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Up-Regulation of p21$^{WAF1/CIP1}$ by Histone Deacetylase Inhibitors Reduces Their Cytotoxicity

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ABSTRACT

Histone deacetylase inhibitors show promise as chemotherapeutic agents and have been demonstrated to block proliferation in a wide range of tumor cell lines. Much of this antiproliferative effect has been ascribed to the up-regulated expression of the cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$. In this article, we report that p21 expression was up-regulated by relatively low doses of the histone deacetylase inhibitor azelaic bishydroxamic acid (ABHA) and correlated with a proliferative arrest. Higher doses of ABHA were cytotoxic. Cells that did not up-regulate p21 expression were hypersensitive to killing by ABHA and died via apoptosis, whereas up-regulation of p21 correlated with reduced sensitivity and a block in the apoptotic mechanism, and these cells seemed to die by necrosis. Using isogenic p21$^{+/+}$ and p21$^{-/-}$ cell lines and direct inhibition of caspase activity, we demonstrate that the reduced sensitivity to killing by ABHA is a consequence of inhibition of apoptosis by up-regulated p21 expression. These data indicate the enormous potential of therapeutic strategies that bypass the cytoprotective effect of p21 and act on the same molecular targets as the histone deacetylase inhibitors.

Histone deacetylase inhibitors (HDI) are potent inducers of terminal differentiation and cell death in immortalized cells and tumor cell lines (Richon et al., 1998; Kim et al., 1999; Qiu et al., 1999; Saito et al., 1999; Saunders et al., 1999) and are being promoted as a potent new treatment for cancer and other hyperproliferative diseases (Kim et al., 1999; Saito et al., 1999; Saunders et al., 1999). The molecular mechanism by which these compounds achieve their antitumor activity is believed to be their ability to modify chromatin structure by increasing the acetylation state of the nucleosomal histones, thereby influencing transcription (Grunstein, 1997). Much of the research on HDIs has focused on the up-regulated expression of the cyclin-dependent kinase (cdk) inhibitor p21$^{WAF1/CIP1}$ (Kim et al., 1999; Saito et al., 1999; Richon et al., 2000). The transcription of p21 is up-regulated as a consequence of increased acetylation of the chromatin at the Sp1 binding sites in the promotor region of p21 (Sambucetti et al., 1999; Richon et al., 2000). The increased levels of p21 protein bind and inhibit G_1/S-phase cdk2 activity, reducing the retinoblastoma protein to its hypophosphorylated form, and this correlates with the G_1 phase arrest observed in a range of cell types after treatment with diverse HDIs (Kim et al., 1999; Saito et al., 1999; Sambucetti et al., 1999; Qiu et al., 2000). Knocking out p21 expression reduces the growth inhibitory effect of HDI treatment (Archer et al., 1998). A number of other cell cycle regulators are also affected by HDI treatment, and together with the up-regulated p21 expression, these effectively block proliferation (Sambucetti et al., 1999). These findings suggest that up-regulation of p21 expression may play a critical role in the antiproliferative activity of this class of drugs.

Whereas increased p21 expression is correlated with a block in proliferation, a number of reports have demonstrated that HDIs can also induce apoptosis (McBain et al., 1997; Bernhard et al., 1999; Glick et al., 1999). Our own work has demonstrated that the HDI azelaic bishydroxamic acid (ABHA) kills a wide range of immortalized cells and tumor cell lines, but cultures of primary cells and a small number of tumor cell lines are resistant to the cytotoxic effects of these

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ABBREVIATIONS: HDI, histone deacetylase inhibitor; cdk, cyclin-dependent kinase; ABHA, azelaic bishydroxamic acid; IPTG, isopropyl β-D-thiogalactoside; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; PARP, poly ADP-ribose polymerase; Z-DEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone.

drugs (Parsons et al., 1997; Qiu et al., 1999; Qiu et al., 2000). The selective cytotoxicity is the consequence of loss of an HDI-sensitive G_2-phase cell cycle checkpoint arrest in the ABHA-sensitive cells, resulting in the cells undergoing an aberrant mitosis. Reintroduction of a G_2 phase arrest rescued the sensitive cells from ABHA-induced cell death (Qiu et al., 2000). ABHA-induced up-regulation of p21 expression was also found and correlated with a G_1 phase arrest in both ABHA-sensitive and -resistant cell lines (Qiu et al., 2000). We found that the cell line most sensitive to killing by ABHA did not up-regulate p21 after drug treatment. We reasoned that ABHA-induced up-regulation of p21 and the consequent G_1 phase arrest may provide some degree of protection from the cell cycle-dependent cytotoxic effects in the ABHA-sensitive cell lines by reducing the proportion of cells passing through the faulty G_2 checkpoint.

In this study, we investigated the effect of up-regulated p21 expression on the sensitivity of cells to killing by ABHA and also the mechanism of cell death induced by drug treatment. We report that cell lines that did not increase p21 protein levels in response to ABHA treatment were hypersensitive to killing by the drug, and cell death occurs exclusively via apoptosis. However, a large proportion of the tumor cell lines tested up-regulated p21, and this correlated with a reduced sensitivity to killing by ABHA and a block in the apoptotic pathway. We discuss the implications of these findings on the future development of novel chemotherapeutic agents.

Experimental Procedures

Materials. ABHA was synthesized by Mike West (Center for Drug Design and Development, University of Queensland, Brisbane, Queensland, Australia). All other chemicals used were of analytical grade.

Cell Lines and Culture Conditions. The cell lines used were a human cervical cancer cell line (HeLa); spontaneously immortalized keratinocyte (HaCaT); human melanoma cell lines SK-Mel-13, MM96L, A2058, HT144, ME10538, MM170, MM384, SK-Mel-28, and JA; ovarian cancer cell lines c180–135 and OvCar; breast cancer lines T47D, ZR-75–1, and SKBr3; and two SK-Mel-13 lines engineered for stable inducible expression of p16: pOPRSVp16wt-4 and -6. These cells used the Lac Switch system (Stratagene, Cambridge, UK) to allow IPTG-inducible expression of p16. The HCT-116 wild-type ($p21^{+/+}$) and p21-deleted ($p21^{-/-}$) human colon carcinoma cells were kindly provided by B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD) (Waldman et al., 1995). All cells were cultured in Dulbecco's modified Eagle's medium containing 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 3 mM HEPES and supplemented with 5% (HeLa) or 10% (remaining cell lines) (v/v) Serum Supreme (BioWhittaker Europe, Verviers, Belgium). In addition, the p16-inducible lines were cultured with 375 μg/ml Geneticin (Roche Molecular Biochemicals, Mannheim, Germany) and 100 μg/ml hygromycin (Invitrogen, Carlsbad, CA) to maintain selection of the stable transfected cells. Assays for mycoplasma were performed to ensure that the cultured cells were free of contamination. Asynchronous cultures of each cell line were treated with 100 μg/ml ABHA for 24 h or 48 h and then harvested for immunoblotting or flow cytometry.

Cell Proliferation Assay. Cells in log-phase growth were seeded into 96-well plates at a density of 2 to 5×10^3 cells on the day before addition of 100 μg/ml ABHA. Cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), which measures the mitochondrial activity of viable cells. MTT was added to the culture medium at a final concentration of 0.5 μg/ml, and the plated cells were incubated for 4 h at 37°C. The insoluble formazan product was then precipitated by centrifuging the plates, removing the supernatant, and redissolving the formazan crystals in 100 μl of dimethyl sulfoxide. Absorbance at 570 nm was measured using a microplate reader (Bio-Rad, Hercules, CA).

Flow Cytometry. For flow cytometric analysis, floating and attached cells were collected. Cells were fixed in ice-cold 70% ethanol and stored at −20°C. Samples were then washed once in PBS and resuspended in a solution of propidium iodide (5 μg/ml) and RNase A (0.5 mg/ml) in PBS. The stained cells were filtered through 37-μm gauze, and the single-cell suspensions were analyzed on a FACSCalibur system (BD Biosciences, San Jose, CA) using Cell Quest (BD Biosciences) and ModFit (Verity Software, Topsham, ME) data analysis software. In some cases, a one-tailed unpaired Student's t test was used to determine whether treated and untreated samples were significantly different. P values less than 0.05 were considered significant and evidence of population differences.

TUNEL Staining of Apoptotic Cells. A terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) analysis cell death detection kit (Roche Molecular Biochemicals) was used to detect apoptotic cells. Asynchronous cultures were treated with ABHA (100 μg/ml) for 24 h and 48 h and then harvested at indicated time points, washed once in PBS, fixed in 70% ethanol, and stored at −20°C. Cells were then washed once in PBS and resuspended in 100 μl of a solution containing 0.1% sodium citrate and 0.1% (v/v) Triton X-100 in PBS for 2 to 5 min on ice. Cells were then washed with PBS and stained with 50 μl of reaction mixture containing 5 μl of enzyme and 45 μl of fluorescein isothiocyanate (Roche Molecular Biochemicals) labeling solution for 1 h.

Cells were then washed twice with PBS and resuspended in a solution of propidium iodide (5 μg/ml) and RNase A (0.5 mg/ml) in PBS. The stained cells were filtered through 37-μm gauze, and the single-cell suspensions were analyzed on a FACSCalibur system using Cell Quest data analysis software.

Annexin V Staining of Apoptotic cells. Annexin V staining was performed essentially as described previously by van Engeland et al. (1996). Briefly, asynchronous cell cultures were treated with ABHA (100 μg/ml) and harvested at the indicated time points. Both the floating and attached cells were harvested. The cell pellet was washed in 1× PBS, resuspended in 100 μl of annexin buffer (10 mM HEPES, 10 mM NaOH, 140 mM NaCl, and 5 mM $CaCl_2$), and incubated with 4 μl of the annexin V protein labeled with fluorescein isothiocyanate for 15 min in the dark at room temperature. After incubation, an additional 400 μl of annexin buffer was added to each sample, along with 5 μg/ml of propidium iodide. The solution was then filtered through 37-μm gauze and analyzed by flow cytometry on a FACScan or FACSVantage system (BD Biosciences) using Cell Quest data analysis software.

Two-Dimensional Dye Exclusion Apoptosis Assay. A modified version of the method described by Pollack and Ciancio (1990) was used to discriminate between viable, apoptotic, and necrotic cells. Asynchronous cell cultures were treated with ABHA (100 μg/ml) and harvested at indicated time points. Both the floating and attached cells were collected and washed once in 1× PBS before resuspending the cells in 1 to 2 ml of 5% media containing 20 μg/ml propidium iodide for 30 min at 37°C. Samples were then washed twice with PBS, fixed with 70% ethanol, and stored at −20°C. The fixed samples were then washed once with PBS and resuspended in a solution of Hoechst 33342 (1 μg/ml) and RNase A (0.5 mg/ml) in PBS. The stained cells were filtered through 37-μm gauze, and the single-cell suspensions were analyzed on a FACSVantage system using Cell Quest data analysis software.

Immunoblotting. Cells were lysed in buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 20 mM Tris, pH 8) supplemented with 5 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 0.1 mM sodium orthovanadate. The cleared supernatants were stored at −70°C until use. Protein quantification was performed using bicinchoninic acid

(Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard. Samples (20 μg of protein) were resolved on 10% or 12% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Amersham Pharmacia Biotech UK, Ltd., Little Chalfont, Buckinghamshire, UK). Antibodies were then used to detect the levels of p21 (Calbiochem, San Diego, CA) and poly ADP-ribose polymerase (PARP) (BD PharMingen, San Diego, CA) with the appropriate horseradish peroxidase-conjugated secondary antibody (Silenus, Melbourne, Australia) using enhanced chemiluminescence detection (PerkinElmer Life Sciences, Boston, MA).

Results

ABHA Inhibits Proliferation at Low Doses but Is Cytotoxic at High Doses. HDIs have been reported to have both cytostatic and cytotoxic properties. We were interested to examine whether these two properties were related to the dose of drug used. When cell lines were treated with a relatively low dose of ABHA (10 μg/ml), there was a reduction in proliferation in all of the cell lines tested (Fig. 1A). FACS analysis showed that HeLa and SK-Mel-13 cells were arrested in the G_1 phase after 24 h, as demonstrated by the reduction in S-phase cells, but little cell death was detected, which is normally indicated by the presence of cells with <2n DNA content (Fig. 1B; Qiu et al., 2000). By 48 h, these cell lines had resumed cycling. A similar transient G_1 phase arrest was observed in a range of cell lines, including ABHA-resistant neonatal foreskin fibroblasts, melanoma cell line MM229, and the drug-sensitive A2058 and HT144 cell lines after treatment with 10 μg/ml ABHA (data not shown). There was no evidence of a G_2/M arrest with low-dose drug treatment in the ABHA-sensitive cell lines or in ABHA-resistant cell lines in which a G_2/M arrest was observed with high-dose treatment (Qiu et al., 2000). The proportion of S-phase cells in the melanoma cell line MM96L did not decrease to the same extent as the HeLa and SK-Mel-13 cells after 24-h low-dose ABHA treatment, although their S-phase content reduced further by 48 h and their proliferation was reduced (Fig. 1, A and B). Again, little cell death was detected at this dose of ABHA. At a dose of 100 μg/ml, we observed both proliferative arrest by 24 h and cell death by 48 h in the HeLa and SK-Mel-13 cells, with cell death demonstrated by the reduction in metabolically viable cells to lower than the day-0 levels and high proportion of subdiploid cells (Fig. 1, A and B). MM96L cultures seemed to be more sensitive than either HeLa or SK-Mel-13 cells to the cytotoxic effects of this dose of ABHA and contained >40% subdiploid cells by 24 h and >90% by 48 h.

The transient G_1 phase arrest in the HeLa and SK-Mel-13 cells correlated with HDI-induced expression of the cdk inhibitor p21, which acts by binding and inhibiting the G_1/S-phase cyclin/cdk2 complexes (Sambucetti et al., 1999; Qiu et al., 2000). Immunoblotting confirmed a strong increase in p21 levels in HeLa and SK-Mel-13 cells after both low- and high-dose drug treatment at 24 h, which declined at 48 h, although it was still higher than control levels (Fig. 2). However, no p21 expression was detected in MM96L cells, even with high-dose treatment (Fig. 2).

ABHA-Sensitive Cells Can Be Subdivided into Two Classes. Dose-response experiments demonstrated that cell lines sensitive to the cytotoxic effects of ABHA could be further subdivided into two classes: cell lines sensitive to killing by ABHA with a D_{37} value [dose required to lower

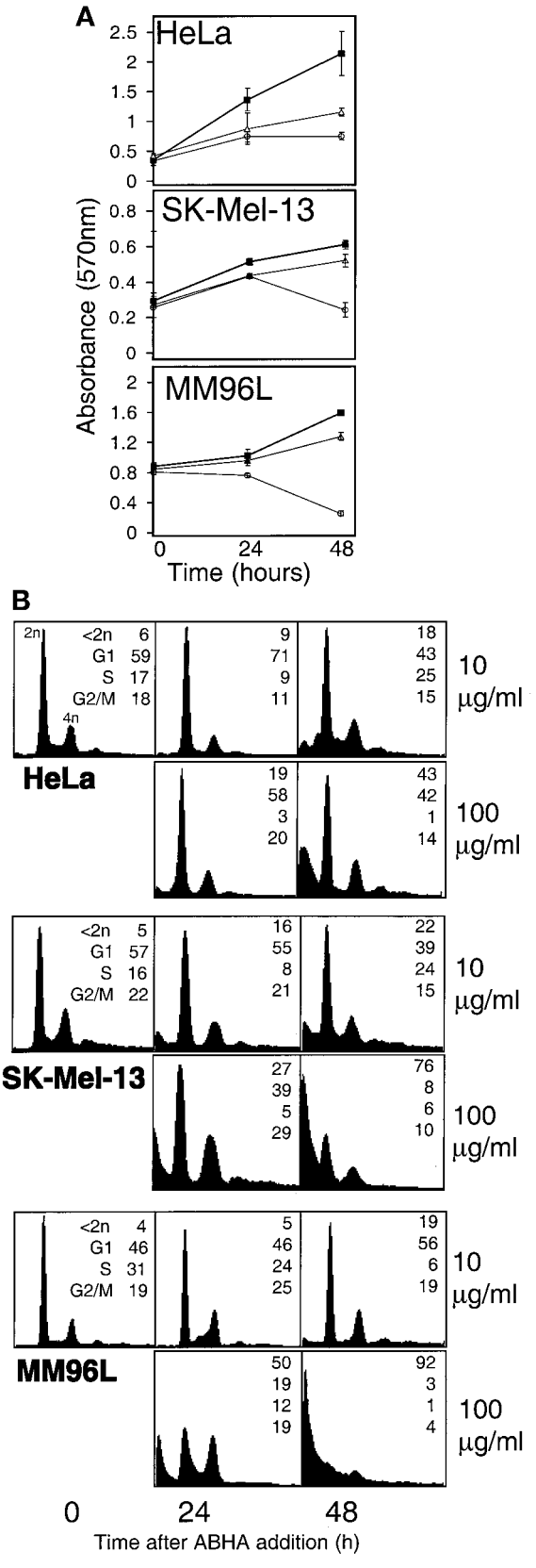


Fig. 1. A, proliferation assay for control (■), low-dose (10 μg/ml; △), and high-dose (100 μg/ml; ○) treatment with ABHA. The data shown are the mean and standard error from triplicate determinations. B, FACS cell cycle profiles corresponding to the proliferation data shown in A. The percentage of cells with subdiploid (<2n) DNA content and the various cell cycle phases are shown.

survival to 37% of control (Qiu et al., 1999)] of 30 to 70 μg/ml (e.g., HeLa and SK-Mel-13) and those hypersensitive to killing by ABHA with a D_{37} value < 20 μg/ml [e.g., MM96L and the immortalized human keratinocyte cell line HaCaT (Fig. 3)]. The common characteristics of the hypersensitive cell lines were their lack of G_1 phase arrest and p21 induction and >50% and >90% subdiploid cells by 24 and 48 h of treatment, respectively, with high doses of ABHA. A panel of 16 cell lines was tested at a single cytotoxic dose of ABHA (100 μg/ml), and the level of p21 expression and their cell cycle status, including the subdiploid population as a measure of cell death, were assessed at time points to 48 h. Using criteria established from detailed study of proliferation, cell cycle profile, dose response, and p21 expression of the HeLa, SK-Mel-13, MM96L, and HaCaT cell lines, we identified only four cell lines that fit the criteria for hypersensitivity: MM96L, HaCaT, JA, and OvCar (Fig. 4). The remaining cell lines were all sensitive to killing by high doses of ABHA, although the percentage of subdiploid cells at 48 h ranged from 20 to 80%. In every case in which p21 was induced strongly at 24 h, there was a corresponding decrease in the percentage of S-phase cells, indicative of a G_1 phase arrest (Fig. 4).

Mode of Death Varies Between Sensitive and Hypersensitive Cells. HDIs have been reported to induce apoptosis in a range of cell lines (Medina et al., 1997; Bernhard et al., 1999; Glick et al., 1999). We examined whether the differences in cytotoxicity of high-dose ABHA treatment was a result of the efficacy with which ABHA induced apoptosis in the sensitive and hypersensitive cell lines. A number of markers of apoptosis were examined in cells treated with a cytotoxic dose of ABHA. An initial measure of apoptosis was the proteolytic cleavage of PARP, which is cleaved by the apoptotic executioner protease caspase-3 from its full-length 113-kDa form to 89- and 24-kDa products. The extent of PARP cleavage was assessed in all 20 cell lines by immunoblotting. Complete cleavage of the PARP to its 89-kDa fragment was observed 24 h after ABHA treatment in the MM96L, HaCaT, JA, and OvCar cell lines, but only partial cleavage was found after 24 h in the remaining cell lines, although almost complete PARP cleavage was observed in KJD, HT144, ME10538, MM170, MM383, and SkBr3 cell lines by 48 h (Figs. 4 and 5). The cell lines showing complete PARP cleavage by 24 h lacked or had very low levels of p21 after ABHA treatment, whereas cell lines with partial PARP cleavage at 24 h up-regulated p21 expression. This suggested that up-regulation of p21 may influence the ability of cells to undergo apoptotic cell death after high-dose ABHA treatment. To further examine the mechanism of cell death, a representative panel of four cell lines—HeLa and SK-Mel-13, which up-regulated p21, and MM96L and HaCaT, which failed to do so—were analyzed for other markers of apoptosis. These markers were analyzed using flow cytometry to assess the state of individual cells within the total population. TUNEL labeling revealed that only 10 and 20% of HeLa and SK-Mel-13 cells, respectively, were TUNEL-positive (apoptotic) 48 h after treatment with ABHA (Fig. 6). This compared with 60 and >80% displaying TUNEL-positive staining at the same time for MM96L and HaCaT cells, respectively. Annexin V staining revealed the same trend, with only 20% of HeLa cells, compared with 83% of MM96L cells, staining with annexin V after 48 h of ABHA treatment (data not shown). A two-dimensional dye-exclusion FACS assay that measured plasma membrane integrity produced similar results. With HeLa cells, 35% underwent necrosis (i.e., strongly stained with propidium iodide, indicating loss of membrane integrity), and only 19% were apoptotic (low propidium iodide and high H33342 staining) at 48 h, compared with 57% that were apoptotic in MM96L cultures at this same time after high-dose ABHA treatment (data not shown). These data demonstrate that 60 to 80% of MM96L and HaCaT cells died by apoptosis, whereas <20% of HeLa and SK-Mel-13 died via this mechanism.

Induction of p21 Inhibits Apoptosis and Reduces Cytotoxicity of ABHA. To test directly whether the elevated levels of p21 were responsible for the reduced sensitivity to killing by ABHA and inhibiting apoptosis, a set of isogenic cell lines differing only in their p21 status was used (Wald-

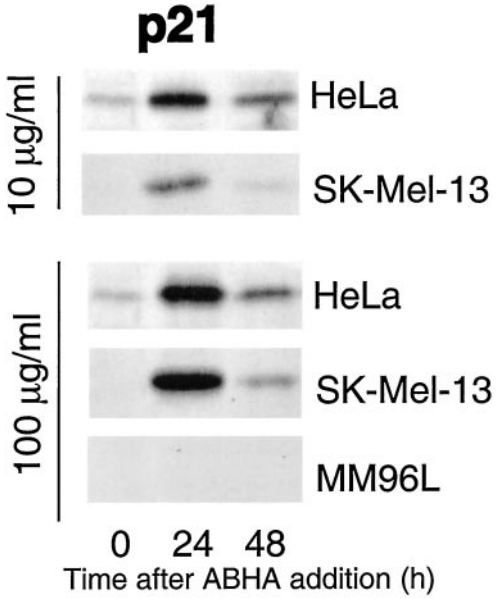


Fig. 2. The level of p21 protein was assessed by immunoblotting lysates from the indicated cell lines, either control (0) or after 24 or 48 h of treatment with either 10 μg/ml or 100 μg/ml ABHA.

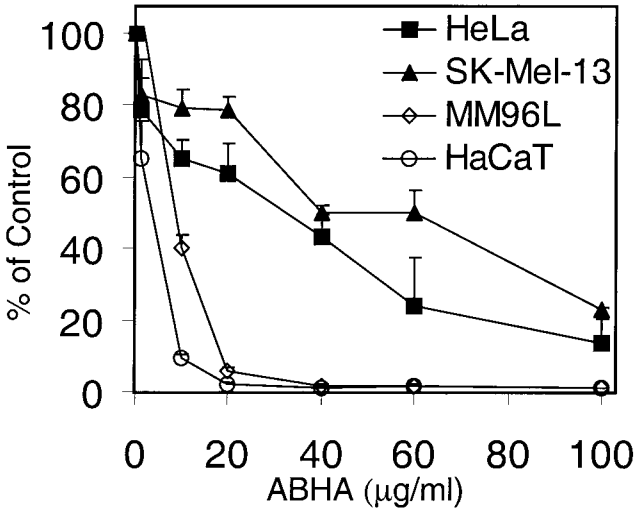


Fig. 3. Dose response of the indicated cells lines with increasing concentrations of ABHA. Cell proliferation was measured by MTT assay after 48 h of treatment. The data shown are mean and S.E. from triplicate determinations.

man et al., 1995). The $p21^{+/+}$ HCT116 cells were sensitive to killing by ABHA, with a D_{37} value of 25 to 30 μg/ml, whereas the $p21^{-/-}$ cells had a dose response that was characteristic of the hypersensitive lines and were much more sensitive to killing by ABHA (D_{37} = 3–5 μg/ml; Fig. 7A). ABHA increased p21 expression in the parental $p21^{+/+}$ cells, although not to the extent seen with the other sensitive cell lines (compare Fig. 7B with Fig. 2). The mechanism of cell death was assessed by analysis of PARP cleavage and TUNEL staining. The $p21^{+/+}$ cells increased to 21% of cells staining TUNEL-positive at 24 h after high-dose ABHA treatment compared with 82% in the $p21^{-/-}$ cells (Fig. 7C). The $p21^{+/+}$ cells also showed incomplete PARP cleavage 24 h after high-dose ABHA addition, whereas complete cleavage was detected in the $p21^{-/-}$ cells (data not shown). Thus, loss of p21 induction increased the sensitivity of the cells to killing by ABHA and increased the proportion of cells dying via an apoptotic mechanism.

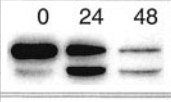
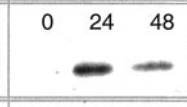
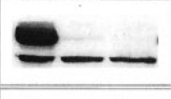
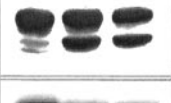
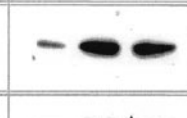
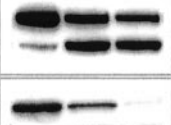
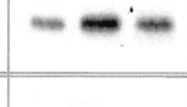
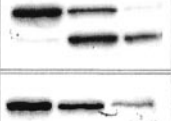
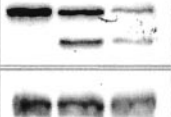
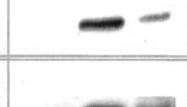
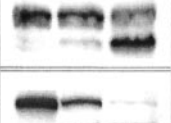
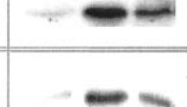
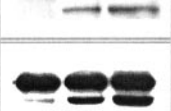
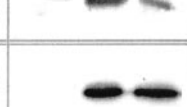
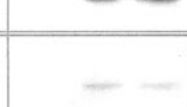
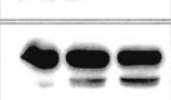
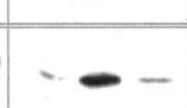
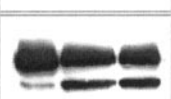
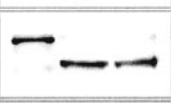
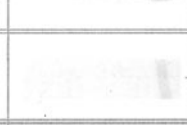
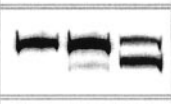
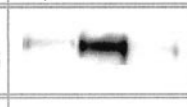
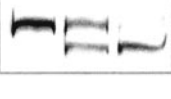
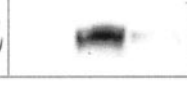
Cell type	PARP	p21	Time	<2n	G1	S	G2/M
	0 24 48	0 24 48					
KJD (SV40 keratinocyte)			0	12	39	28	21
			24	58	18	0	24
			48	83	7	0	10
HaCaT (Immortalized keratinocyte)			0	12	35	40	13
			24	70	10	3	18
			48	95	4	1	1
18-11-Ti (HPV transformed keratinocyte)			0	5	60	12	23
			24	37	38	0	25
			48	53	34	0	13
A2058 (melanoma)			0	1	52	26	21
			24	11	55	1	33
			48	75	15	0	10
HT144 (melanoma)			0	3	49	22	26
			24	22	42	6	30
			48	61	18	7	14
ME10538 (melanoma)			0	2	62	18	18
			24	14	55	5	26
			48	79	16	0	5
MM170 (melanoma)			0	4	45	34	17
			24	40	39	3	18
			48	72	17	4	7
MM383 (melanoma)			0	5	57	19	19
			24	16	53	8	23
			48	26	48	3	23
SK-Mel-28 (melanoma)			0	5	60	19	17
			24	10	56	6	28
			48	20	52	3	26
JA (melanoma)			0	8	47	29	16
			24	59	21	19	1
			48	98	1	1	0
RPMI 7932 (melanoma)			0	2	55	31	12
			24	22	43	14	22
			48	81	10	6	3
c180-135 (ovarian cancer)			0	9	40	27	24
			24	37	23	3	38
			48	84	4	3	8
OvCar (ovarian cancer)			0	22	37	21	20
			24	91	1	5	3
			48	97	2	0	1
T-47D (breast cancer)			0	12	60	21	8
			24	16	51	4	30
			48	63	19	0	18
ZR-75-1 (breast cancer)			0	9	63	17	11
			24	32	39	10	20
			48	56	27	6	11
SkBr3 (breast cancer)			0	18	47	19	16
			24	25	54	10	11
			48	32	57	9	3

Fig. 4. Cell lines were treated with a single dose of ABHA (100 μg/ml) and harvested at the indicated times. Cells were analyzed by FACS to assess cell cycle status and degree of cell death (<2n DNA content). Cell lysates also underwent immunoblotting for p21 levels and PARP cleavage.

The loss of p21 expression correlated with hypersensitivity to killing by ABHA, suggesting that p21 was in some way inhibiting the apoptotic machinery to reduce the sensitivity of cells to the cytotoxic effects of the drug. To test this directly, the hypersensitive cell line MM96L was treated with the caspase-3 inhibitor Z-DEVD-FMK, and a dose-response experiment was performed. The addition of the caspase inhibitor decreased the sensitivity of these cells to ABHA (Fig. 8A), and the difference in sensitivity was of a magnitude similar to that between the cell lines that did and did not express p21 (Figs. 3 and 7). A low level of p21, although insufficient to produce a G_1 phase arrest (A. J. Burgess and B. G. Gabrielli, unpublished observations), was also detected in the caspase inhibitor and in ABHA-treated cells (Fig. 8B).

Cell Cycle Arrest Only Delays Cell Death. We demonstrated previously that sensitivity to ABHA correlated with the loss of a G_2 checkpoint, resulting in cells undergoing a catastrophic mitosis (Qiu et al., 2000). Using synchronized cultures, we also demonstrated that increasing the proportion of cells progressing through the aberrant mitosis increased the rate of cell death, whereas blocking cell cycle progression in G_2 phase had a protective effect (Qiu et al., 2000). The decreased sensitivity of the cell lines with ABHA-induced p21 expression compared with the hypersensitive lines suggested that the G_1 phase arrest induced by p21 may itself be protective against ABHA killing by blocking transit through the defective G_2 checkpoint. To examine this, two SK-Mel-13 cell lines engineered to conditionally express p16 were induced with IPTG for 24 h to produce a G_1 phase arrest. These cells arrest stably for at least 4 days (A. J. Burgess and B. G. Gabrielli, unpublished observations). The ability of a toxic dose of ABHA (100 μg/ml) to induce cell death in these G_1 phase-arrested cells was assessed. Treatment of the arrested cells with ABHA resulted in a reduction in the proportion of subdiploid cells after 24 h of drug treatment to half the level of the normally cycling cultures, but by 48 h, the G_1 phase arrest provided no cytoprotection, with >80% of the cells in the subdiploid fraction (Fig. 9, A and B). In a similar experiment, hydroxyurea was used to block the transit of MM96L cells through the S phase. After 6 h of hydroxyurea treatment, to permit the hydroxyurea-insensitive G_2/M-phase compartment to empty, the cultures were treated with a high dose of ABHA. The results from this experiment mirrored those found with the p16-inducible cell lines. After 24 h of ABHA treatment, the hydroxyurea-blocked cultures contained half the proportion of subdiploid cells compared with the normally cycling cultures, but by 48 h, essentially 100% of cells in both arrested and cycling cultures contained <2n DNA (Fig. 10A).

The consequence of blocking the cell cycle progression on the mechanism of cell death was also examined. MM96L cells from the hydroxyurea arrest experiments underwent immunoblotting to assess the extent of PARP cleavage. Complete cleavage was consistently observed in hydroxyurea-blocked cells after 24 h of ABHA treatment (Fig. 10B), indicating that even though the proportion of subdiploid cells was reduced, early markers of apoptosis were unaffected by the cell cycle

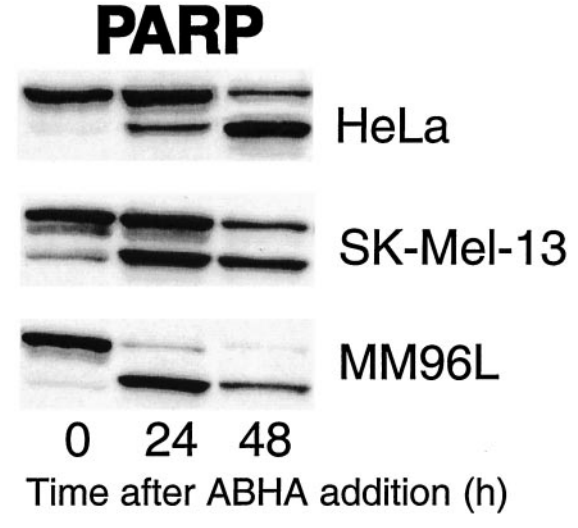


Fig. 5. Lysates similar to those shown in Fig. 2 underwent immunoblotting for PARP. The full-length and 89-kDa product of caspase-3 cleavage are shown.

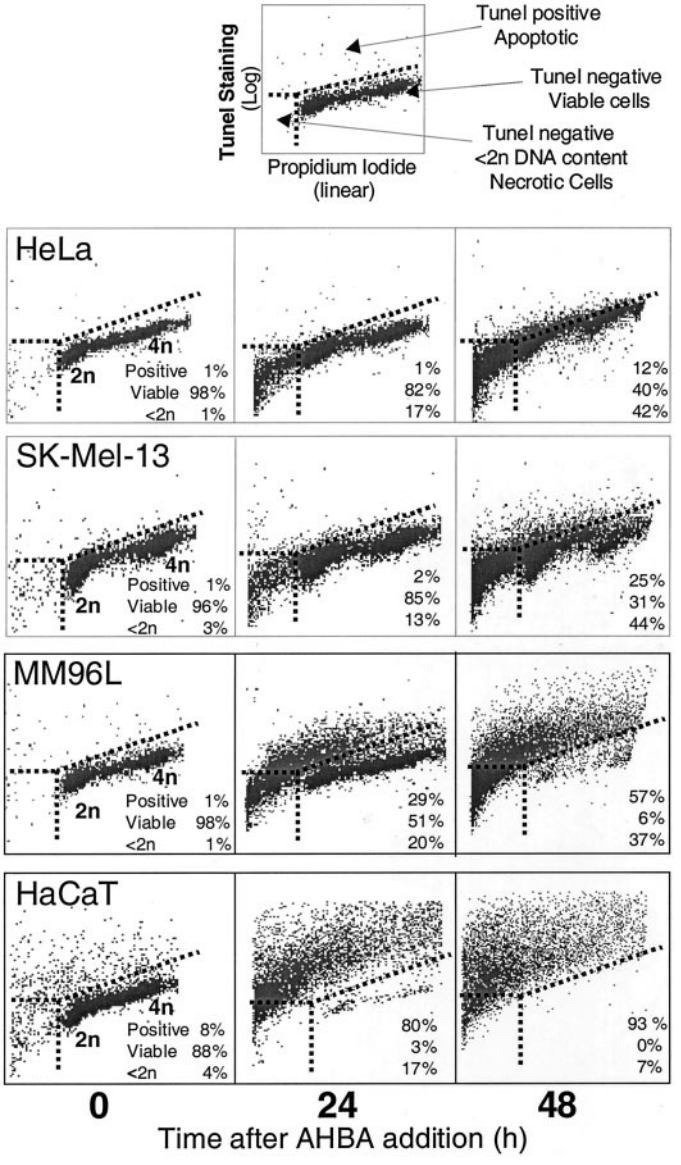


Fig. 6. TUNEL staining was performed on the indicated cell lines, either control (0) or after 24 and 48 h of treatment with 100 μg/ml ABHA. Cells were analyzed by flow cytometry, and the percentages of TUNEL-positive apoptotic (+) and TUNEL-negative viable and necrotic (<2n) cells are given.

arrest. The same result was obtained with the hypersensitive HaCaT cell lines (data not shown). ABHA treatment of the p16-induced, G_1-arrested SK-Mel-13 cells still resulted in increased p21 expression, and only partial cleavage of PARP was observed, essentially identical to the results seen in the parental cell line (Fig. 5).

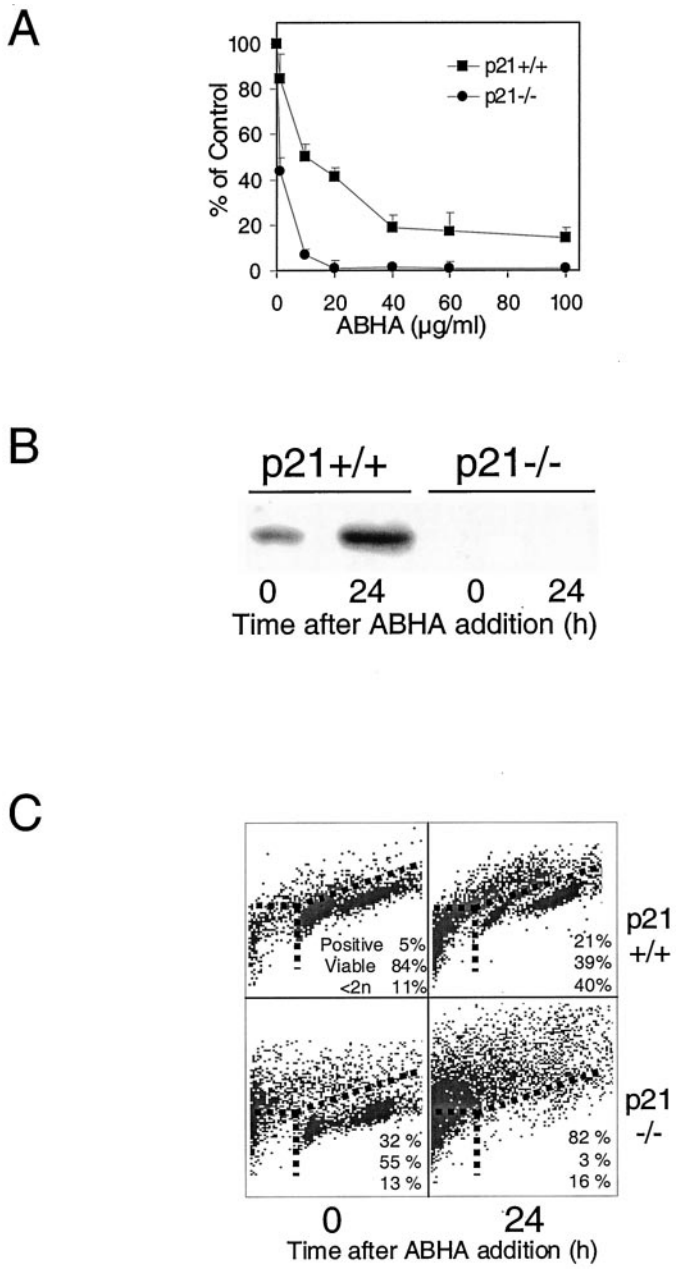


Fig. 7. A, dose response for the HCT116 isogenic p21 wild-type ($p21^{+/+}$) or knockout ($p21^{-/-}$) cell lines with increasing concentrations of ABHA. Cell proliferation was measured by MTT assay after 48 h of treatment. The data shown are mean and standard deviation from triplicate determinations. B, the level of p21 protein was assessed by immunoblotting lysates from the HCT116 cell lines, either control untreated or after 24 h of treatment with 100 μg/ml ABHA. C, TUNEL staining was performed on the indicated HCT116 cell lines, either control or after 24 h treatment with 100 μg/ml ABHA. Cells were analyzed by flow cytometry, and the percentages of TUNEL-positive apoptotic (+) and TUNEL-negative viable and necrotic (<2n) cells (as shown in Fig. 6) are given.

Discussion

In this study, we demonstrated a differential effect with different doses of ABHA. At low doses, this drug can reduce the proliferation of cells, whereas at high doses, the drug is cytotoxic. The up-regulation of p21 by HDIs is likely to be a contributing factor to the reduced proliferation and G_1 phase arrest observed (Archer et al., 1998; Saito et al., 1999; Richon et al., 2000). This is consistent with our observations that all tumor cell lines that increased p21 levels after 24 h of ABHA treatment also displayed a decrease in their S-phase content, indicative of a G_1 phase arrest, whereas cell cycle arrest in cell lines lacking p21 up-regulation did not occur at this time. However, increased p21 expression cannot be solely responsible for the G_1 phase arrest observed with low-dose ABHA treatment, because MM96L cells, which do not increase p21 expression, had reduced proliferation after low-dose drug treatment. The reduced proliferation was not caused by a high level of cell death at this dose; even at a high dose of ABHA, there was little reduction in either DNA synthesis or G_1/S cdk2 activity (Qiu et al., 2000). Therefore, other factors must contribute to this antiproliferative effect.

High-dose treatment with ABHA causes cell death through the failure of a G_2 checkpoint response in sensitive cell lines, and reintroducing a cell cycle arrest in these cells reduced the cytotoxic effect of AHBA (Qiu et al., 2000). Thus, a reduction

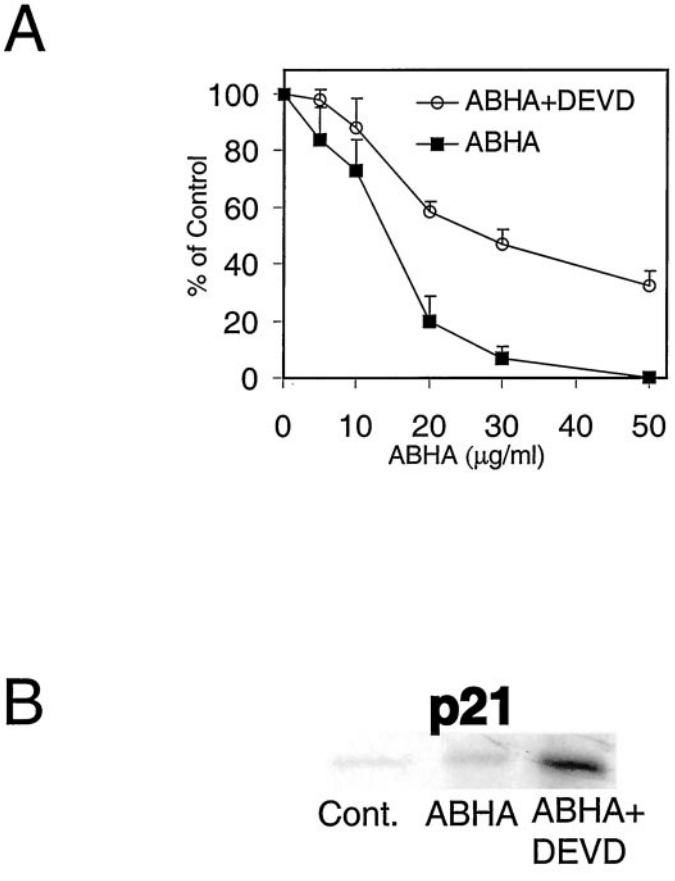


Fig. 8. A, dose response for MM96L cells with increasing doses of ABHA, either in the presence or absence of the caspase-3 inhibitor Z-DEVD-FMK. The data shown are mean and S.E. for triplicate determinations after 48 h. B, immunoblot for p21 in MM96L cells, either control untreated (Cont.) or treated with 100 μg/ml ABHA for 24 h (ABHA), or with ABHA and the caspase-3 inhibitor DEVD (ABHA + DEVD).

in cell death 24 h after high-dose ABHA treatment would be an expected outcome of a block in G_1/S progression and was the observed result of the G_1 phase arrest produced by ectopic p16 expression and the S phase arrest with hydroxyurea. It may also account for the delayed onset of cell death in cells that displayed up-regulated p21 expression and the corresponding G_1/S arrest after high-dose ABHA treatment. Up-regulation of p21 also correlated with a change in the mode of cell death and a reduction in the cytotoxicity of the ABHA dose. The hypersensitive cells died via an apoptotic mechanism, whereas in the sensitive cells, only a small proportion of cells died via apoptosis. The loss of viability of the sensitive cell types, measured by the loss of mitochondrial function (MTT assay) and increased subdiploid population, together with the loss of membrane integrity (dye exclusion) and absence of apoptotic markers, indicated that these cells most probably died by necrosis. We have found that treating the hypersensitive MM96L cells with a caspase-3 inhibitor also reduced sensitivity to the cytotoxic effects of ABHA, although a high level of cell death was still detected with a high dose of drug. The data presented here suggest that ABHA promotes cell death via apoptosis, and factors that compromise the apoptotic mechanism force cells to die via a default, possibly necrotic pathway, rather than avoid cell death. Preventing cleavage of PARP after its activation by apoptosis-promoting agents results in the PARP activity depleting the intracellular NAD+ and ATP pools (Herceg and Wang, 1999), and low intracellular levels of ATP have been linked with a switch from apoptosis to necrosis (Eguchi et al., 1997). Thus, the inhibition of PARP cleavage we have observed in the sensitive cell lines in which p21 expression is up-regulated may directly contribute to the switch from apoptosis to necrosis.

The antiapoptotic effect of p21 may be the result of a number of mechanisms. p21 has been demonstrated to bind to procaspase-3, the inactive precursor of the apoptotic executioner caspase-3, and inhibit its proteolytic activation (Suzuki et al., 1999a). Caspase-3 is responsible for may of the proteolytic cleavage events associated with apoptosis, including PARP cleavage (Wolf and Green, 1999). The reduced ability to rapidly cleave PARP was closely correlated with up-regulated p21 expression in those cells, supporting the role for p21 in inhibiting procaspase-3 activation in these

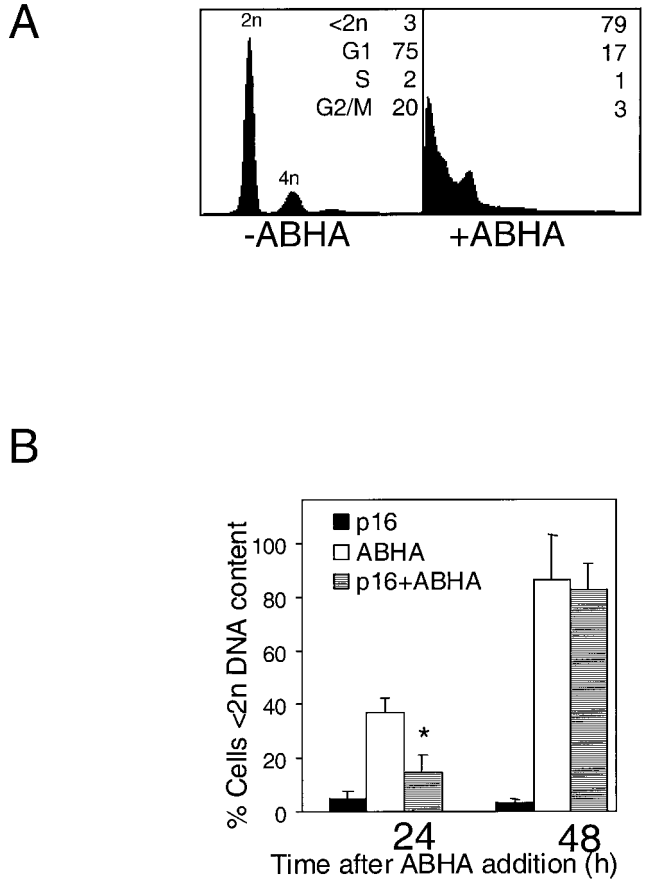


Fig. 9. A, two SK-Mel-13 cell lines engineered to conditionally express p16 with the addition of IPTG were induced for 24 h with IPTG, which induced high-level p16 expression and a G_1 phase arrest. The G_1-arrested cells and control uninduced cells were treated with or without 100 μg/ml ABHA. Their cell cycle status was assessed by FACS analysis after 48 h of treatment with drug. Data for only one cell line is presented, although essentially identical results were obtained with the second cell line. B, the proportion of subdiploid cells in either p16-induced only (p16), ABHA-treated only (ABHA), or p16-induced then ABHA-treated (p16 + ABHA) were assessed 24 and 48 h after treatment. These data are the mean and S.E. from five independent experiments. *$p < 0.0003$, significant difference.

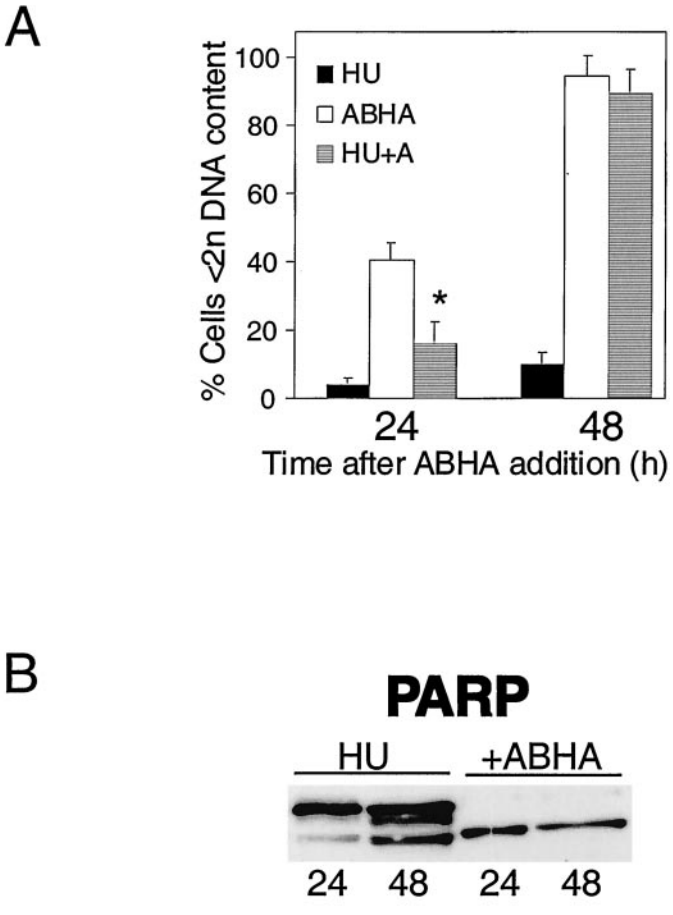


Fig. 10. A, MM96L cells were treated with 2 mM hydroxyurea for 6 h, then 100 mg/ml ABHA was added to some cultures. The proportion of subdiploid cells in either hydroxyurea-arrested (HU), asynchronously growing cells treated with ABHA (ABHA), or hydroxyurea- and ABHA-treated (HU + A) MM96L cells after 24 and 48 h of ABHA treatment. *$p < 0.0001$, significant difference. B, immunoblots for PARP and its cleavage products in lysates from either hydroxyurea-arrested (HU) and hydroxyurea- and ABHA-treated (+ABHA) MM96L cells after 24 and 48 h of ABHA treatment.

cells. p21 has also been reported to inhibit apoptosis by forming a complex with the apoptosis signal-regulating kinase 1, thereby blocking the activation of the stress-activated protein kinase/c-Jun NH_2-terminal kinase pathway (Asada et al., 1999). Overexpression of p21 has been reported to block initiator caspase cleavage and activation by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors (Xu and El-Deiry, 2000), and p21 expression seems to induce the expression of antiapoptotic factors (Chang et al., 2000). Antisense ablation of HDI-induced p21 expression resulted in a significant increase in HDI-induced apoptosis in the myelomonocytic cell line U937 (Vrana et al., 1999).

The accumulation and reduction in p21 levels after both low- and high-dose ABHA treatment is likely to be caused by a combination of factors. At low doses of ABHA and other HDIs, histone hyperacetylation peaks at 4 to 8 h and then declines rapidly to almost control levels by 24 h (Saunders et al., 1999; R. Warrener and B. G. Gabrielli, unpublished observations), and this transient hyperacetylation is likely to result in a transient increase in p21 expression. At higher doses of ABHA, hyperacetylated histone has been detected at 24 h (R. Warrener and B. G. Gabrielli, unpublished observations); thus, a sustained up-regulation of p21 expression would be expected. However, p21 is also a substrate for caspase-3 cleavage, producing 14- and 7-kDa fragments (Gervais et al., 1998). The 14-kDa p21 fragment was detected in cells that expressed high levels of p21 after treatment with a high dose of ABHA, with the cleavage of the full-length p21, and corresponding abundance of the 14-kDa fragment, mirroring PARP cleavage. The low levels of p21 detected in the DEVD- and ABHA-treated MM96L cells demonstrate that caspase-3 activity proteolyzes the low level of p21 that is expressed in these cells. Although it is unclear whether the proteolytic cleavage reduces the cdk and/or caspase-3 inhibitory function of p21, it may effect the mitochondrial localization of p21, which is essential for its procaspase-3 inhibitory effect (Suzuki et al., 1999b)

These observations lead to a model for ABHA-induced cell death of tumor cells. Cytotoxic doses of ABHA cause some form of "damage," which is normally responded to by a checkpoint mechanism. This checkpoint mechanism is defective in a high proportion of immortalized and tumor cell lines (Qiu et al., 2000), resulting in cells initiating an apoptotic response. In cells in which ABHA also induces strong p21 expression, p21 blocks procaspase-3 activation and full expression of the apoptotic phenotype (Fig. 11). It is possible that the reduced caspase-3 activation may be sufficient to cleave and inactivate p21, which in turn permits further activation of caspase-3. Thus, the kinetics and level of p21 induction and caspase-3 activation by ABHA may determine whether individual cells die via apoptosis or necrosis. This provides an explanation for the cell lines with strong induction of p21 showing varying degrees of PARP cleavage and apoptosis, as well as cell lines such as KJD and JA, which have 50 to 60% subdiploid cells by 24 h after high-dose ABHA treatment but some level of p21 up-regulation.

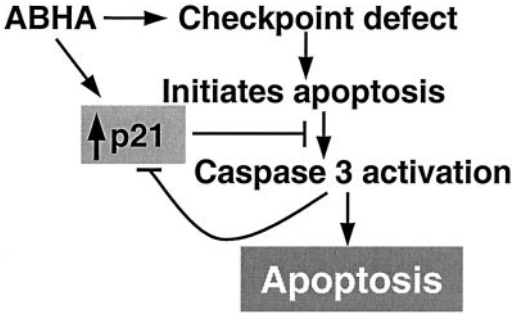


Fig. 11. Model of the role of p21 in blocking ABHA-induced apoptosis. The defective checkpoint response to high-dose ABHA treatment results in the initiation of apoptosis. In cells in which p21 expression is up-regulated, the p21 blocks procaspase-3 activation and full expression of the apoptotic response. These cells died via a default mechanism, possibly necrosis.

One consequence of the inhibition of caspase-3 seems to be a reduction in the sensitivity of cells to killing by ABHA. The reduced sensitivity of the $p21^{+/+}$ HCT116 compared with their isogenic $p21^{-/-}$ derivative and of the MM96L cells upon treatment with the caspase-3 inhibitor Z-DEVD-FMK point to p21 up-regulation as a major factor in reducing the sensitivity to the cytotoxic effects of ABHA, and probably all HDIs, by directly blocking the proteolytic activation of procaspase-3. The higher level of apoptotic cells in the untreated controls is likely to be a consequence of the absence of any p21 expression, which may normally regulate procaspase-3 activation during proliferation. The findings reported here suggest that use of ABHA and other HDIs as chemotherapeutics may be more effective in tumors in which these drugs do not induce p21 expression. In these tumors, relatively low doses of HDIs would be required for the cytotoxic effects and would consequently have fewer potential side effects. Also, the possible complications of massive necrosis of the tumor and associated immune responses to the rapid accumulation of cellular debris from lytic cell death would be avoided by promoting apoptosis. The potency of HDIs in killing hypersensitive cells also points to the potential of drugs that target the same molecular defect as the HDIs but do not up-regulate p21 expression. As a first step, identifying the molecular basis of this HDI-sensitive G_2 checkpoint mechanism will provide new targets for developing more specific and potent inhibitors, which may circumvent the problems associated with p21 up-regulation. The targeting of this defective checkpoint mechanism has the additional benefit of tumor-specific toxicity, because normal cells and tissues seem resistant to the effects of these drugs because of their competent checkpoint mechanisms (Parsons et al., 1997; Qiu et al., 2000).

In summary, we have shown that ABHA-induced p21 expression is correlated with reduced sensitivity to the drug and a switch from apoptotic to necrotic cell death. These data suggest that the currently available HDIs may be useful chemotherapeutic agents, but drugs that can more specifically target the molecular defect triggered by the HDIs, without increasing p21 expression, may provide very potent and specific treatments for a wide range of cancers and hyperproliferative diseases. The clinical potential of drugs with a high degree of tumor-selective toxicity is enormous.

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References

Archer SY, Meng S, Shei A, and Hodin RA (1998) p21(WAF1) is required for butyrate-mediated growth inhibition of human colon cancer cells. *Proc Natl Acad Sci USA* **95:**6791–6796.

Asada M, Yamada T, Ichijo H, Delia D, Miyazono K, Fukumuro K, and Mizutani S

(1999) Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. *EMBO (Eur Mol Biol Organ) J* **18:**1223–1234.
Bernhard D, Ausserlechner MJ, Tonko M, Loffler M, Hartmann BL, Csordas A, and Kofler R (1999) Apoptosis induced by the histone deacetylase inhibitor sodium butyrate in human leukemic lymphoblasts. *FASEB J* **13:**1991–2001.
Chang BD, Watanabe K, Broude EV, Fang J, Poole JC, Kalinichenko TV, and Roninson IB (2000) Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. *Proc Natl Acad Sci USA* **97:**4291–4296.
Eguchi Y, Shimizu S, and Tsujimoto Y (1997) Intracellular ATP levels determine cell death fate by apoptosis or necrosis. *Cancer Res* **57:**1835–1840.
Gervais JL, Seth P, and Zhang H (1998) Cleavage of CDK inhibitor p21(Cip1/Waf1) by caspases is an early event during DNA damage-induced apoptosis. *J Biol Chem* **273:**19207–19212.
Glick RD, Swendeman SL, Coffey DC, Rifkind RA, Marks PA, Richon VM, and La Quaglia MP (1999) Hybrid polar histone deacetylase inhibitor induces apoptosis and CD95/CD95 ligand expression in human neuroblastoma. *Cancer Res* **59:**4392–4399.
Grunstein M (1997) Histone acetylation in chromatin structure and transcription. *Nature (Lond)* **389:**349–352.
Herceg Z and Wang ZQ (1999) Failure of poly(ADP-ribose) polymerase cleavage by caspases leads to induction of necrosis and enhanced apoptosis. *Mol Cell Biol* **19:**5124–5133.
Kim YB, Lee KH, Sugita K, Yoshida M, and Horinouchi S (1999) Oxamflatin is a novel antitumor compound that inhibits mammalian histone deacetylase. *Oncogene* **18:**2461–2470.
McBain JA, Eastman A, Nobel CS, and Mueller GC (1997) Apoptotic death in adenocarcinoma cell lines induced by butyrate and other histone deacetylase inhibitors. *Biochem Pharmacol* **53:**1357–1368.
Medina V, Edmonds B, Young GP, James R, Appleton S and Zalewski PD (1997) Induction of caspase-3 protease activity and apoptosis by butyrate and trichostatin A (inhibitors of histone deacetylase): dependence on protein synthesis and synergy with a mitochondrial/cytochrome c-dependent pathway. *Cancer Res* **57:**3697–3707.
Parsons PG, Hansen C, Fairlie DP, West ML, Danoy PA, Sturm RA, Dunn IS, Pedley J, and Ablett EM (1997) Tumor selectivity and transcriptional activation by azelaic bishydroxamic acid in human melanocytic cells. *Biochem Pharmacol* **53:**1719–1724.
Pollack A and Ciancio G (1990) Cell cycle phase-specific analysis of cell viability using Hoechst 33342 and propidium iodide after ethanol preservation. *Methods Cell Biol* **33:**19–24.
Qiu L, Burgess A, Fairlie DP, Leonard H, Parsons PG, and Gabrielli BG (2000) Histone deacetylase inhibitors trigger a G2 checkpoint in normal cells that is defective in tumor cells. *Mol Biol Cell* **11:**2069–2083.
Qiu L, Kelso MJ, Hansen C, West ML, Fairlie DP, and Parsons PG (1999) Anti-tumour activity in vitro and in vivo of selective differentiating agents containing hydroxamate. *Br J Cancer* **80:**1252–1258.
Richon VM, Emiliani S, Verdin E, Webb Y, Breslow R, Rifkind RA, and Marks PA (1998) A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. *Proc Natl Acad Sci USA* **95:**3003–3007.
Richon VM, Sandhoff TW, Rifkind RA, and Marks PA (2000) Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. *Proc Natl Acad Sci USA* **97:**10014–10019.
Saito A, Yamashita T, Mariko Y, Nosaka Y, Tsuchiya K, Ando T, Suzuki T, Tsuruo T, and Nakanishi O (1999) A synthetic inhibitor of histone deacetylase, MS-27–275, with marked in vivo antitumor activity against human tumors. *Proc Natl Acad Sci USA* **96:**4592–4597.
Sambucetti LC, Fischer DD, Zabludoff S, Kwon PO, Chamberlin H, Trogani N, Xu H, and Cohen D (1999) Histone deacetylase inhibition selectively alters the activity and expression of cell cycle proteins leading to specific chromatin acetylation and antiproliferative effects. *J Biol Chem* **274:**34940–34947.
Saunders N, Dicker A, Popa C, Jones S, and Dahler A (1999) Histone deacetylase inhibitors as potential anti-skin cancer agents. *Cancer Res* **59:**399–404.
Suzuki A, Tsutomi Y, Miura M, and Akahane K (1999a) Caspase 3 inactivation to suppress Fas-mediated apoptosis: identification of binding domain with p21 and ILP and inactivation machinery by p21. *Oncogene* **18:**1239–1244.
Suzuki A, Tsutomi Y, Yamamoto N, Shibutani T, and Akahane K (1999b) Mitochondrial regulation of cell death: mitochondria are essential for procaspase 3-p21 complex formation to resist Fas-mediated cell death. *Mol Cell Biol* **19:**3842–3847.
van Engeland M, Ramaekers FC, Schutte B, and Reutelingsperger CP (1996) A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. *Cytometry* **24:**131–139.
Vrana JA, Decker RH, Johnson CR, Wang Z, Jarvis WD, Richon VM, Ehinger M, Fisher PB, and Grant S (1999) Induction of apoptosis in U937 human leukemia cells by suberoylanilide hydroxamic acid (SAHA) proceeds through pathways that are regulated by Bcl-2/Bcl-XL, c-Jun, and p21CIP1, but independent of p53. *Oncogene* **18:**7016–7025.
Waldman T, Kinzler KW, and Vogelstein B (1995) p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res* **55:**5187–5190.
Wolf BB and Green DR (1999) Suicidal tendencies: apoptotic cell death by caspase family proteinases. *J Biol Chem* **274:**20049–20052.
Xu SQ and El-Deiry WS (2000) p21 (WAF1/CIP1) inhibits initiator caspase cleavage by TRAIL death receptor DR4. *Biochem Biophys Res Commun* **269:**179–190.

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