CANCER DISCOVERY

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RESEARCH ARTICLE

Discovery of Mdm2-MdmX E3 Ligase Inhibitors Using a Cell-Based Ubiquitination Assay

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ABSTRACT

E3 ubiquitin ligases are of interest as drug targets for their ability to regulate protein stability and function. The oncogene Mdm2 is an attractive E3 ligase to target, as it is the key negative regulator of the tumor suppressor p53, which controls the transcription of genes involved in cell fate. Overexpression of Mdm2 facilitates tumorigenesis by inactivating p53, and through p53-independent oncogenic effects. We developed a high-throughput cellular Mdm2 auto-ubiquitination assay, which we used to discover a class of small-molecule Mdm2 ligase activity inhibitors. These compounds inhibit Mdm2 and p53 ubiquitination in cells, reduce viability of cells with wild-type p53, and synergize with DNA-damaging agents to cause cell death. We determined that these compounds effectively inhibit the E3 ligase activity of the Mdm2-MdmX hetero-complex. This mechanism may be exploitable to create a new class of anti-tumor agents.

SIGNIFICANCE: We identified a class of small-molecule inhibitors of the Mdm2-MdmX hetero-complex E3 ligase activity through a high-throughput cell-based Mdm2 ubiquitination screen. This is a new target for small-molecule therapeutics and may be developed to treat specific cancers.
Cancer Discovery; 1(4); OF1–OF14.

INTRODUCTION

The murine double minute 2 protein (Mdm2, also used to denote the human protein) is a RING-domain-containing E3 ubiquitin ligase of paramount importance in cancer biology because of its ability to regulate the p53 tumor suppressor protein. The p53 protein is a transcription factor that controls the activation of multiple genes involved in apoptosis and growth arrest following a wide range of cellular stresses (1). The importance of the Mdm2-p53 pathway in tumor progression is shown by the fact that approximately 50% of tumors have a mutation in the *TP53* gene (2), and many other tumors have a deregulated p53 pathway (3).

In unstressed cells, p53 levels are kept low and it is held in a latent state by its negative regulator Mdm2, via two primary mechanisms. First, Mdm2 causes rapid degradation of p53 through ubiquitination and proteasomal degradation (4–6); second, Mdm2 binds the N-terminal transactivation domain of p53, preventing transcriptional activation of p53 target genes (7). Upon stress signals, such as hypoxia, DNA damage, or expression of oncogenes, post-translational modifications on both p53 and Mdm2 inhibit their interaction, stabilizing p53 and activating p53-mediated transcription (8).

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://www.cancerdiscovery.aacrjournals.org).

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Mdm2 also has auto-ubiquitination activity, whereby it can regulate itself via protein degradation (9). In addition, a transcriptional target of p53 is the *Mdm2* gene, creating an autoregulatory negative feedback loop between Mdm2 and p53 (10). Once a stress response is removed or resolved, p53 returns to basal levels due to Mdm2-mediated degradation and inhibition. Although there are other E3 ligases capable of targeting p53 (3), Mdm2 is of primary importance in p53 regulation. This relationship is illustrated by the rescue of embryonic lethality of *Mdm2*$^{-/-}$ mice by the concomitant deletion of *p53* (11, 12).

The Mdm2 homolog MdmX (also known as Mdm4 and used to denote both the human and mouse forms of the protein) is a nonredundant and essential p53 regulator (13–15). Similarly to Mdm2, MdmX is overexpressed in human tumors generally distinct from those containing p53 mutations (3). Evidence suggests that Mdm2 and MdmX function together to inhibit p53 activity. MdmX can interact with p53 and inhibit its transactivation ability (16). Although MdmX has no intrinsic E3 ligase activity on its own, Mdm2 and MdmX can form hetero-oligomers through their RING domains (17), whereby MdmX can increase Mdm2 E3 ligase activity (18, 19). Mdm2 can also directly ubiquitinate and degrade MdmX upon DNA-damage stimuli (20–22).

Mdm2 may be a promising target for therapeutics. Small molecule inhibitors of the Mdm2-p53 protein-protein interaction, such as Nutlin-3, and inhibitors of Mdm2 E3 ligase activity, such as the HLI series of compounds, have been identified (23, 24). These compounds revealed that inhibiting Mdm2 has therapeutic potential by reactivating p53 *in vitro* and in cell-based assays. However, Nutlin-3 does not inhibit Mdm2 E3 ligase activity and so does not block p53-independent functions of Mdm2; the HLI compounds have nonspecific effects at higher concentrations. For these reasons, there is a need for more specific and novel inhibitors of Mdm2 E3 ligase activity.

Here, we used a novel high-throughput cell-based auto-ubiquitination assay to identify inhibitors of Mdm2 E3 ligase activity. This is the first cell-based screen used to identify inhibitors of the E3 ligase activity of Mdm2 and can be readily adapted in order to identify inhibitors of other E3

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ligases. This screen yielded two related compounds, which we named Mdm2 E3 Ligase Inhibitors 23 and 24 (MEL23 and MEL24). Treatment of multiple cell lines with MEL23 and MEL24 inhibited Mdm2 and p53 ubiquitin conjugates and increased the stability of Mdm2 and p53. MEL activity was shown to be dependent upon Mdm2–MdmX in *in vitro* and in cell-based studies. MEL compounds decreased cell survival in a p53-dependent manner and increased sensitivity to DNA-damaging agents, and therefore represent a useful class of Mdm2-targeted small molecule inhibitors.

RESULTS

High-Throughput Screening Using a Cell-Based Mdm2 Stability Assay

To identify Mdm2 E3 ligase inhibitors, we designed a high-throughput cellular assay to measure changes in Mdm2 auto-ubiquitination and degradation. We took advantage of the fact that Mdm2 can regulate itself in cells through ubiquitination and degradation (9). In this assay, we used 2 luciferase fusion proteins: a wild-type Mdm2-luciferase fusion protein, which can auto-ubiquitinate and thereby target itself for degradation, and a mutant Mdm2(C464A)-luciferase fusion protein. Both fusion proteins were constructed with luciferase N-terminal to Mdm2. The C464A mutation disrupts a metal-binding site in the RING domain, thereby inhibiting Mdm2 E3 ligase activity (9). Small molecules that increase the luminescence of the Mdm2(wt)-luciferase fusion protein without increasing the luminescence of the Mdm2(C464A)-luciferase fusion protein likely inhibit Mdm2 E3 ligase activity or proteasomal degradation of Mdm2. Alternatively, small molecules that increase the luminescence of both fusion proteins are likely affecting transcription, translation, or have a general cellular impact that is not dependent on the E3 ligase activity of Mdm2 or on proteasomal degradation.

We first compared the expression levels of the two fusion proteins in 293T cells. Consistent with auto-ubiquitination, Mdm2(wt)-luciferase protein is expressed at lower levels than the mutant Mdm2(C464A)-luciferase protein (Fig. 1A). To confirm that the difference in luminescence levels between the two constructs was the result of a difference in degradation rates, we made 293T cell lines stably expressing both constructs and treated them with a proteasome inhibitor, MG132 (Z-Leu-Leu-Leu-aldehyde). In multiple clonal cell lines, proteasome inhibition increased the levels of the Mdm2(wt)-luciferase protein, while Mdm2(C646A)-luciferase levels remained unaffected (Fig. 1B; and Supplementary Fig. S1). This is consistent with the wild-type protein being degraded through a ubiquitin-dependent mechanism. Treatment with cycloheximide selectively decreased the luminescence of the Mdm2(wt)-luciferase

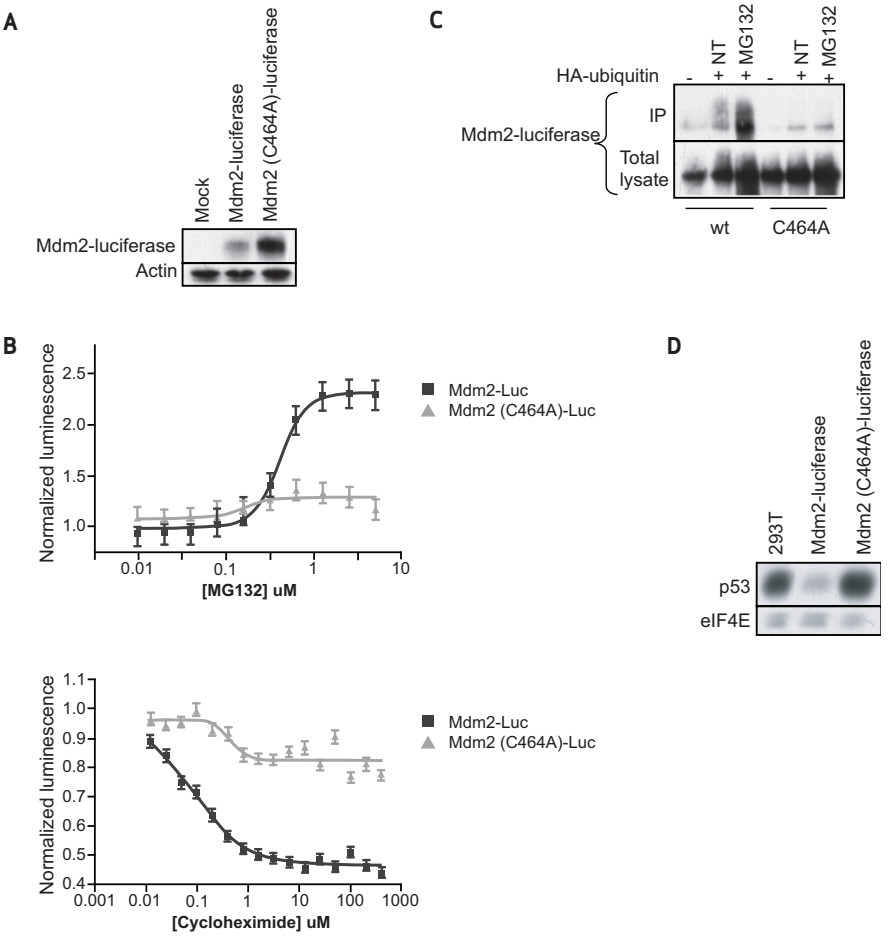

Figure 1. Validation of cell-based luminescence assay for Mdm2 E3 ligase activity. **A**, Mdm2(C464A)-luciferase expression levels are higher than Mdm2(wt)-luciferase expression levels. 293T cells were transfected with Mdm2(wt)-luciferase, Mdm2(C464A)-luciferase, or a mock transfection. Cells were lysed 48 hours later and Mdm2 levels were analyzed by Western blotting with anti-Mdm2 and anti-actin antibodies. **B**, Mdm2(wt)-luciferase protein is degraded by the proteasome. Stable cell lines were seeded in 384-well plates and 24 hours later treated with MG132 for 2 hours or cycloheximide for 30 minutes at the indicated concentrations. Cells were lysed with a luminescence buffer and analyzed on a Victor3 Plate Reader. Each graph represents the median of 10 replicates normalized to a no-treatment control; one standard deviation is shown. **C**, Mdm2(wt)-luciferase, but not Mdm2(C464A)-luciferase, is ubiquitinated. Stable cell lines were transfected with HA-ubiquitin (5 μg) or a mock transfection. Forty-eight hours following transfection, cells were treated with 10 μM MG132 for 3 hours. Cells were lysed and immunoprecipitated with an anti-HA affinity matrix. Total lysates and immunoprecipitated samples were analyzed by Western blotting using an anti-Mdm2 antibody. **D**, levels of p53 protein are lower in Mdm2(wt)-luciferase cells than in Mdm2(C464A)-luciferase cells. 293T and stable cell lines were lysed and equal amounts of cell extracts were analyzed by Western blotting with anti-p53 and anti-eIF4E antibodies. NT, no-treatment control.

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protein, suggesting that the observed decrease in stability was due to a post-translational mechanism (Fig. 1B).

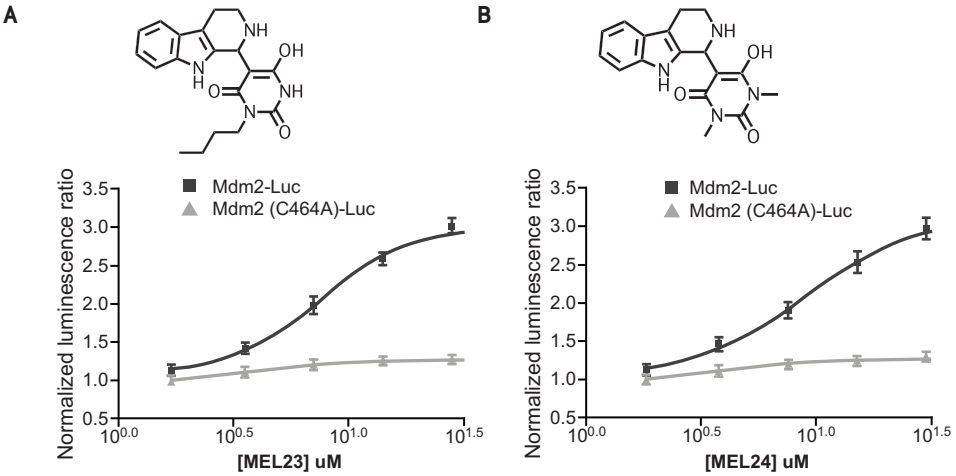
To examine the ubiquitin ligase activity of the Mdm2-luciferase constructs, wild-type and mutant Mdm2-luciferase cell lines were transfected with HA-ubiquitin and cell lysates were immunoprecipitated with an anti-HA antibody. The Mdm2(wt)-luciferase protein was immunoprecipitated with the anti-HA affinity matrix, indicating that it is ubiquitinated in cells, whereas the Mdm2(C464A)-luciferase protein was immunoprecipitated to a significantly lesser extent (Fig. 1C). Furthermore, the Mdm2(wt)-luciferase cell line had overall lower endogenous p53 levels, compared to the Mdm2(C464A)-luciferase cell line, suggesting that the Mdm2(wt)-luciferase fusion protein was able to regulate endogenous p53 (Fig. 1D). Together, these results confirmed that Mdm2(wt)-luciferase is rapidly degraded after auto-ubiquitination through the proteasome, and its stability is regulated by an intact RING domain.

Screening and Identification of MEL23 and MEL24

The above experiments verified that the cell lines we engineered could function as a valid system to screen for Mdm2 E3 ligase inhibitors. We proceeded to screen 270,080 compounds; 51,356 were screened at 5.33 μg/mL in 384-well format and 218,724 were screened at 5 μM in 1536-well format [Molecular Libraries Screening Center Network (MLSCN) library, Pubchem BioAssay ID 1442, 1230, 1444, and 1394]. All compounds were incubated with the Mdm2(wt)-luciferase cell line for 2 hours, after which relative luminescence was detected by the addition of a luminescence buffer. The time point of 2 hours was chosen because it is possible to see increases in the Mdm2 protein level due to its short half life of approximately 20 minutes (25), and it minimizes secondary effects that affect luminescence levels. Compounds that increased luminescence in the primary screen were tested in a counter screen in the mutant Mdm2(C464A)-luciferase cell line to eliminate compounds with effects not dependent on Mdm2 E3 ligase activity or proteasomal degradation.

We identified 57 selective compounds. A series of secondary assays was performed to validate these potential hits. First, these compounds were tested in a dilution series in both luciferase cell lines. Then, the effects of the compounds on endogenous p53 and Mdm2 levels, and on Mdm2 ubiquitination activity, were tested in multiple cell lines. Two structurally similar compounds, MEL23 and MEL24, were validated to be effective Mdm2 inhibitors in these assays and further studied.

MEL23 and MEL24 increased the luminescence of Mdm2(wt)-luciferase cells to a similar level as MG132 treatment, and did not significantly affect the luminescence of the control Mdm2(C464A)-luciferase cell line (Fig. 2A and B). The EC_{50}



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Compound	R_1	R_2	R_3	R_4	Activity at 15 μM
MEL23	$-CH_3(CH_2)_3$	H	H	H	*
MEL24	$-CH_3$	$-CH_3$	H	H	**
1	-t(bu)	H	H	H	*
2	$-CH_2CH{=}CH_2$	H	H	H	**
3	$-CH_2CH_2OCH_3$	H	H	H	**
4	-Ph	-Ph	H	H	*
5	H	-Bz	H	H	*
6	H	-Sec-butyl	H	H	**
7	$-CH_2CH_2{=}CH_2$	H	$-OCH_3$	H	***
8	$-p\text{-}PhOCH_3$	H	H	H	-
9	$-CH_3$	$-CH_3$	-Cl	H	-
10	$-CH_3$	-Sec-butyl	H	$-CH_3$	-
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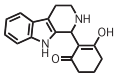
12					
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Figure 2. Structure and activity of MEL23, MEL24, and MEL analogs. **A** and **B,** structures of MEL23 and MEL24 and their activities in the cell-based auto-ubiquitination assay. Cells were seeded in 384-well plates and treated with MEL23 or MEL24 for 2 hours at the indicated concentrations. Cells were lysed with a luminescence buffer and analyzed on a Victor3 Plate Reader. Each graph represents the median of 5 replicates normalized to a no-treatment control; one standard deviation is shown. The EC_{50} for MEL23 and MEL24 are 2.7 μg/mL and 3.0 μg/mL (7.5 μM and 9.2 μM), respectively. The graphs and EC_{50} values were generated using GraphPad Prism. **C**, structure activity relationship of MEL23 and MEL24. MEL analogs were tested in the cell-based luminescence assay for activity (ranked from ***, most active to –, no activity).

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values of MEL23 and MEL24 were determined to be 2.7 μg/mL and 3.0 μg/mL (7.5 μM and 9.2 μM), respectively.

The MEL compounds are composed of tetrahydro-β-carboline and barbituric acid moieties with slightly different substituents (Fig. 2A and B). Testing a series of analogs revealed that both the tetrahydro-β-carboline and barbituric acid moieties are necessary for activity. Structure activity relationship (SAR) studies identified substituents that could increase, decrease, or eliminate the activity (Fig. 2C; Supplementary Fig. S2).

MEL23 and MEL24 Increase Levels of Mdm2, p53, and MdmX and Activate p53

An Mdm2 E3 ligase inhibitor should prevent the degradation of Mdm2 substrates, including Mdm2, p53, and MdmX. Accordingly, Mdm2 and p53 levels increased in 3 wild-type p53 cell lines (U2OS, RKO, HCT116), following incubation with 5 μg/mL MEL23 or MEL24 for 6 hours, to similar levels as with MG132 treatment (Fig. 3A; Supplementary Figs. S3 and S4). This increase in Mdm2 and p53 levels occurred in a concentration-dependent manner (Fig. 3B). Additionally, we were able to show that p53 mRNA levels were not affected by MEL compound treatment (Supplementary Fig. S5). Furthermore, we were able to show that the MEL compounds are specific for the p53 substrate as they did not affect the levels of c-Myc or c-Jun, two short-lived proteins involved in cell survival (Supplementary Fig. S6).

MdmX is stable in unstressed cells, but upon DNA-damage, MdmX is rapidly degraded by Mdm2 E3 ligase activity (26). Accordingly, when MCF7 cells were treated with etoposide (30 μg/mL), MdmX levels decreased substantially. This degradation could be prevented when cells were co-treated with 5 μg/mL MEL23 or MEL24 for 16 hours or 50 μM ALLN (N-Acetyl-Leu-Leu-Nle-CHO), a proteasome inhibitor (Fig. 3C). Thus, MEL compounds inhibit endogenous MdmX degradation.

We wanted to determine if the increases in p53 protein levels led to a corresponding increase in the transcription of p53 target genes. Therefore, several p53 target genes were analyzed by quantitative real-time PCR (qRT-PCR). In RKO cells treated with 5 μg/mL MEL23, mRNA levels of *Mdm2, p21, Bax,* and *puma,* all established p53 target genes (27), increased within 48 hours of treatment (Fig. 3D). This increase in transcription was less than observed with doxorubicin treatment, a DNA-damaging and p53-activating agent, but more than with ALLN, a proteasome inhibitor (Supplementary Fig. S7). Therefore, MEL compounds activate transcription of at least a set of p53 target genes by increasing p53 protein levels.

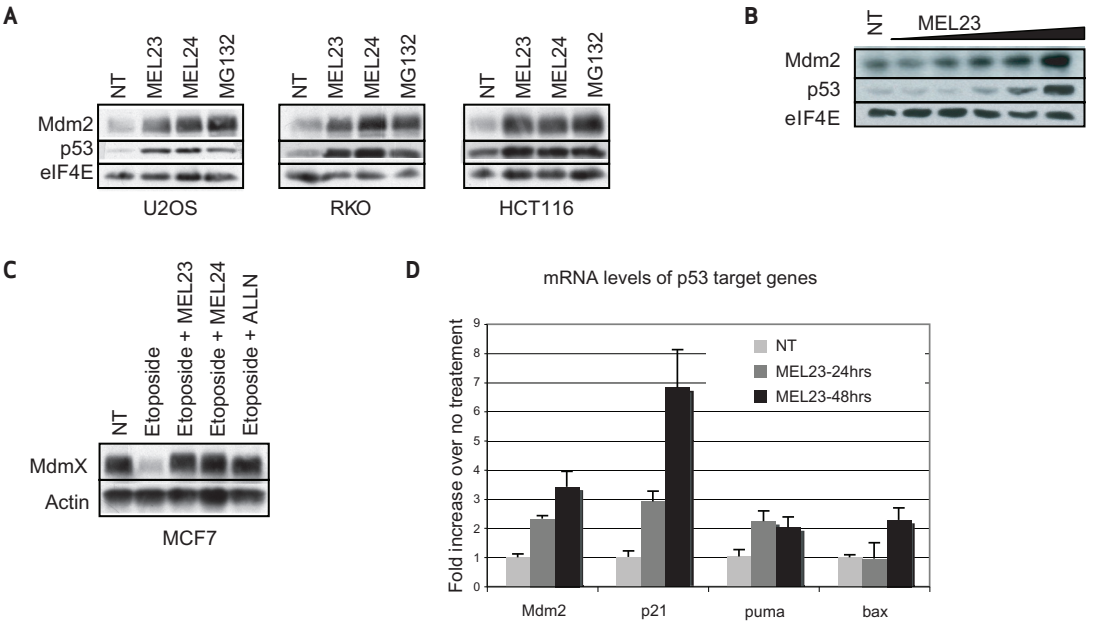

Figure 3. Effects of MEL23 and MEL24 on Mdm2, p53, and MdmX protein levels and on p53 target genes. **A**, Mdm2 and p53 protein levels are increased with MEL compound treatment. U2OS, RKO, and HCT116 cells (p53 wild-type) were incubated with 5 μg/mL (14 μM) MEL23, 5 μg/mL (15 μM) MEL24, or 10 μM MG132 for 6 hours. Cells were lysed and analyzed by Western blotting using anti-Mdm2, anti-p53, and anti-eIF4E antibodies. **B**, Mdm2 and p53 protein levels increase with MEL treatment in a concentration-dependent manner. RKO cells were incubated with decreasing concentrations of MEL23, starting from 10 μg/mL (28 μM) in a 2-fold, 5-point dilution series for 6 hours. Cells were lysed and analyzed by Western blotting using anti-Mdm2, anti-p53, and anti-eIF4E antibodies. **C**, MEL compounds inhibit etoposide-induced MdmX degradation. MCF7 cells (p53 wild-type) were incubated with 30 μg/mL (51 μM) etoposide alone or in combination with 50 μM ALLN, 5 μg/mL (14 μM) MEL23, or 5 μg/mL (15 μM) MEL24 for 16 hours. Cells were lysed and analyzed by Western blotting with anti-MdmX and anti-actin antibodies. **D**, MEL23 increases the transcription of p53 target genes, *Mdm2, p21, puma,* and *bax*. RKO cells were treated with 5 μg/mL (14 μM) MEL23 for 24 or 48 hours. qRT-PCR was used to analyze the mRNA levels of the *Mdm2, p21, puma,* and *bax* transcripts. All values are normalized to no-treatment, DMSO control. The figure is representative of three independent experiments and standard error bars represent intra-experimental error. NT, no-treatment control.

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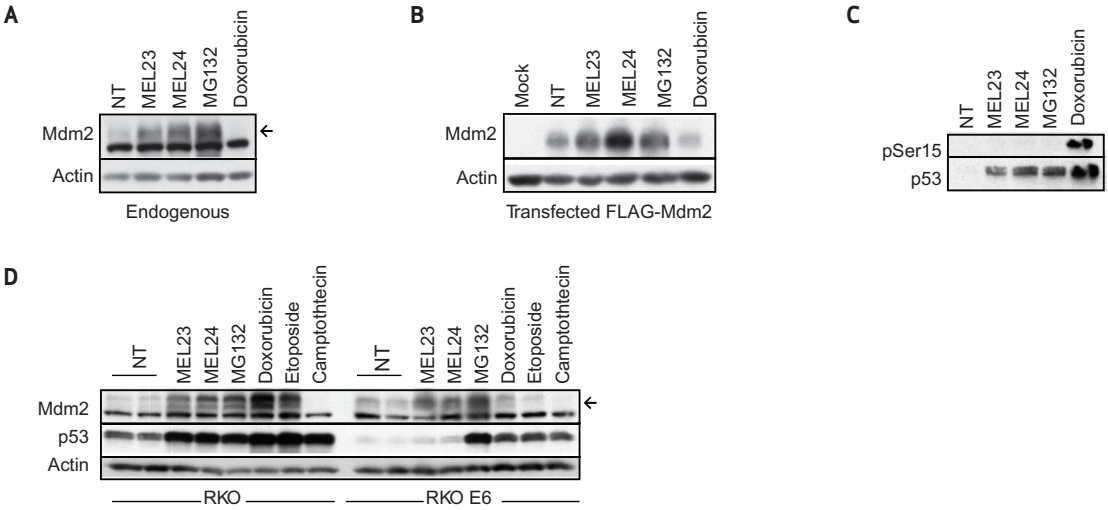

Figure 4. MEL23 and MEL24 are specific toward Mdm2 and cause Mdm2 accumulation independent of p53 transcription. **A** and **B**, MEL23 and MEL24 stabilize Mdm2 protein in p53-null cells. H1299 cells were transfected with Flag-Mdm2 (5 μg) or left untransfected. Cells were treated with 5 μg/mL (14 μM) MEL23, 5 μg/mL (15 μM) MEL24, 10 μM MG132, or 1 μg/mL (1.7 μM) doxorubicin for 6 hours. Cells were lysed and analyzed by Western blotting using anti-Mdm2 and anti-actin antibodies. The upper band indicated with an arrow is the Mdm2 protein. **C**, MEL23 and MEL24 do not induce p53-ser15 phosphorylation. RKO cells were treated with 5 μg/mL (14 μM) MEL23, 5 μg/mL (15 μM) MEL24, 10 μM MG132, or 0.5 μg/mL (0.9 μM) doxorubicin for 6 hours. Cells were lysed and analyzed by Western blotting using anti-p53 and anti-phospho-ser15 p53 antibodies. **D**, MEL23 and MEL24 increase p53 levels in RKO cells, but not in RKO-E6 cells. RKO and RKO-E6 cells were treated with 5 μg/mL (14 μM) MEL23, 5 μg/mL (15 μM) MEL24, 10 μM MG132, 0.5 μg/mL (0.9 μM) doxorubicin, 0.5 μg/mL (1.4 μM) camptothecin, or 25 μg/mL (42 μM) etoposide for 6 hours. Cells were lysed and analyzed by Western blotting using anti-Mdm2, anti-p53, and anti-actin antibodies. The upper band indicated with an arrow is the Mdm2 protein. NT, no-treatment control.

Activity of MEL23 and MEL24 Is Mdm2-Specific and Independent of p53 Transcription

Many types of compounds lead to increases in both Mdm2 and p53 protein levels by inducing a DNA-damage response. A series of experiments was performed to exclude the possibility that the MEL compounds were inducing p53-mediated stress, thereby leading to the accumulation of p53 and Mdm2. First, p53-null H1299 cells were treated with 5 μg/mL MEL23 or MEL24 for 6 hours. The levels of both endogenous Mdm2 protein and ectopically expressed Mdm2 increased after treatment, whereas no effect on Mdm2 levels was observed following doxorubicin treatment (Fig. 4A and 4B). This indicates that the MEL compounds stabilize Mdm2 in a p53-independent manner. Further verification that MEL23 and MEL24 are not genotoxic and are not inducing a p53-stabilizing stress response was revealed by the lack of detectable phosphorylation of p53 at serine-15 (Fig. 4C) or phosphorylation of histone H2A.X at serine-139 (Supplementary Fig. S8) with MEL23 or MEL24 (5 μg/mL); these phosphorylation events were strongly induced by doxorubicin-mediated DNA damage.

Next, we tested the specificity of MEL23 and MEL24 for Mdm2 in two isogenic cell lines, RKO and RKO-E6. RKO and RKO-E6 cells have wild-type p53 and Mdm2, but RKO-E6 cells express the HPV-E6 protein. The HPV-E6 protein forms an active E3 ligase in association with E6AP, a HECT domain E3 ligase, and targets p53 for degradation (28). As a result, p53 levels are significantly reduced in RKO-E6 cells compared to RKO cells (Fig. 4D, NT lanes). A compound that specifically inhibits Mdm2 E3 ligase activity should increase p53 levels in RKO cells, but not RKO-E6 cells. Consistently, treatment with 5 μg/mL MEL23 or MEL24 for 6 hours increased the levels of p53 in RKO cells, but not RKO-E6 cells, whereas treatments with MG132, doxorubicin, etoposide, and camptothecin increased p53 in both cell lines through proteasome inhibition or through the activation of a DNA-damage response (Fig. 4D). These data demonstrate that MEL23 and MEL24 do not inhibit the proteasome, do not inhibit E6AP activity, and do not induce a DNA-damage response, suggesting that MEL23 and MEL24 stabilize Mdm2 via a mechanism independent of p53 transcription.

Some compounds can react nonspecifically with proteins, DNA and glutathione, leading to off-target affects (29). In order to determine if MEL compounds generate a Michael acceptor (an electophile) *in situ* and react nonspecifically, we conducted experiments to determine if a MEL-glutathione adduct would form. MEL24 did not form an adduct with glutathione as determined by mass spectrometry, whereas a known Michael acceptor, phenylisothiocyanate, formed a glutathione adduct under the same reaction conditions (Supplementary Fig. S9).

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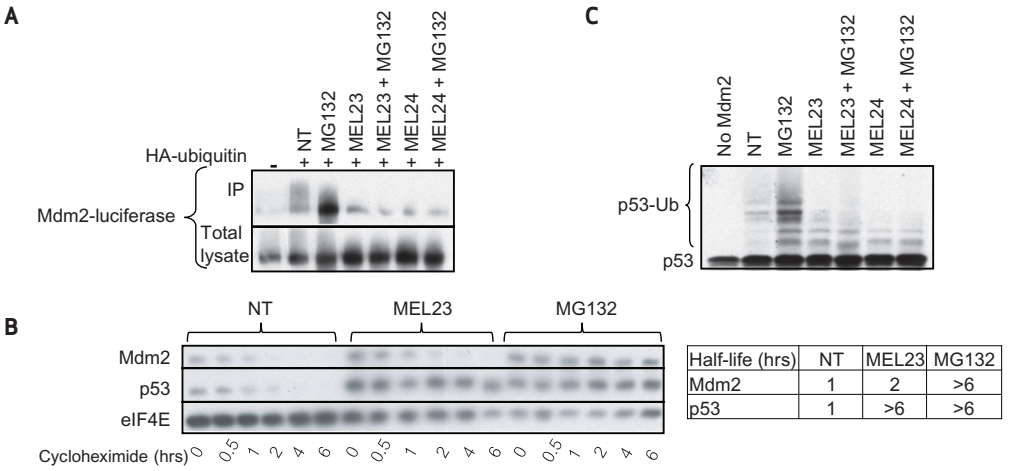
MEL23 and MEL24 Inhibit Mdm2-MdmX E3 Ligase Activity

To determine whether the MEL compounds inhibit Mdm2 and p53 ubiquitination in cells, we analyzed Mdm2 and p53 ubiquitin conjugates in cell-based ubiquitination assays. The Mdm2(wt)-luciferase cell line was transfected with HA-ubiquitin and pretreated with 10 μg/mL MEL23 or MEL24, followed by 10 μM MG132. An increased concentration of the compounds, 10 μg/mL, was used to facilitate complete inhibition of ubiquitination. Cell lysates were immunoprecipitated with an anti-HA antibody and analyzed by Western blotting. In cells treated with MEL23 or MEL24, significantly fewer Mdm2-ubiquitin conjugates were immunoprecipitated, indicating that the MEL compounds inhibit ubiquitination of Mdm2 (Fig. 5A). The inhibition of Mdm2 ubiquitination led to a stabilization of Mdm2 protein, as indicated by its increased half-life from 1 hour to 2 hours with 5 μg/mL MEL23 treatment (Fig. 5B).

To test the effects of the MEL compounds on p53 ubiquitination, H1299 cells were transfected with plasmids expressing p53, Mdm2, and ubiquitin and pre-treated with 10 μg/mL MEL23 or MEL24, followed by treatment with 10 μM MG132. Ubiquitination of p53 was analyzed by Western blotting. Cells treated with MG132 showed a significant increase in p53 ubiquitination that was eliminated by pre-treatment with MEL23 or MEL24 (Fig. 5C). Additionally, treatment with MEL23 or MEL24 alone demonstrated that the compounds stabilized p53 without ubiquitination. In fact, the half-life of p53 increased from 1 hour in this cell line to greater than 6 hours with 5 μg/mL MEL23 treatment (Fig. 5B). Thus, treatment with MEL23 and MEL24 inhibits ubiquitin conjugation to Mdm2 and p53 thus stabilizing these proteins.

We then tested the specificity of the MEL compounds in *in vitro* ubiquitination reactions, which included purified E1, UbcH5C, ^{32}P-labeled ubiquitin, and ATP and determined the extent of ubiquitination by autoradiography. The addition of increasing amounts of MEL23 to ubiquitination reaction mixtures containing full-length flag-Mdm2 had only modest inhibitory effect on the Mdm2 ligase activity (Fig. 6A). Because Mdm2 is able to make a functional complex with MdmX, and this complex is reported to have different activity and specificity in cells, we tested the effect of MEL23 on the purified Flag-Mdm2-HA-MdmX hetero-complex (purification method outlined in Supplementary Fig. S10). Indeed, MEL23 markedly decreased the ligase activity of the Mdm2-MdmX complex (Fig. 6A). Of note, Mdm2 is less active than the Mdm2-MdmX complex as an E3 ligase, but the inhibitory effects of MEL23 are still not seen with Mdm2 when equally active amounts of protein are used (Supplementary Fig. S11, 50 ng Mdm2/X vs. 100 ng Mdm2).

Because Mdm2 and MdmX interact through their RING domains (17), we hypothesized that the target of the MEL



Half-life (hrs)	NT	MEL23	MG132
Mdm2	1	2	>6
p53	1	>6	>6

Figure 5. MEL23 and MEL24 inhibit Mdm2 and p53 ubiquitination in cells. **A**, MEL compounds inhibit Mdm2 ubiquitination in cells. Mdm2(wt)-luciferase cells were transfected with HA-ubiquitin (5 μg) and treated 8 hours later with 10 μg/mL (28 μM) MEL23, 10 μg/mL (31 μM) MEL24, or a DMSO control. After 16 hours, cells were additionally treated with 10 μM MG132 or a DMSO control. Three hours later cells were lysed and immunoprecipitated for 3 hours with an anti-HA affinity matrix. Total lysates and immunoprecipitated samples were analyzed by Western blotting using an anti-Mdm2 antibody. **B**, MEL23 increases the half-life of p53 and Mdm2 proteins. RKO cells were treated with 5 μg/mL (14 μM) MEL23, 10 μM MG132, or NT control for 4 hours, followed by treatment with 5 μg/mL (18 μM) cycloheximide for 30 minutes or 1, 2, 4, or 6 hours. Cell lysates were analyzed by Western blotting with anti-Mdm2, anti-p53 and anti-eIF4E antibodies. Protein levels were measured by densitometry using ImageJ and normalized to eIF4E. Half-life was calculated by plotting the protein levels in the treated cells to the protein level in the cells not treated with cycloheximide. **C**, MEL compounds inhibit p53 ubiquitination in cells. H1299 cells were transfected with Flag-Mdm2 (5 μg), p53 (0.5 μg), and HA-ubiquitin (2 μg). After 8 hours, cells were treated with 10 μg/mL (28 μM) MEL23, 10 μg/mL (31 μM) MEL24, or a DMSO control. After 16 hours cells were additionally treated with 10 μM MG132 or a DMSO control. Three hours later cells were lysed and analyzed by Western blotting using an anti-p53 antibody. Brackets indicate the slower migrating species of the protein that are the ubiquitin-conjugated forms. NT, no-treatment control.

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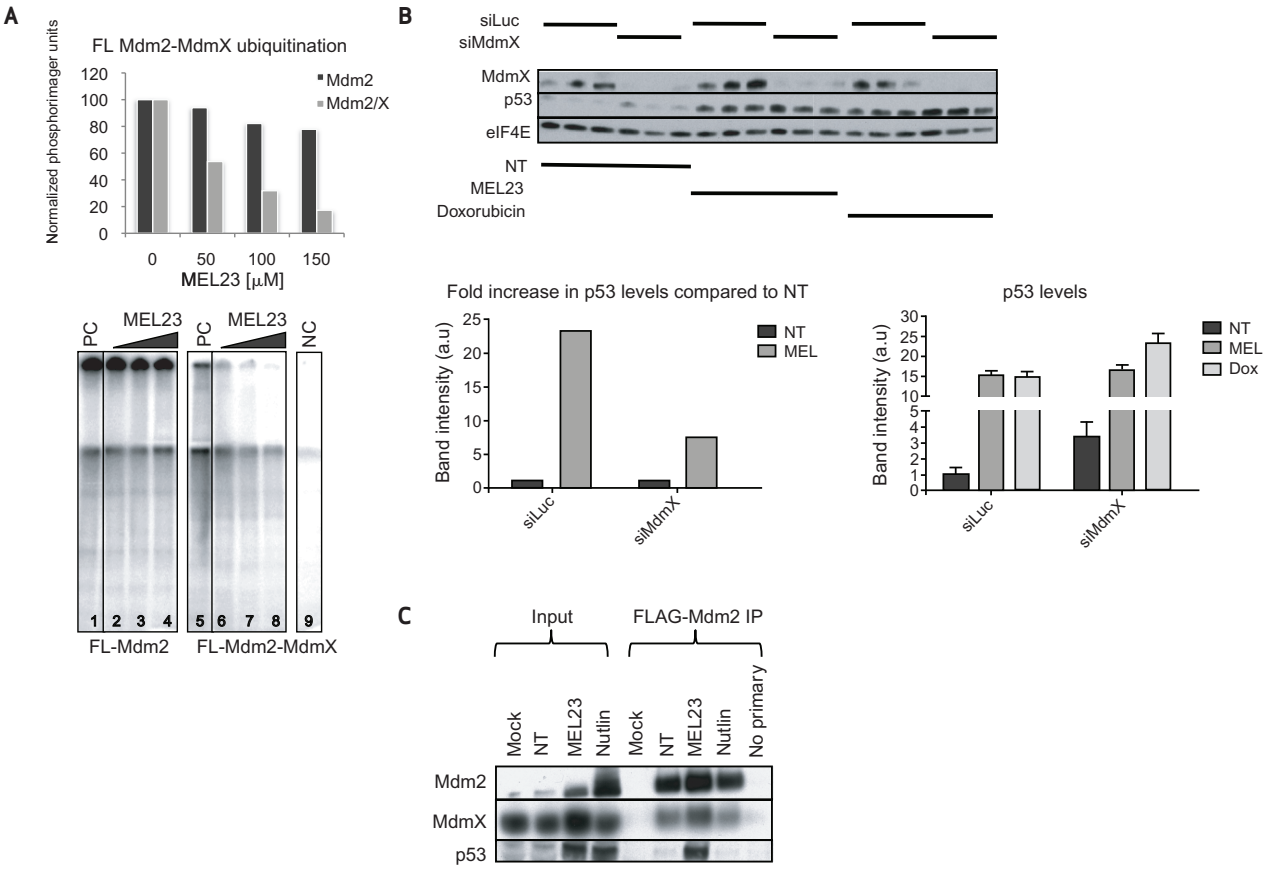

Figure 6. MEL23 and MEL24 inhibit Mdm2 and p53 ubiquitination through the Mdm2-MdmX hetero-complex. **A**, MEL23 inhibits Mdm2-MdmX E3 ligase activity *in vitro*. Bottom panel: Ubiquitination assays in the presence of increasing concentrations of MEL compounds were performed with 100 ng of full-length Flag-Mdm2 or Flag-Mdm2/HA-MdmX complexes, ^{32}P-labeled ubiquitin, UbcH5C, and E1. Following 30 minutes' incubation at 37°C, reactions were terminated and resolved on a SDS-PAGE gel followed by autoradiography. Top panel, the gels were quantified by phosphorimaging using ImageQuant software. PC, positive control; NC, negative control with UbcH5C eliminated from the reaction. **B**, MdmX is needed for efficient MEL23 activity in cells. Top panel, RKO cells were transfected with siRNAs against MdmX or luciferase, as a control. Forty-eight hours after transfection, cells were treated with 10 μg/mL (28 μM) MEL23, 0.5 μg/mL (0.9 μM) doxorubicin, or DMSO for 4 hours before harvesting. Lysates were analyzed by Western blotting using anti-MdmX, anti-p53, and anti-eIF4E antibodies. Bottom left panel, fold increase in p53 levels of NT and MEL23 treated cells normalized to the NT control under two different siRNA co-treatment conditions. Bottom right panel, raw p53 levels in each condition. Protein levels were measured by densitometry using ImageJ and graphed using GraphPad Prism. Error bars represent the SEM of 3 samples. **C**, MEL23 does not inhibit Mdm2-p53 and Mdm2-MdmX complex formation. MCF7 cells were transfected with FLAG-Mdm2 (12 μg) and p53 (2.5 μg). After 24 hours, cells were treated with 10 μg/mL (28 μM) MEL23, 5.3 μg/mL (10 μM) Nutlin-3a, or DMSO for 6 hours. Cell lysates were incubated with anti-FLAG M2 antibody for 2 hours, followed by incubation with Protein-A/G sepharose beads for 1 hour at 4°C. Cells were lysed and analyzed by Western blotting using anti-Mdm2, anti-MdmX, and anti-p53 antibodies. NT, no-treatment control.

compounds is the interface between the Mdm2 RING and the MdmX RING domains. We tested the activity of the compounds on the purified RING domains of the proteins. The compounds inhibited the ubiquitination of the Mdm2(RING)-MdmX(RING) hetero-complex, but not that of the Mdm2 RING homo-complex (Supplementary Fig. S12). However, unlike the full-length complex, which was produced through co-expression of Mdm2 and MdmX in SF9 cells followed by 2 consecutive purification steps (Supplementary Fig. S10), the RING domain ubiquitination reactions were performed by mixing individually expressed and purified Mdm2 RING and MdmX RING proteins. Thus, it is likely that both active hetero- and homo-RING domain complexes were present in the reactions. As predicted by the fact that the Mdm2-MdmX hetero-complex is preferentially inhibited, the overall extent of the inhibition by MEL23 was lower in this format than with the full-length hetero-complex.

In order to further test the specificity of the MEL compounds, we analyzed their effect on Roc1-Cul1 E3 RING domain ligase activity, a multi-subunit ligase (30). We saw no inhibition when the complex was treated with MEL23 or MEL24 (Supplementary Fig. S13). Additionally, we saw no effect of the MEL compounds on the E3 ligase activity of the BRCA1/BARD1 protein complex (31) (Supplementary Fig. S14). Of note, Roc1-Cul1 and BRCA1/BARD1 share the same ubiquitin conjugating enzyme as Mdm2, UbcH5C, providing an additional specificity control (32).

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To confirm that the MEL compounds act on the Mdm2-MdmX complex in cells, we decreased MdmX levels via siRNA transfection in RKO cells. Cells transfected with a nontargeting siRNA and then treated with MEL23 showed an increase of greater than 20-fold in p53 protein levels compared to dimethylsulfoxide (DMSO)-treated control cells. By contrast, siMdmX-transfected cells that were then treated with MEL23 showed an increase of approximately 7-fold in p53 protein levels compared to DMSO control cells (Fig. 6B). Cells were also treated in parallel with doxorubicin to ensure we were not saturating p53 levels with the MdmX knockdown and/or MEL treatment. We found that p53 levels increased more (albeit modestly so) with doxorubicin than MEL23 treatment in cells transfected with siMdmX. This result demonstrates that with reduced MdmX levels, the MEL compounds are less efficient at preventing p53 degradation.

In order to further validate the mechanism of action of the MEL compounds, we overexpressed p53, Mdm2, and/or MdmX in U2OS cells, treated with MEL23 and analyzed p53 levels. We used Mdm2 and MdmX at concentrations at which synergy was observed in the destabilization of p53 (19). We monitored p53 levels in cells under 4 DNA transfection conditions treated with MEL23; nontransfected, p53 only, p53 and Mdm2, and p53, Mdm2 and MdmX transfections. The most dramatic stabilization occurred when all 3 proteins (p53, Mdm2, and MdmX) were overexpressed (Supplementary Fig. S15). An increase in p53 in the nontransfected cells occurred, although significantly less than in the transfected cells, as expected since U2OS cells have wild-type p53. This result suggests that the MEL compounds work most efficiently in the presence of both Mdm2 and MdmX.

Although the data suggest that the MEL compounds interfere with the Mdm2-MdmX E3 ligase activity, they were not found to inhibit formation of the complex between Mdm2 and MdmX in co-immunoprecipitation experiments (Fig. 6C; Supplementary Fig. S16). MEL23 also did not inhibit p53-Mdm2 complex formation. These results suggest that the MEL compounds have a different mode of action than Nutlin-3, an inhibitor of p53-Mdm2 interaction (23).

Taken together, these results suggest that while the MEL compounds may inhibit Mdm2 alone to some extent, their primary target in cells is the E3 ligase activity of the Mdm2-MdmX hetero-complex. Additionally, the compounds function via a mechanism other than interfering with the formation of this protein complex.

MEL23 Leads to Cell Death that Is p53-dependent and Synergistic with DNA Damaging Agents

To test the physiological outcomes and potential therapeutic efficacy of the MEL compounds, we examined the survival of RKO and RKO-E6 cells after treatment with MEL23. Because treatment of RKO cells with the MEL compounds led to an increase in p53 levels and activity (Fig. 3A, 3C, and 4D), we expected a corresponding increase in p53-dependent cell death. Consistent with this, RKO cell survival decreased in a dose-dependent manner following MEL23 treatment, while RKO-E6 cells remained largely unaffected (Fig. 7A). MEL23 treatment increased the sub-G_1 population and caused a slight G_2 cell-cycle arrest in RKO, mouse embryonic fibroblast (MEF), and HT-1080 cells (Supplementary Fig. S17; data not shown). These cells stained positive for TUNEL and exhibited caspase activation (Supplementary Fig. S17), suggesting an apoptotic cell death process.

To further test the p53- and Mdm2-dependence of MEL23-induced cell death, we analyzed 3 MEF cell lines: wild-type, *p53*$^{-/-}$, and *p53*$^{-/-}$; *mdm2*$^{-/-}$ (Fig. 7B; Supplementary Fig. S18). Consistently, MEL compounds showed p53-dependent activity, as the survival of the wild-type cells was decreased in a dose-dependent manner by MEL23. The survival of the *p53*$^{-/-}$ MEFs was also decreased by MEL23 treatment, indicating some p53-independent, Mdm2-dependent activity. This is consistent with reports that Mdm2 has oncogenic effects independent of p53 (33). Only a small decrease in cell survival was seen in the *p53*$^{-/-}$; *mdm2*$^{-/-}$ MEFs treated with MEL23. Therefore, consistent with the Mdm2-MdmX complex being the target of the MEL compounds, the majority of growth inhibition was dependent on the presence of Mdm2 and p53.

We tested the ability of MEL23 to cooperate with DNA-damaging agents to potently induce cell death. U2OS (p53 wild-type) and H1299 (p53-null) cells were treated with increasing amounts of MEL23 and either camptothecin or etoposide in combination for 48 hours and cell viability was measured. The combination of MEL23 with these DNA-damaging agents led to a synergistic decrease in viability (Fig. 7C; Supplementary Fig. S19). The results are presented as a decrease in cell viability over that predicted by the Bliss independence model. Bliss independence was determined by the formula $C = A + B - A*B$, where A is the treatment with one compound alone and B is treatment with the second compound alone (34). The excess over Bliss independence was determined by subtracting the predicted Bliss effect from the experimentally determined effect, yielding a synergistic effect of the combined treatment.

Because MEL23 can inhibit p53 ubiquitination, and Nutlin-3 can prevent Mdm2 from inhibiting p53 transcriptional activation, we also tested the ability of MEL23 and Nutlin-3 to cooperatively decrease cell viability. Synergy between MEL23 and Nutlin-3 occurred, but to a lesser extent than the combination of MEL23 and DNA-damaging agents (Fig. 7C; Supplementary Fig. S19).

Interestingly, MEL23 also synergized with DNA-damaging agents in *p53*-null H1299 cells. Nutlin-3, on the other hand exhibited synergy with camptothecin and etoposide only in wild-type p53 cells. This may be because the MEL compounds are inhibiting additional functions of Mdm2 that are p53-independent, but still potentially oncogenic. These synergistic effects of MEL23 with low concentrations of DNA damaging agents that do not cause deleterious effects on their own may increase the therapeutic utility of the MEL compounds.

DISCUSSION

In this study, we conducted a screen to identify inhibitors of Mdm2 E3 ligase activity. We found 2 closely related analogs out of 270,080 tested compounds—MEL23 and MEL24. Using full-length Mdm2 allowed us to take advantage of the

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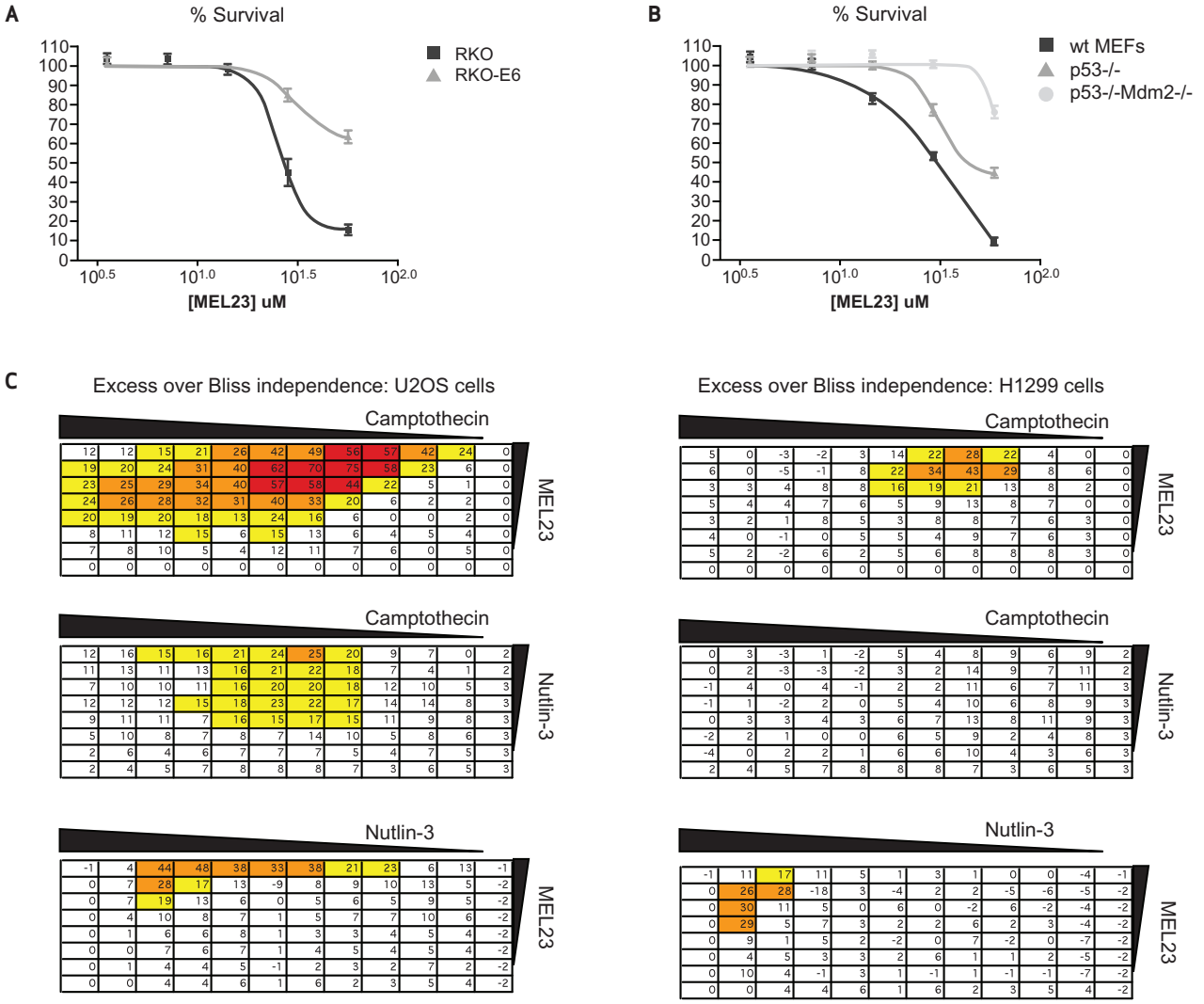

Figure 7. MEL23 decreases cell viability alone and in combination with DNA-damaging agents. **A**, MEL23 specifically decreased RKO cell viability. RKO and RKO-E6 cells were seeded in 384-well plates and treated with a dilution series of MEL23 for 48 hours as indicated. Alamar Blue was added for 16 hours and fluorescence determined on a Victor3 Plate Reader. The median percent inhibition of 5 replicates was determined and normalized to a no-treatment control. One standard deviation is shown. **B**, percent survival of wild-type, $p53^{-/-}$, and $p53^{-/-}$;$mdm2^{-/-}$ MEFs. Cells were seeded in 384-well plates and treated with a dilution series of MEL23 for 48 hours at the indicated concentrations. Alamar Blue was added for 16 hours and fluorescence determined on a Victor3 Plate Reader. The median percent inhibition of 5 replicates was determined and normalized to a no-treatment control. One standard deviation is shown. **C**, MEL23 synergizes with DNA-damaging agents. U2OS (p53 wild-type) and H1299 (p53 null) cells were seeded in 384-well plates and treated with compound(s) for 48 hours. The starting concentrations of the compounds diluted vertically in the plate are MEL23 at 10 μg/mL (28 μM) and Nutlin-3 at 10 μg/mL (17 μM). The starting concentrations of the compounds diluted horizontally in the plate are camptothecin at 25 μg/mL (72 μM), Nutlin-3 at 50 μg/mL (86 μM), and etoposide at 100 μg/mL (170 μM). Compounds were diluted in a 2-fold, 7-point dilution vertically down the plate or in a 2-fold, 11-point dilution horizontally across the plate. After the incubation period, Alamar Blue was added for 16 hours, fluorescence was measured on a Victor3 Plate Reader, and the percent growth inhibition determined. Bliss independence was determined by the formula $C = A + B - A*B$, where A is the treatment with one compound alone and B is the treatment with the second compound alone. The excess over Bliss independence was determined by subtracting the predicted Bliss effect from the experimentally determined effect, yielding the synergistic effect of the combined treatment. Highlighted values in red have > 50% growth inhibition over that predicted by Bliss independence, values in orange have > 25%, and values in yellow have > 15%.

presence of all domains of Mdm2 in order to improve the likelihood of discovering inhibitors. Additionally, the use of a cell-based assay enabled targeting of potential cellular co-factors necessary for ligase activity. The high-throughput screen we present here could be modified to discover inhibitors of other E3 ligases. The ease of this type of high-throughput screen and its adaptability may therefore be valuable in the identification of new small-molecule inhibitors directed against different E3 ligases, which have previously been considered challenging targets for drug discovery.

Mdm2 inhibitors described to date include peptide inhibitors (35) and small molecules such as Nutlin-3 (23) and

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RITA (36). Mdm2 E3 ligase inhibitors identified by *in vitro* screens, such as the HLI series of compounds, have also been described (24). Although the HLI compounds were able to induce p53-dependent apoptosis, they lack specificity towards Mdm2 (24). Other Mdm2 E3 ligase inhibitors identified in *in vitro* ubiquitination assays, such as sempervirine and lissochlinidine B, have not been shown to inhibit Mdm2-MdmX E3 ligase activity (37, 38). More recently, the first small-molecule inhibitor of MdmX binding to p53 was identified (39). Unlike other Mdm2 and MdmX inhibitors identified, MEL23 and MEL24 are a unique class of Mdm2 inhibitors identified in a cell-based assay that are able to inhibit the E3 ligase activity of the Mdm2-MdmX complex.

These data show that MEL23 and MEL24 preferentially inhibit the E3 ligase activity of the Mdm2-MdmX complex, although they do have some activity against Mdm2 alone. The inhibition of the Mdm2-MdmX complex *in vitro* and stabilization of p53 and Mdm2 in cells indicate that the Mdm2-MdmX complex is centrally involved in regulating the degradation of these proteins. These observations are in agreement with multiple studies that describe the importance and activity of the Mdm2-MdmX complex. While Mdm2 can catalyze p53 ubiquitination *in vitro* (40), in cells MdmX is needed along with Mdm2 for efficient p53 degradation (19). MdmX can lower the concentration of Mdm2 needed for both p53 ubiquitination and autoubiqutination, and the Mdm2-MdmX complex has been shown to be a better ligase for p53 than Mdm2 alone (41). Additionally mouse studies have shown that the interaction between Mdm2 and MdmX is essential for regulating p53 during early embryogeneisis (42, 43). Enhancement of activity of RING domain hetero-oligomers has also been demonstrated for BRCA1-BARD1 complexes, where the presence of BARD1 in the complex increases the ligase activity of BRCA1 (44). Therefore our results support studies suggesting that although Mdm2 is able to function as an E3 ligase on its own, MdmX augments the nature and activity of the ligase with profound functional consequences in cells.

One caveat in these mechanistic studies was that high concentrations of MEL compounds were necessary to see inhibition of ubiquitination *in vitro*. It is possible that the enzymatic activity of the Mdm2-MdmX ligase is difficult to inhibit *in vitro*; the well-known Nutlin-3 compound does not inhibit Mdm2 ubiquitination (45). Perhaps the MEL compounds only partially inhibit E3 ligase activity in cells, and this is sufficient to cause p53 accumulation, consistent with mouse data showing that just a 20% to 30% reduction in Mdm2 can lead to p53 activation (46). It is tempting to speculate that compounds that completely inhibited Mdm2 or Mdm2-MdmX E3 ligase activity would activate p53 in both cancer and normal cells to such a large extent that the compounds would not be tolerated and would therefore not be useful as therapeutics. The MEL compounds, on the other hand, have a small but consistent differential activity between tumor derived and nontransformed cell lines, decreasing the survival of the tumor-derived cells to a greater extent (Supplementary Fig. S20).

Because Mdm2 can inhibit p53 through 2 independent mechanisms—E3 ligase-mediated degradation and binding-mediated functional inactivation—it is formally possible that inhibitors of Mdm2 E3 ligase activity would not be sufficient to activate p53. In this model, Mdm2 E3 ligase inhibitors would increase both p53 and Mdm2 levels, but would not prevent Mdm2 from binding p53 and thereby inhibiting its activity. Based on qRT-PCR data (Fig. 3D; Supplementary Fig. S7) and also co-immunoprecipitation data (Fig. 6C; Supplementary Fig. S16), it is likely that Mdm2 and p53 are still interacting to some extent upon MEL compound treatment and that Mdm2 is partially inhibiting p53 activity. However, we observed increased p53 activity upon MEL treatment compared to proteasome inhibition, which also increases Mdm2 and p53 levels. In RKO cells, such increases in p53 activity caused by MEL compound treatment appear to be sufficient for p53-dependent cell death, albeit at higher concentrations of MEL23 than are needed for p53 stabilization. Indeed, HLI compounds, another class of Mdm2 E3 ligase inhibitors, also induce p53-dependent cell death, suggesting that complete disruption of the Mdm2-p53 interaction may not be necessary for activation of p53 (24). Additionally, mutant knock-in mice studies have demonstrated that Mdm2-p53 binding, without Mdm2-mediated p53 ubiquitination, is not sufficient to control p53 activity (47). Furthermore, although MEL compounds do not induce p53 post-translational modifications, some cancer cells may already have such modifications in place as a consequence of oncogenic stress. Nevertheless, we predict that the combination of the MEL compounds and DNA-damaging agents would enhance their therapeutic potential. This could also allow for use of DNA-damaging agents at concentrations that do not cause some of their normal deleterious side effects.

MEL23 cooperated with DNA-damaging agents in p53-null cells to a small, yet reproducible, extent. Mdm2 has been shown to have p53-independent oncogenic effects, for example, (1) overexpression of Mdm2 in mice causes tumors independent of p53 status (33); (2) splice variants of Mdm2 that cannot bind to p53 have been shown to be oncogenic (48); (3) Mdm2 destabilization of Rb (49, 50) and p21 (51) may contribute to tumor growth; and (4) Nutlin-3-mediated disruption of p73-Mdm2 binding enhances p73 function (52). Therefore, the MEL compounds and other specific Mdm2 E3 ligase inhibitors may be beneficial in p53-null or p53-mutant tumors. This hypothesis, although intriguing, requires further study.

As Mdm2-MdmX ligase inhibitors, MEL compounds may provide further insight into the function of the Mdm2-MdmX E3 ligase and allow for investigation of the differences in activity between the hetero-complex and the Mdm2 homo-complex. It will be informative to ultimately determine the precise biophysical mechanism of action of the MEL compounds, as well as the binding site of the compounds. Additionally, the MEL compounds may be used as molecular tools to validate novel targets of Mdm2-MdmX. Finally, although these compounds were not suitable for testing in mice, we have demonstrated that targeting Mdm2-MdmX with small molecules is feasible and future studies could either optimize these compounds or use this high-throughput assay to discover additional drug-like scaffolds with similar activity. An improved understanding of the mechanism of action of the MEL compounds, and/or future inhibitors of this pathway, may lead to new ways to inhibit E3 ligases, which could be beneficial in diverse applications.

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METHODS

Reagents

MG132 (Sigma, C2211), ALLN (Calbiochem, 208719), cycloheximide (Sigma, C7698), doxorubicin hydrochloride (Sigma, D1515), etoposide (Sigma, E1383), camptothecin (Sigma, C9911), and Nutlin-3 (Sigma, N6287) were used for cell treatments at the indicated concentrations.

Antibodies to p53 (Calbiochem, OP43 for Western blotting, and Calbiochem, OP03 for co-immunoprecipitation), Mdm2 (Calbiochem, OP115), anti-M2 (FLAG) (Sigma, F1804), phospho-p53 ser15 (Cell Signaling, 9284s), phospho-histone H2A.X (Millipore, 05-636), histone H2A.X (Millipore, 07-627), MdmX (Bethyl, A300-287A), c-Myc (5605S, Cell Signal), c-jun (9165S, Cell Signal), BRCA1 (Calbiochem, OP92), eIF4E (BD Transduction Laboratories, 610270), and actin (Santa Cruz Biotech, sc-1616-R) were used. Anti-Mdm2 SMP14, 2A10, 3G5 mix, used as supernatants from hybridoma cultures, was used for *in vitro* Mdm2 ubiquitination assays.

Cell Lines and Transfections

293T (human embryonic kidney), H1299 (non–small-cell lung carcinoma), MCF7 (mammary gland adenocarcinoma), wild-type MEFs, $p53^{-/-}$ MEFs, and $p53^{-/-}mdm2^{-/-}$ MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) (Fisher, MT-15-018-CM) with 10% fetal bovine serum (FBS) and 100 μg/mL penicillin/streptomycin (P/S). Mdm2(wt)-luciferase and Mdm2(C464A)-luciferase stable cell lines (in 293T cells) were additionally treated with 300 μg/mL of zeocin (Invitrogen, R250-05). Wild-type MEFs were a kind gift from Craig B. Thompson at the University of Pennsylvania. $P53^{-/-}$MEFs and $p53^{-/-}$;$mdm2^{-/-}$ MEFS were a kind gift from Guillermina Lozano at the University of Texas. RKO (colon carcinoma) and RKO E6 (colon carcinoma) cells were maintained in Minimum Essential Medium (MEM) Eagle (Sigma, M5650) with 10% FB and 100 μg/mL P/S. U2OS (osteosarcoma) and HCT116 (colorectal carcinoma) were maintained in McCoy's 5a Medium (Invitrogen, 16600-108) with 10% FBS and 100 μg/mL P/S. HT-1080 cells were maintained in DMEM, 10% FB, 100 μg/mL P/S, and 1% Non-essential amino acids (Invitrogen, 11140-050). All cells were grown at 37°C in 5% CO_2.

Cells were transfected using FuGENE 6 (Roche, 814-442-001) in accordance with the manufacturer's protocol. Transfections were performed in 6-well dishes, except for the co-immunoprecipitation experiment performed in 15-cm dishes or as noted.

To knock down MdmX expression, cells were transfected with 20 nM control siRNA (AACTTACGCTGAGTACTTCGA) or MdmX siRNA (AGAGATTCAGCTGGTTATTAA) using DharmaFECT1 (Dharmacon, T-2001-01) according to manufacturer's instructions. After 48 hours of knockdown, cells were treated with the indicated compounds.

Plasmids

Mdm2(wt)-luciferase and Mdm2(C464A)-luciferase plasmids were cloned into the pcDNA3.1 vector (Invitrogen, P/N 35-0574) using the Nhe 1 and Xho 1 restriction sites. Luciferase is on the N-terminus of Mdm2 with a linker (Gly Ser Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Thr Gly Ser) cloned between the Mdm2 and the luciferase DNA sequence. This was confirmed by sequencing. Primer sequences for cloning are in the Supplementary Data.

Flag-Mdm2 in pcDNA3 (53), HA-Ubiquitin in pcDNA3 (53), and p53 in pcDNA3 (54) have been previously described. PK-ubiquitin (55) and His-Ubch5C (55) in pET-15a, His-p53 in pRSETB (56), GST-Mdm2 (400-491) (55), and GST-MdmX (410-491) (55) in pGEX-4T1 have been previously described. For constructing HA-MdmX baculovirus, PCR was performed to insert an HA tag upstream of MdmX. The PCR product was purified and digested with RsrII and KpnI and cloned into the pFastBac HTa plasmid (Invitrogen). Flag-Mdm2 baculovirus was a kind gift from Dr. S. Grossman at the University of Massachusetts Medical School. Both Flag-Mdm2 and HA-MdmX baculoviruses were prepared according to the Bac-to-Bac baculovirus expression system (Invitrogen).

Compound Libraries

A total of 51,356 compounds were screened in the Stockwell Lab. These included synthetic compounds and natural products from InterBioScreen, Timtec, Chembridge, MicroSource, and Life chemicals (details available upon request). These compounds were stored in DMSO in 384-well plates at 4 mg/mL. MEL23 and MEL24 were identified from InterBioScreen and the analogs were also obtained from InterBioScreen.

A total of 218,724 compounds were screened through the NIH MLSCN at the University of Pennsylvania.

Screening (Stockwell Lab)

The Mdm2(wt)-luciferase cell line was seeded at 7,500 cells per well, in 384-well white plates (PerkinElmer, 6007688), in 27 μL of media (DMEM, FB, P/S). Assay plates with cells were incubated at 37°C overnight to allow cells to adhere. After 24 hours, 2 μL from the "mother" plate (4 mg/mL) was transferred to a "daughter plate" containing 148 μL media in order to dilute the compound 1:75. Next, 3 μL from each well of the daughter plates was added to triplicate assay plates for a final concentration of 5.33 μg/mL. Assay plates were incubated at 37°C for 2 hours. Then, the cells were lysed with the addition of 30 μL of luminescence buffer (PerkinElmer, 6016989) and the plates were incubated at RT for 30 minutes before being analyzed on a Victor3 Plate Reader (PerkinElmer) for luminescence. Compounds that caused an increase in luminescence $>$ 30% were re-ordered for further analysis. All repurchased compounds were tested in a 2-fold dilution series via the same protocol as the primary screening. All transfers were conducted using a BioMek (Beckman Coulter). The final optimized screen has a Z′ value of 0.63 (57). This was determined by comparing the negative control, no treatment, to the positive control, 10 μM MG132.

Screening (MLSCN library)

Described in Pubchem (BioAssay AID 1442, AID 1230, AID 1444, and AID 1394). The final optimized screen has a Z′ value of 0.49 (57).

Western Blot Analysis

Cells were seeded and treated with the indicated compound(s). Cells were lysed after each time point in a buffer of 50 mM TrisHCl pH 7.5, 200 mM NaCl, 0.5% NP-40, and 1 complete mini EDTA-free protease inhibitor cocktail tablet per 10 mL of buffer (Roche, 8360170). Protein content was quantified using a Bio-Rad protein assay reagent (Bio-Rad, 500-0006). SDS sample buffer was added to the samples and they were boiled for 5 minutes. Equal amounts of protein were resolved on a 1D SDS-PAGE. Following separation, proteins were transferred to a nitrocellulose membrane, blocked with 5% milk, and then incubated with primary antibody overnight at 4°C. The membrane was incubated with secondary-HRP antibody for 45 minutes at RT and developed with SuperSignal West Pico Substrate (Pierce, 34080).

Immunoprecipitation

Cells were lysed in 150 mM NaCl, 10 mM TrisHCl pH 7.5, 0.5% NP-40, 10% glycerol, 1 mM EDTA, 1 mM DTT, and 1 complete mini EDTA-free protease inhibitor cocktail tablet per 10 mL of buffer. FLAG-tagged Mdm2 immunoprecipitation experiments were performed by incubating cell lysates with anti-M2 (FLAG) antibody (Sigma, F1804) for 2 hours at 4°C and with Protein-A/G sepharose beads (Pierce, 20421) for 1 hour at 4°C. HA-ubiquitin immunoprecipitation experiments were performed by incubating cell lysates with HA-affinity matrix (Roche, 11815016001) for 3 hours at 4°C.

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All lysates were washed 3 times with lyses buffer before eluting the protein with SDS sample buffer.

In Vitro *Ubiquitination Assay*

Full-length Mdm2 *in vitro* ubiquitination reactions were performed in 15–μl reactions mixtures containing 50 mM TrisHCl pH 7.5, 5 mM $MgCl_2$, 2 mM NaF, 2 mM ATP, 0.6 mM DTT, 50 ng E1 (Boston Biochem, E-305), 1 μg PK-ubiquitin, 50 ng His-UbcH5C, and 100 ng Flag-Mdm2. 50 ng His-p53 was used for *in vitro* p53 ubiquitination assay. 100 ng Flag-Mdm2 and 100 ng Ha-MdmX were used for ubiquitination reactions with the Mdm2-MdmX complex. After incubation for 30 minutes at 37°C, SDS sample buffer was added to the samples and they were boiled for 5 minutes. The products were resolved by SDS-PAGE and analyzed by Western blot with anti-p53, anti-Mdm2 SMP14, 2A10, 3G5 mix, or anti-MdmX antibodies.

Radiolabeled *in vitro* ubiquitination assays were done in 30 μl reaction mixtures with ^{32}P-labeled ubiquitin. 500 ng GST-Mdm2(400-491) and GST-MdmX(410-490) were used for the RING-RING reactions. The products of the reaction were resolved on a SDS-PAGE gel and visualized by autoradiography.

Roc1-Cul1 protein was a kind gift from Zhen-Qiang Pan at the Mount Sinai School of Medicine; 3 μg of protein was used per ubiquitination reaction.

Purified His-BARD1/BR304 (31) was incubated with 5 pmol Ubch5C and 0.2 pmol E1 enzyme (Boston Biochem) along with 300 pmol ubiquitin in reaction buffer (without BSA) for 2 hours at 37°C. The products were resolved by SDS-PAGE and analyzed by Western blotting with anti-BRCA1. BRCA1/BARD1 was a kind gift from Dr. Richard Baer.

Additional Materials and Methods

Additional information on methods and materials is available in the Supplementary Data, including Alamar Blue viability assay, Bliss independence model, protein purification, RNA extraction and qRT-PCR analysis, glutathione reactivity assay, TUNEL stain, cell cycle profiling, and caspase activity.

Disclosure of Potential Conflicts of Interest

B.R. Stockwell has equity in and serves as a consultant to Solaris Therapeutics, a startup company that has an option to license the Mdm2 inhibitors described in this manuscript. No potential conflicts of interest were disclosed by the other authors.

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