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Inhibiting Hdm2 and Ubiquitin-Activating Enzyme: Targeting the Ubiquitin Conjugating System in Cancer

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Abstract. The ubiquitin conjugating system represents a rich source of potential molecular targets for cancer and other diseases. One target of great interest is the RING finger ubiquitin ligase (E3) Hdm2/Mdm2, which is frequently overexpressed in cancer and is a critical E3 for the tumor suppressor p53. For those 50% of tumors that express wild-type p53, agents that inhibit Hdm2 have great potential clinical utility. We summarize our ongoing efforts to identify inhibitors of Hdm2 E3 activity by high-throughput screening of both defined small molecules and natural product extracts. Employing a strategy using both enzymatic and cell-based assays, we have identified inhibitors that block the E3 activity of Hdm2, activate a p53 response, preferentially kill p53-expressing cells, and have the capacity to differentially cause death of transformed cells. Therefore, screening for inhibitors of Hdm2 ubiquitin ligase activity through in vitro assays represents a powerful means of identifying molecules that activate

p53 in cancer cells to induce apoptosis. We also discuss the potential of inhibitors of ubiquitin-activating enzyme (E1) that were discovered during these screens. E1 inhibitors may similarly serve as the basis for novel therapeutics. Additionally, they represent unique tools for providing new insights into the ubiquitin conjugating system.

1 Introduction

Ubiquitylation occurs as the result of a multienzyme process involving ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin protein ligases (E3s). It is a potentially reversible process with deubiquitylating enzymes serving to remove ubiquitin from substrates (Hershko and Ciechanover 1998). Among ubiquitin ligases, there are two major classes. There are approximately 50 HECT (homologous to E6-AP carboxyl terminus) domain E3s in the human genome. HECT E3s possess a large catalytic domain of approximately 350 amino acids, the hallmark of which is a cysteine residue to which ubiquitin is transesterified from E2 prior to transfer to heterologous substrates. In contrast, RING finger and RING finger-like E3s, which constitute well over 500 different E3s in humans, are not known to function as catalytic intermediates. Instead, they promote the transfer of ubiquitin from E2 to substrates or to ubiquitin molecules that have already been bound to substrate. The RING finger is a compact structure of approximately 50 amino acids defined by eight cysteines and histidines that coordinate two zinc ions in a cross-braced pattern. This large family includes proteins having a canonical RING finger, those with RING finger variants such as the PHD/LAP finger and also the U-box, which conforms like a RING finger but does so through hydrophilic interactions rather than by coordinating zinc (Fang and Weissman 2004; Lorick et al. 2006).

The ubiquitin conjugating system is involved in virtually all cellular processes in eukaryotes. Numerous proteins that are either components of this system or substrates for ubiquitylation are implicated in cancer and other diseases. For this reason, the ubiquitin conjugating system represents a rich source of clinical molecular targets. The most extensively studied role for ubiquitylation is in the regulated destruction

of proteins by the 26S proteasome. Proof of principle for targeting the ubiquitin-proteasome system (UPS) in cancer has been established with the demonstrated efficacy of the proteasome inhibitor Bortezomib in multiple myeloma (Adams and Kauffman 2004; Leonard et al. 2006). However, as proteasome inhibitors result in the stabilization of many ubiquitylation substrates, it is desirable to target more substrate-specific steps, particularly ubiquitin ligases that are largely responsible for conferring specificity to ubiquitylation.

2 Targeting Hdm2

The discovery that RING fingers are, in general, ubiquitin ligase domains, greatly expanded the number of known potential molecular targets (Joazeiro and Weissman 2000; Lorick et al. 1999). Along with our collaborators, we became interested in whether we could identify small molecule inhibitors of the RING finger-dependent ubiquitin ligase activity of Hdm2 (Mdm2 in mouse). Hdm2 is a ubiquitin ligase that leads to proteasomal degradation of the tumor suppressor p53, the guardian of the genome (Aylon and Oren 2007; Levine et al. 2006; Vousden and Lane 2007; Yang et al. 2004). Our logic was that inhibition of the E3 activity of Hdm2 could result in an increase in p53 activity in the roughly 50% of tumors that retain wild-type p53, a substantial number of which have amplified the Hdm2 gene (Momand et al. 1998). An additional premise was that reactivation of p53 would differentially cause apoptosis, as opposed to growth arrest, in tumor cells (Lowe et al. 1993).

To develop screens for inhibitors of Hdm2 activity, we took advantage of the fact that Hdm2 targets itself as well as p53 for ubiquitin-mediated proteasomal degradation (Fang et al. 2000; Honda and Yasuda 2000). The ability to utilize a decrease in Hdm2 ubiquitylation as a readout for inhibition of its E3 activity simplified assay development by eliminating the need to include p53 in high-throughput screens. Together with our collaborator at NCI (Dr. Karen Vousden, now at the Beatson Institute, Glasgow, UK) and with IGEN (now Bioveris and Meso Scale Discovery), we adapted the gel-based autoubiquitylation assay extensively utilized in our laboratory (Fang et al. 2000; Lorick et al. 1999) to a high-throughput format using GST-Hdm2 bound to mag-

netic beads. Instead of using radioactive ubiquitin or immunoblotting for ubiquitin, an antibody tagged with a ruthenium chelate and IGEN's proprietary ORIGENE technology was employed to carry out our first-generation screens. Inhibition of activity reduced electrochemiluminescence and was scored as a positive hit in this assay (Davydov et al. 2004).

3 The Hdm2 Ligase Inhibitor Family of 5-Deazaflavins

Our initial screen of a 10,000 compound small molecule library resulted in approximately 20 hits that showed over 50% inhibition of Hdm2 autoubiquitylation. However, most of these did not show selective inhibition of Hdm2 relative to a HECT domain E3, Nedd4 (neural precursor cell expressed developmentally downregulated 4), when further assessed in our SDS-PAGE-based autoubiquitylation assays. Others appeared to represent nonspecific inhibitors, which we surmised to likely be acting nonspecifically on thiol-active enzymes (i.e., E1 and E2). However, this screen resulted in the isolation of three closely related 7-nitro 5-deazaflavin compounds, referred to as the HLI98s (Hdm2 ligase inhibitors) (Fig. 1), that inhibited Hdm2 autoubiquitylation in a dose-dependent manner in vitro. These also caused accumulation of p53 and Hdm2 in cells (Yang et al. 2005).

Since HDM2 is a p53 responsive gene and p53 activity increases in response to genotoxic stress (Aylon and Oren 2007; Levine et al. 2006; Vousden and Lane 2007; Yang et al. 2004), it was important to know that the increases in cellular p53 and Hdm2 observed with the HLI98s were not due to DNA damage. To evaluate this, Hdm2 levels were directly assessed by transfection of Hdm2 under the control of a heterologous promoter into *p53*$^{-/-}$ *mdm2*$^{-/-}$ mouse embryonic fibroblasts. Hdm2 accumulated under these conditions, suggesting that the effect observed with the HLI98s could not be accounted for by genotoxic stress. The relative specificity of these compounds was assessed by comparing their inhibition of Hdm2 to effects on other ubiquitin ligases. While there was significant evidence of specificity in cells, this was not complete as there was evidence that the HECT E3, E6-AP, could be partially inhib-

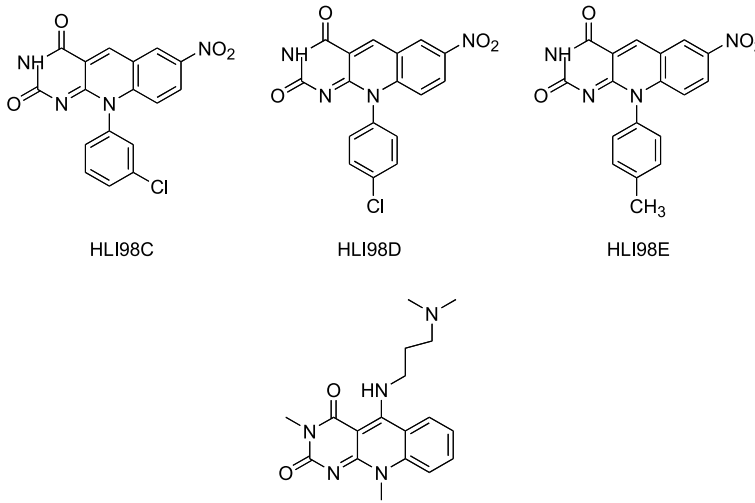

Fig. 1. Structure of the HLI compounds. HLI98A-C were identified in an in vitro screen for inhibitors of the Hdm2 ubiquitin ligase activity. HLI373 was evaluated based on its similarity to the HLI98s

ited at higher doses of the HLI98s, as assessed by accumulation of p53 in cells expressing HPV-E6 (Yang et al. 2005).

An additional concern regarding the efficacy of these compounds that stabilize both Hdm2 and p53 relates to the known overlap between the p53 transactivation domain and the site of Hdm2 binding on p53. Thus, the potential existed that stabilized p53 would be functionally inactive as a consequence of being bound to accumulated Hdm2. This concern was alleviated by the finding that there was a clear activation of a p53 response when cells were treated with the HLI98s, although it was not as substantial as that seen with an optimal dose of the DNA damaging agent adriamycin (Yang et al. 2005).

The HLI98s behaved as expected in cell-based assays with differential killing of E1A-transformed tert-immortalized retinal pigment epithelial (RPE) cells as compared to untransformed RPE cells. Further-

more, they showed a differential capacity to kill p53 expressing transformed mouse embryonic fibroblasts compared to untransformed cells (Yang et al. 2005). Together these results established proof of principle for inhibiting the E3 activity of Hdm2 to reactivate p53 in order to induce apoptosis in tumor cells.

One of the limiting features of the HLI98s was their poor solubility in aqueous solutions and their relatively low potency, even at concentrations of 20–50 μM. Issues of solubility are a particularly significant problem in development of pharmaceuticals (Dimond 2005). Therefore, we evaluated related 5-deazaflavin compounds for potential activity. A compound referred to as HLI373 was identified (Fig. 1). The characteristics of this compound in vitro and in cells have recently been described (Kitagaki et al.). Most striking is its improved solubility, approximately 200 mM in PBS, its increased potency in stabilizing Hdm2 and p53 (IC_{50} = 3 μM), and a level of transactivation of a p53 response element-driven reporter (el-Deiry et al. 1993) that was substantially greater than either the HLI98s or adriamycin (Fig. 2). Also important was its capacity to target transformed cells for degradation without causing DNA damage and to differentially kill tumor cells expressing wild-type p53 (Kitagaki et al.). Thus, HLI373 is a promising lead for further development.

4 Natural Products Screens

To maximize the potential utility of our novel screening system for inhibitors of Hdm2 autoubiquitylation, we adapted the assay for use in screening natural product extracts. Approximately 50% of pharmaceuticals developed for use in cancer over the last 65 years are derived from natural products (Newman and Cragg 2007). Nevertheless, many large pharmaceutical companies no longer screen crude natural product extracts as part of their high-throughput screening programs. Part of the reason for this dichotomy is that natural product extracts contain many nuisance compounds that interfere with commonly used fluorescent and colorimetric assay endpoints. To take advantage of the rich chemical diversity of natural compounds contained within the NCI natural product extract repository, the Hdm2 screen described in Sect. 2 was adapted

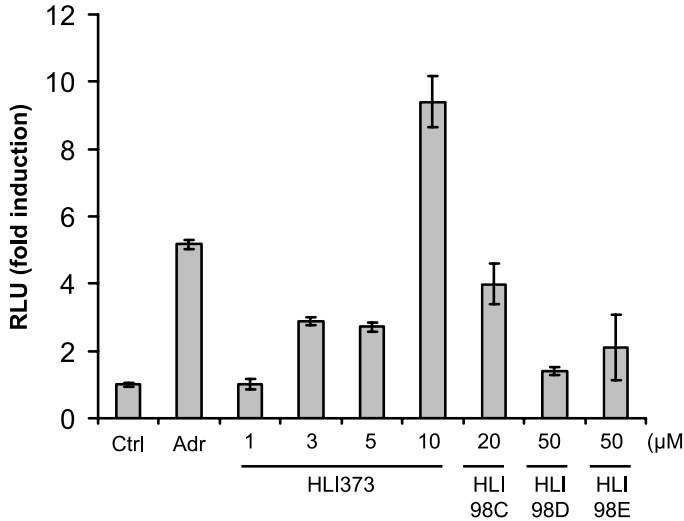

Fig. 2. p53 transactivation by HLI compounds. pG13 stably transfected U2OS cells, stably expressing a luciferase reporter under the control of multiple copies of a p53 response element (U2OS-pG13), were incubated with 1 μg/ml adriamycin or with the HLI98s, as indicated for 22 h and then evaluated for luciferase activity. Data represent average and standard deviation of three independent experiments. Data previously published in Kitagaki et al. (2008)

for use in a screen of natural products extracts by optimizing a variation of the IGEN ORIGENE technology that had been developed by Meso Scale Discovery. This system utilized plate-based electrochemiluminescent technology rather than the previous bead-based system. Optimizing the plate-based technology required adaptations in the kinetics of the ubiquitylation reactions to allow for efficient 384-well high-throughput screening. This assay was then further modified to the necessities of screening natural product extracts. The changes made to optimize the assay system included: (a) modification of the kinetics of the ubiquitylation reaction by precharging E2 with ubiquitin; (b) prebinding of Hdm2 to the assay plate to prevent identification of extracts that inhibit Hdm2 binding; and (c) the addition of BSA to the assay mixture to prevent false-positive results due to nonspecific protein binding. The assay

already took advantage of electrochemiluminescence as an endpoint, which helped eliminate false-negative results from inherently fluorescent or colored natural compounds (Sasiela et al. 2008).

To test the new assay system, we undertook the screening of the NCI's Structural Diversity Set. This is a group of roughly 1,900 compounds selected to represent the overall chemical diversity present in the NCI Developmental Therapeutics Program's synthetic compound library. Screening of this library resulted in the identification of six compounds that displayed concentration-dependent inhibition of Hdm2 autoubiquitylation (Fig. 3a). The majority of these compounds were alkaloids that demonstrated moderate activity against Hdm2 (Fig. 3b). The fact that the active compounds appeared to be natural products or natural product-derived, validated our overall strategy of screening natural product extracts. These active compounds were further evaluated in a series of cell-based assays that we developed based on studies with the HLI compounds, so as to further prioritize lead compounds and extracts (Fig. 4). This provided the means to differentiate compounds based on their ability to induce desirable cellular responses. As can be seen in Fig. 5a, at a concentration of 5 μM, three of these compounds—NSC311152, NSC311153, and NSC354961—resulted in increased levels of endogenous p53 and of Hdm2. As NSC354961 had the most significant effect on p53, it was evaluated further and found to increase both p53 and Hdm2 in a dose-dependent manner (Fig. 5b). This increase was not due to a genotoxic effect resulting in activation of a p53 response, as stabilization of Hdm2 was also observed in *p53*$^{-/-}$*mdm2*$^{-/-}$ mouse embryo fibroblasts (Lowe et al. 1993) (Fig. 5c). To directly evaluate whether p53 ubiquitylation was being inhibited, HCT116 cells expressing p53 were evaluated for accumulation of ubiquitylated p53 (Fig. 5d). While the proteasome inhibitor, ALLN, resulted in the accumulation of p53 as well as ubiquitylated p53 the addition of NSC354961 prevented the proteasome-dependent accumulation of ubiquitylated forms, consistent with inhibition of ubiquitylation. The results presented thus far are all similar to the HLIs in demonstrating clear effects on accumulation of p53 and on inhibition of its ubiquitylation. However, for compounds to have possible clinical utility, they need to differentially kill transformed cells and induce a p53 response. Unfortunately, this agent showed poor induction of a p53-driven reporter

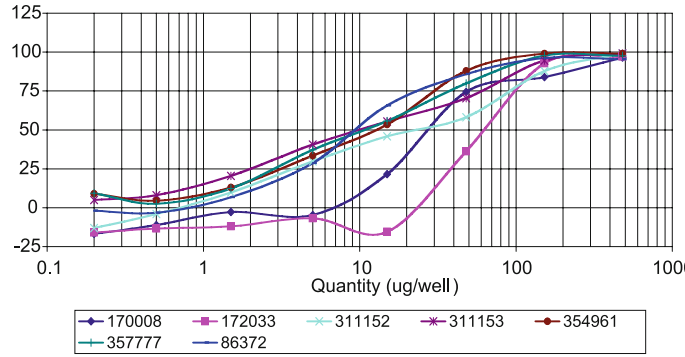

Fig. 3a. In vitro inhibition of Hdm2 E3 ligase activity by select pure compounds. Compounds from the NCI Developmental Therapeutics Program's Structural Diversity Set were tested for their ability to inhibit Hdm2 autoubquitylation in an electrochemiluminescent assay system. Concentration response curves were determined for seven active compounds

and significant toxicity was found in nontransformed cells at concentrations necessary to obtain over 50% killing of transformed cells (data not shown). Thus, while NSC354961 has the potential in stabilize p53, it is less than ideal for further development.

Initial validation of the Hdm2 screen with the Structural Diversity Set and the identification of natural compound inhibitors encouraged further screening of natural product extracts to identify additional compounds with more favorable characteristics. We proceeded to screen more than 140,000 natural product extracts. This resulted in the identification of over 2,800 extracts that inhibited autoubiquitylation of Hdm2 in the primary screen and were scored as positive hits. Greater than 2,200 of these extracts were confirmed by subsequent screening (~80% confirmation rate).

As more than 2,200 extracts were initially identified as inhibiting Hdm2, additional assays were designed, utilizing other ubiquitin ligases, to help prioritize extracts with the ability to selectively inhibit Hdm2. For these secondary assays, the RING finger E3 ligases X-linked inhibitor of apoptosis (XIAP) (Salvesen and Duckett 2002) and muscle ring finger 1 (MuRF1) (Glass 2003) along with the HECT domain E3

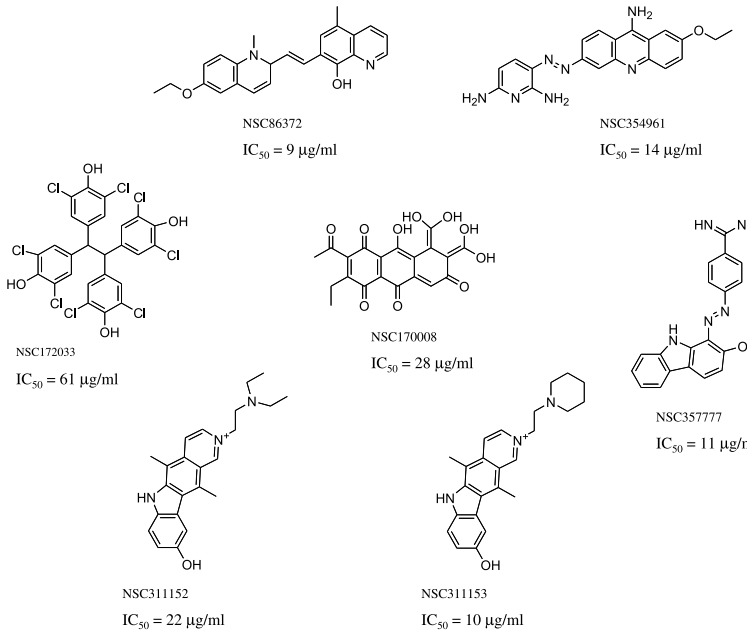

Fig. 3b. In vitro inhibition of Hdm2 E3 ligase activity by select pure compounds. Chemical structures and calculated IC_{50} values for the seven Hdm2 inhibitory compounds identified from the Diversity Set

Nedd4 (Kumar et al. 1997) were selected. Of the more than 2,200 confirmed hits, 472 were found to selectively inhibit only the RING finger ligases; having little or no activity against Nedd4. Furthermore, of those 472 RING finger-selective extracts, 94 displayed selectivity for Hdm2 (Sasiela et al. 2008). Finally, in order to enhance the opportunity to find selective E3 inhibitors, these extracts were evaluated for inhibition of E2. As shown in Fig. 6, many of the extracts showed the desired selectivity for Hdm2 while showing little or no activity against XIAP, MuRF1, Nedd4, or E2. An additional advantage of the strategy of prioritizing extracts is that this also leads to the identification of extracts that show activity against these other E3 ligases. As XIAP, MuRF1, and Nedd4 are all interesting targets in their own right, the identifica-

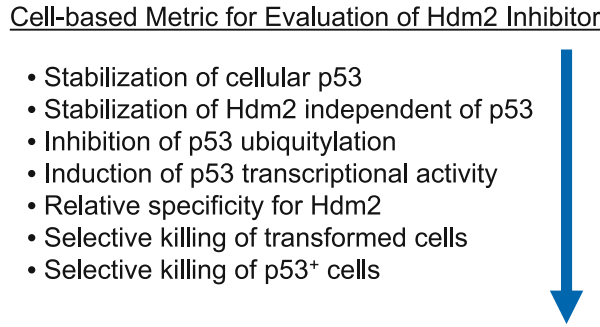

Fig. 4. Cell-based assays used to evaluate potential inhibitors of Hdm2. Cell-based secondary assays were utilized to evaluate the ability of compounds, identified in cell-free primary assays, to recapitulate Hdm2 inhibitory activity in cells. Direction of *arrow* indicates increasing stringency in the selection process for desirable compound attributes

tion of pure compounds that were found to target these ligases is potentially significant. Isolation of active compounds from these extracts that targets each of these ligases, as well as those that specifically inhibit Hdm2, is ongoing.

One of the results of our screen of natural products was the identification of a pure natural product with inhibitory activity against Hdm2. This compound, sempervirine (Fig. 7A), inhibited Hdm2 autoubiquitylation with an IC_{50} of 8 μg/ml. Sempervirine was evaluated more thoroughly employing the aforementioned algorithm of cell-based assays (Fig. 4) to determine if its activity in a cellular context was consistent with its ability to inhibit Hdm2 autoubiquitylation. The results, as recently reported (Sasiela et al. 2008), clearly showed that sempervirine leads to the accumulation of Hdm2 in a manner that is independent of genotoxic stress-mediated activation of p53 and that, in accord with its predicted function, it leads to accumulation of p53 and an inhibition of p53 ubiquitylation in cells. In addition, increasing doses of this plant-derived compound induces a p53 response and selectively induces apoptosis in transformed cells in a p53-dependent manner.

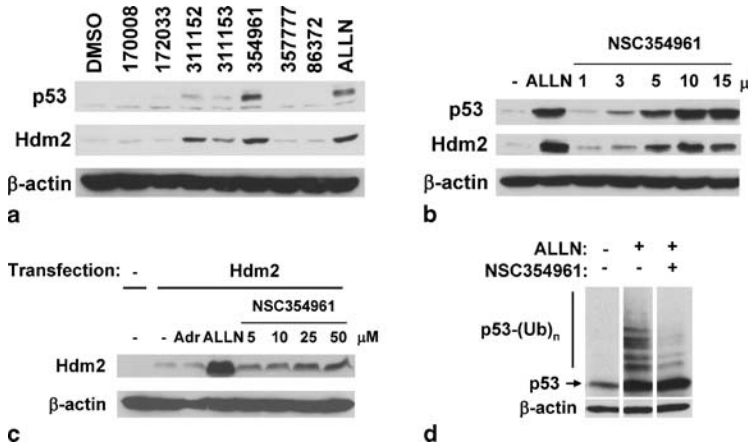

Fig. 5a–d. NSC354961 inhibits Hdm2 activity in cells. **a** RPE cells were incubated with vehicle control, 5 μM of each of seven compounds assessed in Fig. 3, or 50 μM of the proteasome inhibitor ALLN for 8 h. This was followed by lysis of cells, resolution by SDS-PAGE and immunoblotting for p53, Hdm2 and β-actin as a loading control. **b** RPE cells were incubated as in **a** and accumulation of p53 and Hdm2 assessed. **c**, *p53*$^{-/-}$*mdm2*$^{-/-}$ mouse embryo fibroblasts (MEFs) were transfected with plasmid encoding Hdm2 for 48 h prior to incubation with 1 μg/ml adriamycin (Adr), 50 μM ALLN or 5–50 μM NSC354961 for 8 h. Hdm2 was analyzed by immunoblotting. **d** Human colon cancer HCT116-p53^{+} cells were pretreated with 15 μM NSC354961 for 1 h and then for an additional 7 h with 50 μM ALLN. p53 was assessed by immunoblotting

5 Inhibiting Ubiquitin-Activating Enzyme, Identification of PYR-41

While our initial screening of a 10,000-compound small molecule library resulted in the identification of the HLI98s and led to the identification of HLI373, a subsequent screen of a 100,000-member-small-molecule library from Chembridge resulted in hits that appeared promising in the high-throughput assay but did not specifically inhibit Hdm2 when evaluated further. However, among these compounds was one that demonstrated inhibition of loading of the ubiquitin E1 (UBA1) but not of E2s. Further assessment led to the conclusion that this pyrazone-

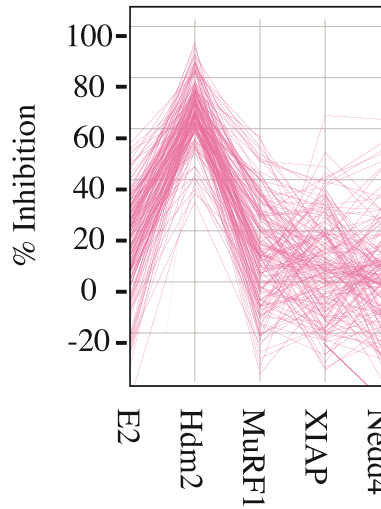

Fig. 6. Specificity of selected natural product extracts for inhibition of Hdm2. Organic and aqueous extracts (68.5 μg/ml) were tested in a cell-free electrochemiluminescent assay system that measured E3 ligase activity via quantification of autoubiquitylation of four ubiquitin ligases. Additional control assays determined the ability of the same extracts to inhibit E2 (UbcH5B) activity utilizing a similar electrochemiluminescent assay system

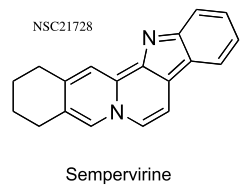

Fig. 7. The chemical structure of sempervirine

derived compound, PYR-41 (pyrazone-41) (Fig. 8) modified E1 in an irreversible manner. This inhibition was prevented by co-incubation with reducing agent (Yang et al. 2007). This suggests that PYR-41 may be acting on the active site cysteine of E1 rather than on the ATP binding site required for initial activation of ubiquitin (Haas and Rose 1982;

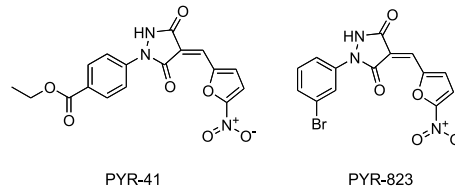

Fig. 8. The chemical structures of E1 inhibitory pyrazone derivatives

Haas et al. 1982). Similar results were obtained with a related compound, PYR-823 (Fig. 8) (Yang et al. 2007).

PYR-41 is active in cells, resulting in a marked decrease in E1~Ub thiolester formation as well as inhibition of a number of proteasomal and nonproteasomal ubiquitin-mediated processes. The potential for this reagent to be of therapeutic efficacy is underscored by its inhibition of NF-κB activation, which correlated with inhibition of both K63 ubiquitylation of TRAF6 and inhibition of K48 IκB ubiquitylation. Further, much like the Hdm2 inhibitors, PYR-41 resulted in stabilization of p53 and a p53 response, as assessed by transactivation of a reporter gene. This response was substantially greater than that seen with the HLI98s and sempervirine, although not as striking as that seen with HLI373. This activation correlated with other findings observed with Hdm2 inhibitors, including differential killing of transformed cells and particularly transformed cells expressing p53 (Yang et al. 2007).

A striking finding with PYR-41 was a marked increase in total cellular sumoylation. While this could represent an off-target effect of this compound by, for example, inhibiting desumoylating enzymes, a similar increase in sumoylation was observed using two different well-characterized cells expressing a temperature-sensitive form of the ubiquitin E1, UBA1 (Yang et al. 2007). The relationship between ubiquitylation and sumoylation uncovered in these studies now becomes an interesting area for future study. One possible explanation relates to the known competition between ubiquitylation and sumoylation on targets such as IκB and p53 (Ulrich 2005); our findings might suggest that this relationship is more general than previously appreciated. However, there is another highly intriguing non-mutually exclusive possibility. At least one ubiquitin ligase, RNF4 (SNURF) (Hakli et al. 2004) has re-

cently been found to recognize and ubiquitylate certain sumoylated proteins (Prudden et al. 2007; Sun et al. 2007). Thus, part of the explanation for the findings observed could be a consequence of failure to degrade sumoylated species. There are clearly many issues to be explored with inhibitors of the ubiquitin E1 both with regard to basic scientific issues and potential clinical utility.

6 Discussion

The ubiquitin conjugating system is a rich source of potential molecular targets in cancer (Fang et al. 2003; Nalepa et al. 2006). The general disruption of the system through the use of proteasome inhibitors represents a relatively nonspecific means of disrupting this system. Nevertheless, proteasome inhibitors are proving to have a significant therapeutic index in at least some malignancies (Adams and Kauffman 2004; Leonard et al. 2006).

p53 plays a central role in response to genotoxic stress, has clear importance in inducing cell growth arrest and apoptosis in cancer cells, and is regulated to a great degree by ubiquitylation and proteasomal degradation. As Hdm2 has consistently proven to be a major and essential ubiquitin ligase for p53, there is significant interest in preventing Hdm2 from targeting p53 for degradation. One approach has been to reactivate p53 by disrupting its interaction with Hdm2 (Issaeva et al. 2004; Li et al. 2005; Vassilev et al. 2004). Indeed the identification of the nutlins, which block the interaction between the two proteins, suggests that this approach may be efficacious (Vassilev 2007). Another approach utilized by us and others has focused on identifying small molecules that might decrease the ubiquitin ligase activity of Hdm2 and thereby increase p53 levels and increase its cellular activity (Davydov et al. 2004; Kitagaki et al.; Lai et al. 2002; Sasiela et al. 2008; Wilson et al. 2007; Yang et al. 2005).

In our studies, we have identified multiple members of the 5-deazaflavin family as inhibitors of Hdm2 that activate p53. Active members of this family now include a potent water-soluble version. Thus, there is great potential for this compound to serve as a lead for development of additional reagents.

As natural products provide the opportunity to explore an unparalleled range of chemical space and represent a rich source of reagents either for direct use or to serve as the basis for generating synthetic compounds, we have expanded our screening to include the more than 140,000 natural product extracts maintained by the NCI. Purification and characterization of hits from screening of natural products extracts is ongoing at NCI and it is evident that, using this approach, there is significant potential to identify new potent inhibitors of Hdm2 that will reactive p53. Proof of principle for the potential utility of natural products is borne out with results achieved with sempervirine and with the demonstration that at least one alkaloid contained within the NCI diversity set has the potential to stabilize Hdm2 and p53. We believe, based on our results to date, that there is great potential for natural products to yield novel ubiquitin ligase inhibitors with therapeutic potential in cancer and other diseases.

As conjugation of proteins with ubiquitin is a multienzyme process, screens for inhibitors of substrate-specific ubiquitin ligases always have the potential to result in the identification of inhibitors of E1 or E2, the proximal enzymes in this cascade. We have isolated at least one family of compounds that include PYR-41 and PYR-823 that show relative selectivity in inhibiting E1. These represent the first members of a potential new set of tools to explore the ubiquitin system and it relationship to sumoylation. However, what is also apparent from our in vitro studies is that PYR-41 can have desirable effects that may be useful in cancer treatment. These include inhibition of the pro-survival NF-κB family of transcription factors and reactivation of p53. Studies to determine how PYR-41 and related compounds function to inhibit E1 are currently ongoing; however, data accumulated to date suggest that it is acting on the active site of E1 (Yang et al. 2007).

Our efforts began to provide proof of principle for inhibiting RING finger E3s. We now find that we have agents in hand with real potential for reactivating p53 in cancer that may serve as leads for new therapeutics. At the same time, we have come full circle in showing that we can nonspecifically inhibit the ubiquitin system by blocking E1 and that this too has the potential to serve as a basis for therapeutics. These are still early days and further advancement will await additional structure–activity relationship studies, purification of natural products from the

extracts that have now been identified, and the testing of these inhibitors in the appropriate in vivo models.

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