Small molecules that bind the Mdm2 RING stabilize and activate p53

Introduction

Tp53 is a tumor suppressor gene that encodes the transcription factor p53. In response to cellular stress (such as genotoxic damage, oncogene activation, hypoxia and telomere erosion), p53 is activated and mediates responses including cell cycle arrest, apoptosis, senescence and differentiation. In this way, p53 prevents the accumulation of damaged cells and therefore limits malignant progression. In normal tissues, p53 is kept at very low levels due to the rapid degradation of the p53 proteins through the proteasome. The main regulator of p53 is the E3 ubiquitin ligase MDM2, which binds to p53’s transactivation domain (1) and functions by both preventing p53’s transcriptional activity and targeting it for degradation (2,3). The regulation of p53 function by MDM2 is essential for normal growth and development, and the absence of MDM2 leads to an early embryonic lethality that is the result of uncontrolled p53-dependent apoptosis (4).

In the event of cellular stress, several mechanisms that inhibit MDM2 to allow stabilization of p53 have been described. The mechanism of p53 activation depends on the particular stress stimulus. Both p53 and MDM2 can be extensively modified in response to stress, and phosphorylation of both proteins by DNA damage-induced kinases such as ATM and ATR can disrupt their interaction and so prevent the ubiquitination of p53 by MDM2 (5–7). In response to oncogene activation, the alternative reading frame (ARF) protein binds to the central domain of MDM2, directly inhibiting its E3 activity (8,9). A third mechanism of p53 activation involves the response to ribosomal stress in which ribosomal proteins bind MDM2, again inhibiting E3 activity without blocking the interaction of MDM2 with p53 (10).

Abbreviations: ARF, alternative reading frame; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; HLI98, HDM2 ligase inhibitor 98 class.

MDM2 with p53 (10). Under conditions when MDM2 is no longer required to keep p53 in check, MDM2 may continue to act as its own E3 ubiquitin ligase and target itself for degradation (11).

Dysfunction of the p53 pathway has been shown to contribute to the development of most human cancers. In over 50% of tumors, this is due to mutation of the p53 gene that frequently results in expression of a mutant p53 protein (12,13). In many of the remaining cancers, the p53 pathway is defective due to aberrations in the mechanisms that allow for the stabilization of p53 (14), including the overexpression of MDM2 (15) or loss of ARF (16). The potential for the reactivation of p53 functions such as apoptosis or senescence is an attractive therapeutic strategy for cancer. Three different mouse models have demonstrated that reactivation of wild-type p53 in mouse models results in tumor regression (17–19), providing support for the development of agents that reactivate the p53 pathway for tumor therapy. One strategy to reactivate wild-type p53 is to inhibit MDM2 in cancers that retain wild-type p53 but have suffered perturbations in the pathways that normally allow p53 activation in response to stress (for example loss of ARF or DNA damage signaling). Several different approaches have been taken to develop small molecule MDM2 inhibitors, with most effort focused on the development of agents designed to inhibit the interaction between MDM2 and p53 (e.g. Nutlins, spiro-oxindoles, benzodiazepines and reactivation of p53 and induction of tumor cell apoptosis) (20–28). However, a complementary approach is to develop agents designed to inhibit the E3 ligase activity of MDM2 directly to mimic the effects of ARF or the ribosomal protein L11. A screen for inhibitors of MDM2 E3 activity identified a family of 7-nitro-5-deazaflavin compounds with IC50 values for inhibition of ubiquitination of MDM2 in vitro at 20 μM (29). This group of compounds was named the MDM2 ligase inhibitor 98 class (HLI98) and the synthesis of further 5-deazaflavin compounds (here referred to as MPD compounds), based on the HLI98 class, demonstrated that the nitro group is not required for the inhibitory activity (30).

Using almost 100 newly synthesized MPD compounds, we have been evaluating the biological action and mechanism of action of the MPD compounds. Here, we describe further analogs of the MPD class of compounds and provide evidence that the mechanism of action of these compounds is through direct binding and inhibition of the MDM2 RING domain, the functional domain essential for MDM2’s E3 ligase activity (11).

Materials and methods

In vitro ubiquitination assay

The expression of recombinant glutathione-S-transferase-tagged MDM2 (GST-MDM2) was induced in 25 ml culture of exponentially growing Echerichia coli BL21 cells (OD600 0.6) by 1 mM isopropyl-thio-β-D-galactoside for 3 h. Glutathione-S-transferase-MDM2 was purified on glutathione-sepharose beads (Amersham). The beads were washed with 50 mM Tris (pH 7.5), Fluorometer ubiquitin (5 μg; Invitrogen), 50 μg mammalian E1 (Enzo), 200 ng human recombinant UbeH5B E2 (Enzo) and 200 ng His-p53 (Enzo) were mixed with reaction buffer [50 mM Tris pH 8, 2 mM dithiothreitol, 5 mM MgCl2, 2 mM adenosine triphosphate (ATP)]. A dose titration of each MPD compound or dimethyl sulfoxide (DMSO) was added to the mixture and the mixture was pipetted onto GS4b-MDM2 beads. The reaction was incubated at 37°C, shaking at 1200 rpm, for 1 h and then stopped by the addition of 3× sodium dodecyl sulfate sample buffer. Free fluorescent ubiquitin was washed off and total fluorescent ubiquitin signal was measured on a monochromator plate reader (Safire).

For non-fluorescent in vitro ubiquitination assays, the procedure was as above except 5 μg unlabeled ubiquitin (Enzo) was used. Ten micrometers of each MPD compound or DMSO was added to the mixture and the mixture was pipetted onto GS4b-MDM2 beads. After incubation as described previously, reaction products were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by western blotting with anti-p53 DO-1.

For the MDM2 RING auto ubiquitination assay, GS4b-MDM2 RING beads were prepared as above and used in place of the full-length MDM2.
For the MDM2 autoubiquitination assay, the procedure was performed as detailed above except that no His-p53 was added to the reaction and western blotting was with Ab1 and Ab2 antibodies. For the Cbl autoubiquitination assay, bacterially expressed full-length Cbl was used in a reaction with the previously described quantities of E1, E2 and ubiquitin. Ubiquitinated Cbl was detected by western blotting using anti-ubiquitin antibody (Sigma clone 6C1).

Surface plasmon resonance spectroscopy
Sensor chip surfaces were prepared on a Biacore T100 instrument (Biacore), using reagents obtained from the manufacturer. After FLAG antibodies where cross-linked on a CMS chip via amine coupling, individual flowcells where injected with FLAG-MDM2 RING where appropriate or glutathione-S-transferase as a control protein. Measurements were performed at 25°C, 30 μl/min and a collection rate of 10 Hz. Various concentrations of compounds were prepared in 100 mM Hepes buffered saline with a final DMSO concentration of 5%. The experimental data were corrected for instrumental and bulk artifacts by double referencing a control sensor chip surface and buffer injections using Biacore software package, V 2.0.1.

Cells and transfections
H1299 cells (p53-null human non-small-cell lung adenocarcinoma cells) were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum, 1% glutamine, 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate at 37°C and 5% CO2. Retinal pigment epithelial cells were obtained from ATCC and cultured in DMEM/F12 HAM (Invitrogen), supplemented with 10% fetal calf serum, 1.6% sodium bicarbonate, 1% glutamine, 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate at 37°C and 5% CO2. U2OS-GFP-MDM2 TetOn cells were made by transfecting a U2OS TetOn cell line with a pTRE2 GFP-MDM2 plasmid and pBabe Eco Puro plasmid. Cells were then selected for 2 weeks using puromycin. Colonies resistant to puromycin were picked and grown up in DMEM (Invitrogen) supplemented with 10% fetal calf serum, 1% glutamine, 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate at 37°C and 5% CO2. Several clones were then tested by fluorescent microscopy to ensure that GFP-positive nuclei were visible after doxocycline induction and tested for inducible GFP-MDM2 expression on western blot. Clone 2 was selected for further work. RKO cells (wild-type and null for p53) were a gift from Bert Vogelstein and were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum, 1% glutamine, 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate at 37°C and 5% CO2.

Cells were transfected with GeneJuicer (Merck Biosciences) according to the manufacturer’s instructions. Cells were treated with 10 μM Nutlin-3a (Roche) and MPD compounds at the indicated doses.

Plasmids
Plasmid expressing human wild-type p53 has been described previously (31). Wild-type MDM2 has been described previously (32). HA-tagged Ubiquitin was kindly provided by R. Hay. The GFP-MDM2 plasmid used to make the U2OS GFP-MDM2 TetOn cell line described above was cloned from pHIS-C1 p53M2 (wild-type MDM2 cloned into Clontec backbone) into the pTRE2 plasmid (Clontec).

Protein analysis
Western blot analysis was performed as described previously (33). p53 was detected using the DO1 antibody (1:10000) (34), actin (1:5000; Milipore) and HA 16B12 antibody (1:2500; Covance). MDM2 was detected using the AB-1 and AB-2 antibodies (1:2000, Calbiochem), p21 was detected using either the rabbit polyclonal p21 antibody sc-397 (1:2000; Santa Cruz) or the anti-p21 mouse monoclonal antibody AB-1 (1:1000; Calbiochem).

In vivo ubiquitination of p53
Cells were seeded in 10 cm plates to reach 50% confluence 24 h prior to transfection of 1 μg p53, 4 μg MDM2 and 1 μg HIS-ubiquitin with GeneJuicer reagent. After 20 h, Cells were treated with MPD compounds or controls for 6 h and MG132 for 4 h, washed and collected in phosphate-buffered saline and 5% was kept as input. The rest was centrifuged for 5 min and the cell pellet lysed in 700 μl of ubiquitin buffer A (6 M guanidinium HCl, 300 mM NaCl, 50 mM phosphate pH 8.0, 100 μg/ml N-ethylmaleimide) and sonicated for 5 min at 20% amplitude. Lysates were incubated overnight with Invitrogen Dynabeads His-Tag matrix, once washed with ubiquitin buffer A, B and C buffer and phosphate-buffered saline (ubiquitin buffer B: Mix ubiquitin buffer A and ubiquitin buffer C 1:1; ubiquitin buffer C: 300 mM NaCl, 50 mM phosphate pH 8.0, 100 μg/ml N-ethylmaleimide) and resolved by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by immunoblotting with p-p53 (DO-1) antibody.

Flow cytometry
To investigate the effect on MPD compounds on stability of MDM2, U2OS GFP-MDM2 TetOn cells were plated in 10 cm tissue culture dishes and induced with doxycycline. After 48 h, cells were incubated with doxycycline plus DMSO, MPD compound (5 μM) or MG132 (10 μM) for 4 h. Attached and floating cells were collected by trypsin-ethylenediaminetraacetic acid. Cells were then fixed in 0.05% paraformaldehyde for 30 min and then rehydrated with phosphate-buffered saline. Cells were propidium iodine stained and treated with 50 μg/ml RNase A or 2 h. GFP signal was measured in each condition by flow cytometric analysis (FACScan, Becton Dickinson). Sub G1 cells were excluded.

To study the effect of MPD compounds on cell cycle profile, cells were treated with DMSO, MPD compound (5 μM) or Nutlin (10 μM) for 16 h. Cells were harvested and stained for flow cytometric analysis (FACScan, Becton Dickinson) as described previously except cells were fixed in methanol (35).

Results
MPD compounds specifically inhibit MDM2 and the ubiquitination of p53
Previous studies have demonstrated that the MPD class of compounds can inhibit MDM2’s ubiquitination activity both against itself and p53 (29). We synthesized 84 new analogs of these compounds and tested them all in an in vitro fluorescent ubiquitination assay that measured the overall ability of the compounds to inhibit MDM2 and p53 ubiquitination simultaneously. These results showed that despite all compounds having a very similar structural backbone, 73% were not detectably active in the inhibition of MDM2/p53 ubiquitination, whereas 11 showed some activity, with IC50 values between 1.5 and 50 μM (Figure 1). Details of the chemical synthesis and inhibition of MDM2 ubiquitination structure–activity relationship will be published elsewhere; in this study, we have explored in more detail the possible mechanism of function of a subset of the MPD compounds. We have focused on a core set of compounds including MPD20 to represent the moderately activate compounds, MPD37 to represent the highly active compounds and MPD39 to represent the inactive compounds, as assessed in the fluorescent assay.

We first sought to determine whether the compounds were efficient in inhibiting the ubiquitination of p53, MDM2 or both. Using a gel-based assay, we examined the impact of each compound on the ubiquitination of p53 by MDM2 in an in vitro ubiquitination assay (Figure 2A). Consistent with the results from the screen, active compounds (20, 37 and 131) were effective in inhibiting ubiquitination of p53. A number of inactive compounds (26, 39, 46 and 47) did not affect p53 ubiquitination by MDM2 (Figure 2A).

To further explore the activity of the compounds on the activity of MDM2, we examined their effect on autoubiquitination of MDM2. Consistent with their activity in the p53 ubiquitination assays, the active compounds inhibited MDM2 autoubiquitination and the inactive compounds had no effect (Figure 2B). Multiple domains of MDM2 have been shown to be responsible to ubiquitinate p53 in cells. These include the C-terminal RING domain, the N-terminal p53-binding domain and the central acidic domain. However, in in vitro assays, co-incubation of the isolated RING domain with p53 is sufficient to drive ubiquitination (11,36). Using this assay, we showed that the active compounds (20, 37 and 159) retained the ability to inhibit ubiquitination of p53, whereas the inactive compounds (26, 39, 46 and 47) have no effect (Figure 2C). These results demonstrate that the compounds function to inhibit the RING domain of MDM2 directly.

MPD compounds do not inhibit Cbl autoubiquitination
To determine the specificity of the compounds for MDM2, we tested a highly active compound (37) and an inactive compound (39) in an in vitro assay of autoubiquitination of a RING E3 ubiquitin ligase with a similar structure to MDM2, Cbl (Figure 2D), an assay that uses the same E1 and E2 as the MDM2 assay. At a dose of 10 μM, active compound 37 is able to inhibit autoubiquitination of MDM2 but not autoubiquitination of Cbl, whereas inactive compound 39 cannot
Fig. 1. Chemical structures for the 11 active MPD compounds and the inactive MPD39 are shown in addition to their ability to inhibit in vitro ubiquitination. Glutathione sepharose-MDM2 beads were added to a mixture of fluorescent ubiquitin, E1, E2 and p53 in the presence of indicated concentrations of MPD compound. Free fluorescent ubiquitin was washed away. Intensity of fluorescent was measured on Safire and IC50s were calculated using PRISM 5 software. For the core compounds (20 and 37), the assay was performed three times. Error bars shown represent the standard error of the three independent experiments.
inhibit ubiquitination of either Cbl or MDM2. These data show selectivity of the compounds toward MDM2 and also confirm that they are not functioning through inhibition of other components of the pathway such as the E1 or E2 enzymes, which are the same in both MDM2 and Cbl assays.

Active MPD compounds bind to the MDM2 RING
The in vitro assays have shown that active MPD compounds inhibit the isolated RING domain of MDM2, indicating that they function by directly targeting this region of MDM2 (29). Using surface plasmon resonance spectroscopy, we tested whether the MPD compounds (20, 37, 39 and 159) could bind to the RING finger of MDM2. With this approach, we demonstrated a specific binding of the active MPD compounds to the MDM2 RING at affinities close to the IC50 values for inhibition of MDM2 activity in vitro (Kd = 3.7 μm for MPD37) (Figure 3). Unfortunately, the chemical properties of compound 159 limited the testing of this compound by surface plasmon resonance and therefore, no surface plasmon resonance data for this compound can be presented. Of note, the active MPD compounds demonstrate fairly fast on- and off-rates of binding MDM2, with the more active compound 37 showing a slightly slower off-rate, which is more desirable in terms of further drug development.

MPD compounds stabilize and activate p53
Previous analyses have shown that MPD-related compounds can stabilize and activate p53 in cells (29). We therefore examined the consequences of treatment of tissue culture cells with the MPD compounds described in this study. In support of our in vitro analyses, we could demonstrate that active compounds (20, 37 and 159) inhibit ubiquitination of p53 with an efficiency that correlates well with their in vitro inhibition IC50 values, whereas inactive compounds (26, 39, 46 and 47) do not inhibit ubiquitination of p53 (Figure 4A). By comparison, Nutlin (a small molecule that interrupts the interaction of MDM2 and p53) reduces, but does not abolish, p53 ubiquitination. The active compounds (20, 37 and 159) can be shown to increase the overall levels of endogenous p53 (consistent with an inhibition of ubiquitination and degradation) and this increased expression of p53 correlates with enhanced expression of the p53-transcriptional targets p21 and MDM2. Inactive compounds (39, 47 and 165) fail to alter either p53 levels or activity (Figure 4B). The more potent MPD compounds present challenges in cell-based assays because they induce significant levels of cell death, as demonstrated by an increased sub-G1 fraction on cell cycle analysis of RKO cells (Figure 6B) and this is reflected in an apparent decrease in p21 levels in cells treated with high concentrations of compound 37 (Figure 4B).

Although the activation of p53 would drive increased expression of MDM2, we would predict that the ability of the MPD compounds to inhibit autoubiquitination of MDM2 would also contribute to the elevation of protein levels. To investigate directly the effect of the MPD compounds on MDM2 protein levels, we treated U2OS cells that have been engineered to express GFP-MDM2 via a tet-inducible promoter with MPD compounds. Active MPD compounds (20, 37 and 159) induced a modest but significant increase in GFP signal, indicating that the inhibition of MDM2 autoubiquitination results in the stabilization of the MDM2 protein in cells (Figure 4C).

To confirm that the p53 stabilization and activation of p21 caused by MPD compounds have functional effects, we quantified the cell cycle profile following MPD treatment of untransformed wild-type p53 expressing retinal pigment epithelial cells (Figure 5A). Consistent with the effect of p53 stabilization, active MPD compounds (20, 37 and 159) caused a G1 cell cycle arrest similar to that seen following treatment of the cells with Nutlin (Figure 5A and B), whereas the inactive compound (39) had no effect on cell cycle progression. There was no clear correlation between the efficiency of the compounds in cells and their activity in vitro, which may reflect both uptake of the compounds and toxicity (see below). However, unlike Nutlin, the active MPD compounds also led to a slight decrease of cells in G2. To investigate the p53 dependence of the response to the MPD compounds, we turned to isogenic matched p53+/− and p53−/− RKO cells (Figure 6A and B). As seen in retinal pigment epithelial cells, the moderately active MPD compound (20) induced a G1 arrest that was
Fig. 3. FLAG antibody was amine coupled to a Biacore CM5 series sensor chip and subsequently loaded with FLAG-tagged RING domains of MDM2. Relative units were measured with various doses of compounds. Data are normalized to an antibody only surface to exclude MDM2-independent effects.

Fig. 4. (A) U2OS cells were cotransfected with p53, Mdm2 and HIS-ubiquitin, 20 h after transfection, cells were treated with DMSO, Nutlin or the indicated MPD compounds (10 μM each) and MG132 and harvested and lysates were precipitated with HIS-tag isolation Dynabeads and western blotted for p53. The input level of p53, MDM2 and actin was assessed by western blotting. (B) Retinal pigment epithelial cells were treated with DMSO, Nutlin (10 μM), MPD47 (10 μM), MPD20, 37, 39, 159 or 165 at 2.5 μM, 5 μM and 10 μM for 16 h. MDM2, p53 and p21 were detected by western blotting using AB1, AB2, DO1 and p21 antibodies. Actin was used as loading control. (C) U2OS GFP-MDM2 TetOn cells were induced with doxycycline for 48 h. Cells were next treated with DMSO, indicated MPD compound or MG132 for 4 h. Cells were then harvested, fixed with 0.05% paraformaldehyde, rehydrated and stained with propidium iodine for flow cytometric analysis. Sub G1 cells were excluded from analysis and mean GFP signal was quantified for each condition. Error bars represent standard error of 3- to 9-independent experiments.
p53 dependent (Figure 6A), although loss of p53 revealed a modest increase in cells with a G2 DNA content after treatment with compound 20. Further analysis of possible p53-independent activities of the MPD compounds showed clearly that compounds such as 37 that were most active in vitro efficiently induced p53-independent cell death (Figure 6B).

Discussion

For tumors that retain wild-type p53, inhibition of MDM2 provides an attractive therapeutic strategy for several reasons. First, mouse models have shown that reactivation of wild-type p53 is effective in causing tumor regression and second, following restoration of p53, there is tumor cell-specific killing without toxicity to normal cells (17–19). To date, Nutlin, the inhibitor of p53-MDM2 interaction, is the MDM2 inhibitor at the most advanced stage in development (21). Recently, results of a phase I study of a Nutlin derivative in hematological malignancy have been presented (37). Encouragingly, maximum tolerated dose was yet to be reached and even at doses below maximum tolerated dose, responses had been observed.

We have chosen to take an alternative approach to MDM2 inhibition by identifying and characterizing inhibitors of the E3 ligase activity of MDM2 (29,30,38). Clearly the licensing of the proteasome inhibitor bortezomib for treatment of multiple myeloma has demonstrated that targeting the ubiquitin proteasome system is feasible (39). In the case of MDM2 inhibition, we have an added degree of specificity since the substrate specificity of the ubiquitination reaction is determined by the E3 ligase (40). This should allow a more targeted stabilization of p53, which should minimize off-target side effects. Indeed, we show in this study that the MPD compounds show some selectivity of inhibition of MDM2 compared with Cbl, a similar RING domain E3 ligase.

The MPD compounds have been shown to inhibit both MDM2 autoubiquitination and p53 ubiquitination. This has raised concerns that the continued binding of MDM2 and p53 could prevent p53 from mounting its full transcriptional program. Reassuringly, our present data and data previously published on the original HLI98 compounds...
show that despite an increase in MDM2 expression, p53 stabilization by these compounds is able to cause a cell cycle arrest (29). While we found a good correlation between the activity of the compounds in the in vitro assay and their ability to inhibit ubiquitination and stabilize p53 in cells, compounds that were most active in vitro did not necessarily retain this relative activity in cells. This may reflect differential ability to be taken up by cells and remain active, but we also found that the compounds that were most active in vitro were more toxic to cells. The effect on p53-dependent G\textsubscript{1} arrest therefore reflects a combination of the ability of the compounds to activate p53 and the non-specific p53-independent toxicity. Furthermore, mouse models in which the MDM2 RING domain is mutated show a phenotype very similar to those in which MDM2 is completely deleted, suggesting that the E3 activity is critically important to regulate p53 (41). Interestingly, ARF has been shown to selectively inhibit MDM2’s activity toward p53 while allowing MDM2 autoubiquitination and degradation (42,43). This raises the possibility that small molecule mimetics of ARF might retain the degradation of MDM2 and other groups have reported small molecules that selectively inhibit p53 ubiquitination without affecting MDM2 autoubiquitination (44,45). Both ARF and L11 function by binding to the acidic domain of MDM2 and may disturb a second p53/MDM2 interaction domain (46,47). However, we show that the MPD compounds are able to target the isolated RING domain of MDM2 and show that the active compounds can bind directly to the RING domain, thus providing insight into their mechanism of action. Further studies will show whether this drug/RING domain interaction disrupts binding of E2 to MDM2 or prevents dimerization of MDM2 with either itself or MDMX (48–50).

Our results show that targeting the MDM2 RING is feasible and effective at inhibiting the E3 ligase activity of MDM2. Although the MPD compounds lack the problematic nitro group of the original HL198 class, they clearly still retaining E3 ligase inhibitory activity. However, this group of compounds still has limitations in terms of poor solubility, relatively low potency and (for the most active compounds) clear p53-independent activities. While demonstrating a proof of concept—that a drug targeting the RING domain of MDM2 can lead to p53 activation—it seems likely that 5-deazaflavin derivatives with better pharmacological and pharmaceutical properties, or a different class of compounds, will be needed before this approach can then be tested more extensively in vivo.

Fig. 6. (A) RKO cells either wild-type or null for p53 were treated with DMSO, 5 μm of compound 39 or 20 or 10 μm Nutlin for 16 h. Cells were then harvested and propidium iodide (PI) stained for cell cycle analysis by fluorescence-activated cell sorting (FACS). Experiments were performed at least in triplicate. Error bars show the standard error. (B) RKO cells either wild-type or null for p53 were treated with DMSO or 5 μm of compound 37 for 16 h. Cells were then harvested and PI stained for cell cycle analysis by FACS. Experiments were performed at least in triplicate. Representative histograms are shown.

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**References**