Histone Deacetylase Inhibitor–Mediated Radiosensitization of Human Cancer Cells: Class Differences and the Potential Influence of p53

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Abstract Histone deacetylase inhibitors (HDI) are emerging as potentially useful components of the anticancer armamentarium and as useful tools to dissect mechanistic pathways. HDIs that globally inhibit histone deacetylases (HDAC) have radiosensitizing effects, but the relative contribution of specific HDAC classes remains unclear. Newly characterized HDIs are now available that preferentially inhibit specific HDAC classes, including SK7041 (inhibits class I HDACs) and splitomicin (inhibits class III HDACs). We investigated in human cancer cells the relative radiosensitizations that result from blocking specific HDAC classes. We found that trichostatin A (TSA; inhibitor of both class I and II HDACs) was the most effective radiosensitizer, followed by the class I inhibitor SK7041, whereas splitomicin (inhibitor of class III) had least effect. Interestingly, radiosensitization by TSA in cell lines expressing p53 was more pronounced than in isogenic lines lacking p53. Radiosensitization of cells expressing p53 by TSA was reduced by pifithrin-α, a small-molecule inhibitor of p53. In contrast, the radiosensitization by TSA of cells expressing low levels of p53 was enhanced by transfection of wild-type p53–expressing vector or pretreatment with leptomycin B, an inhibitor of nuclear export that increased intracellular levels of p53. These effects on radiosensitization were respectively muted or not seen in cells treated with SK7041 or splitomicin. To our knowledge, this may be among the first systematic investigations of the comparative anticancer effects of inhibiting specific classes of HDACs, with results suggesting differences in the degrees of radiosensitization, which in some cell lines may be influenced by p53 expression.

Histone deacetylases (HDAC) belong to a deacetylase superfamily and play major roles in chromatin remodeling, control of gene expression, and epigenetics (1–3). HDACs have been classified into three major classes based on their homologies to yeast orthologues (4–6). Class I HDACs are generally nuclear proteins homologous to yeast protein Rpd3 and include HDACs 1, 2, 3, and 8. Class II HDACs, homologous to the yeast HDA1, include HDACs 4, 5, 6, 7, 9, and 10; this class is further divided into subclasses on the basis of deacetylase domain structure: class IIa HDACs (HDACs 4, 5, 7, and 9) have one major deacetylase domain whereas class IIb HDACs (HDACs 6 and 10) have two in tandem, with different substrate preferences [HDAC6 targets histones (the HDAC site) and HDAC10 targets tubulin (TDAC site)]. HDAC11 contains conserved residues in the catalytic core region uniquely shared by both class I and II enzymes (6–8). The third class of HDACs includes the NADH-dependent Sir family of deacetylases, which are implicated in chromatin-dependent silencing in yeast (9, 10). The class III HDACs are resistant to inhibition by trichostatin A (TSA) and suberoylanilide hydroxamic acid, unlike class I and II HDACs, which are susceptible to these drugs.

HDACs play critical roles in an immense number of biological pathways, which is reflected in part by the increasing number of HDACs being identified; there are likely many more HDACs and HDAC-dependent intracellular roles that remain to be discovered. Histone deacetylase inhibitors (HDI) have also emerged as potentially useful therapeutics in the clinic, including for the treatment of cancer (11, 12). The widespread interest in HDIs as research tools and as therapeutic drugs has spurred the development and characterization of HDIs. The diversity of HDACs and their intracellular roles suggest that class-specific HDAC inhibitors should offer greater clinical usefulness. Specific HDIs are now available that preferentially inhibit specific HDAC classes; TSA inhibits class I and II HDACs (13, 14), SK7041 inhibits class I HDACs (15, 16), and splitomicin inhibits class III HDACs (17–19).

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Received 6/8/05; revised 10/2/05; accepted 11/11/05.
Grant support: Seoul National University Hospital Research Fund grant 03-2005-009.
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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).
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doi:10.1158/1078-0432.CCR-05-1230

In the investigations described here, we tested the differential cytotoxicities and radiosensitizations induced by these three different types of HDIs. We found that at isotoxic concentrations, TSA led to the greatest degree of radiosensitization, followed by the class I inhibitor SK7041, whereas the class III HDAC inhibitor splitomicin had least effect on radiosensitization. In assessing isogenic human cancer cell lines that differed only in the expression of p53, we were surprised to discover that TSA-mediated radiosensitization was greater in cells expressing p53 than in those negative for p53. The radiosensitization by TSA was reduced when p53 was targeted by the specific inhibitor pifithrin-α. In contrast, TSA-mediated radiosensitization was enhanced by transfection with wild-type (WT) p53–expressing vector or pretreatment with leptomycin-B, which blocks nuclear export of p53 and thus prevents its degradation. These effects were not observed in p53-negative cells, or for SK7041 or splitomicin. Taken together, these results suggest that the targeting of different HDAC classes may have differential effects for radiosensitization, which in some cell lines may be influenced by p53 expression. To our knowledge, this may be among the first systematic investigations of the comparative anticancer effects of inhibiting specific classes of HDACs.

Materials and Methods

Cell culture. All cell lines were maintained at 37°C in water saturated with 5% CO_2. HeLa and HCT116 cell lines were purchased from the American Type Culture Collection (Rockville, MD) and grown in either DMEM (Welgene, Daegu, Korea) or RPMI (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum. A549 lung carcinoma cells stably expressing E6 constructs (pLXSN-16E6SD) and overexpressing human papilloma virus E6 protein, which constantly degrades p53 protein, were generously provided by Dr. Eun-Kyung Choi (Ulsan University, Ulsan, Korea). The functional knockout of p53 was confirmed by Western blot analysis; A549 cells transfected with empty vector alone (hereafter referred to as "A549 control cells") express p53 protein at levels similar to that of parental cells whereas E6-expressing A549 cells show significantly lower p53 protein levels. These were maintained under conditions identical to those of the other cell lines, except for the addition of 12.5 μg/mL gentamicin (Life Technologies). WT p53 expression vector was kindly supplied by Dr. B. Vogelstein (19) and was transfected into HeLa cell using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Pharmacologic inhibitors. TSA was obtained from Sigma Chemical Co. (St. Louis, MO) and splitomicin, a class III HDAC inhibitor, was obtained from Tocris (Ellisville, MO). 4-Dimethylamino-*N*-[4-(2-hydroxylcarbamoyl-vinyl) benzyl] benzamide 1 (SK7041), a novel class I inhibitor, was kindly provided by Dr. Young-Jue Bang (Seoul National University, Seoul, Korea). Pifithrin-α, a specific small-molecule inhibitor of p53, was obtained from A.G. Scientific (San Diego, CA). Leptomycin B, a specific inhibitor of CRM1-mediated nuclear export, was obtained from Sigma. Inhibitors were dissolved as concentrated stock solutions in DMSO, stored at −20°C, and diluted at the time of use in culture medium. Control cells were treated with medium containing an equal concentration of drug carrier, DMSO.

Clonogenic assays. For the clonogenic assays, identical numbers of cells were plated across the different treatment groups for each radiation dose. A specified number of cells were seeded into each wells of six-well culture plates and treated with HDIs for 18 hours. After exposure of HDIs, cells were irradiated with 4-MV X-ray from a linear accelerator (Clinac 4/100, Varian Medical Systems, Palo Alto, CA) at a dose rate of 2.46 Gy/min and were incubated for colony formation for 14 to 21 days. Colonies were fixed with methanol and stained with 0.5% crystal violet; the number of colonies containing at least 50 cells was determined and surviving fraction was calculated. Radiation survival data were fitted to a linear-quadratic model using Kaleidagraph version 3.51 (Synergy Software, Reading, PA). Each point on the survival curves represents the mean surviving fraction from at least three dishes. Sensitizer enhancement ratio (SER) was calculated as the ratio of the isoeffective dose at surviving fraction 0.5 and surviving fraction 0.05 in the absence of HDIs to that in the presence of HDIs.

Western blot analysis. Cells were washed, scraped, and resuspended in lysis buffer (iNtRON Biotechnology, Seoul, Korea). Proteins were solubilized by sonication and equal amounts of protein were separated on SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Membranes were blocked in PBS containing 0.1% Tween 20 and 5% powdered milk and probed with primary antibody directed against polyclonal rabbit anti–acetyl-histone H3 immunoglobulin G (Upstate, Lake Placid, NY) at 1:1,000 dilution, monoclonal anti-α-tubulin antibody (Sigma) at 1:5,000 dilution, and polyclonal rabbit p53 (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed and incubated with secondary antibody consisting of peroxidase-conjugated goat anti-rabbit or mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:2,000 dilution for 1 hour. Antibody binding was detected using an enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ) using the appropriate secondary antibody supplied with the kit.

Flow cytometric analysis. Cells were harvested at the indicated times and fixed in 1 mL of 80% ethanol (1×10^6-2×10^6 cells per sample). Cells were then washed twice with PBS and incubated in dark for 30 minutes at 37°C in 1 mL of PBS containing 5 μg/mL propidium iodide (Molecular Probes, Eugene, OR) and 0.1% RNase A (Sigma). At least 1×10^4 events were counted. Flow cytometric analysis was done with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). At least 1×10^4 events were counted. To evaluate for nonviable apoptotic cells after treatment, the proportion of cells of each treatment group with less than G_1 DNA content was assessed via fluorescence-activated cell sorting, which was done in the absence of gating to include all cells and minimize bias.

Results

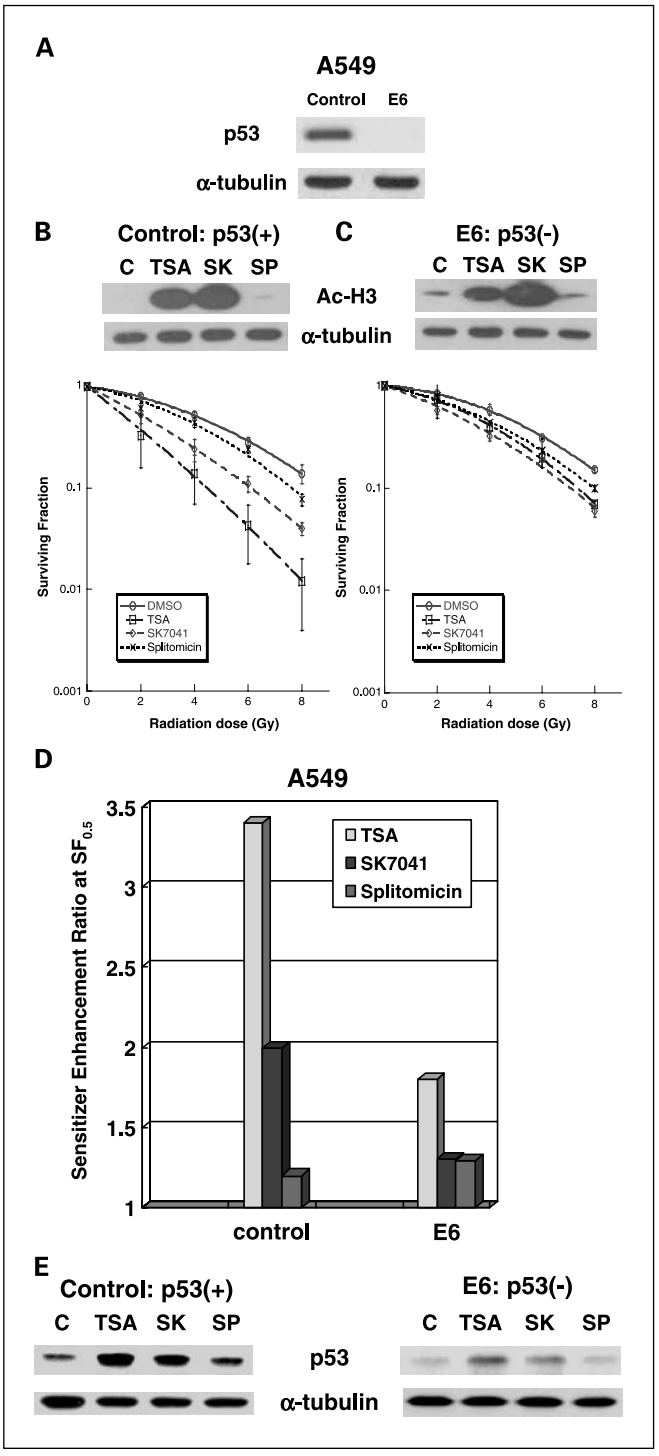
Determination of IC_{50}s of HDIs and optimal treatment schedule. In preparation for comparing the relative radiosensitizing effects of these drugs, we first determined the concentrations at which each of these drugs inhibited the growths of cells by 50% (i.e., the IC_{50}; Table 1). This enabled us to choose for our subsequent experiments concentrations of each HDI that had only minor effects on cell growth.

Table 1. IC_{50}* for three different HDIs

Cell line	HDI		
	TSA (nmol/L)	SK7041 (nmol/L)	Splitomicin (μmol/L)
A549			
Control	100	200	400
E6	200	200	400
HCT116	100	200	400
HeLa	200	200	400

*Concentration of HDIs that lead to 50% inhibition of cell proliferation.

To determine the optimal treatment schedule for assessing the effects of combining HDI and irradiation, we also compared in preparatory experiments the effects of pretreating the cells with HDI before radiation for 18 hours, HDI immediately after radiation, or 3, 6, and 12 hours after irradiation. The greatest degree of radiosensitization was observed when cells are pretreated with TSA (data shown in Supplementary Fig. S1). Based on these results, all subsequent experiments were done with HDI added before radiation.



Differences between the radiosensitivities of A549 cells treated with these HDIs, and the influence of p53. Having determined the concentrations of TSA, SK7041, and splitomicin that had equivalent effects on proliferation in the absence of radiation, we assessed the relative effects of isotoxic concentrations of these drugs on radiosensitization. Initially, we investigated their effects A549 non–small-cell lung carcinoma cells expressing either high (control) or low (E6) levels of p53 protein (Fig. 1A). In A549 cells expressing high p53 levels, TSA caused the highest degree of radiosensitization, as determined by clonogenic survival assays, followed by SK7041 then splitomicin. Interestingly, immunoblots done on mock-treated A549 cells or those treated with each of the three HDI showed the highest degree of histone hyperacetylation (an indirect biochemical marker of HDI biological activity; ref. 20) for SK7041, which was discernibly greater than that of TSA and markedly more than that of splitomicin (Fig. 1B and C). Moreover, in contrast to the marked radiosensitization associated with TSA in p53(+) A549 control cells, the effect of TSA was considerably less in A549 cells expressing low levels of p53 (E6). The SER for TSA in A549 cells expressing high versus low levels of p53 was 3.2 and 1.8, respectively (Fig. 1D). The SERs for SK7041 in high versus low p53 A549 cells was 2 and 1.3, respectively, whereas that of splitomicin was essentially unchanged (at 1.2) regardless of p53 level.

Finally, the radiosensitizing effect of TSA was evident after high as well as low doses of irradiation (SER for surviving fractions of 0.05 are presented in Table 2, which are comparable to SER for surviving fractions of 0.5).

The effects of pifithrin-α on p53 level and radiosensitization in A549 cells. Having found that TSA more potently radiosensitizes A549 control cells expressing higher levels of p53, we examined the effect of reducing p53 protein levels using pifithrin-α, a specific small-molecule inhibitor of p53 (21), on radiosensitization. Treatment of A549 control cells with 30 μmol/L pifithrin-α for 24 hours was found to lead to the near abrogation of p53 expression (Fig. 2A). Clonogenic survival assays were repeated on cells mock treated or cotreated with pifithrin-α and HDIs. As mentioned above, in A549 cells not exposed to pifithrin-α, TSA led to the greatest degree of radiosensitization, followed by SK7041. However, radiosensitization by TSA was muted in cells cotreated with pifithrin-α in which p53 levels were greatly reduced. Interestingly, histone hyperacetylation by TSA was somewhat muted in cells treated with pifithrin-α (compare immunoblots in Fig. 2A versus

Fig. 1. Radiosensitization by HDI pretreatment in A549/control (expressing p53) and A549/E6 (lacking p53) cells. *A,* A549 cells stably transfected with human papillomavirus 16 E6 showed almost no p53 expression compared with A549 transfected with control vector (A549 control versus E6). *B* and *C,* clonogenic survival assays of A549 control and E6 cells pretreated with the respective HDI. TSA-induced radiosensitization assessed by clonogenic survival was greater in A549 cells expressing higher amounts of p53 than in A549/E6 (lacking p53). Radiosensitization by SK7041 and splitomicin was less affected by p53 expression. All drugs were washed off immediately after radiation. Points, mean surviving fractions calculated from cells treated in triplicate. Each experiment was also repeated thrice with similar results. Inset, immunoblots of lysates from cells pretreated with the respective HDI followed by radiation, probing for acetylated histone H3 (*Ac-H3*). *D,* $SER_{0.5}$ calculated from the data shown in (*B* and *C*). TSA-treated cells showed greater radiosensitization than SK7041- or splitomicin-treated cells, but the radiosensitization was muted by reduction of p53 protein in the E6 cells. *E,* immunoblots of lysates from cells which had been pretreated with the respective HDI followed by radiation, showing that the effect of E6 in reducing p53 protein persist.

Table 2. Sensitizer enhancement ratio at $SF_{0.05}$

Cell line	HDI		
	TSA	SK7041	Splitomicin
A549			
Control	1.72	1.28	1.14
E6	1.13	1.21	1.10
Control	1.55	1.32	0.99
Pifithrin-α	1.22	1.14	1.02
HCT116			
Control	1.65	1.33	1.04
Pifithrin-α	1.40	1.30	1.00
HeLa			
Control	1.24	1.16	0.95
WT p53	1.37	1.10	1.04
Control	1.23	1.20	0.91
Leptomycin B	1.43	1.10	1.03

Fig. 2B) and this was not appreciably changed by radiation treatment (data not shown). Radiosensitization by SK7041 was also reduced in A549 cells pretreated with pifithrin-α although the degree of hyperacetylation in SK7041-treated cells seemed to be equivalent. These results together suggest that an inhibitor of p53 protein may mute the radiosensitizing effects of TSA and possibly SK7041. Interestingly, the decreased TSA- and SK7041-induced radiosensitization that was brought about by pifithrin-α did not seem to correlate with changes in histone acetylation levels. Whereas histone acetylation by TSA was somewhat muted in cells treated with pifithrin-α, histone acetylation in SK7041-treated cells seemed to be equivalent even after pifithrin-α treatment (compare immunoblots in Fig. 2A versus Fig. 2B).

The effects of pifithrin-α on p53 level and radiosensitization in HCT116 cells. To extend our findings that pifithrin-α reduced radiosensitization by TSA in p53-expressing A549 cancer cells (Fig. 1C), we assessed these effects in HCT116 cells, which, like A549 cells, also express considerable levels of p53 protein. Treatment of HCT116 cells with pifithrin-α under conditions identical to that previously described for A549 cells led to ~80% reduction in p53 protein expression (Fig. 3A). Pifithrin-α did not prevent hyperacetylation following HDI treatment although, interestingly, SK7041 once again led to the greatest degree of hyperacetylation both in the absence and presence of pifithrin-α (Fig. 3B and C) versus TSA or splitomicin. In terms of radiosensitization, TSA showed greater radiosensitization in HCT116 cells than SK7041 whereas splitomicin had little effect.

SERs for TSA, SK7041, and splitomicin for cells not exposed to pifithrin-α were respectively 2.1, 1.5, and 1.2. TSA-mediated radiosensitization was considerably reduced by cotreatment with pifithrin-α, with SER reduced from 2.1 to 1.3. In contrast, the SER of cells SK7041 cotreated with pifithrin-α was not appreciably different versus without pifithrin-α (1.3 versus 1.5; Fig. 3D). No radiosensitization was observed in cells cotreated with splitomicin, either alone or with pifithrin-α. Finally, we verified that each of the HDI in itself did not impede the reduction of p53 protein levels by pifithrin-α (Fig. 3E). These results suggest that reduced

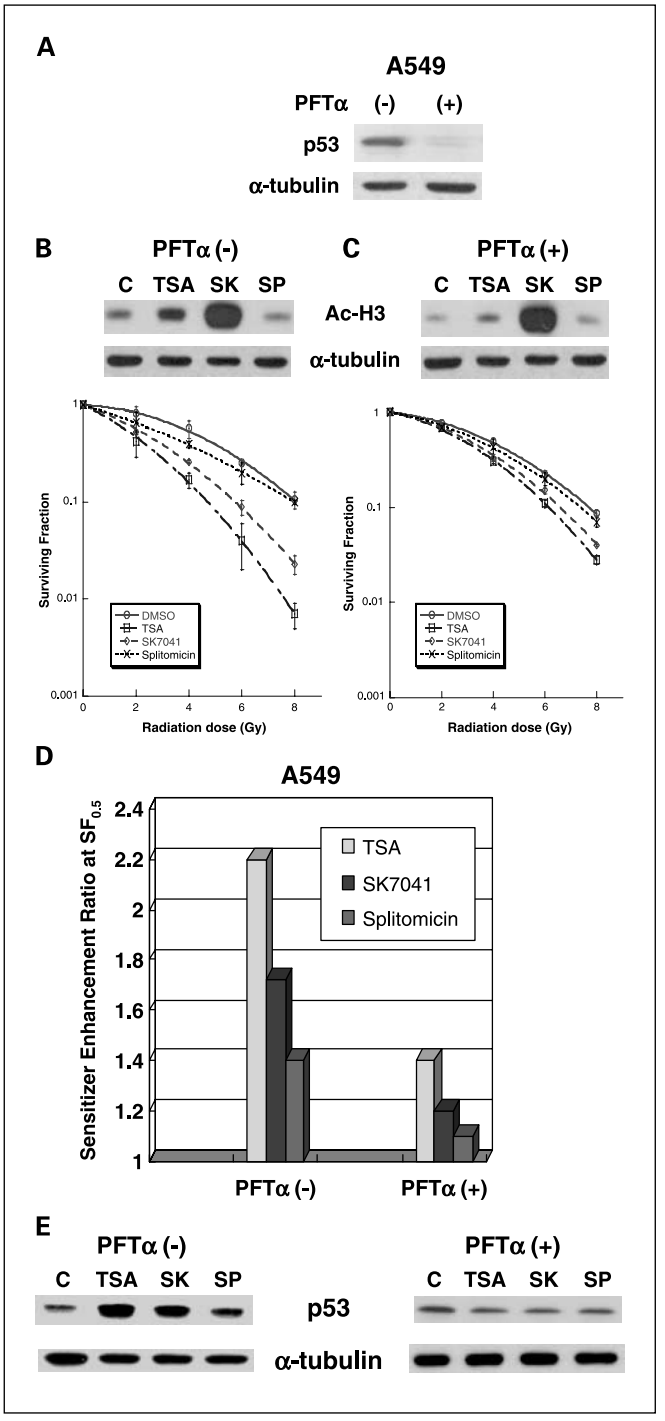


Fig. 2. Treatment of A549 cells with pifithrin-α leads to decreased levels of p53 protein and mutes the radiosensitization induced by TSA. *A,* A549 cells were mock treated or treated with pifithrin-α (30 μmol/L) for 24 hours, followed by harvesting and immunoblotting for p53 protein (showing decreased levels with pifithrin-α). *B* and *C,* clonogenic survival assays of A549 cells mock treated or pretreated with pifithrin-α followed by the respective HDI, with all drugs washed off immediately after irradiation. TSA-induced radiosensitization was decreased in cells in which p53 protein was decreased. Points, mean surviving fractions calculated from cells treated in triplicate. Each experiment was also repeated thrice with similar results. Inset, immunoblots of lysates from cells pretreated with the respective HDI followed by radiation, probing for acetylated histone H3. *D,* $SER_{0.5}$ calculated from the data shown in (*B* and *C*). TSA-treated cells showed greater radiosensitization than SK7041- or splitomicin-treated cells, but the radiosensitization was muted by reduction of p53 protein in the cells pretreated with pifithrin-α. *E,* immunoblots of lysates from cells following radiation, which had been mock treated or pretreated with pifithrin-α, followed by the respective HDI, showing that the decrease in p53 protein by pifithrin-α persisted.

p53 protein levels in HCT116 cells were associated with reduced radiosensitization by TSA.

The effects of leptomycin B on p53 and radiosensitization in HeLa cells. Having found that reducing p53 protein expression reduced radiosensitization by TSA, we were interested in testing whether the converse was true; i.e., would the restoration of p53 in a cell line formerly expressing low levels of the protein increase radiosensitization by TSA? Thus, we treated HeLa cells (which express low levels of p53 protein due to the effects of E6 oncoprotein) with leptomycin B, a specific inhibitor of CRM1-mediated nuclear export (22). This inhibition prevents p53 degradation, which effectively elevates the overall and nuclear levels of p53 protein. Treatment of HeLa cells with 5 ng of leptomycin B for 24 hours led to a considerable increase in p53 level (Fig. 4A). Interestingly, this was associated with increased radiosensitization by TSA (SER of 1.5 without leptomycin B and SER of 2.2 with leptomycin B; Fig. 4C and D). In the absence of leptomycin B, radiosensitization by TSA and SK7041 in the HeLa cells was comparable (SERs of 1.5 and 1.55, respectively) whereas splitomicin caused no radiosensitization (Fig. 4A and D). In the presence of leptomycin B, radiosensitization by SK7041 was actually reduced and that associated with splitomicin was only 1.25. These effects are in accord with our results with pifithrin-α and together suggest that leptomycin B, possibly related with the accumulation of p53 associated with leptomycin B, has the largest effect in increasing radiosensitization by TSA.

The effects of WT p53–expressing vector on HDI-mediated radiosensitization of HeLa cells. A potential concern with any pharmacologic intervention is the possibility of nonspecific effects. Therefore, as an additional test of the potential influence of p53 on TSA-mediated radiosensitization, we transfected HeLa cells with either empty vector or cDNA expressing WT p53. The level of p53 protein was considerably increased 48 hours after transfection with the p53-expressing vector and resulted in an increase of the SER associated with TSA from 1.2 to 1.6 compared with control cells transfected with empty vector (Fig. 5A-D). In contrast, the SER associated with SK7041 was essentially similar in cells transfected with either p53 or empty vector (1.08 and 1.1, respectively) and no radiosensitization was associated with splitomicin. To exclude the possibility that the HDI treatment might reverse the vector-driven expression of p53, we also assessed for p53 protein after HDI and radiation under these conditions and confirmed that p53 protein remained substantial (Fig. 5E). The results with this genetic approach therefore support the results obtained via pharmacologic manipulation of p53, and together suggest that high levels of p53 protein expression lead to greater radiosensitization by TSA, with less effect on SK7041 and splitomicin.

The effects on cell cycle profile and nuclear fragmentation after combined treatment with HDI and radiation. To further investigate the effects of combining HDI with radiation, we followed cell cycle changes after treatment with HDIs. There was relatively little difference among TSA, SK7041, and splitomicin during pretreatment of cells with these HDIs (Supplementary Fig. S2A). We assessed the respective responses of A549 control cells (expressing higher p53 protein levels) and A549 E6 cells (which express little p53) after each form of treatment. Given the role of p53 in

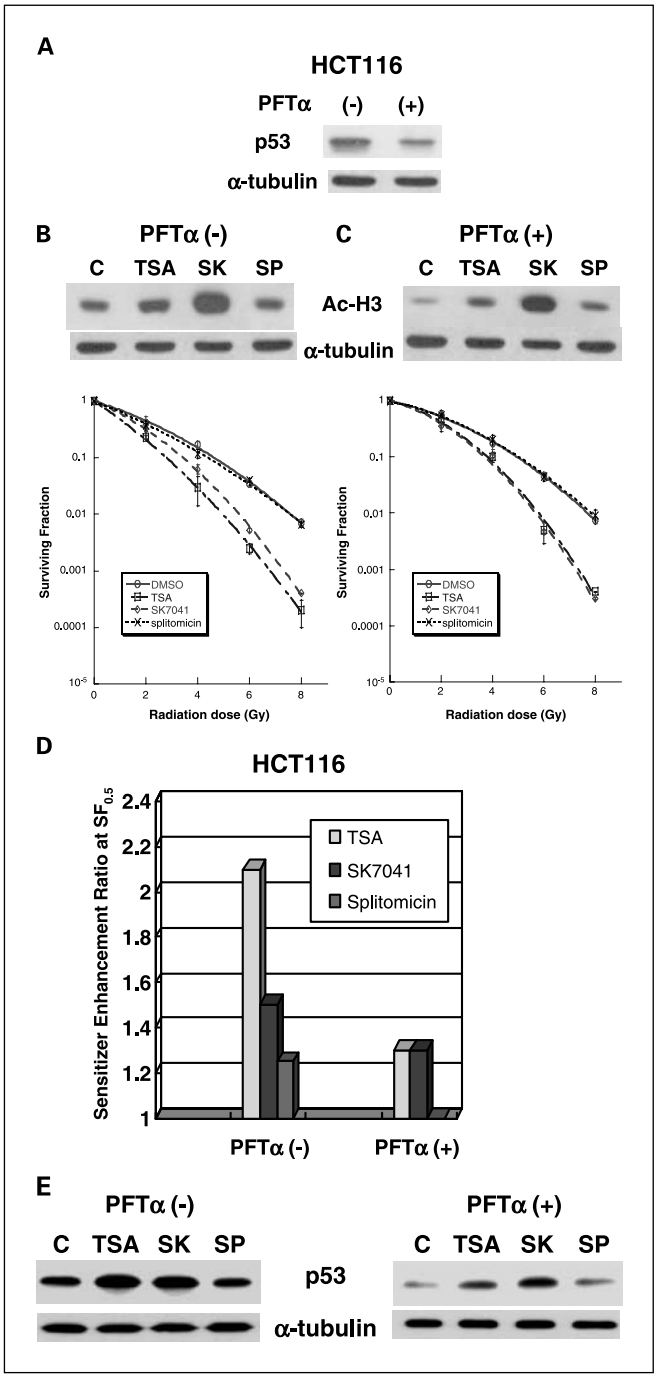


Fig. 3. Radiosensitization by TSA of HCT116 cells (expressing p53) was muted by pifithrin-α. *A,* HCT116 cells were mock treated or treated with pifithrin-α (30 μmol/L) for 24 hours, followed by harvesting and immunoblotting for p53 protein (showing decreased levels with pifithrin-α) or α-tubulin. Pifithrin-α led to substantially reduced p53 protein levels. *B* and *C,* clonogenic survival assays of HCT116 cells mock treated or pretreated with pifithrin-α followed by the respective HDI, with all drugs washed off immediately after irradiation. HCT116 cells, which express high levels of p53, are efficiently radiosensitized by TSA. TSA-induced radiosensitization was decreased in cells in which p53 protein was decreased. Points, mean surviving fractions calculated from cells treated in triplicate. Each experiment was also repeated thrice with similar results. Inset, immunoblots of lysates from cells pretreated with the respective HDI followed by radiation, probing for acetylated histone H3. *D,* $SER_{0.5}$ calculated from the data shown in (*B* and *C*). TSA-treated cells showed greater radiosensitization than SK7041- or splitomicin-treated cells, but the radiosensitization was muted by reduction of p53 protein in the cells treated with pifithrin-α. *E,* immunoblots of lysates from cells following radiation, which had been mock treated or pretreated with pifithrin-α, followed by the respective HDI, showing that the decreased p53 protein levels after pifithrin-α persisted.

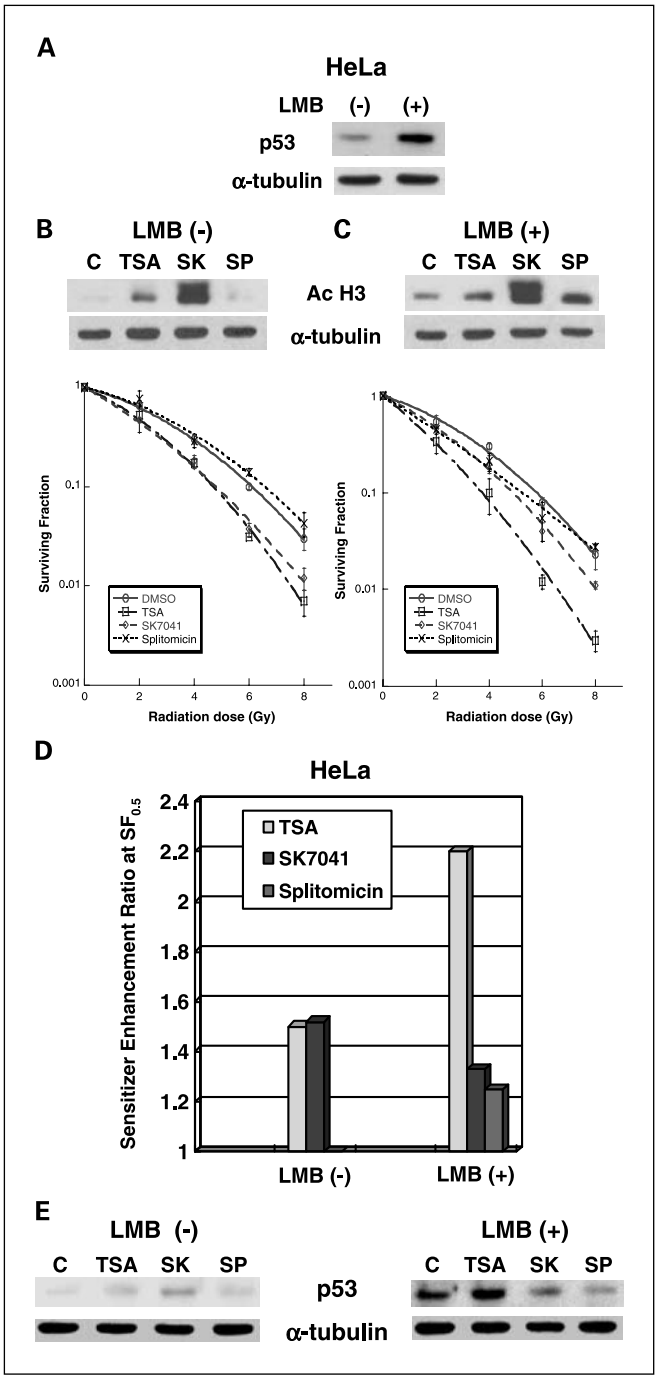


Fig. 4. Treatment of HeLa cells by leptomycin B (*LMB*) leads to increased p53 protein levels and accentuates radiosensitization by TSA. *A,* HeLa cells were mock treated or treated with leptomycin B (5 ng) for 24 hours, followed by harvesting and immunoblotting for p53 protein showing increased p53 protein levels after leptomycin B. *B* and *C,* clonogenic survival assays of HeLa cells mock treated or pretreated with leptomycin B followed by the respective HDI, with all drugs washed off immediately after irradiation. TSA-induced radiosensitization was increased in cells in which p53 protein was increased. Points, mean surviving fractions calculated from cells treated in triplicate. Each experiment was also repeated thrice with similar results. Inset, immunoblots of lysates from cells pretreated with the respective HDI followed by radiation, probing for acetylated histone H3. *D,* $SER_{0.5}$ calculated from the data shown in (*B* and *C*). TSA-treated cells showed radiosensitization similar to SK7041, but the radiosensitization by TSA was substantially increased by leptomycin B. *E,* immunoblots of lysates from cells following radiation, which had been mock treated or pretreated with leptomycin B followed by the respective HDI, showing that the increased p53 protein levels after leptomycin B persisted.

mediating G_1-early S phase checkpoints, the A549 control cells, as expected, retained a greater number of cells in G_1 after irradiation than A549 E6 cells. Also as expected, both cell lines showed a robust G_2-M delay after irradiation alone, consistent with the well-characterized G_2 delay of cancer cells after irradiation. This arrest was, however, strikingly abrogated by pretreatment with TSA. SK7041-treated cells also showed abrogation of G_2 arrest, but less than TSA, whereas little effect was seen in splitomicin-treated cells. These findings were seen in both A549 control as well as A549 E6 cells (Fig. 6A).

We extended our investigations to include analyses of the percentages of cells with sub-G_1 DNA content after treatment. These represent cells with fragmented chromatin that are likely nonviable (data not shown). Whereas the clonogenic survival assays shown in Figs. 1 to 5 are more comprehensive and rigorous in reflecting ultimate tumor control after treatment, fluorescence-activated cell sorting analyses can measure short-term responses to treatment. TSA treatment led to an appreciable increase the proportion of sub-G_1 cells, which was further increased by irradiation (Fig. 6B). In contrast, the proportion of sub-G_1 cells after SK7041 and splitomicin was considerably less. The increase in the proportion cells with sub-G_1 DNA content as a result of treatment was significantly higher in A549 control compared with that in A549 E6 cells. These results suggest that rapid induction of apoptosis induced by TSA in the presence of p53 protein may contribute ultimately to decreased colony formation and therefore increased radiosensitization.

Discussion

Anticancer therapy is a prime example of the clinical application of HDIs, but a key question remains to be answered: which of the HDACs offer optimal treatment efficacy and lowest morbidity? One important characteristic of HDIs is their selectivity in terms of altering gene expression in transformed cells (23). The HDIs available induce the accumulation of hyperacetylated histones in chromatin regions but only a small subset of expressed genes show transcription expression changes after HDI treatment. A DNA microarray analysis–based study found that <10% of genes are affected (24). This selectivity might be due to other covalent modifications of histone tails that can similarly affect gene expression (25). Thus, these observations may be meaningful in the context of the development of strategies targeting the clinical use of HDIs.

The other important point is the increasing evidence of class and isotype specificity of HDACs. Eighteen human HDACs have been identified to date and can be divided into three distinct classes (4–8): class I includes HDACs 1, 2, 3, and 8. The selective disruption of HDAC1 was found to result in embryonic lethality despite its elevating HDAC2 and HDAC3 expressions (26). HDAC2 seems to preferentially act to prevent apoptosis, and not act in cell cycle control similar to the specific importance of HDAC1 in cell cycle regulation (27). HDAC8 associates with the smooth muscle actin cytoskeleton and may regulate the contraction of smooth muscle cells (28). Class IIa HDACs, which include HDACs 4, 5, 7, and 9, mainly function as transcriptional corepressors

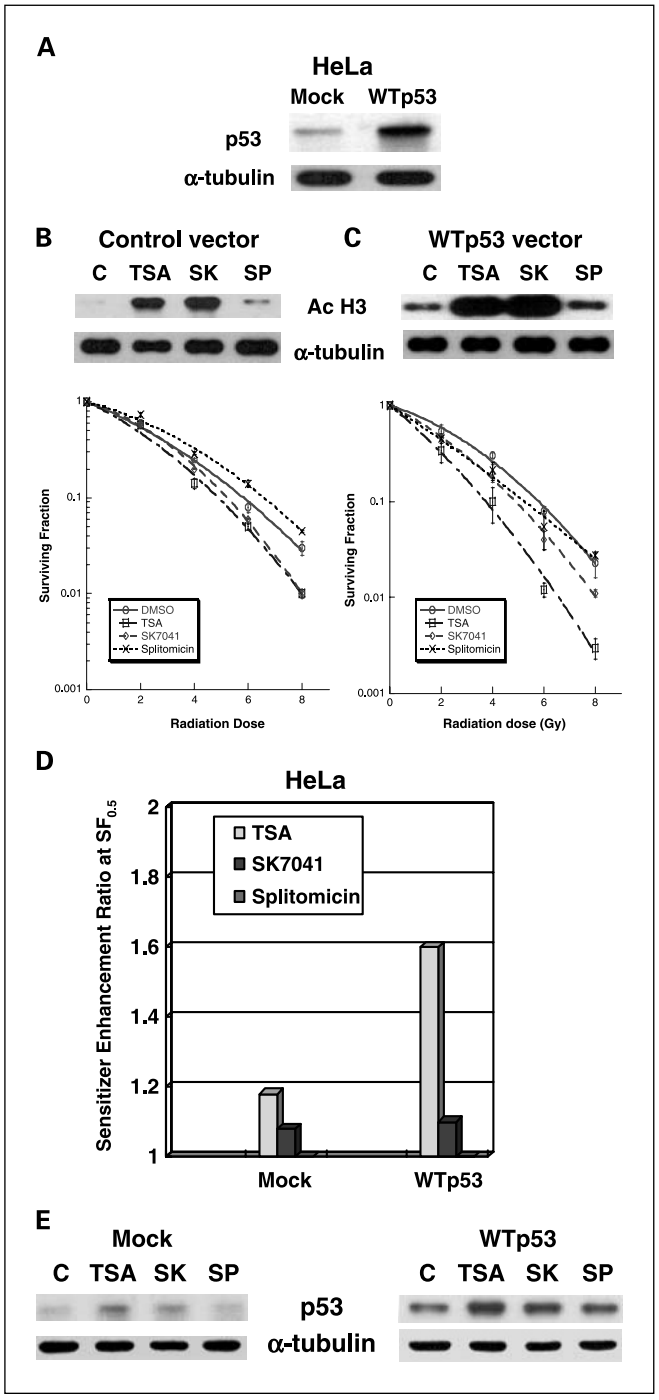


Fig. 5. Overexpression of p53 protein in HeLa cells leads to increased radiosensitization by TSA. *A,* HeLa cells were transfected with either empty vector (*Control*) or a vector expressing WT p53 (*WTp53*). The level of p53 was considerably increased 48 hours after the transfection. Immunoblots were done on lysates from cells, probing for p53 protein (showing increased p53 protein levels). *B* and *C,* clonogenic survival assays of HeLa cells transfected with either empty vector or a vector expressing WT p53, followed by the respective HDI, with all drugs washed off immediately after irradiation. TSA-induced radiosensitization was increased in cells in which p53 protein was increased. Points, mean surviving fractions calculated from cells treated in triplicate. Each experiment was also repeated thrice with similar results. Inset, immunoblots of lysates from cells pretreated with the respective HDI followed by radiation, probing for acetylated histone H3. *D,* $SER_{0.5}$ calculated from the data shown in (*B* and *C*). TSA-treated cells showed substantially increased radiosensitization after overexpression of p53. *E,* immunoblots of lysates from cells following radiation, which had been pretreated with the respective HDI, showing that the effect of WT p53 vector in increasing p53 protein persisted.

(7, 8). HDAC5 can bind to and repress the activity of myocyte enhancer factor-2 transcription factors, which are important for muscle differentiation (29). HDAC9 has an important role in development and stress response of heart (30) and HDAC4 acts downstream of p53 tumor suppressor (31). HDAC7 is known to localize to the mitochondrial inner membrane space and relocalizes to the cytoplasm in response to initiation of the apoptotic cascade (32). Class IIb members, HDAC6 and HDAC10, may be important in breast and lung cancer progression, respectively, and are potentially useful prognostic indicators (33, 34). The deacetylase activity of Sir2p is required continuously for maintenance of the silenced state in nondividing cells, development, and aging in a number of species (16). Moreover, deacetylation of p53 by Sir2 can down-regulate the transcriptional and proapoptotic activities of p53 in response to DNA damage (13, 14). More recently, it has become apparent that Sir2 may act as a negative regulator of the aging process through the transcriptional inactivation of p53 (35).

Class II HDACs differ from class I proteins in terms of their tissue expressions, subcellular localizations, and biological roles. Class I HDACs are ubiquitously expressed whereas class II enzymes display tissue-specific expression in humans and mice. Human HDAC4 is most abundant in skeletal muscle and shows modest expression in brain, heart, and ovary but is undetectable in liver, lung, spleen, and placenta, whereas HDAC5 is expressed in mouse heart, brain, liver, and skeletal muscle but not in spleen (7, 8). Class I HDACs are found almost exclusively in the nucleus (except HDAC3) whereas class II HDACs shuttle between the nucleus and cytoplasm, depending on their phosphorylation extents and subsequent binding with 14-3-3 chaperone proteins (36).

Given this considerable diversity of HDACs and HDAC-mediated activities, the development of agents that offer specificity in targeting particular classes of HDACs is clearly required. For example, it is probable that at the clinical level, the inhibitors targeting specific classes of HDACs would incur fewer unintended side effects while potentially delivering greater efficacy. At a more basic level, HDIs with high specificity would facilitate the dissection of the relative contributions of specific HDACs in particular intracellular pathways. Whereas HDIs have been available for research applications for several decades, such as TSA, these generally lacked specificity, affecting both class I and II HDACs. The recent development of HDIs that preferentially inhibit specific HDAC classes, such as SK7041 (inhibiting class I HDACs) and splitomicin (inhibiting class III HDACs), has begun to allow investigations of the relative contributions of specific classes of HDACs towards cellular processes and in response to external agents, such as ionizing radiation. Here, we report on the characterization of HDAC class–specific differences in terms of their abilities to radiosensitize human cancer cells. TSA was found to have the greatest radiosensitizing effect, followed by the class I inhibitor SK7041, whereas the class III inhibitor splitomicin had least effect.

Whereas previous studies have found radiosensitization by HDIs (19, 37–45) and are consistent with our observations described here, we believe this report may be the first to compare under equivalent conditions the effects of HDI that target different subsets of HDACs. Our results suggest that inhibiting class I and II HDACs together may confer greater

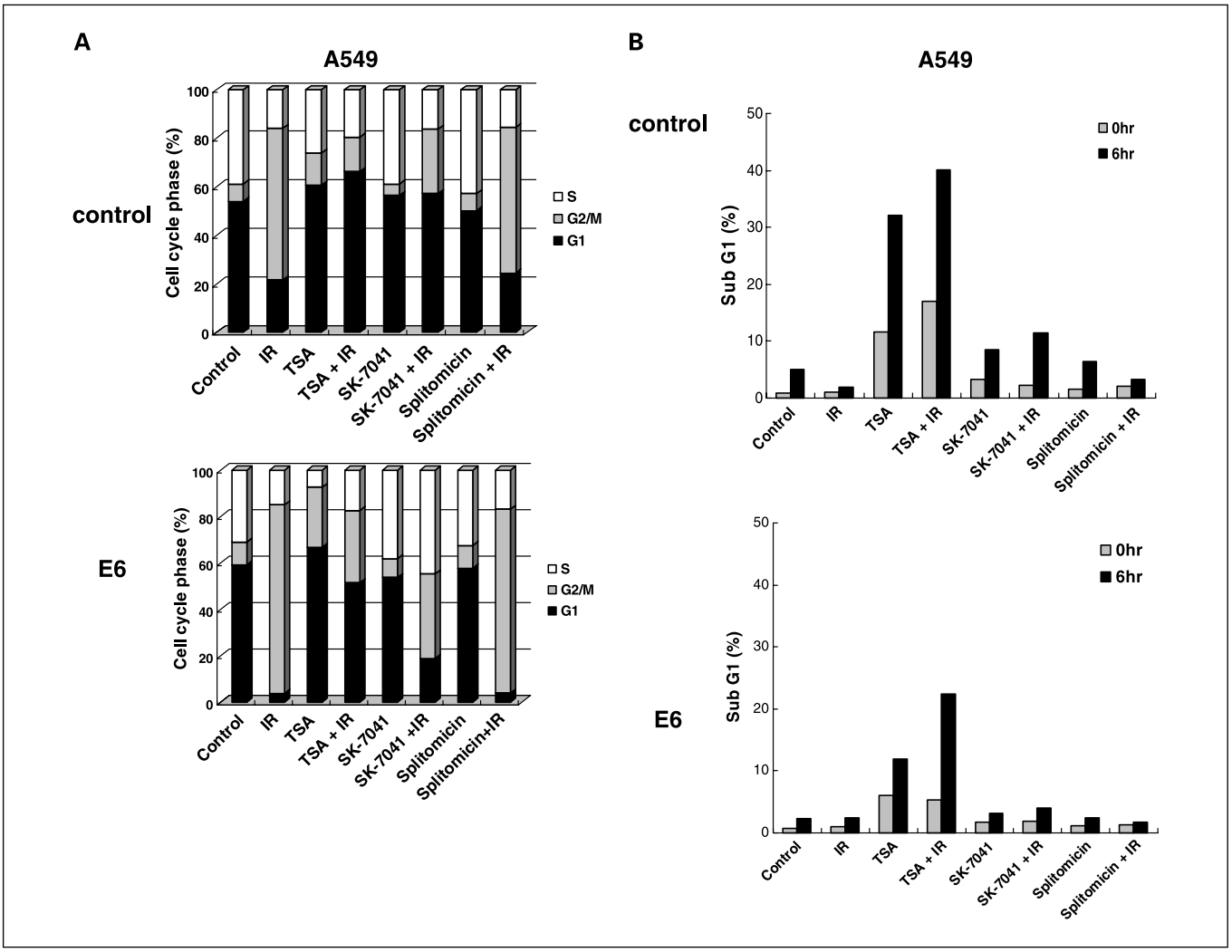


Fig. 6. Cell cycle distribution after HDI and radiation. *A,* cell cycle changes after treatment. A549 control or E6 (p53-deficient) cells were pretreated with HDIs for 18 hours, and then irradiated with 8 Gy. Six hours after irradiation, all cells were collected, fixed, stained with propidium iodide, and analyzed via fluorescence-activated cell sorting for DNA content. At least 1×10^4 cells were counted. As expected, both cell lines showed a robust G_2-M delay after irradiation alone. This arrest was nearly completely abrogated by pretreatment with TSA. SK7041-treated cells also showed lesser but significant abrogation of G_2 arrest. Mock- or splitomicin-treated cells had considerably less effect. *B,* proportion of cells with sub-G_1 DNA content after treatment. A549 control or E6 cells were prepared, treated, and analyzed as in Fig. 5A, with the addition of cells treated only with each respective HDI. Percentage of cells with sub-G_1 DNA content. Cells treated with HDI alone or followed by radiation are grouped together. TSA-treated cells showed highest levels of sub-G_1 cells, followed by those treated with SK7041, whereas splitomicin-treated cells showed least levels. This treatment-induced increase of sub-G_1 population in A549 E6 cells was significantly less compared with that in A549 control cells.

radiosensitization than inhibiting only class I HDACs. It remains to be seen the role of inhibition of class II HDACs alone on radiosensitization and likely awaits the development of class II–specific inhibitors.

Interestingly, SK7041 resulted in the highest acetylation of histone H3 compared with TSA, yet greater radiosensitization was noted with TSA. These findings suggest that TSA and class I inhibitors may influence additional targets, such as nonhistone substrates, possibly related to radiosensitivity. Recent studies suggest that acetylation of nonhistone proteins may have an important role in the biological effect of this class of compounds and may explain the lack of correlation between histone acetylation and induction of cell death by HDIs in some circumstances (46). A number of potential mechanisms of interaction may be postulated. MS-275 and sodium butyrate are reported to radiosensitize human tumor cells by affecting their ability to repair the DNA damage induced by ionizing radiation and that γ-H2AX phosphorylation could be used as a predictive marker of radioresponse (41, 42). Suberoylanilide hydroxamic acid is known to enhance radiation-induced apoptosis and to attenuate several oncoproteins and DNA repair proteins (43). HDAC4, a class II HDAC, was found to mediate radiation DNA damage response in conjunction with p53 binding protein 1. Moreover, the silencing of HDAC4 led to radiosensitization and to the abrogation of the G_2 DNA damage checkpoint (44). More recently, we also observed that TSA abrogated radiation-induced G_2-M arrest and increased apoptosis (45). Abrogation of the radiation-induced G_2-M arrest by class-specific HDI was seen again in this report, which we speculate may decrease the time available for repair of DNA damage or may interfere with repair mechanisms (47). Abrogation of the radiation-induced G_2 delay mediated by caffeine or staurosporin has also been noted to shift the pathway of cell death from mitotic death to apoptotic death (48).

We also describe here observations that suggest that the presence of p53 protein may further augment the radiosensitization of cancer cells by TSA, possibly the first to implicate p53 in sensitizing cancer cells to radiation combined with HDI. HDACs have previously been linked to the regulation of p53, a key molecule in cellular response to DNA damage (49, 50). Susceptibility to HDI-induced cell death has been previously described to be influenced by p53 under experimental conditions that did not involve radiation and therefore different from those described here (51). In a separate report, sodium butyrate suppressed the growth of WT p53–containing cells more efficiently by increasing G_2-M arrest whereas cells without WT p53 accumulated mainly in G_1 phase of the cell cycle. Apoptosis was also considerably reduced in the absence of p53 (52). Clarifying the precise mechanisms by which p53 potentiate radiosensitization by HDIs inhibiting class I and II HDACs, as well as the significance of the abrogation of the radiation-induced G_2 delay by TSA pretreatment, will be fertile topics for future investigation.

In summary, the results presented here add to the growing evidence of differential roles for individual specific classes of HDACs and support the development of new HDAC isotype–specific inhibitors. Such drugs would be expected to confer the most ideal pharmacologic profiles with greatest efficacy and least unintended effects. The work presented here may provide the beginning proof of concept for integrating such drugs into anticancer therapy.

Acknowledgments

We thank Dr. Yung-Jue Bang for providing the novel class I inhibitor SK7041, Dr. Eun-Kyung Choi for helping with the transfection of A549 cells with the E6 construct, Yoon-Sil Lee for providing PCNV vectors, Yun-Jung Choi and Hye Jin Park for technical assistance, and Dr. Eric J. Bernhard for his helpful comments on the manuscript.

References

1. Baylin SB. Tying it all together: Epigenetics, genetics, cell cycle, and cancer. Science 1997;277:1948–9.
2. Mai A, Massa S, Rotili D, et al. Histone Deacetylation in Epigenetics: An attractive target for anticancer therapy. Med Res Rev 2005;25:261–309.
3. Marks PA, Rifkind RA, Richon VM, et al. Histone deacetylases and cancer: Causes and therapies. Nat Rev Cancer 2001;1:194–202.
4. Grozinger CM, Hassig CA, Schreiber SL. Three proteins define a class of human histone deacetylase related to yeast Hda1p. Proc Natl Acad Sci U S A 1999; 96:4868–73.
5. Gray SG, Ekstrom TJ. The human histone deacetylase family. Exp Cell Res 2001;262:75–83.
6. Glaser KJ, Li J, Staver RQ, et al. Role of class I and class II histone deacetylases in carcinoma cells using siRNA. Biochem Biophys Res Commun 2003; 310:529–36.
7. Yang XJ, Gregorie S. Class II histone deacetylases: from sequences to function, regulation and clinical implication. Mol Cell Biol 2005;25:2873–84.
8. Fische W, Kiermer V, Dequiedt F, et al. The emerging role of class II histone deacetylase. Biochem Cell Biol 2001;79:339–48.
9. Luo J, Nikolaev AY, Imai S, et al. Negative control of p53 by Sir2α promotes cell survival under stress. Cell 2001;107:137–48.
10. Vaziri H, Dessain SK, Ng Eaton E, et al. hSIR2^{SIRT1} functions as an NAD-dependent p53 deacetylase. Cell 2001;107:149–59.
11. Marks PA, Richon VM, Rifkind RA. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. J Natl Cancer Inst 2000; 92:1210–6.
12. Zelent A, Waxman A, Carducci M, et al. State of the translational science: summary of Baltimore workshop on gene re-expression as a therapeutic target in cancer January 2003. Clin Cancer Res 2004;10:4622–9.
13. Hoshikawa Y, Kwon HJ, Yoshida M, Horinouchi S, Beppu T. Trichostatin A induces morphological changes and gelsolin expression by inhibiting histone deacetylase in human carcinoma cell lines. Exp Cell Res 1994;214:189–97.
14. Yoshida M, Furumai R, Nishiyama M, Komatsu Y, Nishino N, Horinouchi S. Histone deacetylase as a new target for cancer chemotherapy. Cancer Chemother Pharmacol 2001;48:20–6.
15. Kim TY, Bang YJ. Development of new anticancer agents targeting histone deacetylase. Cancer Res Treat 2002;34 Suppl. 2:9–16S.
16. Park JH, Jung Y, Kim TY, et al. Class I histone deacetylase-selective novel synthetic inhibitors potentially inhibit human tumor proliferation. Clin Cancer Res 2004;10:5271–81.
17. Bedalov A, Gatbonton T, Irvine WP, et al. Identification of a small molecule inhibitor of Sir2p. Proc Natl Acad Sci U S A 2001;98:15113–8.
18. Posakony J, Hirao M, Stevens S, Simon JA, Bedalov A. Inhibitors of Sir2: evaluation of splitomicin analogues. J Med Chem 2004;47:2635–44.
19. Da Casta D, Jen J, He C, Chan A, Kinzler W, Vogelstein B. Converting cancer genes into killer genes. Proc Natl Acad Sci U S A 1996;93:4192–6.
20. Camphausen K, Scott T, Sproull M, Tofilon P. Enhancement of xenograft radiosensitivity by histone deacetylase inhibitor MS275 and correlation with histone hyperacetylation. Clin Cancer Res 2004; 10:6066–71.
21. Komarov PG, Komarova EA, Kondratov RV, et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 1999;285: 1733–7.
22. Lain S, Xirodima D, Lain DP. Accumulating active p53 in the nucleus by inhibition of nuclear export: a novel strategy to promote the p53 tumor suppressor function. Exp Cell Res 1999;253:315–24.
23. Van Lint C, Emiliani S, Verdn E. The expression of small fraction of cellular gene is changed in response to histone hyperacetylation. Gene Expr 1996;5:245–54.
24. Marladson JM, Corner GA, Augenlicht LH. Genetic reprogramming in pathways of colonic cell maturation induced by short chain fatty acids: Comparison with trichostatin A, sulindac, and curcumin and implication for chemoprevention of colon cancer. Cancer Res 2000;60:4561–72.
25. Zhang Y, Reinberg D. Transcription regulation by histone methylation: Interplay between different covalent modifications of core histone tails. Genes Dev 2001; 15:2343–60.
26. Lagger G, O'Carroll D, Rembold D, et al. Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. EMBO J 2002; 21:2672–81.
27. Zhu P, Huber EM, Kiefer F, Gottlicher M. Specific and redundant functions of histone deacetylases in regulation of cell cycle and apoptosis. Cell Cycle 2004;3:1240–2.
28. Waltregny D, Glenisson W, Tran SL, et al. Histone deacetylase HDAC8 associates with smooth muscle α-actin and is essential for smooth muscle cell contractility. FASEB J 2005;19:966–8.
29. McKinsey TA, Zhang CL, Olson EN. Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. Proc Natl Acad Sci U S A 2000;97:14400–5.
30. Chang S, McKinsey TA, Zhang CL, Richardson JA, Hill JA, Olson EN. Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. Mol Cell Biol 2004;24:8467–76.
31. Berns K, Hijimans EM, Mullenders J, et al. A large scale RNAi screen in human cells identifies new components of the p53 pathways. Nature 2004;428: 431–7.
32. Bakin RE, Jung MO. Cytoplasmic sequestration of HDAC7 from mitochondrial and nuclear compartments upon initiation of apoptosis. J Biol Chem 2004; 279:51218–25.
33. Zhang Z, Yamashita T, Toyama H, et al. HDC 6 expression is correlated with better survival in breast cancer. Clin Cancer Res 2004;10:6962–8.
34. Osada H, Tatemasu Y, Saito Y, et al. Reduced expression of class II histone deacetylase genes is associated with poor prognosis in lung cancer patients. Int J Cancer 2004;112:26–32.
35. Lombard DB, Chua KF, Mostoslavsky R, Franco S, Gostissa M, Alt FW. DNA repair, genome stability, and aging. Cell 2005;120:497–512.
36. Grozinger CM, Schreiber SL. Regulation of histone deacetylases 4 and 5 and transcriptional activity by 14–3–3 dependent cellular localization. Proc Natl Acad Sci U S A 2000;97:7825–40.
37. Biade S, Stobbe CC, Boyd JT, Chapman JD. Chemical agents that promote chromatin compaction radiosensitize tumour cells. Int J Radiat Biol 2001;77:1033–42.
38. Zhang Y, Jung M, Dritschilo A, Jung M. Enhancement of radiation sensitivity of human squamous carcinoma cells by histone deacetylase inhibitors. Radiat Res 2004;161:667–74.
39. Kim JH, Shin JH, Kim IH. Susceptibility and radiosensitization of human glioblastoma cells to trichostatin A, a histone deacetylase inhibitor. Int J Radiat Oncol Biol Phys 2004;62:1348–54.
40. Camphausen K, Burgan W, Cerra M, et al. Enhanced radiation-induced cell killing and prolongation of γH2AX foci expression by the histone deacetylase inhibitor MS-275. Cancer Res 2004;64:316–21.
41. Munshi A, Kurland JF, Nishikawa T, et al. Histone deacetylase inhibitors radiosensitize human melanoma cells by suppressing DNA repair activity. Clin Cancer Res 2005;11:4912–22.
42. Camphausen K, Cerna D, Scott T, et al. Enhancement of *in vitro* and *in vivo* tumor cell radiosensitivity by valproic acid. Int J Cancer 2005;114:380–6.

43. Chinnaiyan P, Vallabhaneni G, Armstrong E, Chinnaiyan AM, Huang S, Harari PM. Modulation of radiation response by histone deacetylase inhibition. Int J Radiat Oncol Biol Phys 2005;62:223–9.

44. Kao GD, McKenna WG, Guenther MG, Muschel RJ, Lazar MA, Yen TJ. Histone deacetylase 4 interacts with 53BP1 to mediate the DNA damage response. J Cell Biol 2003;160:1017–27.

45. Kim IA, Kim JH, Shin JH, et al. A histone deacetylase inhibitor, trichostatin A enhances radiosensitivity by abrogating G_2-M arrest in human carcinoma cells. Cancer Res Treat 2005;37:122–8.

46. Rosato RR, Grant S. Histone deacetylase inhibitors: insights into mechanisms of lethality. Expert Opin Ther Targets 2005;9:809–24.

47. Kao GD, McKenna WG, Yen TJ. Detection of repair activity during the DNA damage-induced G_2 delay in human cancer cells. Oncogene 2001;20:3486–96.

48. Bernhard EJ, Muschel RJ, Bakanauskas VJ, McKenna WG. Reducing the radiation-induced G_2 delay causes HeLa cells to undergo apoptosis instead of mitotic death. Int J Radiat Biol 1996;69:575–84.

49. Juan LJ, Shia WJ, Chen MH, et al. Histone deactylases specifically down-regulation p53 dependent gene activation. J Biol Chem 2000;275:20436–43.

50. Luo J, Su F, Chen D, Shiloh A, Gu W. Deacetylation of p53 modulates its effect on cell growth and apoptosis. Nature 2000;408:377–81.

51. Henderson C, Mizzau M, Paroni G, Maestro R, Schneider C, Brancolini C. Role of caspases, Bid, and p53 in the apoptotic response triggered by histone deacetylase inhibitors trichostatin-A (TSA) and suberoylanilide hydroxamic acid (SAHA). J Biol Chem 2003;278:12579–89.

52. Joseph J, Wajapeyee N, Somasundaram K. Role of p53 status in chemosensitivity determination of cancer cells against histone deacetylase inhibitor sodium butyrate. Int J Cancer 2005;115:11–8.