Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells

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Summary

The p53 tumor suppressor protein is regulated by its interaction with HDM2, which serves as a ubiquitin ligase (E3) to target p53 for degradation. We have identified a family of small molecules (HLI98) that inhibits HDM2's E3 activity. These compounds show some specificity for HDM2 in vitro, although at higher concentrations effects on unrelated RING and HECT domain E3s are detectable, which could be due, at least in part, to effects on E2-ubiquitin thiol-ester levels. In cells, the compounds allow the stabilization of p53 and HDM2 and activation of p53-dependent transcription and apoptosis, although other p53-independent toxicity was also observed.

Introduction

Regulation of protein stability through ubiquitin-dependent proteolysis plays important roles in controlling the function of many proteins, including critical regulators of cell proliferation and apoptosis (Clarke, 2002; Yang and Yu, 2003). This proteolytic system depends on the conjugation of ubiquitin onto primary amino groups on substrate proteins, with appropriately linked polyubiquitin chains serving as targeting signals for proteasomal degradation. Ubiquitylation occurs through a multienzyme cascade, where recognition is specified by the ubiquitin ligase (E3). Numerous E3s have been identified, including a large family of proteins that contain a structural motif known as the RING finger (Fang and Weissman, 2004). RING finger E3s mediate the direct transfer of ubiquitin from the ubiquitin conjugating enzyme (E2) to substrate, and in some cases these E3s have also been shown to regulate their own stability through autoubiquitylation (Fang et al., 2000; Fang and Weissman, 2004).

The tumor suppressor protein p53 plays an important role in preventing cancer development by inhibiting the proliferation of cells undergoing tumorigenic stress, such as DNA damage or oncogene activation (Vousden and Lu, 2002). Although the ability of p53 to induce apoptosis plays an important role in tumor suppression (Schmitt et al., 2002; Symonds et al., 1994), additional p53 activities are also important in regulating tumor development (Fazeli et al., 1997; Liu et al., 2004; Ryan et al., 2004). p53 functions both as a transcription factor (Vogelstein et al., 2000) and through transcriptionally independent mechanisms (Chipuk and Green, 2003). Many p53-inducible genes have been identified that play a role in mediating the different responses to p53. The choice of response to p53 depends, at least in part, on which p53-responsive genes are activated following induction of p53 (Vousden and Lu, 2002). There is evidence that transformed cells are more sensitive to p53-induced apoptosis than their normal counterparts, leading to the suggestion that activation of p53 may cause tumor-specific cell killing. As such, activation of the p53 response becomes an attractive therapeutic goal (Lane and Lain, 2002).

The importance of p53 in tumor suppression is highlighted by the observation that almost all human cancers show evidence for loss of normal p53 function. In about half of all cancers, this occurs through mutation within the *p53* gene, leading to the expression of a mutant p53 protein that is defective for growth and tumor inhibition (Selinova, 2001). In many of the cancers that retain wild-type p53, there is evidence for defects

SIGNIFICANCE

Many tumors that retain wild-type p53 show evidence of alterations that prevent efficient activation of p53 in response to stress, linked to a failure to inactivate HDM2. In these tumors, inhibition of HDM2 and reactivation of p53 is an attractive therapeutic strategy. While many chemotherapeutics stabilize p53, the HLI98 compounds, which directly target HDM2's ubiquitin ligase activity, should function to reactivate p53 without accompanying deleterious genotoxic damage that contributes to the toxicity of current therapeutic drugs. Our data establish ubiquitin ligases such as HDM2 as viable targets for drug discovery, and identify a family of compounds that may provide a structural basis for generation of drugs that could be used in the treatment of tumors retaining wild-type p53.

in the mechanisms that allow activation of p53 (Vousden, 2002). The strong growth-suppressive activities of p53 mean that p53 must be kept tightly regulated to allow normal cell growth, and this is achieved to a large extent by regulating the stability of the p53 protein. One of the principal E3s responsible for targeting the degradation of p53, and keeping p53 levels low during normal growth and development, is HDM2 (Mdm2 in mice) (Michael and Oren, 2003). Since *HDM2* is a transcriptional target of p53, a feedback loop exists in which p53 drives expression of the protein that downregulates p53 activity. In response to oncogenic stress, the degradation of p53 by HDM2 is inhibited, leading to a rapid increase in p53 protein levels in the cell. Several pathways leading to the inhibition of HDM2 have been described, including the activation of DNA damage-induced kinases that phosphorylate p53 and HDM2 (Ljungman, 2000; Meek and Knippschild, 2003), and the interaction of HDM2 with proteins involved in ribosome assembly or function; these include ARF, L11, L23, and L5 (Dai and Lu, 2004; Dai et al., 2004; Jin et al., 2004; Lohrum et al., 2003; Sherr and Weber, 2000; Zhang et al., 2003). The binding of these small proteins to HDM2 does not inhibit the interaction of HDM2 with p53, but appears to block the ubiquitylation and degradation of p53. These HDM2 binding proteins can also prevent HDM2 autoubiquitylation in in vitro assays. Defects in the pathways that control the stabilization and activation of p53 in response to stress can contribute to cancer development, without the requirement for mutation within the *p53* gene itself. We have therefore sought to identify small molecule inhibitors of HDM2's E3 activity that may have efficacy in activating p53 in those tumors that retain wild-type p53.

Results

High-throughput screening for E3 ligase inhibitors

Using a previously described high-throughput assay developed for the identification of ubiquitin ligase inhibitors (Davydov et al., 2004), we screened libraries of small molecules for inhibition of HDM2 autoubiquitylation. A library of 10,000 compounds was screened in duplicate, yielding forty compounds that inhibited HDM2 autoubiquitylation by more than 50%. These compounds were further tested in previously described in vitro gel-based assays (Fang et al., 2000) that identified at least four compounds that significantly inhibited E2-dependent HDM2 autoubiquitylation. Three of the compounds, 10-(3-chloro-phenyl)-7-nitro-10H-pyrimido[4,5-b]quinoline-2,4-dione (HLI98C), 10-(4-chloro-phenyl)-7-nitro-10H-pyrimido[4,5-b]quinoline-2,4-dione (HLI98D), and 10-(4-methyl-phenyl)-7-nitro-10H-pyrimido[4,5-b]quinoline-2,4-dione (HLI98E), comprise a family of closely related 7-nitro-5-deazaflavin compounds (Figure 1A). A fourth compound, anthra[1,2-c][1,2,5]oxadiazole-6,11-diol (GLI97H), showed a distinct structure. Using the plate-based assays, IC_{50}s for inhibition of HDM2 autoubiquitylation were determined to be ~20 μM for each of the three 7-nitro-5-deazaflavin compounds (Figure 1B and data not shown).

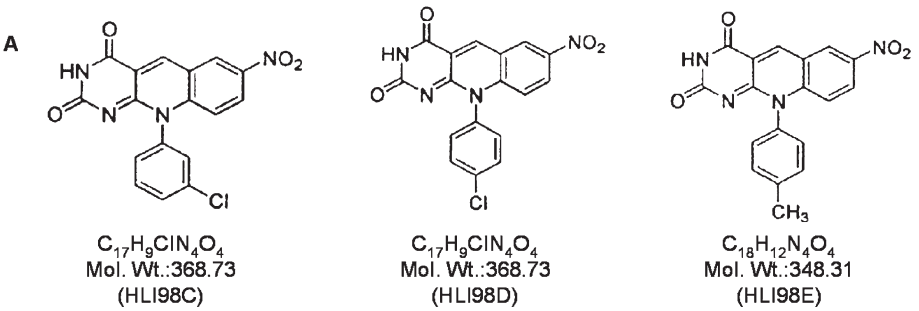
In vitro evaluation of inhibitors

To determine whether these compounds have the capacity to inhibit ubiquitylation of p53 by HDM2, p53 was induced by doxocycline in SAOS cells stably transfected with p53 cDNA under the control of Tet-responsive promoter (Nakano et al., 2000). GST-HDM2 that had been bound to glutathione-Sepharose beads was incubated with cell lysates, unbound material removed, and ubiquitylation reactions carried out in the presence of the compounds (Figure 2A). Both HLI98C and the less potent HLI98D exhibited dose-dependent inhibition of p53 ubiquitylation that becomes apparent for HLI98C at 20–50 μM. To evaluate the specificity for HDM2 relative to other E3s, we assessed the inhibitory effects of HLI98 compounds against both HDM2 and Nedd4, a structurally distinct HECT domain E3 (Figure 2B). GLI97H dramatically blocked both HDM2 and Nedd4 activity. This effect is due to irreversible inactivation of both E1 and E2 (data not shown)—a feature not shared with the HLI98 compounds. The HLI98 series also displayed inhibition of both Nedd4 and HDM2 at 100 μM, although the inhibition of HDM2 was more prominent (Figure 2B and data not shown). Using a different synthesis of HLI98C (Figure 2C), dose-dependent inhibition of both HDM2 and Nedd4 was observed, with HDM2 inhibition manifest at a lower concentration (10 μM compared to 50 μM for Nedd4). On the other hand, another RING finger E3, Siah1, showed little evidence for inhibition by HLI98C under the same conditions, although some inhibition of Siah1 was observed in other experiments where less Siah1 was used (data not shown). The difference in inhibition of HDM2 in Figures 2B (100%) and 2C (89%), is reflective of variation in the range of both E3 activity and inhibition in these semiquantitative in vitro assays. While screening was carried out using the full-length HDM2 molecule, the RING finger by itself is inhibited by HLI98C to a similar degree as the full-length protein (Figure 2D); thus, more N-terminal regions, including those involved in interactions with p53, are dispensable for the function of this inhibitor. Since the RING finger is all that is required for the inhibition of HDM2 E3 activity, and the same regions of E2s that interact with HECT E3s interact with RING fingers, this may provide an explanation for the overlap in inhibition between two disparate classes of E3s. Unfortunately, stable binding of HDM2 to E2 has yet to be reproducibly observed; thus, formal testing of effects of the HLI98 series on this binding is currently problematic.

Not only are interaction sites on E2s shared between HECT and RING finger E3s, but the regions on E2s that bind RING fingers and HECT domains are also crucial for interactions of E2s with E1. Thus, it might be the case that the HLI98s also inhibit the transfer of ubiquitin from E1 to E2. Consistent with this, HLI98C also shows some inhibition of E2-ubiquitin thiolester linkages (Figure 2E). Overall, these results suggest that there is some selectivity for the HLI98 compounds in the inhibition of HDM2 E3 activity, but that an effect on unrelated RING and HECT domain E3s could also be measured. This could be due, at least in part, to effects on loading of E2 with ubiquitin.

Activity of the HLI98 inhibitors in cells

The in vitro evidence suggests that the HLI98 family compounds could function as E3 inhibitors, with at least some degree of specificity for HDM2. To determine whether these effects are manifest in cells, we initially tested all 40 compounds identified in the high-throughput screen for effects on cellular p53 and HDM2. Primary human fibroblasts were treated with 50 μM of each compound for 6 hr, after which levels of p53 and HDM2 were determined by Western blotting (an example of this is shown in Figure 3A). Of the 40 compounds tested, the HLI98 series exhibited the greatest ability to increase p53 and HDM2 protein levels, functioning with efficiency similar to



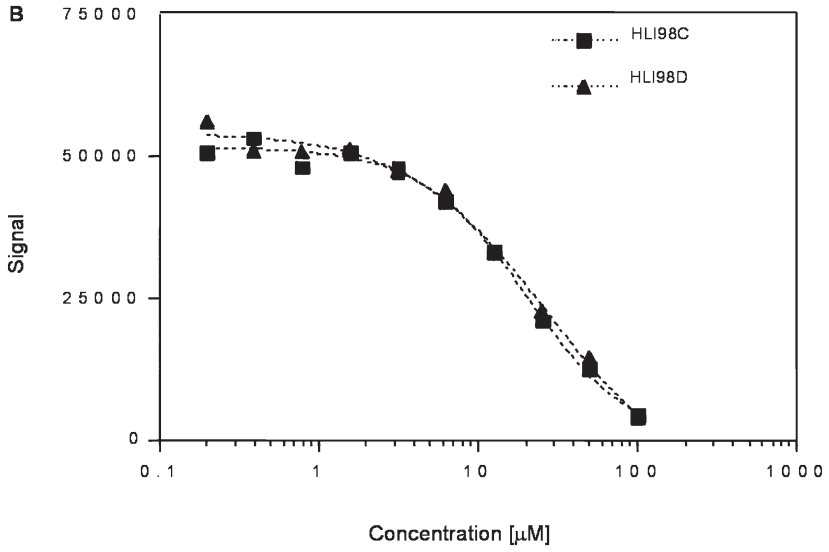


Figure 1. Small molecule inhibitors of HDM2

A: The structures of 10-(3-chloro-phenyl)-7-nitro-10H-pyrimido[4,5-b]quinoline-2, 4-dione (HLI98C), 10-(4-chloro-phenyl)-7-nitro-10H-pyrimido[4,5-b] quinoline-2,4-dione (HLI98D) and 10-(4-methyl-phenyl)-7-nitro-10H-pyrimido[4,5-b]quinoline-2, 4-dione (HLI98E).

B: Dose-dependent inhibition of HDM2 auto-ubiquitylation by HLI98C and HLI98D.

that seen with MG132, a peptide aldehyde proteasome inhibitor. A direct comparison of each of the compounds with another peptide aldehyde proteasome inhibitor (ALLN) and adriamycin, a DNA-damaging chemotherapeutic agent known to induce stabilization of p53, showed that each efficiently increased cellular p53 (Figure 3B). As expected, the HLI98 compounds and ALLN also inhibited the degradation of HDM2, leading to an increase in HDM2 levels. However, treatment of cells with adriamycin led to the stabilization of p53 without increasing the expression of HDM2, reflecting the increase in HDM2 autodegradation in response to DNA-damage induced kinase activation (Stommel and Wahl, 2004). Characteristic of proteasome inhibitors, treatment with ALLN resulted in accumulation of p53 primarily in its nonubiquitylated form, but with clear evidence of a characteristic ladder of higher molecular weight forms of the protein representative of ubiquitylation (Figure 3B). In contrast, and consistent with the predicted mechanism of action in inhibiting ubiquitylation rather than proteasome function, no evidence for ubiquitylated p53 was detected with the HLI98 compounds. Adriamycin, which reduces expression of HDM2, also showed p53 accumulation without ubiquitylation (Figure 3B).

An increase in both p53 and HDM2 is the predicted effect of an HDM2 inhibitor. However, a possibility that must be excluded is that the increase in HDM2 is a result of p53-dependent transcriptional activation of *HDM2*. To rule out this possibility, *p53/mdm2* null mouse embryo fibroblasts were transiently transfected with plasmid encoding HDM2 driven by a p53-independent CMV promoter. As expected, adriamycin had no effect on the level of transfected HDM2 (Figure 3C). However, both proteasome inhibition (with ALLN) and treatment with HLI98 compounds significantly increased HDM2 levels (Figure 3C). These findings indicate that the compounds can lead to an accumulation of HDM2 by inhibition of HDM2 autoubiquitylation, as well as through the activation of p53.

Specificity of the HLI98 compounds in cells

As in vitro assays showed only relative selectivity of the HLI98s toward HDM2, and potential for inhibition at the level of E2-ubiquitin thiol-ester bond formation, several approaches were undertaken to assess their specificity in cells. In vitro studies showed that the compounds more efficiently inhibited HDM2 E3 activity than that of the unrelated RING finger E3 Siah1. The stabilizing effect of HLI98C and HLI98D on HDM2 and Siah1 in cells was therefore examined in transfected *p53*$^{-/-}$ *mdm2*$^{-/-}$ cells (Jones et al., 1995). Under these conditions, no stabilization of Siah1 was observed with any of the HLI98 compounds, whereas a RING finger mutant form of Siah1 was stable even without proteasome inhibitor (Figure 4A and data not shown).

To further examine the effect of the compounds on E3 activ-

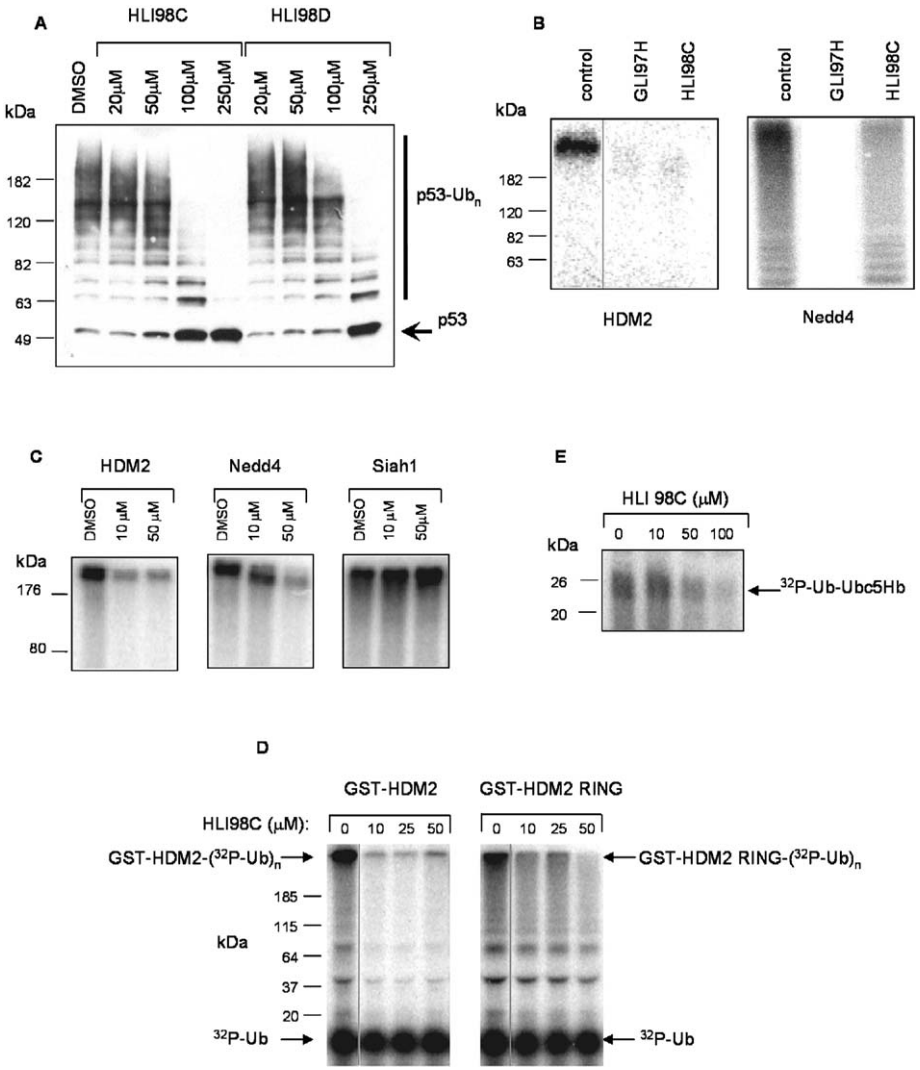


Figure 2. Inhibition of HDM2-mediated ubiquitylation by HLI98 compounds in vitro

A: Inhibition of p53 ubiquitylation by HLI98C and HLI98D. Transfected *p53* expressed in SAOS cells after induction with doxocycline was bound to GST-HDM2 and excess material removed by washing. Ubiquitylation reactions were then carried out in the presence of indicated concentrations of HLI98C and HLI98D. Ubiquitylated p53 was detected by Western blotting using the anti-p53 antibody DO-1.

B: Inhibition of HDM2 and Nedd4 autoubiquitylation in a gel-based assay. Autoubiquitylation of HDM2 and Nedd4 was detected using ^{32}P-labeled ubiquitin. 20 μM HLI98C and 50 μM GLI97H were used.

C: Evaluation of relative inhibition of HDM2, Nedd4, and Siah 1 using a gel-based assay and ^{32}P-labeled ubiquitin as in **B**.

D: Inhibition of GST-HDM2 and GST-HDM2 RING finger autoubiquitylation. Assays were carried out as in **B** and **C**.

E: The effect of HLI98C on the formation of E1-dependent E2 thiol-ester conjugates with ubiquitin.

ity in cells, expression of the RING finger E3 Cbl-b was examined. Autoubiquitylation and degradation of Cbl-b is seen following stimulation of cells with EGF, a response that requires the E3 activity of Cbl-b and is blocked by MG132 (Ettenberg et al., 2001) (Figure 4B). However, treatment of cells with HLI98D failed to prevent EGF-induced Cbl-b loss, suggesting that the HLI98 inhibitors do not block Cbl-b ubiquitin ligase activity. In similar experiments, the HLI98 compounds also failed to block TNFα-induced ubiquitin-dependent degradation of IκBα (Chen et al., 1995b) under conditions where an E1 inhibitor under development did inhibit IκBα degradation (Y.Y. and A.M.W., unpublished observations). Taken together, these studies suggest that the HLI98 compounds show specificity in vivo, inhibiting the autoubiquitylation of HDM2 more efficiently than the activity of other RING finger E3s.

To determine whether the compounds also inhibit the degradation of other proteins regulated by ubiquitylation, we evaluated the effect of the compounds on the stability of endogenous p21$^{WAF1/CIP1}$ (Figure 4C), a protein targeted to the proteasome by both ubiquitin-dependent and ubiquitin-independent mechanisms (Bendjennat et al., 2003; Bloom et al., 2003; Coulombe et al., 2004; Sheaff et al., 2000). As expected, proteasome inhibition by treatment with ALLN resulted in the stabilization of HDM2, p53, and p21$^{WAF1/CIP1}$. In contrast, treatment of cells with adriamycin resulted in stabilization of p53, but had no effect on levels of either HDM2 or p21$^{WAF1/CIP1}$. It should be noted that the failure of adriamycin to elevate levels of HDM2 or p21$^{WAF1/CIP1}$ protein in these wild-type p53 expressing cells is a reflection of the early time point (6 hr) following treatment at which these cells were evaluated. Since both *HDM2* and *p21$^{WAF1/CIP1}$* are transcriptional targets of p53, stabilization of p53 by adriamycin leads to the transcriptional activation of both *HDM2* and *p21$^{WAF1/CIP1}$* (Figure 6B), which is seen as an increase in protein levels at later time points. The absence of stabilization of p21$^{WAF1/CIP1}$ further supports a degree of selectivity of the compounds for HDM2 E3 activity. In addition to HDM2, other E3s such as Pirh2 (Leng et al., 2003) and COP1 (Dornan et al., 2004) can target p53 for degradation. Since the HLI98 compounds can potentially inhibit E3s other than Mdm2 we examined the contribution of Mdm2 expression to the ability of these compounds to stabilize p53 by comparing *p53* null or *p53/mdm2* null mouse embryo fibroblasts that

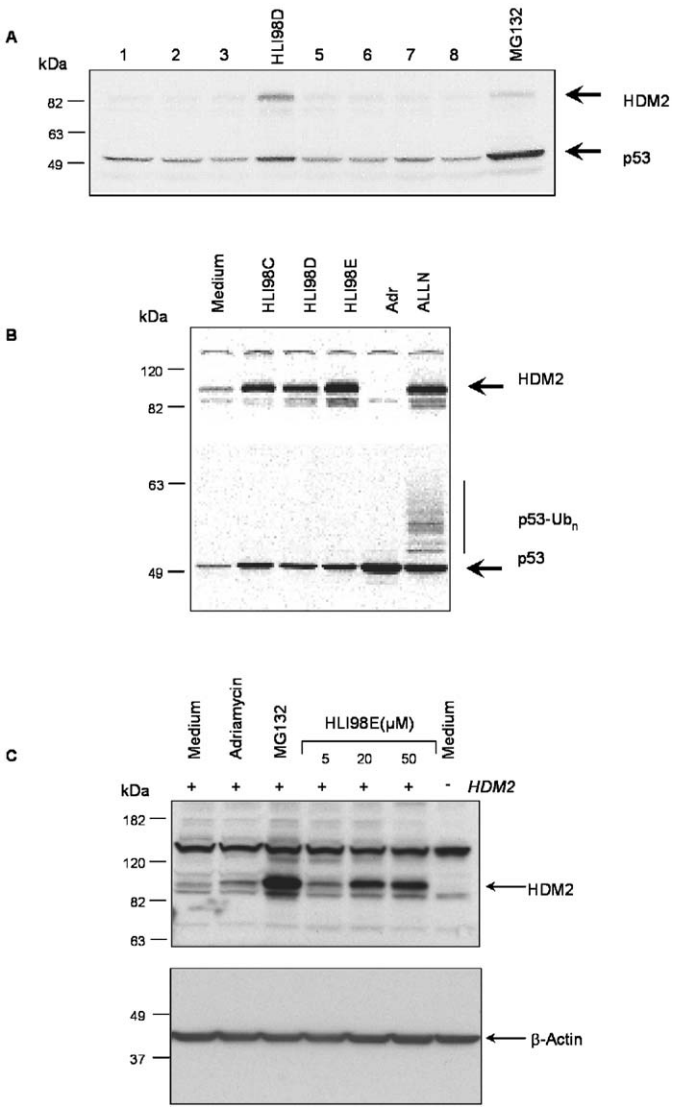


Figure 3. HLI98 family compounds accumulate p53 and HDM2 in cells

A: Screening for compounds that increase p53 and HDM2 in cells. Hits from high-throughput screening were added to cultured human MRC5 fibroblasts for 6 hr at a final concentration of 50 μM. Cells treated with 50 μM MG132 were used as a control. HDM2 and p53 in the cells were examined by Western blotting with anti-HDM2 and anti-p53 antibodies.

B: Human RPE cells were treated with 20 μM HLI98C, 50 μM HLI98D, 50 μM HLI98E, 1 μg/ml adriamycin, and 50 μM ALLN for 6 hr. HDM2 and p53 in cells were assessed by Western blot using anti-HDM2 and anti-p53 antibodies.

C: Fibroblasts from *p53*$^{-/-}$*mdm2*$^{-/-}$ mice were transfected with HDM2 cDNA under the control of a CMV promoter. After 24 hr, the cells were treated with the indicated concentrations of HLI98E, 50 μM MG132, or 0.2 μg/ml adriamycin for 8 hr, and the level of HDM2 was determined by Western blot. Expression of β-actin was monitored as a control for loading.

had been transfected with p53. In the cells expressing Mdm2, both HLI98C and adriamycin led to p53 stabilization (Figure 4D, left panels). By comparison, in cells lacking Mdm2, the basal levels of p53 were significantly higher, as previously described (Kubbutat et al., 1997). Adriamycin treatment could further stabilize p53, despite the absence of Mdm2, consistent with a contribution of inhibition of Pirh2 or COP1 in the stabilization of p53 in response to DNA damage. However, HLI98C treatment showed little stabilization of p53 in the absence of Mdm2, supporting a selectivity for inhibition of Mdm2 activity.

Finally, to determine whether the compounds could affect the degradation of p53 mediated by E3s other than HDM2, an isogenic cell system was utilized. In this system, p53 degradation is mediated either by HDM2 (RKO cells) or by stably expressed HPV-E6 from an oncogenic strain of HPV that functions with the cellular HECT domain E3, E6-AP (RKO/E6 cells) (Scheffner et al., 1993) (Figure 4E). In this system, proteasome inhibitor resulted in the stabilization of p53 regardless of which E3 was targeting degradation. When HLI98C was evaluated, selective stabilization of HDM2-mediated p53 loss was observed at 10 μM, although when the concentration was increased to 20 μM, evidence of inhibition of E6-AP-mediated degradation was also observed. Collectively, these results are consistent with the in vitro data in demonstrating relative selectivity for HDM2, but as demonstrated in Figure 4E, there is clearly the potential to inhibit a HECT E3, in this case E6-AP.

Recently, small molecules that can inhibit the HDM2-mediated degradation of p53 by blocking the HDM2-p53 interaction have been described (Issaeva et al., 2004; Vassilev et al., 2004). Although the HLI98 compounds were selected for their ability to inhibit HDM2 directly, and also inhibit the isolated HDM2 RING finger (Figure 2D), we wished to confirm that these compounds do not interfere with the binding of HDM2 to p53. Both transfected p53 and transfected HDM2 were stabilized by treatment with MG132 or HLI98C (Figure 5A, lower two panels). HDM2 was found to be efficiently coimmunoprecipitated with Flag-p53 in cells cotransfected with p53 and HDM2 and treated with either MG132 or HLI98C (Figure 5A). These results are consistent with a role for the HLI98 compounds that does not involve inhibition of the p53-HDM2 interaction.

Many drugs that induce p53 stabilization also activate stress responsive kinases that lead to N-terminal phosphorylation of p53 (Xu, 2003). These phosphorylation events have been proposed to play a role in allowing both the stabilization and activation of p53. The induction of p53 in response to both DNA damage and oxidative stress is accompanied by phosphorylation of p53 on serine 15 (Chen et al., 2003; Shieh et al., 1997; Siliciano et al., 1997). As shown previously, this modification can be detected, using a serine 15 phosphospecific antibody, in response to camptothecin treatment (Figure 5B), but not in p53 stabilized by proteasome inhibition (MG132) or by treatment with low levels of actinomycin D (Ashcroft et al., 2000). Importantly, treatment of cells with the HLI98 compounds also failed to stimulate serine 15 phosphorylation. Furthermore, while the presence of the nitro group in each of the HLI98 compounds raises some possibility for the activation of oxidative stress, we have recently synthesized and tested a number of HLI98 analogs lacking this nitro group that show no reduction in the ability to stabilize p53 in cells (D. Robins, G. Henderson, J. Wilson, R.L.L., and K.H.V., unpublished observations). Taken together, these results indicate that the means by which these compounds stabilize p53 and increase HDM2 levels is not through genotoxic or oxidative stress.

Induction of p53 transcriptional activity in response to treatment with the inhibitors

One of the principal activities of p53 is as a transcription factor, inducing expression of a number of genes that can contribute

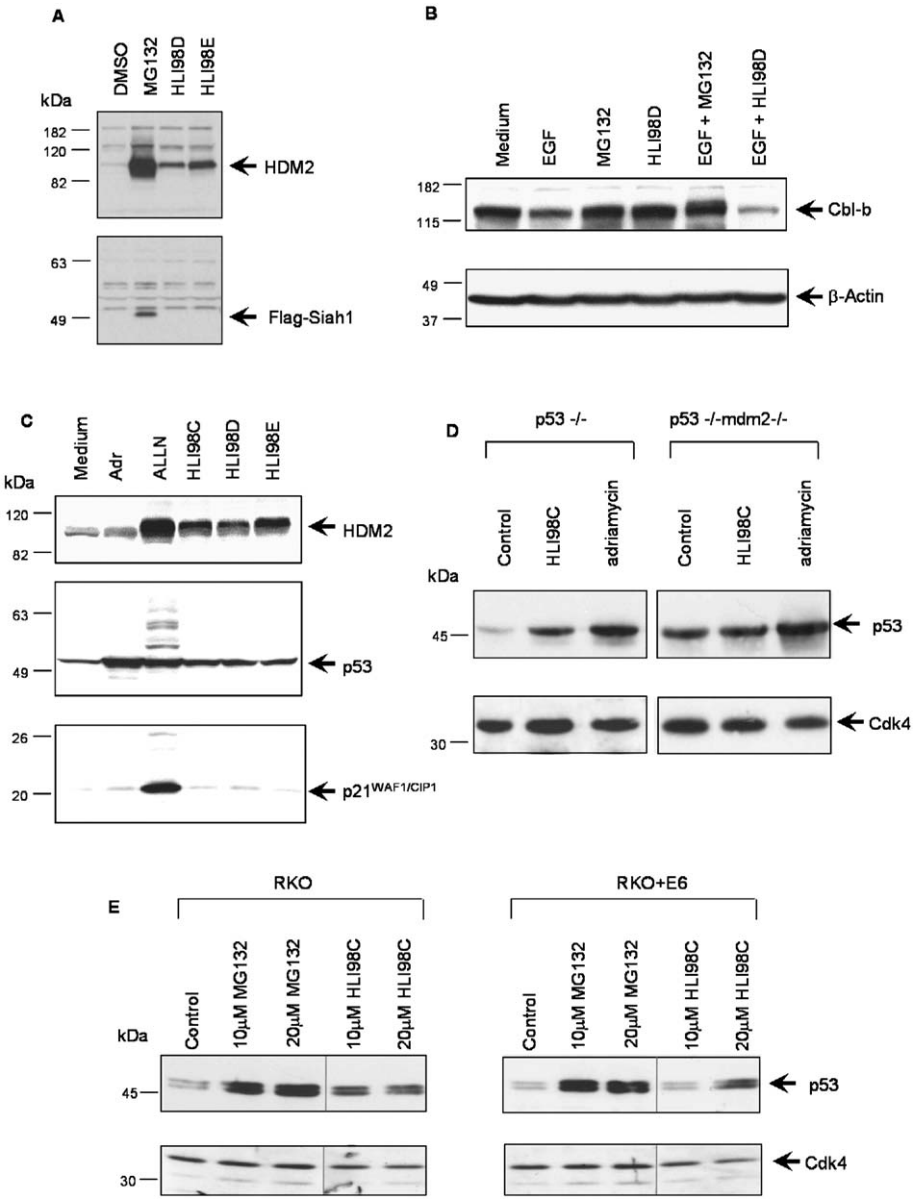


Figure 4. HLI98 family compounds show specificity toward HDM2-mediated ubiquitylation

A: Fibroblasts from *p53*$^{-/-}$*mdm2*$^{-/-}$ mice were transfected with cDNAs encoding HDM2 or Siah1. Twenty-four hours after transfection, cells were treated with 50 μM HLI98D, HLI98E, or MG132 for 8 hr. The levels of HDM2 and Siah1 were determined by Western blot using anti-HDM2 and anti-Flag antibodies.

B: MDA-MB-468 cells stably expressing Cbl-b were first treated with 50 μM MG132 or 50 μM HLI98D for 30 min. 100 μg/ml EGF was then added to the cultures as indicated for 2 hr. The levels of Cbl-b and β-actin in cells were assessed by Western blotting with anti-Cbl-b and anti-β-actin antibodies.

C: p21$^{WAF1/CIP1}$ degradation was not blocked by HLI98 family compounds. RPE cells were treated with 0.2 μg/ml adriamycin, 50 μM ALLN, and 20 μM HLI98C or 50 μM HLI98D or HLI98E for 6 hr. Cellular HDM2, p53, and p21$^{WAF1/CIP1}$ were analyzed by immunoblotting with specific antibodies.

D: Fibroblasts from *p53*$^{-/-}$ or *p53*$^{-/-}$*mdm2*$^{-/-}$ mice were transfected with p53 and treated with 20 μM HLI98C or 0.2 μg/ml adriamycin. Levels of p53 and Cdk4 were determined after 16 hr by Western blotting.

E: Carcinoma cell line RKO and RKO cells expressing E6 from oncogenic HPV were treated with 10–20 μM of MG132 or 10–20 μM of HLI98C for 6 hr, as indicated. p53 and Cdk4 levels were determined by Western blotting.

to the cell cycle arrest and apoptotic response. To test the ability of the HLI98 compounds to activate p53-dependent transcription, we made use of a cell line expressing endogenous wild-type p53 and a luciferase reporter gene under the transcriptional control of a synthetic p53-responsive promoter (Figure 6A). Treatment of these cells with the HLI98 compounds resulted in a reproducible increase in luciferase induction, albeit substantially less than that seen with adriamycin (Figure 6A). This weaker activation of p53-dependent transcription in response to the HLI98 compounds correlated to some extent with a lower induction of p53 protein levels compared to that seen with adriamycin (data not shown). We next examined the effect of the HLI98 compounds on the transcription of endogenous p53 target genes in cells expressing wild-type p53 (Figure 6B). The transcription of both the cell cycle arrest p53 target gene *p21*$^{WAF1/CIP1}$ and the apoptotic p53 target gene *PUMA* was efficiently induced in response to treatment of cells with HLI98C, to an extent similar to that seen following treatment with camptothecin (Figure 6B). This activation of *p21*$^{WAF1/CIP1}$ expression by two independent syntheses of HLI98C is also seen at the protein level (Figure 6C). These results indicate that in cells, the stabilization of p53 by the HLI98 compounds results in an activation of p53-dependent transcription.

Induction of p53-dependent apoptosis

The ability of p53 to induce apoptosis is likely to play an important role in tumor suppression. A well-established system for measuring p53-dependent cell death is in mouse embryo fibroblasts (MEFs) transformed with the adenovirus E1A protein and activated Ha-ras (Lowe et al., 1993). Analysis of caspase activation in cells following treatment with the HLI98 compounds showed an efficient induction of DEVDase activity in the 5–20

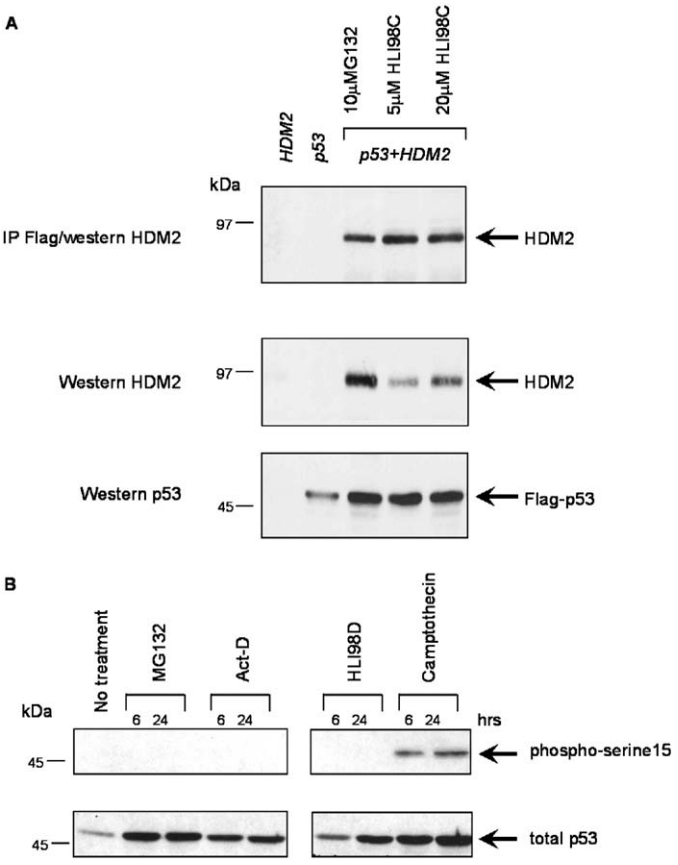


Figure 5. Effects of the HLI98 compounds on p53/HDM2 interaction and p53 phosphorylation

A: HLI98C does not inhibit p53/HDM2 interaction. U2OS cells were transfected with *HDM2* and *Flag-p53*, then treated with HLI98C or MG132 for 6 hr as indicated before harvesting. p53/HDM2 complexes were immunoprecipitated with an anti-flag antibody, and the amount of HDM2 in the complex determined by Western blotting (top). Total levels of HDM2 and p53 were assessed by Western blotting the lysate before immunoprecipitation (middle and bottom).

B: HLI98 family compounds do not induce phosphorylation of p53. RPE cells were treated with 20 μM MG132, 2.5 μM actinomycin-D, 50 μM HLI98D, and 0.5 μM camptothecin for 6 or 24 hr as indicated. The overall p53 levels were determined by Western blotting with the DO1 antibody, and the extent of serine 15 phosphorylation assessed using a phosphoserine 15-specific anti-p53 antibody.

μM range (Figure 7A). This activity was dependent on p53, since a similar activation was not seen in p53-deficient MEFs expressing Ha-ras and E1A. Similarly, treatment with the HLI98 compounds resulted in p53-dependent PARP cleavage selectively in the p53-expressing cells (Figure 7B). The compounds also induced cell death in a p53-dependent manner as measured by trypan blue exclusion (Figure 7C).

Previous studies have shown that certain oncogenes sensitize cells to p53-induced apoptosis (Lowe et al., 1993). To determine whether this differential is also seen in human epithelial cells (the cell type from which the majority of human cancers arise), we established Tert-immortalized human retinal pigment epithelial cells expressing E1A. Activation of p53 with the genotoxic chemotherapeutic adriamycin induced a cell cycle arrest but little apoptosis in the parental RPE cells. However, as seen previously with MEFs, expression of E1A strongly sensitizes these cells to p53-dependent apoptosis in response to adriamycin (data not shown). Similarly, treatment of these cells with the HLI98 compounds only induced significant apoptosis in the cells expressing E1A (Figure 8A). This death occurred in a dose-dependent manner, with 50% of the cells undergoing apoptosis at 10 μM HLI98C (Figure 8B). These results suggest that the compounds may lead to p53-dependent death selectively in cancer cells. Interestingly, these studies suggest that induction of p53-mediated death in cells is achieved at concentrations of the HLI98 compounds lower that those necessary for clear inhibition of p53 ubiquitylation in vitro (Figure 2A). This may reflect differences in levels of HDM2 or threshold activity of the compounds required to achieve a detectable effect. Evidence for the p53 dependence of the growth-inhibitory effect of the HLI98 compounds was also seen in human tumor cell lines (Figure 8C). Using a colony assay and low concentrations of the compounds, p53-containing tumor cells such as RKO and U2OS showed greater sensitivity to growth inhibition than p53 null cells such as H1299 or RKO cells expressing E6. However, at higher concentrations, the HLI98 compounds inhibited the growth of all cells, regardless of their p53 status, most likely reflecting additional, p53-independent activities of these compounds. To begin to investigate this, we examined the effect of the compounds on cell cycle progression in cells that would not be expected to undergo apoptosis in response to p53. FACS analysis of HCT116 ($p53^{+/+}$ and $p53^{-/-}$) and H1299 cells revealed a complex and variable accumulation of cells in S phase and G2 that was not correlated to p53 expression (data not shown). At higher concentrations of the compounds, p53-independent apoptosis also became evident. In RPE cells, a concentration-dependent difference in response to the compounds could be observed (Figure 8D). At lower concentrations (10 μM HLI98C), where HDM2 is preferentially inhibited, a classic p53 response of G1 arrest is observed. By contrast, at higher concentrations (20 μM HLI98C), the response of a strong G2 arrest is similar to that seen following more general inhibition of ubiquitin-dependent degradation by treatment with MG132. A similar G2 arrest was also seen in these cells in response to treatment with the E1 inhibitor (Y.Y., R.L.L., K.H.V., and A.M.W., unpublished observations). These results clearly show that the compounds can have activities detrimental to cell growth and survival that are independent of p53, and probably reflect off-target activities such as the inhibition of other E3s or perhaps a subset of E2s.

Discussion

Intensive study of the p53 protein has revealed that loss of p53 function is virtually a prerequisite for cancer development and that induction of p53 is likely to lead to tumor cell specific killing, two key observations that have prompted several attempts to identify small molecules that can activate p53 (Haupt and Haupt, 2004). In tumors that retain wild-type p53, these efforts have concentrated on the development of drugs that protect p53 from the inhibitory effects of HDM2. Although other ubiquitin ligases—such as COP1 and Pirh2—can also target p53 (Dornan et al., 2004; Leng et al., 2003), downregulation of HDM2 appears to be sufficient to activate a p53 response in cells and in vivo (Mendrysa et al., 2003). Most attempts to in-

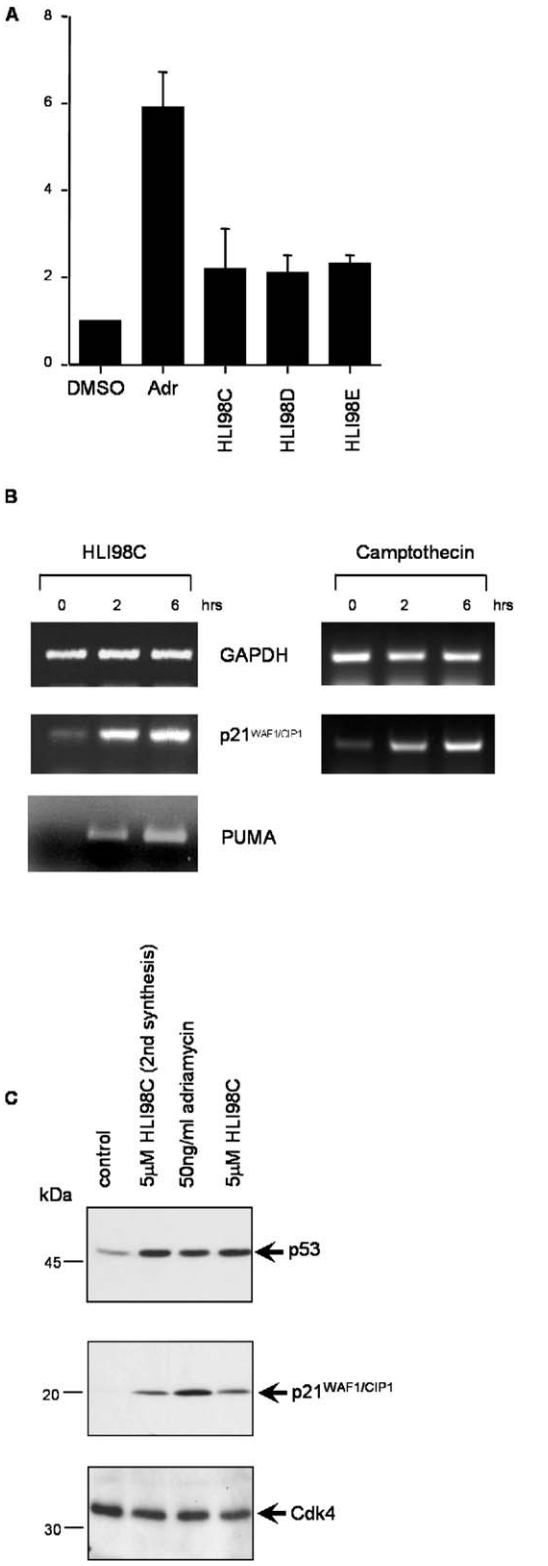


Figure 6. Activation of p53-dependent transcription by the HLI98 compounds

A: HLI98 family compounds activate p53-dependent transactivation. U2OS cells harboring a luciferase gene under the control of p53-response elements were treated with 0.3 μg/ml adriamycin or HLI98 family compounds (20 μM HLI98C, 50 μM HLI98D, and 50 μM HLI98E) for 20 hr and luciferase activity assessed. Data represents averages of three independent experiments. The differences between the DMSO-treated cells, and those treated with adriamycin and HLI98C, D, and E are all significant, with p values of <0.01. There are no significant differences between HLI98C, D, or E.

B: Induction of p53-responsive genes by HLI98 compounds. RPE cells were treated with 20 μM HLI98C or 2 μM camptothecin for the indicated times. Expression of *p21*$^{WAF1/CIP1}$ and *PUMA* was determined by RT-PCR; *GAPDH* expression was monitored as a control for RNA integrity.

C: Induction of p53-reponsive gene expression by two different syntheses of HLI98C. RPE cells were treated as indicated for 24 hr, then levels of p53 and p21$^{WAF1/CIP1}$ protein analyzed by Western blotting. Cdk4 levels were monitored as a control.

hibit HDM2 have concentrated on preventing the interaction with p53, and significant success has been achieved with peptides (Chene, 2003) and more recently with the identification of small molecules such as the Nutlins (Vassilev et al., 2004) and RITA (Issaeva et al., 2004). We have taken an alternative approach, which is to identify small molecule inhibitors of HDM2's E3 activity. A potential concern with this approach is that the binding of HDM2 to p53 can inhibit p53's transcriptional activity, and so elevation of HDM2 and p53 protein levels without perturbing their interaction may not allow full activation of p53. One approach to circumvent this issue has been to identify small molecules with selective ability to inhibit p53 ubiquitylation, without blocking HDM2 autoubiquitylation in vitro (Lai et al., 2002). Our inhibitors, which were selected for their ability to inhibit HDM2 autoubiquitylation, clearly lead to the activation of p53 function despite allowing the stabilization of both HDM2 and p53. There are various potential explanations for this observation, including the possibilities that p53 levels increase more rapidly than HDM2 levels and so allow for the accumulation of free p53, or that the p53/HMD2 complex retains some activity. A recent study has shown that the proteasome inhibitor bortezomib also leads to the stabilization of p53 without impeding the interaction with HDM2, and, as with the HLI98 compounds, this p53 was transcriptionally active (Williams and McConkey, 2003). In vivo validation to this approach is also provided by the identification of several small proteins—such as p14ARF and the ribosomal proteins L11, L5, and L23. These can bind HDM2 and inhibit p53 degradation without directly preventing the HDM2/p53 interaction (Dai and Lu, 2004; Dai et al., 2004; Lohrum et al., 2003; Sherr and Weber, 2000; Zhang et al., 2003).

In addition to the regulation of p53 stability, the ubiquitylation of p53 by HDM2 has also been shown play a role in allowing nuclear export of p53 (Gu et al., 2001; Lohrum et al., 2001). Depending on levels of HDM2, p53 can become monoubiquitylated and exported from the nucleus, or polyubiquitylated and degraded (Li et al., 2003). As expected, the p53 that is stabilized by the HLI98 compounds also remains localized to the nucleus (data not shown). While degradation of p53 clearly inhibits p53 activity, the consequences of nuclear export on p53 function are less predictable. While preventing transcriptional activity, cytoplasmic localization could favor the mitochondrial function of p53 in promoting apoptosis (Mihara et al., 2003). Presumably, inhibition of ubiquitylation by the HLI98 compounds would also reduce nuclear export of p53, although our results suggest that this is not accompanied by a defect in its apoptotic activity.

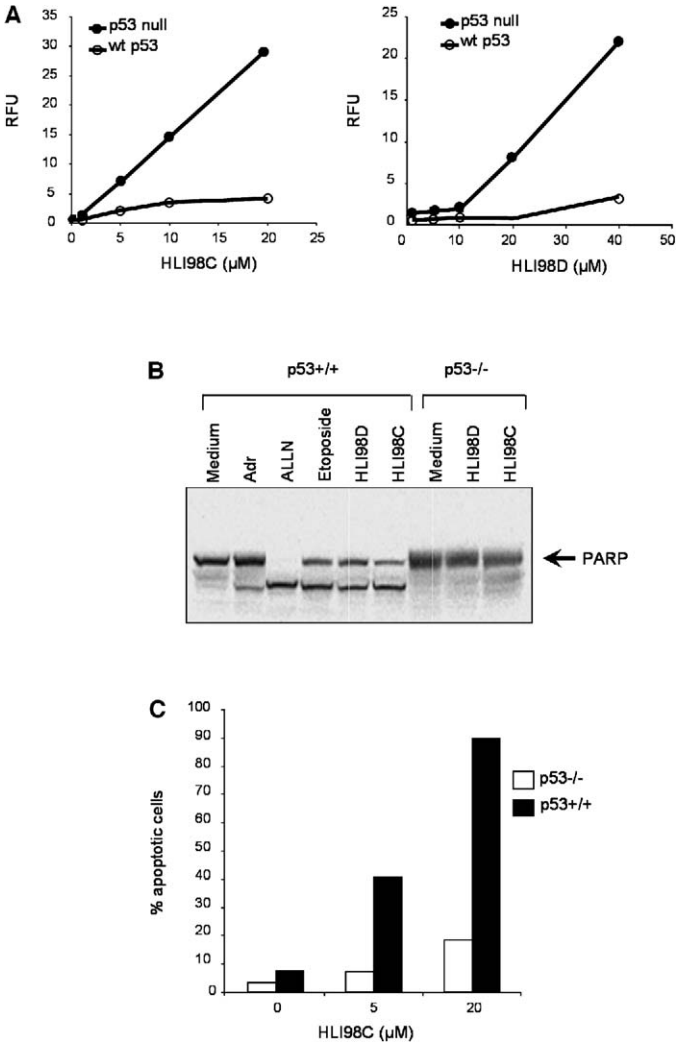


Figure 7. HLI98 family compounds activate caspases in a p53-dependent manner

Wild-type or *p53*-deficient MEFs transformed with E1A and Ha-ras were treated with HLI98 family compounds for 20 hr.

A: Caspase activity measured by FMC-DEVD.

B: Cleavage of PARP following treatment of cells with 20 μM ALLN, 5 μg/ml etoposide, 20 μM HLI98C, and 50 μM HLI98D, revealed by immunoblotting.

C: Cell death measured by trypan blue exclusion.

Although we have concentrated on the analysis of the HLI98 inhibitors on HDM2's ability to degrade p53, it is also possible that these compounds can prevent HDM2 mediated neddylation of p53 (Xirodimas et al., 2004). Further analysis of the mechanism of function of these compounds, particularly an identification of whether and where they interact with HDM2, will be essential in understanding how p53 stability and function can be modulated. The HLI98 compounds described here are relatively insoluble, which has hindered further analysis of their activity in vivo.

While attempts to identify novel drugs for cancer treatment have concentrated mainly on kinase inhibitors, there is much interest in developing the therapeutic potential of modulators of protein stability. Success in clinical trials has been reported for proteasome inhibitors (Adams, 2004), and bortezomib has now been approved for treatment of multiple myeloma, a particularly refractory cancer. This is despite their lack of specificity for any specific protein or family of proteins. The explosion in knowledge of E3s and their many obvious links to disease has spurred considerable interest in targeting E3 activity as a much more specific means of developing therapeutics by altering the stability of critical specific substrates, such as p53. This enthusiasm is, however, tempered by concerns as to whether specific inhibitors can be developed given the similarity among RING fingers, the level of similarity in E2 interaction sites between and among RING finger and HECT domain E3s, and the potential to inhibit E2 loading with ubiquitin by E1 by pharmaceuticals that might affect E2-E3 interactions (Weissman, 2001). Our data reinforce the potential significance of these issues in that, although the HLI98 compounds are relatively specific for HDM2/Mdm2, we provide evidence for effects on HECT E3s both in vitro and in cells, which may well reflect the inhibition of E2 loading seen in vitro. Consistent with such off-target effects, there is clear p53-independent toxicity in several different cell lines.

Off-target activity is of course both a bane and a potential benefit in therapeutic development, and when known beforehand to be present, is a factor that must be carefully weighted in making decisions as to whether to move a lead compound forward. The compounds described here themselves have little potential as therapeutics due to limited solubility and the need for micromolar levels. This can potentially be addressed by identifying more "druggable" analogs. Should this be successful, the issue of specificity will need to be addressed in animal models and weighted against potential benefits in patients with wild-type p53 tumors. Regardless, our results should be viewed as heartening, as they establish proof of principle, in that relatively selective inhibitors of E3 ligases, such as HDM2, can in fact be identified, and that they have the predicted cellular effects.

Experimental procedures

Reagents

Proteasome inhibitors ALLN and MG132, creatine phosphokinase, and rabbit E1, were from Calbiochem (La Jolla, CA). Adriamycin, actinomycin D, creatine phosphate, and camptothecin were obtained from Sigma (St. Louis, MO). Recombinant EGF was from R&D Systems (Minneapolis, MN). Glutathione Sepharose was from Amersham Biosciences (Piscataway, NJ). Anti-HDM2 antibodies Ab-1 and Ab-2 were from Oncogene (Boston, MA). Anti-p53 monoclonal antibody DO-1, anti-$p21^{WAF1/CIP1}$, anti-PARP, and anti-Cbl-b polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p53 (serine15) antibody was from Cell Signaling (Beverly, MA). Anti-β-actin and anti-Flag monoclonal antibodies were from Sigma (St. Louis, MO).

Plasmids

Bacterial expression plasmids for HDM2, Nedd4, and Siah1 have been described previously (Fang et al., 2000; Lorick et al., 1999). CMV-driven mammalian expression constructs for HDM2 and Siah1 have also been described (Chen et al., 1995a; Hu and Fearon, 1999).

Cell lines

RPE, a human retinal pigment epithelial cell line that stably expresses human telomerase reverse transcriptase (hTert) was from Clontech (Palo Alto, CA) and cultured with DME/F-12 (HyClone, Logan, UT) supplemented with 10% FCS, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 0.3% sodium bicarbonate. E1A-expressing RPE cells (Balint et al., 2002) have been described. Mouse embryo fibroblasts (MEFs) from *p53*- and *mdm2*-

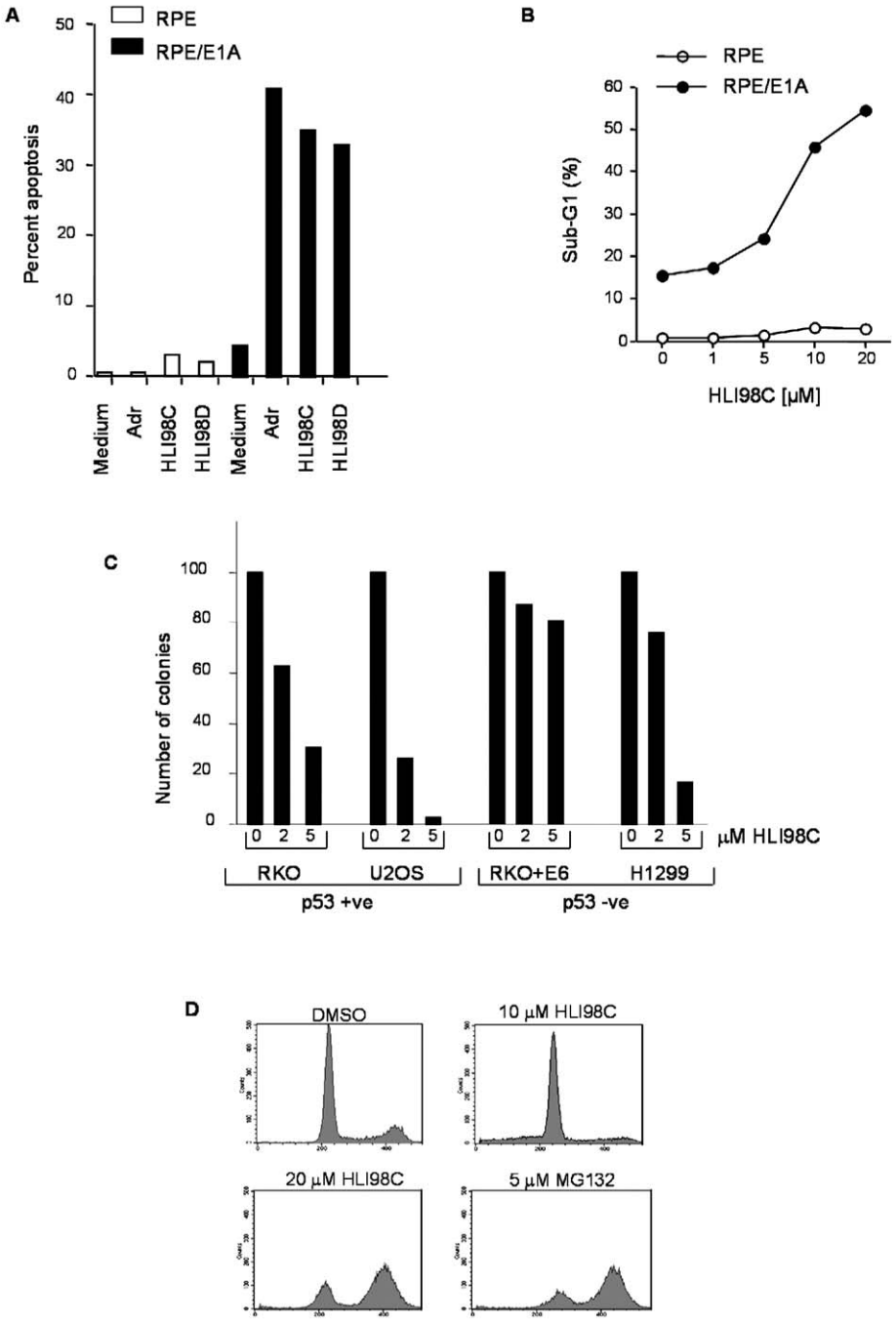


Figure 8. Transformed cells are susceptible to HLI98 family compound-induced apoptosis

A: HLI98 family compounds induce apoptosis in E1A-transformed cells but not untransformed RPE cells (1 μg/ml adriamycin, 20 μM HLI98C, and 50 μM HLI98D).

B: HLI98C induces dose-dependent apoptosis of E1A-transformed RPE cells, but not untransformed RPE cells.

C: Colony assay with human tumor cell lines. Wild-type p53 expressing cells (RKO and U2OS) and cells null for p53 (H1299) or expressing E6 (RKO+E6) were grown in the presence of the indicated concentrations of HLI98C for 5–7 days, before fixing and counting colonies.

D: FACS analysis of RPE cells following treatment with the indicated concentrations of HLI98C and MG132.

deficient mice and MEFs transformed with both E1A and Ras were maintained in DMEM supplemented with 10% FCS. U2OS-pG13 are a clone of U2OS cells stably expressing the luciferase gene under the control of the synthetic p53-responsive promoter (reporter pG13) (Kern et al., 1992). p53-inducible Saos-2 cells have been described (Nakano et al., 2000). These human cells were cultured with DMEM supplemented with 10% FCS. MDA-MB-468 breast cancer cells stably expressing Cbl-b (Ettenberg et al., 2001) were maintained in RPMI 1640 supplemented with 10% FCS.

RT-PCR

Total RNA was isolated from RPE cells grown to 80% confluence and treated for indicated times using TRIzol reagent (Invitrogen). First strand synthesis was carried out using 5 μg total RNA using SuperScript First-Strand Synthesis Systems for RT-PCR (Invitrogen) or GC-Rich PCR System (Roche). 10 ng of the reaction was used for PCR using AmpliTaq (Applied Biosytems).

Western blot

Cells were lysed with RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/ml aprotinin, 100 μg/ml PMSF, and 5 μg/ml leupeptin) and centrifuged at 15,000 × g for 20 min. Superanatants were mixed with 4× SDS-PAGE sample buffer (200 mM Tris [pH 6.8], 40% glycerol, 8% SDS, 400 mM DTT, and 0.2% bromophenol blue) and heated at 100°C for 3 min prior to resolution by SDS-PAGE and

transfer to nitrocellulose membranes. Membranes were preincubated with 5% nonfat dry milk in TBST (50 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween-20) before incubation with specific antibody for 2 hr. Specific molecules were visualized with horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies and enhanced chemiluminescence (Amersham Biosciences, UK).

In vitro autoubiquitylation

Glutathione sepharose-bound GST fusion proteins (GST-HDM2, GST-Nedd4, and GST-Siah1) were resuspended in 27 μl of ubiquitylation reaction buffer (50 mM Tris [pH 7.4], 0.2 mM ATP, 0.5 mM $MgCl_2$, 0.1 mM DTT, 16 μM creatine phosphokinase, and 1 mM creatine phosphate). Ubiquitylation reactions were carried out by adding 100 ng of rabbit E1, 100 ng of bacterially expressed UbcH5B, and 2×10^4 cpm of ^{32}P-ubiquitin (prepared as described previously [Fang et al., 2000; Scheffner et al., 1994]) to each mixture and incubating at 30°C for 90 min with agitation. Reactions were terminated by addition of 10 μl of 4× reducing SDS-PAGE sample buffer and heating at 100°C for 3 min. Samples were resolved on 8% SDS-PAGE, and ubiquitylated HDM2 visualized by Storm PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

HDM2-mediated p53 ubiquitylation

SAOS2 cells stably transfected with *p53* cDNA under the control of Tet-on promoter were treated with 2 μg/ml of doxocycline for 20 hr to induce the expression of p53. Cells were then harvested and lysed with lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5% NP-40). After a 15,000 × g centrifugation to remove cellular debris, the supernatant was added to GST-HDM2 that had been bound to glutathione Sepharose beads and incubated at 4°C for 2 hr. Following washing 4 times with 50 mM Tris to remove unbound materials, the beads containing HDM2/p53 complexes were resuspended in ubiquitylation reaction buffer and ubiquitylation reaction was carried out as described above. The reaction mixture was separated on 8% SDS-PAGE and transferred to nitrocellulose membrane for immunoblotting with anti-p53 antibody DO-1. The blot was developed with enhanced chemiluminescence.

In vitro E2 ubiquitin conjugation

The reaction was carried out by mixing bacterially expressed UbcH5B (100 ng) with rabbit E1 (100 ng) and 2×10^4 cpm of ^{32}P-ubiquitin in ubiquitylation reaction buffer. After incubating the mixture at room temperature for 5 min, the reaction was stopped by addition of 4× nonreducing SDS sample buffer and heated at 100°C for 3 min. The samples were then separated on 18% SDS-PAGE and visualized by autoradiography.

Caspase activity

DEVDase activity was determined as previously described (Yang et al., 1998). Briefly, the cells were lysed with 50 mM HEPES buffer (pH 7.4) containing 10% sucrose and 0.1% Triton X-100. After centrifugation, the supernatants were incubated with 50 μM ac-DEVD-AFC (Biomol, Meeting, PA) in the presence of 10 mM DTT for 30 min at room temperature. Fluorescence was measured using a CytoFluor multiwell plate reader (PerSeptive Biosystems, Foster City, CA).

Luciferase assay

Cells were lysed with the reporter lysis buffer (Promega, Madison, WI). Following centrifugation, luciferase activity in the supernatant was measured with reagents in the luciferase assay system according to manufacturer's instructions (Promega, Madison, WI).

Cell death assays

In some experiments, cell death was directly assessed by trypan blue exclusion under microscope. In others, the percentage of cells with sub-G1 DNA content was determined as previously described (Rowan et al., 1996). Briefly, cells were harvested, washed with PBS, and fixed with 75% ethanol overnight at –20°C. Following centrifugation and removal of residual ethanol, cell pellets were resuspended in 0.5 ml PBS and incubated with 0.2 mg/ml RNase A and 20 μg/ml propidium iodide for 1 hr at room temperature in the dark. The samples were then analyzed with a FACSCalibur (Becton Dickinson, San Jose, CA).

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References

Adams, J. (2004). The development of proteasome inhibitors as anticancer drugs. Cancer Cell *5*, 417–421.

Ashcroft, M., Taya, Y., and Vousden, K.H. (2000). Stress signals utilize multiple pathways to stabilize p53. Mol. Cell. Biol. *20*, 3224–3233.

Balint, E., Phillips, A.C., Kozlov, S., Stewart, C.L., and Vousden, K.H. (2002). Induction of $p57^{KIP2}$ expression by p73β. Proc. Natl. Acad. Sci. USA *99*, 3529–3534.

Bendjennat, M., Boulaire, J., Jascur, T., Brickner, H., Barbier, V., Sarasin, A., Fotedar, A., and Fotedar, R. (2003). UV irradiation triggers ubiquitin-dependent degradation of p21(WAF1) to promote DNA repair. Cell *114*, 599–610.

Bloom, J., Amador, V., Bartolini, F., DeMartino, G., and Pagano, M. (2003). Proteasome-mediated degradation of p21 via N-terminal ubiquitinylation. Cell *115*, 71–82.

Chen, J., Lin, J., and Levine, A.J. (1995a). Regulation of transcription functions of the p53 tumor suppressor by the mdm-2 oncogene. Mol. Med. *1*, 142–152.

Chen, Z., Hagler, J., Palombella, V.J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995b). Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. Genes Dev. *9*, 1586–1597.

Chen, K., Albano, A., Ho, A., and Keaney, J.F.J. (2003). Activation of p53 by oxidative stress involved platelet-derived growth factor-beta receptor mediated ataxia telangiectasia mutated (ATM) kinase activation. J. Biol. Chem. *278*, 39527–39533.

Chene, P. (2003). Inhibiting the p53–MDM2 interaction. Nat. Rev. Cancer *3*, 102–109.

Chipuk, J.E., and Green, D.R. (2003). p53's believe it or not: Lessons on transcription-independent death. J. Clin. Immunol. *23*, 355–361.

Clarke, D.J. (2002). Proteolysis and the cell cycle. Cell Cycle *1*, 233–234.

Coulombe, P., Rodier, G., Bonneil, F., Thibault, P., and Meloche, S. (2004). N-terminal ubiquitination of thet extracellular signal-regulated kinase 3 and p21 directs their degradation by the proteasome. Mol. Cell. Biol. *24*, 6140–6150.

Dai, M.S., and Lu, H. (2004). Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. J. Biol. in press.

Dai, M.S., Zeng, S.X., Jin, Y., Sun, X.X., David, L., and Lu, H. (2004). Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition. Mol. Biol. Cell *24*, 7654–7668.

Davydov, I.V., Woods, D., Safiran, Y.J., Oberol, P., Fearnhead, H.O., Fang, S., Jensen, J.P., Weissman, A.M., Kenten, J.H., and Vousden, K.H. (2004). Assay for ubiquitin ligase activity: High-throughput screen for inhibitors of HDM2. J. Biomol. Screen. *9*, 695–703.

Dornan, D., Wertz, I., Shimizu, H., Arnott, D., Frantz, G.D., Dowd, P.,

O'Rourke, K., Koeppen, H., and Dixit, V.M. (2004). The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature *429*, 86–92.

Ettenberg, S.A., Magnifico, A., Cuello, M., Nau, M.M., Rubinstein, Y.R., Yarden, Y., Weissman, A.M., and Lilowitz, S. (2001). Cbl-b-dependent coordinated degradation of the epidermal growth factor receptor signaling complex. J. Biol. Chem. *276*, 27677–27684.

Fang, S., and Weissman, A.M. (2004). A field guide to ubiquitylation. Cell. Mol. Life Sci. *61*, 1546–1561.

Fang, S., Jensen, J.P., Ludwig, R.L., Vousden, K.H., and Weissman, A.M. (2000). Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. J. Biol. Chem. *275*, 8945–8951.

Fazeli, A., Steen, R.G., Dickinson, S.L., Bautista, D., Dietrich, W.F., Bronson, R.T., Bresalier, R.S., Lander, E.S., Costa, J., and Weinberg, R.A. (1997). Effects of p53 mutations in apoptosis in mouse intestinal and human colonic adenomas. Proc. Natl. Acad. Sci. USA *94*, 10199–10204.

Gu, J., Nie, N., Wiederschain, D., and Yuan, Z.-M. (2001). Identification of p53 sequence elements that are required for MDM2-mediated nuclear export. Mol. Cell. Biol. *21*, 8533–8546.

Haupt, S., and Haupt, Y. (2004). Improving cancer therapy through p53 management. Cell Cycle *3*, 912–916.

Hu, G., and Fearon, E.R. (1999). Siah-1 N-terminal RING domain is required for proteolysis function, and C-terminal sequences regulate oligomerization and binding to target proteins. Mol. Cell. Biol. *19*, 724–732.

Issaeva, N., Bozko, P., Enge, M., Protopopova, M., Verhoef, L.G., Masucci, M., Pramanik, A., and Selinova, G. (2004). Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. Nat. Med. *10*, 1321–1328.

Jin, A., Itahana, K., O'Keefe, K., and Zhang, Y. (2004). Inhibition of HDM2 and activation of p53 by ribosomal protein L23. Mol. Cell. Biol. *24*, 7669–7680.

Jones, S.N., Roe, A.E., Donehower, L.A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature *378*, 206–208.

Kern, S.E., Pietenpol, J.A., Thiagalingam, S., Seymour, A., Kinzler, K.W., and Vogelstein, B. (1992). Oncogenic forms of p53 inhibit p53-regulated gene expression. Science *256*, 827–830.

Kubbutat, M.H.G., Jones, S.N., and Vousden, K.H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299–303.

Lai, Z., Yang, T., Kim, Y.B., Sielecki, T.M., Diamond, M.A., Strack, P., Rolfe, M., Caligiuri, M., Benfield, P.A., Auger, K.R., and Copeland, R.A. (2002). Differentiation of Hdm2-mediated p53 ubiquitination and Hdm2 autoubiquitination activity by small molecular weight inhibitors. Proc. Natl. Acad. Sci. USA *99*, 14734–14739.

Lane, D.P., and Lain, S. (2002). Therapeutic exploitation of the p53 pathway. Trends Mol. Med. *8*, S38–S42.

Leng, R.P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J.M., Lozano, G., Hakem, R., and Benchimol, S. (2003). Pirh2, a p53-inducible ubiquitin-protein ligase, promotes p53 degradation. Cell *112*, 779–791.

Li, M., Brooks, C.L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003). Mono- versus polyubiquitination: Differential control of p53 fate by Mdm2. Science *302*, 1972–1975.

Liu, G., Parant, J.M., Lang, G., Chau, P., Chavez-Reyes, A., El-Naggar, A.K., Multani, A., Chang, S., and Lozano, G. (2004). Chromosomal stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. Nat. Genet. *36*, 63–68.

Ljungman, M. (2000). Dial 9-1-1 for p53: Mechanisms of p53 activation by cellular stress. Neoplasia *2*, 208–225.

Lohrum, M.A.E., Woods, D.B., Ludwig, R.L., Bálint, E., and Vousden, K.H. (2001). C-terminal ubiquitination of p53 contributes to nuclear export. Mol. Cell. Biol. *21*, 8521–8532.

Lohrum, M.A., Ludwig, R.L., Kubbutat, M.H.G., Hanlon, M., and Vousden, K.H. (2003). Regulation of HDM2 activity by the ribosomal protein L11. Cancer Cell *3*, 577–587.

Lorick, K.L., Jensen, J.P., Fang, S., Ong, A.M., Hatakeyama, S., and Weissman, A.M. (1999). RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. Proc. Natl. Acad. Sci. USA *96*, 11364–11369.

Lowe, S.W., Ruley, H.E., Jacks, T., and Housman, D.E. (1993). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell *74*, 957–967.

Meek, D.W., and Knippschild, U. (2003). Posttranslational modifications of MDM2. Mol. Cancer Res. *1*, 1017–1026.

Mendrysa, S.M., McElwee, M.K., Michalowski, J., O'Leary, K.A., Young, K.M., and Perry, M.E. (2003). mdm2 is critical for inhibition of p53 during lympopoiesis and the response to ionizing irradiation. Mol. Cell. Biol. *23*, 462–472.

Michael, D., and Oren, M. (2003). The p53-Mdm2 module and the ubiquitin system. Semin. Cancer Biol. *13*, 49–58.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U.M. (2003). p53 has a direct apoptogenic role at the mitochondria. Mol. Cell *11*, 577–590.

Nakano, K., Balint, E., Ashcroft, M., and Vousden, K.H. (2000). A ribonucleotide reductase gene is a transcriptional target of p53 and p73. Oncogene *19*, 4283–4289.

Rowan, S., Ludwig, R.L., Haupt, Y., Bates, S., Lu, X., Oren, M., and Vousden, K.H. (1996). Specific loss of apoptotic but not cell cycle arrest function in a human tumour derived p53 mutant. EMBO J. *15*, 827–838.

Ryan, K.M., O'Prey, J., and Vousden, K.H. (2004). Loss of nuclear factor-kappaB is tumor promoting but does not substitute for loss of p53. Cancer Res. *64*, 4415–4418.

Scheffner, M., Huibregtse, J.M., Vierstra, R.D., and Howley, P.M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell *75*, 495–505.

Scheffner, M., Huibregtse, J.M., and Howley, P.M. (1994). Identification of a human ubiquitin-conjugating enzyme that mediates the E6-AP-dependent ubiquitination of p53. Proc. Natl. Acad. Sci. USA *91*, 8797–8801.

Schmitt, C.A., Fridman, S.J., Yang, M., Baranov, E., Hoffman, R.M., and Lowe, S.W. (2002). Dissecting p53 tumor suppressor functions in vivo. Cancer Cell *1*, 289–298.

Selinova, G. (2001). Mutant p53: The loaded gun. Curr. Opin. Investig. Drugs *2*, 1136–1141.

Sheaff, R.J., Singer, J.D., Swanger, J., Smitherman, M., Roberts, J.M., and Clurman, B.E. (2000). Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination. Mol. Cell *5*, 403–410.

Sherr, C.J., and Weber, J.D. (2000). The ARF/p53 pathway. Curr. Opin. Genet. Dev. *10*, 94–99.

Shieh, S.-Y., Ikeda, M., Taya, Y., and Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell *91*, 325–334.

Siliciano, J.D., Canman, C.E., Taya, Y., Sakaguchi, K., Appella, E., and Kastan, M.B. (1997). DNA damage induces phosphorylation of the amino terminus of p53. Genes Dev. *11*, 3471–3481.

Stommel, J.M., and Wahl, G.M. (2004). Accelerated MDM2 auto-degradation induced by DNA-damage kinases is required for p53 activation. EMBO J. *23*, 1547–1556.

Symonds, H., Krall, L., Remington, L., Saenzrobles, M., Lowe, S., Jacks, T., and Van Dyke, T. (1994). p53-dependent apoptosis suppresses tumor growth and progression in vivo. Cell *78*, 703–711.

Vassilev, L.T., Vu, B.T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., King, N., Kammlott, U., Lukacs, C., Klein, C., et al. (2004). In vivo activation of the p53 pathway by small-molecular antagonists of MDM2. Science *303*, 844–848.

Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307–310.

Vousden, K.H. (2002). Activation of the p53 tumor suppressor gene. Biochim. Biophys. Acta *1602*, 47–59.

Vousden, K.H., and Lu, X. (2002). Live or let die: The cell's response to p53. Nat. Rev. Cancer *2*, 594–604.

Weissman, A.M. (2001). Themes and variations on ubiquitylation. Nat. Rev. Mol. Cell Biol. *2*, 169–178.

Williams, S.A., and McConkey, D.J. (2003). The proteasome inhibitor bortezomib stabilizes a novel active form of p53 in human LNCaP-Pro5 prostate cancer cells. Cancer Res. *63*, 7338–7344.

Xirodimas, D.P., Saville, M.K., Bourdon, J.C., Hay, R.T., and Lane, D.P. (2004). Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. Cell *118*, 83–97.

Xu, Y. (2003). Regulation of p53 responses by post-translational modifications. Cell Death Differ. *10*, 400–403.

Yang, Y., and Yu, X. (2003). Regulation of apoptosis: The ubiquitous way. FASEB J. *17*, 790–799.

Yang, Y., Liu, Z., Tolosa, E., Yang, J., and Li, L. (1998). Triptolide induces apoptotic death of T lymphocytes. Immunopharmacology *40*, 139–149.

Zhang, Y., Wolf, G.W., Bhat, K., Jin, A., Allio, T., Burkhart, W.A., and Xiong, Y. (2003). Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. Mol. Cell. Biol. *23*, 8902–8912.