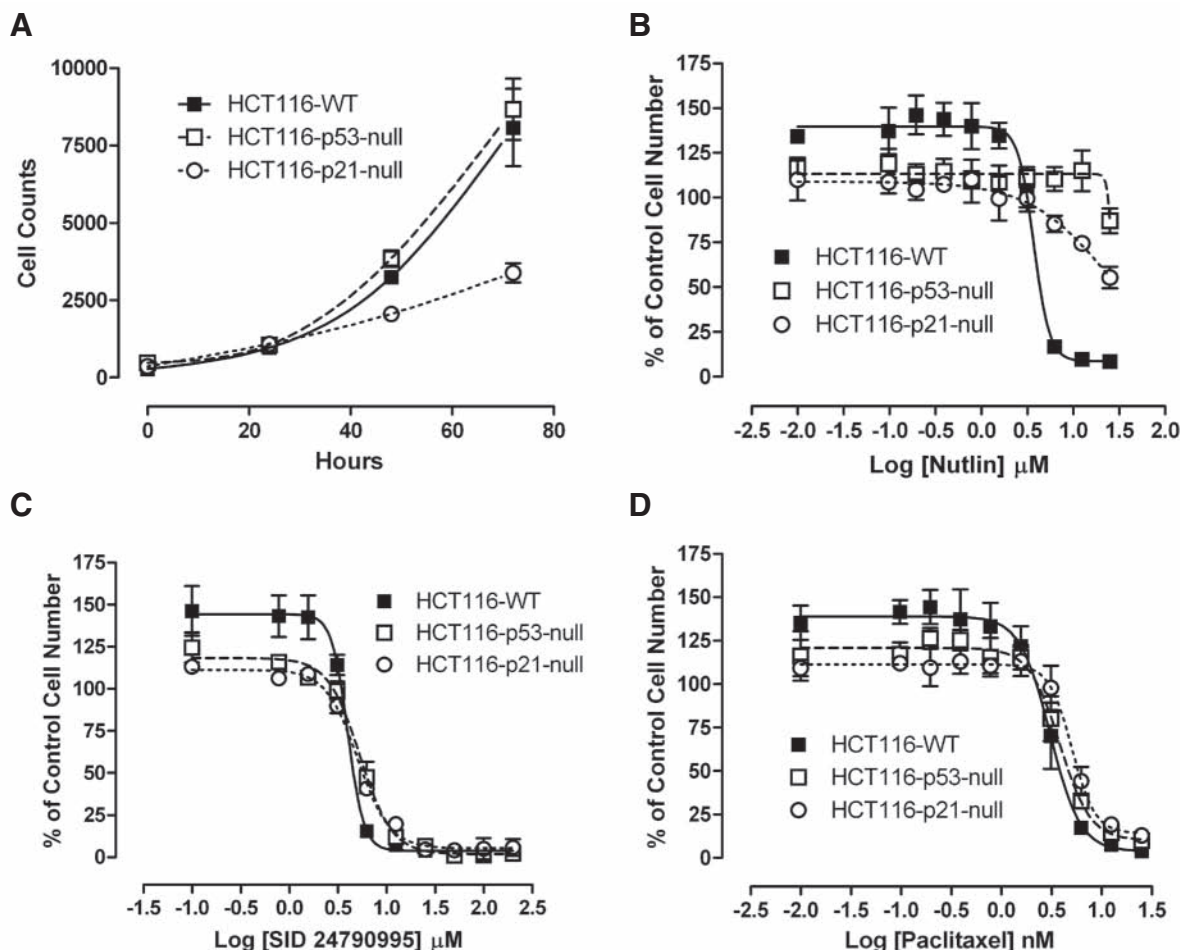


FIG. 5. Inhibition of isogenic HCT116 cell line proliferation by methylbenzo-naphthyridin-5-amines (MBNAs), Nutlin-3, and paclitaxel. HCT116-WT, p53 null, and p21 null cell lines were seeded at 1000 cells/well in collagen-coated 384-well plates. A titration series of Nutlin-3, SID 24790995, or paclitaxel in 0.5% DMSO was added. After allowing 2 h for cells to settle, initial plates were fixed, stained with Hoechst 33342, and counted on the ArrayScanV^{TI}. Subsequent samples were fixed, stained, and scored at 24, 48, and 72 h. (A) Total number of cells for WT (■) and p53 null (□) increased exponentially over time. p21 null cells (○) lagged. (B) Concentration-response curves showing normalized cell numbers after 48 h treatment with Nutlin-3. The p21 null and p53 null cell lines are resistant relative to wild-type (WT). Similar concentration-response curves plotted for (C) SID 24790995 or (D) paclitaxel. All 3 cell lines were equivalently sensitive to the MBNA and paclitaxel.

nuclei, and assign nuclear masks. On the basis of their selective bioactivity profiles and because they had not been flagged as fluorescent outliers in the primary HCS or IC₅₀ confirmation assays, we therefore decided to further characterize the activity of these 3 hits.

Hit characterization experiments

Methylbenzonaphthyridin-5-amines induce stabilization of p53, hDM2, and p21 protein levels independent of DNA damage repair pathways. Activation of p53 leads to stabilization of p53 protein levels and increased expression of p53 target genes, including hDM2 and p21. To demonstrate p53 stabilization and/or

activation, we treated the HCT116 p53 ^{+/+} (HCT-116-WT) cell line with SID 24790995 or Nutlin-3 and measured the total p53, hDM2, and p21 protein levels by Western blotting (Fig. 4A). To demonstrate the specificity of the p53 antibody, total protein/cell lysate samples from control and Nutlin-3-treated U-2 OS and HCT116-WT, p53 ^{-/-} (HCT116-p53 null), or p21 ^{-/-} (HCT116-p21 null) cell lines were subjected to SDS-PAGE, transferred to nitrocellulose, and probed for p53 levels (Suppl. Fig. S2). A 53-kDa anti-p53 stained band that was enhanced by Nutlin-3 treatment was readily detected in the Western blots for all cell lines except the HCT116-p53 null cells. The relative amounts of the protein bands in Figure 4A were quantified for p53, hDM2, and p21 by scanning densitometry (Fig. 4B-D, respectively).

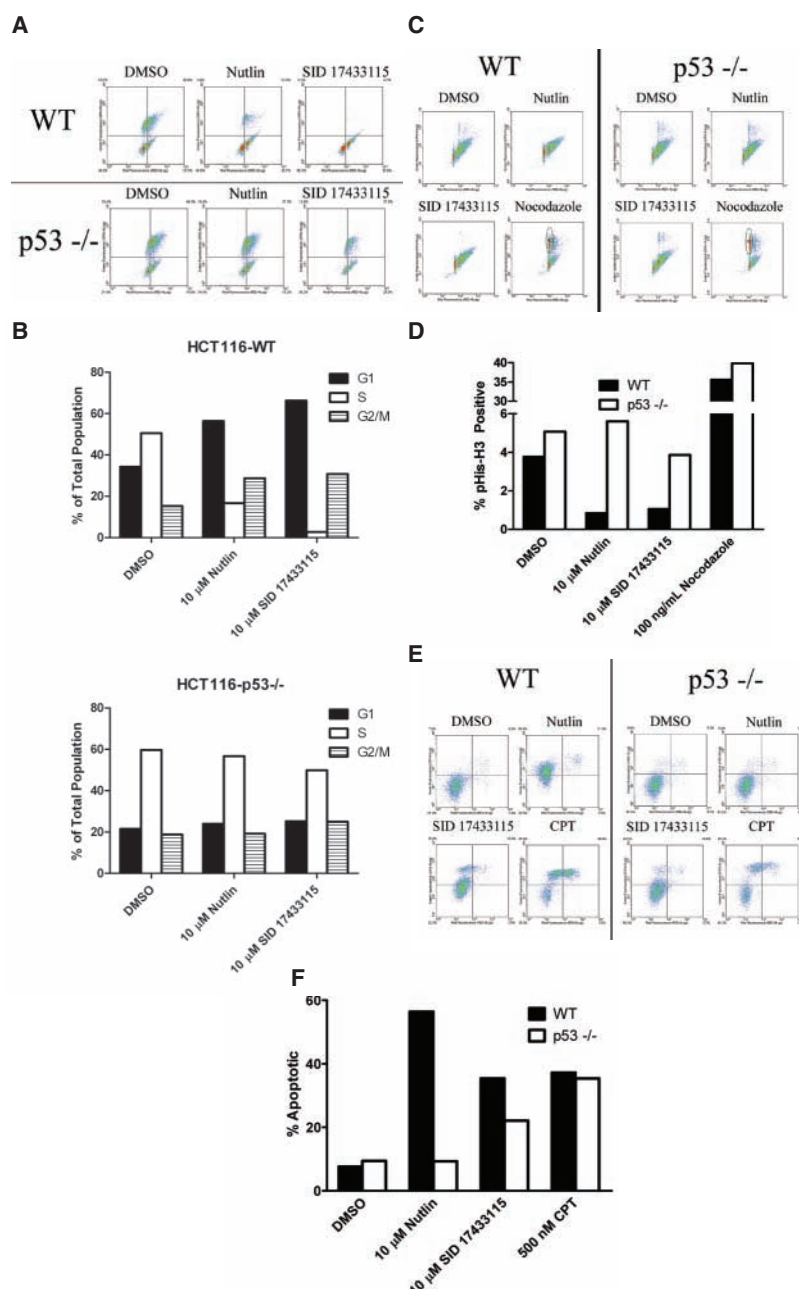


FIG. 6. Methylbenzo-naphthylidin-5-amines (MBNAs) arrest the cell cycle and induce apoptosis in p53-dependent and p53-independent manners. (A) HCT116-WT and HCT116-p53 null cells were treated with 0.1% DMSO, 10 μ M Nutlin-3 in 0.1% DMSO, or 10 μ M SID 17433115 in 0.1% DMSO for 24 h. Cells were treated with 10 μ M BrdU 30 min prior to trypsinization, suspension, fixation, and permeabilization. BrdU was detected by staining with anti-BrdU FITC-conjugated antibody, and DNA content was determined by 7-AAD staining. In total, 20,000 cells were analyzed by 2-color flow cytometry. (B) Quantification of fluorescence-activated cell sorting (FACS) data from A. The depletion of S phase cells by Nutlin-3 or SID 17433115 in wild-type (WT) cells (top panel, white bars) is absent in p53 null cells (bottom panel, white bars). (C) HCT116-WT and HCT116-p53 null cells were treated as in A with the addition of 100 ng/mL nocodazole in 0.1% DMSO. Adherent and suspended cells were collected, fixed, permeabilized, and antibody stained for pH3-H3. DNA content was measured by propidium iodide (PI) staining. In total, 20,000 cells were analyzed by 2-color flow cytometry. (D) Quantification of FACS data from C. The depletion of M phase cells by Nutlin-3 and SID 17433115 in HCT116-WT cells (black bars) is absent in HCT116-p53 null cells (white bars). (E) HCT116-WT and p53 null cells were treated with 0.1% DMSO, 10 μ M Nutlin-3 in 0.1% DMSO, 10 μ M SID 17433115 in 0.1% DMSO, or 500 μ M camptothecin (CPT) in 0.1% DMSO for 48 h. Adherent and suspended cells were collected and stained for apoptosis with Annexin V-FITC and counterstained with PI to determine plasma membrane integrity. Cells (20,000) were analyzed by 2-color flow cytometry. Apoptotic cells were those that were Annexin V-positive and PI-negative (upper left quadrant of each panel). (F) Quantification of data in E. Apoptosis in Nutlin-3 and SID 17433115-treated WT cells (black bars) is decreased in p53 null cells (white bars). Apoptosis for CPT-treated cells is unaffected by p53 status.

Treatment of the HCT116 WT cell line with the tetra-methylbenzonaphthylidene-5-amine SID 24790995 increased endogenous protein levels of p53, hDM2, and p21 relative to DMSO controls (**Fig. 4A-D**). For all 3 proteins, however, the increased expression was less than that observed with Nutlin-3 treatment.

In the p53 DNA damage response, serines 15 and 20 of p53 become phosphorylated upon DNA damage; this results in abrogation of hDM2 binding and stabilization of p53 protein levels. Lysates of cells treated with 50 μ M SID 17433115 were subjected to SDS-PAGE and transferred to nitrocellulose, and Western blots were probed for p53-phospho-ser15. Although p53 protein levels increased as expected with 50 μ M SID 17433115 treatment, the p53-pSer15 levels were significantly less than those observed with 100 nM camptothecin (CPT), a known inducer of the DNA damage response (**Fig. 4E**). A second protein-based indicator of an activated DNA damage response is the phosphorylation of serine 139 on histone H2A by the ATM kinase. Once again, Western blots demonstrated that treatment with 50 μ M SID 17433115 induced significantly less phosphorylation of ser139 on histone H2A than 100 nM CPT and only slightly more H2A-ser139 than was induced by Nutlin-3 treatment (**Fig. 4E**).

The inhibition of cell proliferation by methylbenzonaphthylidene-5-amines is not entirely p53-dependent. One of the hallmarks of compounds that promote p53 stabilization and activation is the ability to inhibit cell proliferation in a p53-dependent fashion. Isogenic HCT116-WT, HCT116-p53 null, and HCT116-p21 null cell lines were treated with a concentration series of SID 24790995, Nutlin-3, or paclitaxel, and the change in cell numbers was monitored over time (**Fig. 5A**). In media control wells, both HCT116-WT and HCT116-p53 null cell lines demonstrated equivalent increases in cell number with time in culture, whereas HCT116-p21 null cells proliferated at a slower rate (**Fig. 5A**). Both the HCT116-p53 null and HCT116 p21 null cell lines exhibited significantly higher resistance to growth inhibition by Nutlin-3 than the HCT116-WT control cell line. In HCT116-WT cells, Nutlin-3 had an IC_{50} of 3.7 μ M compared to extrapolated IC_{50} s of ~30 and 23.5 μ M for the HCT116-p53 null and HCT116-p21 null cell lines, respectively. However, all 3 cell lines were equally sensitive to growth inhibition by either SID 24790995 or paclitaxel treatment. The IC_{50} s for inhibition of proliferation in HCT116-WT, HCT116-p53 null, and HCT116-p21 null cell lines were 3.9, 5.3, and 5.1 μ M, respectively, for SID 24790995 and 3.1, 3.8, and 5.1 nM, respectively, for paclitaxel. Although the treatment of cells with MBNAs increased the levels of p53 and p53 target genes, the ability to inhibit cell proliferation may not entirely be dependent on p53.

Methylbenzonaphthylidene-5-amines induce a p53-dependent cell cycle arrest and apoptosis. Activation of p53 increases the transcription of target genes that promote cell cycle arrest and apoptosis. Cell cycle arrest facilitates DNA repair, and if the DNA

repair process is unsuccessful, proapoptotic genes initiate apoptosis to kill the cell. HCT116-WT and HCT116-p53 null cells were treated with 10 μ M SID 17433115 or Nutlin-3 for 24 h, S phase cells were stained with BrdU, and DNA content was measured by flow cytometry after 7-AAD staining (**Fig. 6A,B**). The flow cytometry analysis revealed that relative to DMSO controls, the percentage of the HCT116-WT population in S phase was significantly decreased in both SID 17433115 and Nutlin-3-treated cells. As the percentage of the HCT116-WT population in S phase declined with compound exposure, there was a corresponding increase in both the G1 and G2 DNA content populations, although the cell cycle arrest appears to be more pronounced at G1 (**Fig. 6A,B**). With the HCT116-p53 null cells, however, neither exposure to SID 17433115 nor Nutlin-3 significantly altered the cell cycle distribution relative to DMSO controls (**Fig. 6A,B**). To investigate potential effects on mitosis, we treated HCT116-WT and HCT116-p53 null cells with 10 μ M SID 17433115 or Nutlin-3 for 24 h and stained for DNA content and the mitotic marker phospho-histone-H3 (pH3; **Fig. 6C,D**). As anticipated, exposure of both cell lines to nocodazole produced a dramatic increase in the percentage of cells in mitosis (G2 DNA content and pH3 positive; **Fig. 6C,D**). The flow cytometry analysis indicated that the treatment of HCT116-WT cells with 10 μ M SID 17433115 or Nutlin-3 significantly depleted the percentage of mitotic cells relative to DMSO controls (**Fig. 6C,D**). Furthermore, this arrest was dose dependent for MBNA exposure (data not shown). In sharp contrast, exposure of the HCT116-p53 null cells to SID 17433115 or Nutlin-3 did not appear to significantly alter the percentage of mitotic cells relative to DMSO controls.

To explore potential effects on apoptosis, we treated HCT116-WT and HCT116-p53 null cells with 10 μ M SID 17433115 or Nutlin-3 for 48 h, stained them with Annexin V-FITC and PI, and analyzed them by 2-color flow cytometry (**Fig. 6E,F**). As expected, exposure of both cell lines to 500 nM CPT for 48 h produced a dramatic increase in the percentage of cells in apoptosis (**Fig. 6E,F**). Exposure of HCT116-WT cells to SID 17433115 and Nutlin-3 dramatically increased the percentage of apoptotic cells (Annexin V positive/PI negative), 37% and 58%, respectively, compared with DMSO controls (6%; **Fig. 6E,F**). In addition, initiation of apoptosis by SID 17433115 was dose dependent (data not shown). In HCT116-p53 null cells, SID 17433115 still elicited a significant increase in the percentage of apoptotic cells (22.9%), whereas exposure to Nutlin-3 did not (**Fig. 6E,F**). The MBNAs induced a p53-dependent cell cycle arrest similar to Nutlin-3 and also appeared to induce apoptosis in both a p53-dependent and p53-independent manner.

DISCUSSION

Although the enhancement or restoration of p53 function has been a major focus of drug discovery efforts to identify novel anticancer therapies, no modulator of p53 activity is currently approved for clinical use. A variety of screening campaigns have

been conducted to identify small molecules that disrupt the protein-protein interactions between p53 and hDM2, inhibit the ubiquitin E₃ ligase activity of hDM2, or restore the thermal stability and DNA binding activity of p53 DNA binding mutants.^{19,28,40-42} We report here the results of screening the NIH's small-molecule diversity library with a novel p53-hDM2 protein-protein interaction biosensor HCS assay to identify novel p53-hDM2 protein-protein interaction disruptor (PPID) chemotypes.

The N-terminal residues 1-131 of p53 encompass the binding site for hDM2 that overlaps with the p53 transactivating domain, and the p53-GFP interaction partner of the biosensor was targeted and anchored in the nucleolus by an NLS.^{17,24,25,34} The N-terminal residues 1-118 of hDM2 encompass the domain for binding to the N-terminal transactivating domain of p53, and the hDM2-RFP interaction partner of the biosensor includes both an NLS and an NES such that the relative distribution of this protein between the nucleus and cytoplasm can be used to measure the interactions between p53 and hDM2.^{17,24,25,34} In U-2 OS cells coinfecting with both of the PPIB adenovirus constructs, p53-GFP:hDM2-RFP complexes colocalized within the nucleolus, but upon exposure to compounds similar to Nutlin-3 (which disrupt the p53-hDM2 interaction), the hDM2-RFP partner translocated into the cytoplasm (**Fig. 1A**). The disruption of the p53-hDM2 interaction biosensor was measured in images acquired on the Array-Scan V^{TI} platform and analyzed with the molecular translocation algorithm to quantify the difference in the average fluorescence intensity of the hDM2-RFP shuttling component within the cytoplasm and nuclear compartments (**Fig. 1B**). The p53-hDM2 PPIB HCS assay performed robustly and reproducibly in HCS (**Fig. 1C,D**), and the 220,017-compound campaign was conducted in 13 independent 2-day operational runs over a 6-week period. The 698 384-well plates screened produced an average Z' factor of 0.64 ± 0.13 and an average S/B ratio of 31.0 ± 9.8 -fold. We employed plate-based Z-score statistical scoring methods to identify and eliminate compounds that were cytotoxic or that were fluorescent outliers in the intensity parameters in 1 or more of the 3 channels (**Fig. 2** and Suppl. Table S1). After confirmation in concentration-response assays, a single cluster of 3 structurally related MBNA hit compounds reproducibly disrupted the interaction between p53 and hDM2 in the PPIB HCS assay and produced IC₅₀s in the 30- to 50- μ M range (**Fig. 3**). A cross-target query of the PubChem database revealed that although the MBNA hits have been screened extensively in >210 bioassays, they have a remarkably narrow bioactivity profile (Suppl. Table S2).

Treatment of HCT116 WT cells with the MBNA hits increased endogenous protein levels of p53 and enhanced the expression of the p53 target genes hDM2 and p21 relative to DMSO controls (**Fig. 4A-D**). The MBNAs did not significantly increase the levels of either p53-phospho-ser15 or H2A-phospho-ser139 (**Fig. 4E**), indicating that these hit compounds do not appear to induce a DNA damage response. Treatment of HCT116 WT cells with the MBNAs significantly decreased the percentage of the population in S phase, with a corresponding increase in both the G1 and G2

DNA content populations, and significantly depleted the percentage of mitotic cells (G2 DNA content and pH3 positive) relative to DMSO controls (**Fig. 6C,D**). Overall, the MBNA-induced cell cycle arrest appears to be more pronounced at G1 (**Fig. 6**). Exposure of HCT116-WT cells to the MBNAs dramatically increased the percentage of apoptotic cells (Annexin V positive/PI negative) compared to DMSO controls (**Fig. 6E,F**) and inhibited the proliferation of HCT116 WT cells in a concentration-dependent manner with an IC₅₀ of 3.9 μ M (**Fig. 5**). In comparison the MBNAs, treatment of HCT116 cells with Nutlin-3 produced equivalent results, save that Nutlin-3 induced higher p53, hDM2, and p21 expression levels and induced more apoptosis.

In HCT116 cells that were null for p53, however, neither the MBNAs nor Nutlin-3 significantly altered the cell cycle distribution of cells with respect to DNA content, S phase, and mitosis (**Fig. 6**). These data suggest that the G1 cell cycle arrest induced by MBNAs is p53 dependent. However, exposure of HCT116-p53 null cells to MBNAs increased the percentage of apoptotic cells relative to both the DMSO controls and Nutlin-3-treated cells (**Fig. 6**), suggesting that the induction of apoptosis by MBNAs was not solely dependent on p53 activation. Consistent with these observations, the MBNAs exhibited equivalent IC₅₀s for the inhibition of cell proliferation in the HCT116-WT, HCT116-p53 null, and HCT116-p21 null isogenic cell lines (**Fig. 5C**). In contrast, HCT116-WT cells were significantly more sensitive to growth inhibition by Nutlin-3 than were the HCT116-p53 null and HCT116-p21 null cell lines (**Fig. 5B**).

The cis-imidazoline Nutlin-3 analogs are the prototype disruptors of p53-hDM2 protein-protein interactions that were identified using a combination of structure-based drug design and surface plasmon resonance technology (Biacore, Piscataway, NJ) to measure p53-hDM2 binding.^{7,21} Nutlin-3s inhibit the p53-MDM2 interaction by mimicking the binding of 3 critical p53 amino acid residues within the hydrophobic cavity of MDM2.^{7,21} Nutlin-3 treatment of human cancer cell lines with WT p53 stabilizes p53 protein levels, leading to activation of p53 target genes, cell cycle arrest, induction of apoptosis, and the inhibition of cell growth. Treatment of established SJSA-1 osteosarcoma xenografts with Nutlin-3 reduced tumor growth by 90% relative to vehicle controls.^{7,21} Mice treated with Nutlin-3 doses causing effective tumor inhibition and regression did not exhibit any significant weight loss or pathological changes.^{7,21} Nutlin-3 treatment of normal human or murine fibroblasts produced transient growth arrest but no apoptosis.^{7,21} Unfortunately, surface plasmon resonance technology has neither the throughput nor the capacity to screen compound libraries that contain more than 10 to 100 compounds. Although Nutlin-3 was ~30-fold more potent than the MBNAs in the p53-hDM2 PPIB, they exhibited equivalent IC₅₀s for the inhibition of cell proliferation in the HCT116-WT cell line (**Fig. 5**).

The MBNA compounds identified in this screen are structurally related to the styrylquinazoline CP-31398 and acridine orange compounds (**Fig. 3**) that have previously been shown to reactivate mutant p53 and to activate WT p53.^{19,39-41,43} CP-31398 was

identified in a temperature-sensitive monoclonal antibody epitope screen to find compounds that could promote the conformational stability of the WT p53 DNA binding domain.¹⁹ CP-31398 promoted the stability of WT p53, enabled mutant p53 to maintain an active conformation, and induced the accumulation of active p53 in cells with mutant p53, thereby allowing mutant p53 to activate transcription and to slow tumor growth in murine xenograft models.¹⁹ CP-31398 was shown to restore the DNA binding activity of mutant p53s in vitro and in treated cell lines.⁴¹ CP-31398 also significantly increased the protein levels and activity of p53 in human cell lines that are WT for p53, and treated cells undergo cell cycle arrest or apoptosis.⁴⁰ CP-31398 blocks the ubiquitination and degradation of p53 without disrupting the physical association between p53 and hDM2.⁴⁰ CP-31398 did not induce a DNA damage response, as indicated by the lack of significant phosphorylation of serines 15 and 20 on p53.⁴⁰ A series of acridine orange derivatives that are structurally related to CP-31398 were also shown to induce high levels of p53 protein and activate its transcriptional activity, indicated by enhanced levels of hDM2 and p21 in cell lines with WT p53.³⁹ The acridine orange derivatives stabilized p53 by blocking its ubiquitination by hDM2 without inducing phosphorylation on serines 15 and 20.³⁹ The acridine orange derivatives induced p53-dependent cell death (apoptosis) in cell lines WT for p53 and induced p53 transcriptional activity in mouse xenograft models.³⁹ Our data suggest that the MBNAs disrupt the interactions between p53 and hDM2 in the PPIB, stabilize and elevate the expression of p53, activate p53 transcriptional activity as indicated by the increased expression of hDM2 and p21 target genes, induce a G1 cell cycle arrest, enhance apoptosis, and inhibit tumor cell proliferation. MBNAs did not induce a significant DNA damage response, leading to increases in either p53-phospho-ser15 or H2A-phospho-ser139. Although the induction of the G1 cell cycle arrest by MBNAs was totally dependent on WT p53, the induction of apoptosis and inhibition of cell proliferation were at least partially independent of p53. We have not tested whether the MBNAs inhibit the hDM2-mediated ubiquitination of p53. The MBNAs are more closely related to the structures of the tricyclic acridine orange derivatives and CP-257042 than the bicyclic CP-31398 (**Fig. 3**). However, in the tricyclic acridine orange derivatives, only the central ring is a nitrogen hetero-cycle, whereas in the MBNA hits, 2 of the 3 rings are nitrogen hetero-cycles, and the central ring is amino-substituted at the 5 position (**Fig. 3**).

A different positional biosensor HCS assay has previously been used to screen a very small compound library of 3165 compounds for inhibitors of the p53-hDM2 protein-protein interactions.⁴² The assay employed a GFP-assisted readout (GRIP) p53-hDM2 redistribution format in which a cell line was constructed where hDM2 was expressed as a fusion protein with cAMP phosphodiesterase (PDE) isoform 4A4B, and p53 was expressed as a GFP fusion protein.⁴² Treatment of the cell line with the PDE-4A4B agonist RS25344 induces the hDM2-PDE-4A4B to relocate to compact intracellular foci. Through its interactions with hDM2-PDE-4A4B, the p53-GFP is recruited to the PDE-4A4B binding sites, and the p53-GFP redistributes from a diffuse cytoplasm staining pattern to a granular foci pattern.⁴² Compounds that disrupt p53-hDM2

interactions such as Nutlin-3 or that inhibit PDE-4A4B such as RP73401 disperse the RS25344-induced GFP foci phenotype. Using the PDE-4A4B inhibitor RP73401 (10 μ M) as a disruption control, 41 compounds (1.3%) exhibited >40% activity at 10 μ M, and only 30 of these were subsequently confirmed in concentration-response assays. Of the 30 confirmed actives, the activity of 10 compounds was deemed too low to be followed up on, 15 produced a PDE-4A4B inhibitor spot dispersion phenotype, and 14 of these were confirmed in a PDE-4A4B redistribution counterscreen.⁴² One of the 5 confirmed p53-hDM2 GRIP redistribution hits was Nutlin-3 spiked into the screening library, and only the Nutlin-3 “hit” was shown to activate a p53-luciferase reporter secondary assay and to differentially inhibit the proliferation of tumor cell lines with WT p53. It was concluded that none of the screening hits from this very limited screen were functional inhibitors of the p53-hDM2 protein-protein interaction.⁴²

In conclusion, we have screened 220,017 compounds from the NIH’s small-molecule library in this novel p53-hDM2 PPIB assay. The p53-hDM2 PPIB assay performed robustly and reproducibly throughout the 6-week HCS campaign, and our statistical analysis of the high-content parameters extracted from the digital images of the 3 fluorescent channels enabled us to identify and eliminate compounds that were cytotoxic or fluorescent artifacts either in solution or within the cells. We identified 3 structurally related MBNA hit compounds with IC_{50} s between 30 and 50 μ M in the p53-hDM2 PPIB biosensor. In HCT116 cells with WT p53, the MBNAs enhanced p53 protein levels, increased the expression of p53 target genes, caused a cell cycle arrest in G1, induced apoptosis, and inhibited cell proliferation with an IC_{50} ~4 μ M. Nutlin-3 was ~30-fold more potent in the p53-hDM2 PPIB assay, and treatment of HCT116 cells with WT p53 produced equivalent biological results, save that Nutlin-3 induced more p53, hDM2, and p21 protein expression and induced more apoptosis. However, unlike the Nutlin-3s, which are dependent on WT p53 for their activity, MBNAs also increased the percentage of apoptosis in p53 null cells and also produced IC_{50} s ~4 μ M for growth inhibition in isogenic cell lines null for p53 or p21. The MBNA compounds share similar biological activities and are more structurally related to acridine orange compounds that reactivate mutant p53 and activate WT p53 by inhibiting the ubiquitination of p53 by hDM2. However, the MBNA structures differ from the acridine orange derivatives in that 2 of the 3 rings are nitrogen hetero-cycles, and the central ring is amino-substituted at the 5 position. In recent years, advances in structure-based drug design and the development of an impressive variety of high-throughput screening assay formats have yielded an expanding list of protein-protein interaction inhibitors. Nevertheless, the perception persists that protein interaction interfaces are flat, occur over very large surfaces, and present challenging targets for drug-like small molecules. Despite the relatively modest size of the screening library, the combination of a novel p53-hDM2 PPIB assay together with an automated imaging HCS platform and image analysis methods enabled us to discover a novel chemotype series that disrupts p53-hDM2 interactions in cells.

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