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A Boronic-Chalcone Derivative Exhibits Potent Anticancer Activity through Inhibition of the Proteasome

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ABSTRACT

Chalcones and their derivatives have been shown to have potent anticancer activity. However, the exact mechanisms of cytotoxic activity remain to be established. In this study, we have evaluated a series of boronic chalcones for their anticancer activity and mechanisms of action. Among the eight chalcone derivatives tested, 3,5-bis-(4-boronic acid-benzylidene)-1-methyl-piperidin-4-one (AM114) exhibited most potent growth inhibitory activity with IC_{50} values of 1.5 and 0.6 μM in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and colony formation assay, respectively. The cytotoxic activity of AM114 was shown to be associated with the accumulation of p53 and p21 proteins and induction of apoptosis. Mechanistic studies showed that AM114 treatment inhibited the chymotrypsin-like activity of the 20S proteasome in vitro, leading to a significant accumulation of ubiquitinated p53 and other cellular proteins in whole cells. In vitro studies showed that AM114 did not significantly disrupt the interaction of p53 and murine double minute 2 protein. It is noteworthy that AM114 as a single agent was preferentially toxic to cells with wild-type p53 expression, whereas combination of this compound with ionizing radiation (IR) significantly enhanced the cell-killing activity of IR in both wild-type p53 and p53-null cells. Together, these results indicate that the boronic chalcone derivative AM114 induces significant cytotoxic effect in cancer cells through the inhibition of the cellular proteasome and provide a rationale for the further development of this class of compounds as novel cancer chemotherapeutic agents.

Chalcones and their derivatives are a group of compounds reported to exhibit promising anticancer activity. These compounds are precursors of flavonoids and isoflavonoids, which are abundant in edible plants. The chemical structure of chalcones (1,3-diphenyl-2-propen-1-ones) consists of two aromatic rings joined by a three-carbon α,β-unsaturated carbonyl system. Previous studies have indicated that chalcones and their derivatives demonstrate anticancer activity in various tumor cells. Natural and synthetic chalcones have been shown to have strong antiproliferative effects in both primary and established ovarian cancer cells (de Vincenzo et al., 1995) and in gastric cancer HGC-27 cells (Shibata, 1994). Hydroxyl chalcones and isoliquiritigenin have been shown to be potent inhibitors of skin carcinogenesis in vivo (Yamamoto et al., 1991; Satomi, 1993). Several studies have demonstrated that chalcones act as chemopreventive agents, capable of inhibiting carcinogenesis induced by chemical agents through enhancement of reduced glutathione levels (Wattenberg et al., 1994; Makita et al., 1996). However, the exact mechanisms of action of chalcones in tumor cells remain to be elucidated. It has been suggested that the isoliquiritigenin inhibits proliferation of lung cancer A549 cells by halting the cell cycle at the G_2/M phase and inducing p21 protein expression (Ii et al., 2004). Studies in HepG2 hepatocellular carcinoma cells indicate that chalcone derivatives function through inhibition of the tyrosine kinase activity of epidermal growth factor receptor (Yang et al., 2001). Another proposed mode of action of hydroxyl chalcones, based on studies in rat hepatocytes, is through the induction of formation of prooxidant radicals (Sabzevari et al., 2004). It is noteworthy that recent studies also suggested that one potential mechanism of cytotoxic activity of carboxylic acid derivatives of chalcones is through binding to the p53-binding pocket of

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ABBREVIATIONS: MDM2, murine double minute 2 protein; AM114, 3,5-bis-(4-boronic acid-benzylidene)-1-methyl-piperidin-4-one; PI, propidium iodide; FITC, fluorescein isothiocyanate; AM58, 3-(4-chloro-phenyl)-1-(phenyl-4-hydroxymethylboronic acid)-propenone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; DMSO, dimethyl sulfoxide; IR, ionizing radiation; wt, wild-type.

MDM2, disrupting the MDM2-p53 interaction to stabilize and activate p53, leading to apoptosis (Stoll et al., 2001).

Based on the structural and biochemical evidence that carboxylic chalcones disrupt the MDM2-p53 interaction by binding the p53-binding domain of MDM2 (Stoll et al., 2001), a series of boronic acid derivatives of chalcones were synthesized for further evaluation. It was hypothesized that these compounds would bind the p53-binding domain of MDM2 with high affinity and would therefore show selective cytotoxicity in MDM2 overexpressing tumors and spare normal cells. Preliminary studies conducted with a primary series of boronic derivatives of chalcones demonstrated that these compounds show 5- to 10-fold greater growth inhibition in human breast cancer cell lines than in normal breast epithelial cells (Kumar et al., 2003). In addition, some of these compounds also induced accumulation of p53 and p21 proteins and showed significantly greater cytotoxicity in cells with wt p53 compared with p53 null cells. Thus, it is important to test whether this class of compounds causes cell death through disruption the MDM2-p53 interaction, or whether other mechanisms of action are involved in inducing p53 accumulation and cell death.

The tumor suppressor p53 is an important molecule involved in regulating cellular response to exogenous and endogenous stress. Depending on the cell types and the nature of the stress, p53 may induce cell cycle arrest or trigger apoptosis. The stabilization and activation of the p53 protein as a transcriptional activator occur by different mechanisms. It is known that p53 is stabilized and activated through a series of phosphorylation and acetylation events, which release it from interaction with MDM2 (Balint and Vousden, 2001; Hofseth et al., 2004). Alternative mechanisms of p53 stabilization include proteasomal inhibition; direct binding by NADPH quinone oxidoreductase, hypoxia inducible factor-1α, or mSin3a; and binding and sequestration of MDM2 by ADP ribosylation factor (Brooks and Gu, 2003). Upon activation, p53 transcriptionally activates a number of target genes involved in regulating cell cycle checkpoints or in inducing apoptosis (Yee and Vousden, 2005).

The cellular proteasome is an enzyme complex that primarily functions in the degradation of misfolded and short-lived regulatory proteins. Intracellular proteins are targeted for proteasomal degradation through ubiquitination by the ubiquitin-conjugating machinery. The ubiquitin-proteasome pathway is essential for many fundamental cellular processes. There has been great interest in targeting the ubiquitin-proteasome pathway for the treatment of cancer, particularly with the introduction of the proteasome inhibitor bortezomib (Velcade or PS 341) into clinical trials. Several studies demonstrated that inhibition of the proteasome by bortezomib leads to the stabilization of proteins involved in cell cycle and apoptosis such as p53, the cyclin-dependent kinase inhibitors p21 and p27, and the proapoptotic proteins Bax and Bid (Breitschopf et al., 2000; Li and Dou, 2000; Shah et al., 2001; Williams and McConkey, 2003). Thus, either disruption of p53-MDM2 interaction or inhibition of proteasome activity can lead to accumulation of p53 and induction of p53-dependent apoptosis.

In the present study, we investigated a series of boronic chalcones for their in vitro anticancer activity and examined the ability of the most potent compound AM114 to disrupt the interaction between p53 and MDM2 and to inhibit proteasome activity.

Materials and Methods

Cell Lines and Chemicals. HCT116 colon carcinoma cells with wild-type p53 and their isogenic cells lacking p53 (HCT116 p53−/−) were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The cells were maintained in McCoy's 5A medium containing 10% fetal bovine serum. Giemsa stain and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies for immunoblot analysis were purchased from the following sources: anti-p53 (Ab-6) and anti-p21 (Ab-1) were from EMD Biosciences (San Diego, CA), anti-ubiquitin was from Cell Signaling Technology Inc. (Beverly, MA), anti-MDM-2 (Ab-1) was from NeoMarkers (Fremont, CA), and anti-actin was from Sigma-Aldrich. Annexin V-FITC conjugate and Annexin binding buffer were purchased from BD Biosciences (San Diego, CA). The proteasome assay kit, 7-amino-4-methylcoumarin, and MG132 were purchased from EMD Biosciences.

Preparation of Boronic Chalcone Derivatives. The chemical synthesis procedures for preparation of boronic chalcone derivatives were described previously (Kumar et al., 2003). To synthesize AM114, a solution of 1-methyl-piperidin-4-one in 10 ml/mmol ethanol was mixed with 4-formylphenylboronic acid (3 Eq), stirred for 15 min, followed by the addition of an ethanolic solution of KOH (6 Eq) at 0°C. The mixture was stirred at room temperature overnight. After the reaction reached completion, it was quenched with 20 ml of water. The resulting mixture was concentrated in vacuo, diluted with 50 ml of water, acidified with 1 M HCl to pH 3, and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residual solid was purified by crystallization to yield AM114, which was confirmed by NMR and mass spectrometric analysis. To prepare AM58, a solution of 4-chloroacetophenone in 10 ml/mmol ethanol was mixed with 4-hydroxybenzaldehyde (1.5 Eq), stirred for 15 min, followed by the addition of an aqueous solution of KOH (3 Eq) at 0°C. The mixture was refluxed overnight. After the reaction reached completion, it was quenched with 20 ml of water. The resulting mixture was concentrated in vacuo, diluted with 50 ml of water, acidified with 1 M HCl to pH 3, and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residual solid was purified by column chromatography to yield the desired product, 3-(4-chlorophenyl)-1-(4-hydroxy-phenyl)-propenone. An ethereal solution of 3-(4-chloro-phenyl)-1-(4-hydroxy-phenyl)-propenone was added dropwise to a cooled solution of KOH (1.8 Eq) in diethyl ether, and the reaction mixture was stirred at room temperature for 40 min. The mixture was cooled to 0°C, and a solution of pinacol (bromomethyl)boronate in diethyl ether was added dropwise, followed by a catalytic amount of 18-crown-6. The mixture was stirred at room temperature overnight. After a standard workup, the product, AM58, was purified by crystallization and confirmed by NMR and mass spectrometric analysis. The chemical structures of AM58 and AM114 are shown in Fig. 1.

Cell Growth Inhibition Assay. HCT116 p53+/+ cells were used to determine the inhibitory effect of boronic chalcone compounds on cell growth using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates at the density of 1500 cells/well, allowed to attach overnight, and then treated with various concentrations of the indicated AM compounds in triplicate. At the end of 72-h incubation, 50 μl of 3 mg/ml MTT reagent was added to each well and incubated for an additional 4 h. The supernatant from each well was then carefully removed, and 200 μl of DMSO was added to each well and mixed thoroughly. The plate was read for optical density at 540 nm, using a Multiskan Ascent plate reader (Thermo Electron Corporation, Waltham, MA). The potency of cell growth inhibition for each AM compound was ex-

pressed as an IC_{50} value, defined as the concentration that caused a 50% loss of cell growth.

Clonogenic Survival Assay. HCT116 p53+/+ and HCT116 p53−/− cells were plated in six-well plates at the density of 1500 cells/well. The cells were allowed to attach overnight, and then they were treated with the indicated concentrations of AM114 and incubated for 14 days. At the end of 14 days, the colonies were fixed with a mixture of 50% methanol, 5% acetic acid, and 45% water; stained; and scored by manual counting of the cell colonies containing 50 cells or greater. The sensitivity of the cells to AM114 was expressed as the percentage of colonies in the treated samples compared with untreated control samples. To assay the efficacy of AM114 in combination with ionizing radiation (IR), HCT116 p53+/+ and HCT116 p53−/− cells were pretreated with AM114 for 12 h and then exposed to 2-Gy IR. After additional 6-h incubation, the cells were trypsinized, plated in six-well plates at a density of 1500 cells/well in the presence of the indicated concentrations of AM114, and incubated for 14 days. Cell colonies were fixed, stained, and scored. The sensitivity of the cells to AM114, IR, and their combination was expressed as the percentage of colonies in the treated samples compared with untreated control samples.

Flow Cytometric Analysis of Cell Death by Annexin V and PI Staining. The extent of cell death induced by AM114 was assayed by Annexin V and PI staining. Cells were treated with 1 μM AM114 for 24, 48, and 72 h. At the end of the incubation period, the cells were collected, washed with 1× Annexin binding buffer (10 mM HEPES-NaOH, pH 7.4, 140 mM sodium chloride, and 2.5 mM calcium chloride), and double-stained with Annexin V-FITC and PI under conditions recommended by the manufacturer. The samples were then analyzed by flow cytometry. The extent of cell death in the samples was determined by measuring the percentage of cells that were positive for Annexin V alone and positive for both Annexin V and PI.

Immunoprecipitation and Immunoblot Analysis. For immunoprecipitation experiments, HCT116 p53+/+ cells were lysed in a buffer containing 150 mM sodium chloride, 1% Nonidet P-40, 0.5% deoxycholate, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, and a cocktail of protease inhibitors. To test the effect of AM114 on the interaction of p53 with MDM2 in vitro, the protein lysates from HCT116p53+/+ cells (without preincubation with AM114) were incubated with the anti-p53 antibody (Ab-6 antibody; EMD Biosciences) at the concentration of 1 μg of antibody/mg protein at 4°C overnight with gentle rotation, followed by incubation with protein G-agarose conjugate for an additional 2 h at 4°C. After centrifugation, the pellets were collected and washed with ice-cold lysis buffer. The precipitated beads with bound proteins were then incubated with various concentrations of AM114 for 30 min at room temperature. The beads were collected and washed four times with lysis buffer. The supernatants and the precipitated fractions were subjected to SDS-PAGE followed by immunoblotting for MDM2. For analysis of the effect of AM114 on p53-MDM2 interaction in whole cells, HCT116p53+/+ cells were first incubated with AM114 for various times, and the protein lysates were then prepared from the control and drug-treated cells, followed by immunoprecipitation with p53 antibody and blotting with a MDM2 antibody. To test the effect of AM114 on p53 ubiquitination, cells were treated with 1 μM AM114 for 12 or 24 h, harvested, and resuspended in the lysis buffer. The cell lysates were immunoprecipitated with p53 antibody as described above. The supernatants and precipitated proteins were subjected to immunoblot analysis for ubiquitin protein.

For analysis of p53, p21 and ubiquitin proteins, HCT116 p53+/+ cells were treated with 1 μM AM114 for the indicated time periods, harvested, and washed with ice-cold phosphate-buffered saline. The cells were then mixed with lysis buffer (1% Triton X-100, 300 mM sodium chloride, 0.5% deoxycholate, 25 mM HEPES, pH 7.5, 20 mM glycerol phosphate, 0.1% SDS, 1 mM orthovanadate, 0.5 mM dithiothreitol, 1.5 mM magnesium chloride, 0.2 mM EDTA, and 1× protease inhibitors), vortexed, and incubated on ice for 20 min. The lysates were centrifuged at 14,000 rpm at 4°C for 20 min. The supernatants were recovered and mixed with an equal volume of loading buffer (50 mM Tris-HCl, 10% glycerol, 2% SDS, 0.025% bromphenol blue, and 2.5% β-mercaptoethanol) and heated to 95°C for 5 min. The samples were then loaded onto a 10% denaturing polyacrylamide gel. The proteins were separated by electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blotted with antibodies specific to the proteins of interest.

Assay of 20S Proteasome Activity. The effect of AM114 on the activity of the 20S proteasome core enzyme was determined using a 20S proteasome assay kit according to the manufacturer's protocol (EMD Biosciences). In brief, a standard curve was first generated using 7-aminomethylcoumarin in the range of 0 to 50 nM. The fluorescence signal of the standards was measured at the excitation wavelength of 355 nm and the emission wavelength of 460 nm. Purified 20S proteasome supplied with the assay kit was diluted with 1× reaction buffer (50 mM HEPES and 1 mM EDTA, pH 7.6) at the concentration of 1 μg/ml and incubated with the indicated concentrations of AM114 or MG132 (as the proteasome inhibitor control) for 30 min at 37°C. The proteasome substrate (Suc-Leu-Leu-Val-Tyr-

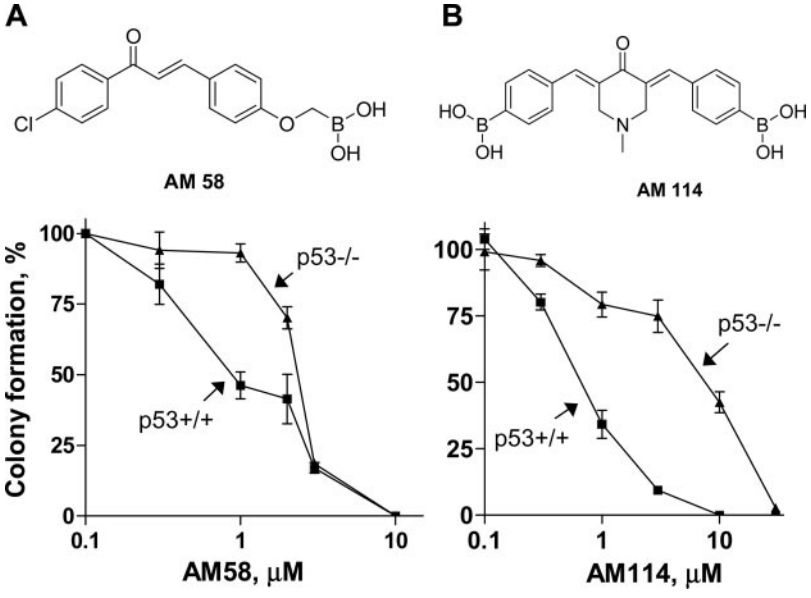

Fig. 1. Cytotoxic effect of boronic chalcone derivatives AM58 and AM114 in p53 isogenic cells. HCT116 p53 wild-type (p53+/+) and HCT116 p53-null (p53−/−) cells were plated in six-well plates and incubated with the indicated concentrations of AM58 (A) or AM114 (B). After a 14-day incubation in the presence of AM compounds, the surviving colonies were fixed, stained, and counted. The cytotoxicity of the boronic chalcone derivatives is expressed as percentage of colony formation relative to the control. The data represent the means ± S.E.M. of triplicate experiments from two sets of repeated experiments. The chemical structures of AM58 and AM114 are shown above the respective cytotoxicity curves.

7-aminomethylcoumarin) was then added to the reaction mixtures at a final concentration of 10 μM and incubated for 60 min at 37°C. The fluorescent signal was measured using an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

Results

In Vitro Anticancer Activity of Boronic Chalcones in Human Colon Cancer HCT116 Cells. We first tested a series of boronic chalcone derivatives (designated as AM compounds) for their growth inhibitory activity in the colon carcinoma cell line expressing wild-type p53 (HCT116 p53+/+ cells) using MTT assay. The IC_{50} values (the drug concentrations that inhibited 50% of cell proliferation) of the AM compounds are listed in Table 1. Among the eight compounds tested, AM114 and AM58 exhibited most potent activity, with the IC_{50} values of 1.5 and 3.9 μM, respectively, as determined by the MTT assay (72 h).

The cytotoxic effect of AM58 and AM114 was further tested in both HCT116 p53+/+ and p53−/− cells, using colony formation and apoptosis assays. As shown in Fig. 1, both compounds were more toxic to the cells expressing wild-type p53. For example, incubation of HCT116 p53+/+ cells with 1 μM AM58 led to a loss of colony formation in the p53+/+ and p53−/− cells by approximately 55 and 10%, respectively (Fig. 1A). Likewise, 1 μM AM114 caused a loss of cell survival in the p53+/+ and p53−/− cells by approximately 70 and 20%, respectively (Fig. 1B). The preferential killing of p53+/+ cells by AM58 and AM114 was observed consistently at the relatively low drug concentrations (<3 μM), whereas at higher concentrations, both compounds were toxic to p53−/− cells in the colony formation assays. Consistent with the MTT assay results show in Table 1, AM114 exhibited potent activity against p53+/+ cells in colony formation assay, with an IC_{50} value of 0.6 μM (Fig. 1B).

The preferential cytotoxic effect of AM114 and AM58 on p53+/+ cells was further evaluated using flow cytometry analysis after the drug-treated cells were double-stained with Annexin V-FITC and PI. As shown in Fig. 2, incubation of HCT116 p53+/+ cells with 1 μM AM114 caused 28% of the cells to exhibit positive Annexin V staining at 48 h, and this fraction of dead cells increased to 76% at 72 h. In contrast, HCT116 p53−/− cells were significantly less sensitive to AM114, with 16 and 17% of the cells positive for Annexin V staining at 48 and 72 h, respectively. Similar preferential induction of cell death was observed in p53+/+ cells incubated with 1 μM AM58, which caused 55 and 20% cell death in p53+/+ and p53−/− cells, respectively, at 72 h (data not shown). These data suggest that boronic chalcone derivatives may have a preferential activity against cells with wt p53 function. However, both AM114 and AM58 caused a significant loss of cell survival in the p53-null cells at higher concentrations in clonogenic survival assays (Fig. 1).

Induction of p53 and p21 Accumulation by AM114 without Disruption of p53-MDM2 Interaction. Based on the observations that AM114 is preferentially active against p53+/+ cells and that certain chalcone derivatives seem to disrupt the p53-MDM2 interaction, leading to accumulation and activation of the p53 protein (Stoll et al., 2001), we tested whether AM114 exerts its cytotoxic effect through disruption of the p53-MDM2 interaction, leading to p53 accumulation and activation of p53-dependent apoptosis. Immunoblot analysis showed that AM114 treatment induced a significant

TABLE 1

Growth inhibitory effect of boronic chalcones in human colon cancer HCT116 cells

HCT116 p53+/+ cells were incubated with 0 to 10 μM of each AM compound for 72 h and subjected to MTT assay as described under *Materials and Methods*, and the IC_{50} values were determined from the growth inhibition curves. The data represent the mean ± S.D. of triplicate experiments.

Compound	IC_{50} in HCT116 p53+/+ Cells
	μM
AM9	>10
AM23	4.96 ± 1.08
AM25	6.61 ± 1.03
AM58	3.85 ± 0.13
AM114	1.49 ± 0.15
AM163	>10
AM170	>10
AM173	>10

AM9, 1-[4-(5-boronic acid-pyridin-2-yl)-piperazin-1-yl]-3-phenyl-propenone; AM23, 3-(4-chloro-phenyl)-1-(4-hydroxy-phenyl)-propenone; AM25, 4-*tert*-butyl-2,6-bis-(4-boronic acid-benzylidene)-cyclohexanone; AM58, 3-(4-chloro-phenyl)-1-(phenyl-4-hydroxymethyl boronic acid)-propenone; AM114, 3,5-bis-(4-boronic acid-benzylidene)-1-methyl-piperdin-4-one; AM163, benzoic acid 3,5-bis-(4-chloro-benzylidene)-1-methyl-piperidin-4-yl ester; AM170, benzoic acid 1-(4-chloro-phenyl)-2-(1*H*-indol-4-ylcarbamoyl)-3-(4-methoxy-phenyl)-allyl ester; AM173, 4-*tert*-butyl-2,6-bis-(4-boronic acid-benzylidene)-cyclohexanol.

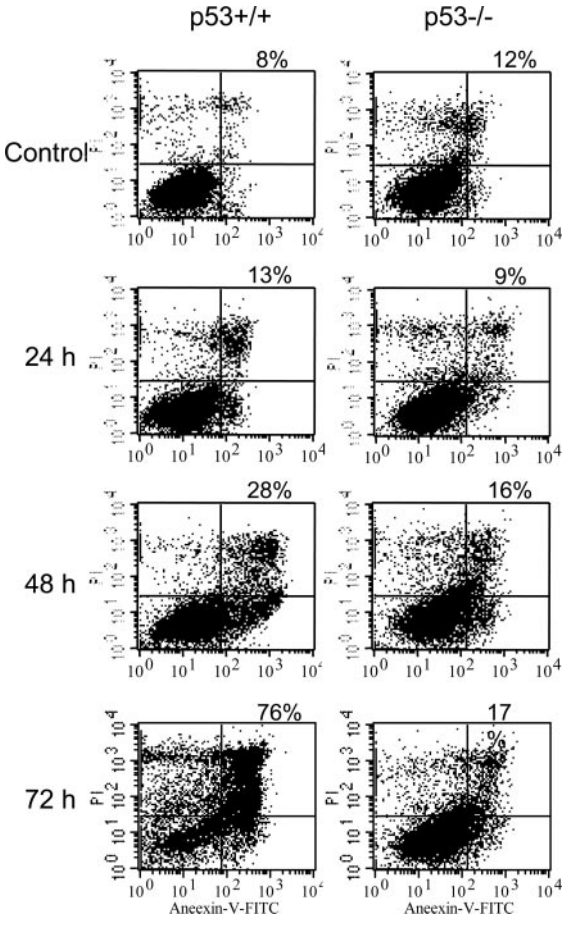

Fig. 2. Induction of cell death by AM114 in HCT116 colon cancer cells. HCT116 wt p53 and p53-null cells were treated with 1 μM AM114 for the indicated times. The untreated and treated cells were double-stained with Annexin V-FITC and PI and analyzed by flow cytometry as described under *Materials and Methods*. The cells that stained positive for Annexin V-FITC alone (in bottom right quarter) or positive for both Annexin V-FITC and PI (in top right quarter) were considered dead cells. The percentage of dead cells for each experimental condition is shown on the top of each panel.

accumulation of p53 protein and its downstream target p21 protein in HCT116+/+ cells (Fig. 3A). Likewise, incubation of HCT116 p53+/+ cells with AM58 also caused a time-dependent accumulation of p53 and p21 protein. The induction of p21 protein expression was p53-dependent, because incubation of HCT116p53−/− cells with AM114 did not cause any significant change in p21 protein expression (data not shown).

We then performed immunoprecipitation experiments to test whether the accumulation of p53 and p21 proteins was due to a disruption of the p53-MDM2 interaction by AM114. Protein extracts from HCTT116 p53+/+ cells were first immunoprecipitated with a p53 antibody. After extensive washing, the precipitated proteins were incubated with 10 to 50 μM AM114 as indicated, followed by immunoblotting for the presence of p53 and MDM2 proteins. As shown in Fig. 3B, MDM2 is coimmunoprecipitated with p53 in the untreated sample, confirming the physical association of these two proteins (lane 1). However, incubation with 10 or 50 μM AM114 did not disrupt this interaction (lanes 2 and 3). The specificity of the immunoprecipitation was confirmed by the use of a control IgG, which did not pull down p53 or MDM2 (lane 4). These results suggest that although AM114 induced an accumulation of p53 protein and its downstream target protein p21, this compound seemed unable to disrupt the p53-MDM2 interaction in vitro. In a separate experiment, HCT116 cells were preincubated with 1 μM AM114 for 12 or 24 h, and the cell lysates were then immunoprecipitated with p53 antibody followed by immunoblotting for MDM2. The results showed that this compound did not disrupt p53-MDM2 interaction in whole cells (Fig. 3C). We also tested the ability of AM58 to disrupt the interaction of p53-MDM2 in vitro using similar assays, and we obtained negative results. These data together suggest that AM114 and AM58 caused p53 accumulation and cytotoxic activity through other mechanisms. We therefore proceeded to examine alternate mechanisms of AM114 cytotoxic activity.

Accumulation of Ubiquitinated Proteins in Cells Treated with AM114. Immunoblot analysis of p53 protein in cells treated with AM114 showed that in addition to the increased accumulation of the p53 protein, there was also an accumulation of protein bands above the 53-kDa region that were detected by the anti-p53 antibody (Fig. 4A). These protein bands seem to suggest that AM114 might induce accumulation of ubiquitinated p53. To test this possibility, we used an anti-p53 antibody to precipitate p53 protein from the

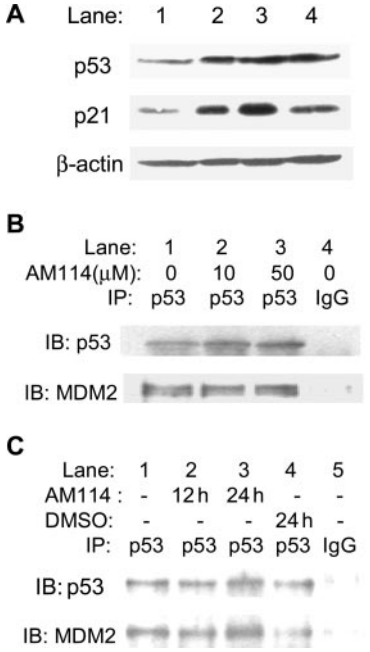

Fig. 3. AM114 induces accumulation of p53 and p21 protein without disrupting p53-MDM2 interaction. A, protein extracts were prepared from the control HCT116 p53+/+ cells or the AM114-treated cells and assayed for p53 and p21 by Western blotting. β-Actin was also measured as a protein loading control. Lane 1, protein extracts from control cells; lanes 2 to 4, protein extracts from cells treated with 1 μM AM114 for 8, 24, and 48 h, respectively. B, protein lysates were prepared from HCT116 p53+/+ cells (without pretreatment with AM114) and subjected to immunoprecipitation with the anti-p53 antibody (lanes 1–3) or IgG as a nonspecific antibody control (lane 4). The immunoprecipitation was carried out in the presence or absence of the indicated concentrations of AM114 as described under *Materials and Methods*. The precipitated proteins were analyzed by gel electrophoresis and immunoblotted for MDM2 and p53. C, HCT116 p53+/+ cells were preincubated with 1 μM AM114 or solvent (DMSO) only in culture for the indicated times. Protein lysates were then prepared and subjected to immunoprecipitation with the anti-p53 antibody (lanes 1–4) or IgG as a nonspecific antibody control (lane 5). The effect of DMSO treatment was tested because stock solutions of AM114 were prepared in DMSO. The precipitated proteins were analyzed by gel electrophoresis and immunoblotted for MDM2 and p53.

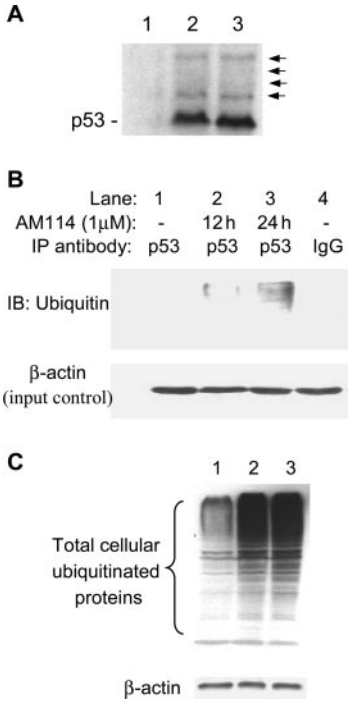

Fig. 4. Accumulation of ubiquitinated proteins in cells treated with AM114. A, protein extracts isolated from the control or AM114-treated HCT116 wt p53 cells were assayed for p53 protein by Western blotting. Lane 1, protein extracts from control cells; lanes 2 and 3, protein extracts from cells treated with 1 μM AM114 for 12 and 24 h, respectively. Note that above the p53 band, there are protein bands detected by p53 antibody (indicated by arrows). B, HCT116 wt p53 cells were treated with 1 μM AM114 for the indicated times. The untreated and treated cells were lysed, and subjected to immunoprecipitation with anti-p53 antibody (lanes 1–3) or IgG as a negative control (lane 4). The immunoprecipitated samples were subsequently blotted for ubiquitin. β-Actin levels were assayed as the protein input control in each sample by immunoblotting before immunoprecipitation. (C) Protein extracts isolated from the control or AM114-treated HCT116 wt p53 cells were assayed for ubiquitin proteins by western blotting using an anti-ubiquitin antibody. Lane 1, protein extracts from control cells; lanes 2 and 3, protein extracts from cells treated with 1 μM AM114 for 12 and 24 h, respectively. β-Actin levels were also measured as a loading control.

lysates of the control cells (without drug treatment) and from cells preincubated with AM114 for various times and then analyzed the precipitates for ubiquitin protein using immunoblotting. As showed in Fig. 4B, upon AM114 treatment, there was a time-dependent increase in the amount of ubiquitin protein that was immunoprecipitated by the anti-p53 antibody, indicating that AM114 induced an accumulation of ubiquitinated p53 protein. This result is consistent with the role of ubiquitination in p53 stability observed previously (Dietrich et al., 2003; Lyakhovich and Shekhar, 2003; Kim et al., 2004).

The above-mentioned observations led us to determine whether the increased accumulation of ubiquitinated p53 was unique to this protein, or whether there was a global accumulation of ubiquitinated proteins in cells treated with AM114. We performed a direct immunoblot analysis of total protein lysates of cells treated with AM114 using an anti-ubiquitin antibody to detect the general uniquitinated proteins. This analysis revealed that AM114 treatment induced a significant increase in the levels of total ubiquitinated proteins in cells (Fig. 4C).

Inhibition of the 20S Proteasome Activity by Boronic Chalcone AM114. Previous studies showed that inhibition of the proteasome activity is a major mechanism that causes increased accumulation of ubiquitinated proteins, which are normally degraded by the cellular proteasome machinery (Chung et al., 2005; Kessova and Cederbaum, 2005; Marteijn et al., 2005; Zhang et al., 2005). We therefore tested whether AM114 might inhibit proteasome activity, leading to the accumulation of ubiquitinated proteins in the cells. Proteasome activity was measured using a proteasome assay kit as described under *Materials and Methods*. MG132, a known inhibitor of the proteasome, was used as a positive control. As the shown in Fig. 5, AM114 inhibited the 20S proteasome activity in vitro with purified enzyme in a concentration-dependent manner. For example, 1 μM AM114 inhibited proteasome activity by 46%, and 3 μM AM114 inhibited proteasome activity by 76%. Under the same assay conditions, 30 μM MG132 inhibited the propeasome activity by 77%. Together, these data indicate that AM114 is an inhibitor of the 20S proteasome, with an IC_{50} value of approximately 1 μM.

Sensitization of Cancer Cells to Ionizing Radiation by AM114. Because p53 plays an important role in cellular response to IR, we tested whether AM114 could affect the sensitivity of cancer cells to IR through its ability to inhibit proteasome and cause p53 accumulation. HCT116 p53+/+ and p53−/− cells were plated in six-well plates and treated with 1 μM AM114, 2-Gy IR, or both. At the end of the 14-day incubation period, cell colonies were fixed and counted. As shown in Fig. 6, the wild-type p53 cells were sensitive to AM114 or IR alone, with a significant decrease in colony formation to only 9 and 22%, respectively. Combination of the two agents decreased the colony formation further to 2%, consistent with an additive effect ($0.09 \times 0.22 = 0.02$). As expected, the HCT116 p53−/− cells were less sensitive to AM114 or IR, which caused a decrease of colony survival to 74 and 36%, respectively. We were surprised to find that the combination of AM114 and IR exhibited a significant synergistic effect on the p53−/− cells, reducing the colony formation to 7%, which was much less than the 27% expected for additive effect ($0.74 \times 0.36 = 0.27$). This synergistic activity seemed p53-independent and may have potential therapeutic implications.

Discussion

Chalcones and chalcone derivatives have been shown to exhibit cytotoxic activity against cancer cells and may have potential applications in cancer treatment (Yamamoto et al., 1991; Satomi, 1993; Shibata, 1994; de Vincenzo et al., 1995; Makita et al., 1996). However, the exact mechanisms by which chalcone compounds exert their cytotoxic effects in cancer cells remains unclear. Based on the observations that carboxylic chalcones may disrupt the MDM2-p53 interaction (Stoll et al., 2001) and that boronic chalcone derivatives induce an accumulation of p53 and p21 proteins and exhibit significantly greater growth inhibitory effect on human breast cancer cell lines than on normal breast epithelial cells

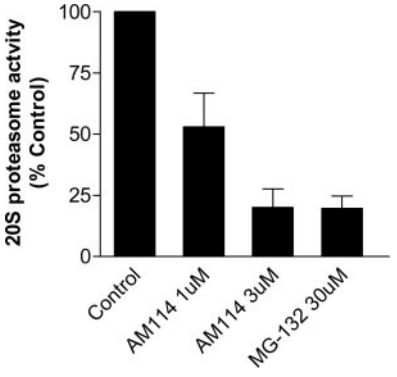

Fig. 5. Inhibition of the 20S proteasome activity by AM114. 20S proteasome activity was measured using the 20S proteasome assay kit as described under *Materials and Methods*. The reaction mixtures containing 1 μg of 20S proteasome were incubated with the indicated concentrations of AM114 or MG-132 (a known inhibitor of proteasome as a positive control) at 37°C for 30 min, followed by addition of the proteasome substrate solution to a final concentration of 10 μM. At the end of 1 h, the fluorescent signal of the reaction products in all samples was determined at 355-nm excitation and 460-nm emission, and the values were plotted on a graph as percentage of the control sample. The data represent mean ± S.D. from three separate experiments.

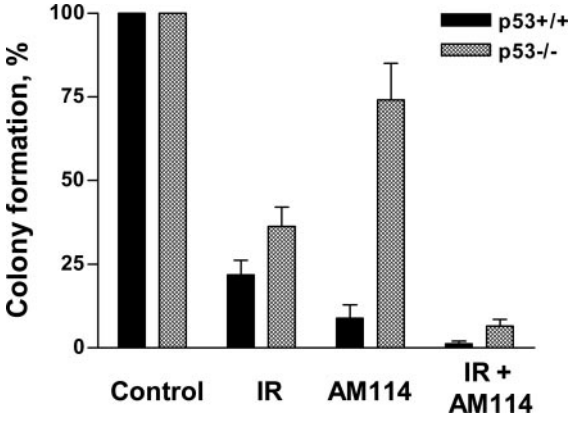

Fig. 6. Cytotoxic effect of AM114 in combination with IR in HCT116 cells. HCT116 p53+/+ and HCT116 p53−/− cells were plated in six-well plates and then treated with 1 μM AM114 (for 12 h before exposing cells to IR), 2-Gy IR, or the combination of 1 μM AM114 + 2-Gy IR as described under *Materials and Methods*. After a 14-day incubation, the surviving colonies were fixed, stained, and counted. The cytotoxicity of AM114, IR, or their combination is expressed as percentage of the control colony formation. Each bar represents mean ± S.D. from three experiments.

(Kumar et al., 2003), we further compared the cytotoxic activity of the potent boronic chalcone derivatives AM114 and AM58 in an isogenic pair of p53+/+ and p53−/− cells and explored its mechanisms of action in the current study. Our studies demonstrated that AM114 and AM58 exhibited greater cytotoxic activity against p53+/+ cells compared with p53−/− cells, as evidenced by clonogenic survival assays (Fig. 1) and Annexin V-PI double staining (Fig. 2).

Although AM114 was able to induce accumulation of p53 and p21 proteins in the p53+/+ cells, no disruption of p53-MDM2 interaction was observed either in vitro or in whole cells treated with this compound. Thus, the observed accumulation of p53 was unlikely to be due to stabilization of p53 through dissociation from MDM2. Our biochemical analysis demonstrated that AM114 significantly inhibited the activity of the 20S proteasome in vitro with an IC_{50} value of approximately 1 μM. In whole cells, this proteasome inhibition led to the accumulation of p53 and other ubiquitinated proteins. These results suggest that AM114 may exert its cytotoxic effect in cancer cells through inhibition of the proteasome, leading to the accumulation of p53 and other ubiquitinated proteins. This is consistent with the observations in previous studies using other proteasome inhibitors such as bortezomib, which induced greater cytotoxicity in cells with functional p53 (MacLaren et al., 2001; Ling et al., 2003; Williams and McConkey, 2003). However, the relationship between p53 status and sensitivity to proteasome inhibition seems to be cell type-dependent, because in certain cell lines such as prostate cancer and multiple myeloma cells induction of cell death by bortezomib seemed to be independent of p53 (Adams et al., 1999; Hideshima et al., 2001). In HCT116 colon cancer cells, AM114 preferentially killed p53+/+ cells at relatively low concentrations (0.3–10 μM), whereas at a higher concentration (30 μM), this compound effectively killed both p53+/+ and p53−/− cells (Fig. 1). This concentration-dependent cell killing might provide an explanation for the apparent discrepancy reported in the literature on the relationship between p53 status and cellular sensitivity to proteasome inhibitors.

It is currently unclear whether AM114 differs from the other boronic chalcone derivatives in its mechanism of cytotoxic action. We have tested another boronic chalcone derivative, AM58, and obtained results similar to those observed with AM114. AM58 also exhibited preferential killing of wt p53 cells and caused accumulation of p53 and p21 proteins, without disruption of MDM2-p53 interaction (data not shown). Earlier studies performed with other boronic chalcones using multidimensional NMR spectroscopic analysis suggested that some of these compounds might bind a region of the p53-binding site of MDM2 and disrupt the MDM2-p53 protein interaction (Kumar et al., 2003). Considering the diverse chemical modifications on these boronic chalcone derivatives, some of these compounds may interfere with p53-MDM2 interaction, whereas other derivatives may inhibit proteasome activity, and/or even have other mechanisms of action. Thus, boronic chalcones are likely to have multiple targets in cells. It is unclear at the present time whether the boronic chalcone derivatives such as AM114 and AM58 could also cause DNA damage and trigger p53 activation and accumulation of p21. Because the p53/MDM-2 pathway and the proteasome play essential roles in multiple cellular functions and represent important targets for anticancer drug development, it would be interesting to determine the chemical structural features responsible for proteasome inhibition or for interruption of p53/MDM2 interaction. The limited number of AM compounds available for this study did not allow us to conduct such a structure-activity relationship analysis. It would be important in future studies to synthesize a larger number of chalcone derivatives for structure-activity relationship determination.

It is interesting to note that AM114 as a single agent alone exhibited preferential cytotoxicity against p53+/+ cells, whereas its combination with IR significantly enhanced the killing of both p53+/+ and p53−/− cells. The combination of AM114 with IR seems to have a greater than additive effect in p53−/− cells (Fig. 6). It is possible that in p53-null cells, accumulation of the ubiquitinated proteins due to inhibition of proteasome by a low concentration of AM114 is insufficient to trigger cell death in the absence of p53. However, such proteasome inhibition may be sufficient to render the cells sensitive to ionizing radiation. Although the precise mechanisms responsible for the p53-independent sensitization of cells to IR by AM114 remain to be further elucidated, the ability of this compound to enhance IR-induced killing of p53-null cancer cells is promising and may have significant therapeutic implications.

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