

## Histone Deacetylase Inhibitors Radiosensitize Human Melanoma Cells by Suppressing DNA Repair Activity

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**Abstract Purpose:** Histone deacetylase (HDAC) inhibitors have emerged recently as promising anticancer agents. They arrest cells in the cell cycle and induce differentiation and cell death. The antitumor activity of HDAC inhibitors has been linked to their ability to induce gene expression through acetylation of histone and nonhistone proteins. However, it has recently been suggested that HDAC inhibitors may also enhance the activity of other cancer therapeutics, including radiotherapy. The purpose of this study was to evaluate the ability of HDAC inhibitors to radiosensitize human melanoma cells *in vitro*.

**Experimental Design:** A panel of HDAC inhibitors that included sodium butyrate (NaB), phenylbutyrate, tributyrin, and trichostatin A were tested for their ability to radiosensitize two human melanoma cell lines (A375 and MeWo) using clonogenic cell survival assays. Apoptosis and DNA repair were measured by standard assays.

**Results:** NaB induced hyperacetylation of histone H4 in the two melanoma cell lines and the normal human fibroblasts. NaB radiosensitized both the A375 and MeWo melanoma cell lines, substantially reducing the surviving fraction at 2 Gy (SF2), whereas it had no effect on the normal human fibroblasts. The other HDAC inhibitors, phenylbutyrate, tributyrin, and trichostatin A had significant radiosensitizing effects on both melanoma cell lines tested. NaB modestly enhanced radiation-induced apoptosis that did not correlate with survival but did correlate with functional impairment of DNA repair as determined based on the host cell reactivation assay. Moreover, NaB significantly reduced the expression of the repair-related genes *Ku70* and *Ku86* and DNA-dependent protein kinase catalytic subunit in melanoma cells at the protein and mRNA levels. Normal human fibroblasts showed no change in DNA repair capacity or levels of DNA repair proteins following NaB treatment. We also examined  $\gamma$ -H2AX phosphorylation as a marker of radiation response to NaB and observed that compared with controls,  $\gamma$ -H2AX foci persisted long after ionizing exposure in the NaB-treated cells.

**Conclusions:** HDAC inhibitors radiosensitize human tumor cells by affecting their ability to repair the DNA damage induced by ionizing radiation and that  $\gamma$ -H2AX phosphorylation can be used as a predictive marker of radioresponse.

Local remodeling of chromatin is a key step in the regulation of gene expression, consequently affecting many cell functions. One important mechanism in chromatin remodeling is the post-translational modification of the NH<sub>2</sub>-terminal tails of

histones by acetylation. In transcriptionally silent chromatin, the histones that comprise the nucleosomes have low levels of acetylation on the lysine residues of their NH<sub>2</sub>-terminal tails (1, 2). However, acetylation of these histone proteins neutralizes the positive charge on the lysine residues thereby disrupting nucleosome structure. This allows unfolding of the associated DNA and subsequent access by transcription factors leading to changes in gene expression (1, 2).

Acetylation of core nucleosomal histones is regulated by histone acetyltransferases and histone deacetylases (HDAC; refs. 3, 4). A controlled balance between histone acetylation and deacetylation seems essential for normal cell growth and aberrant HDAC activity has been associated with the development of certain human cancers (4). Perturbations in histone acetylation have been associated with a number of well-characterized oncogenes and tumor suppressor genes prompting the development of histone deacetylase inhibitors (HDAC-I) as a strategy for treating cancer (3, 4). This emerging class of anticancer drugs can induce growth arrest, differentiation, and apoptotic cell death in many different types of tumor cells

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*in vitro* and *in vivo* (4, 5). Despite the growing interest in these drugs, the molecular basis for their antitumor effects are poorly understood although changes in gene expression is one possible mechanism to explain their effects. Specifically, HDAC-I induce hyperacetylation of core histone proteins (H3 and H4) in chromatin and nonhistone proteins, thereby disrupting their interactions with the adjacent DNA and allowing transcription factors to activate certain specific genes (1, 2). Although all HDAC-I induce histone acetylation, they differ with regard to their antitumor activity, stability, and toxicity.

Sodium butyrate (NaB), a naturally occurring short-chain fatty acid that is a byproduct of carbohydrate metabolism in the gut, is one of the most widely studied HDAC-I (6). However, its short half-life of 6 minutes makes it ineffective as a therapeutic agent (7, 8). Other butyrate derivatives that have longer half-lives *in vivo* have been developed, among them phenylbutyrate, and more specific HDAC-I such as trichostatin A, MS-275, and suberoylanilide hydroxamic acid (9). NaB induces cell cycle arrest, differentiation, and apoptosis in human tumor cell lines (10–15). In addition, it enhances the radiosensitivity of tumor cells. Although Leith et al. (16–18) first showed in the early 1980s that low doses of NaB radiosensitized colon cancer cells treated *in vitro*, these observations were not further extended to any great extent. In the present study, we investigated the ability of HDAC-I to modulate the response of human melanoma cells to ionizing radiation and tested the prototypic agent, NaB, along with the more specific HDAC-I: trichostatin A, phenylbutyrate, and tributyrin. In an attempt to establish the molecular mechanism underlying the radiosensitizing ability of NaB, we determined whether NaB-mediated radiosensitization was associated with a decreased capacity for repair of radiation-induced DNA double-strand breaks (DSB).

## Materials and Methods

**Cells.** The human melanoma cell lines, A375 and MeWo, were obtained from the American Type Culture Collection (Manassas, VA) and routinely maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 10,000 units/mL of penicillin-streptomycin, and 2 mmol/L L-glutamine. The normal human lung fibroblast cell line, MRC-9, was used as a control and maintained in  $\alpha$ -MEM supplemented with 10% fetal bovine serum, 10,000 units/mL of penicillin-streptomycin, 2 mmol/L L-glutamine, nonessential amino acids, and MEM essential vitamins.

**Chemicals.** NaB, tributyrin, and trichostatin A were obtained from Sigma-Aldrich Co. (St. Louis, MO) and phenylbutyrate was obtained from Alexis (San Diego, CA). A 10 mmol/L solution of trichostatin A in absolute ethanol was prepared and stored at  $-20^{\circ}\text{C}$  until use. NaB was prepared fresh before each use as a 100 mmol/L stock in PBS. Phenylbutyrate was resuspended in PBS as a 10 mmol/L stock. Tributyrin was obtained as a solution and stored at  $-20^{\circ}\text{C}$  in aliquots until further use.

**Cell cycle analysis.** Cell cycle arrest was assessed by propidium iodide staining and fluorescence-activated cell sorting analysis. Cells were harvested after 24 hours of treatment with NaB, pelleted by centrifugation, and resuspended in PBS containing 50  $\mu\text{g}/\text{mL}$  propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. Samples were stored at  $4^{\circ}\text{C}$  for 16 hours and vortexed before fluorescence-activated cell sorting analysis (BD PharMingen, San Diego, CA; FACScan, FL-3 channel). The cell cycle calculations were done using the MultiCycle Program from Phoenix Flow Systems (San Diego, CA).

**DNA fragmentation.** Apoptosis-specific DNA fragmentation was measured by a modification of a published procedure. The assay is based on the solubility of low molecular weight DNA in solutions of low salt concentration (19). Briefly, cells labeled by [ $^{14}\text{C}$ ] TdR incorporation for one cycle were treated with NaB for 24 hours and irradiated. NaB was then removed and the cells irradiated for an additional 4 hours to allow apoptosis. After treatment, cells were washed with PBS and lysed with 0.5 mL of lysis buffer [10 mmol/L Tris, 1 mmol/L EDTA, 0.2% Triton X-100 (pH 7.5)] on ice for 20 minutes. The chromatin was pelleted by centrifugation at  $14,000 \times g$  for 10 minutes. The supernatant (fragmented DNA) was removed, and the chromatin pellet was solubilized in 1 mL of Soluene (Packard, Meriden, CT). Radioactivity was determined using a liquid scintillation counter (Packard Instruments, Downers Grove, IL). DNA fragmentation was expressed as the percentage of radioactivity found in the supernatant fraction compared with the total radioactivity (pellet plus supernatant).

**Clonogenic survival.** The effectiveness of the combination of HDAC-I and ionizing radiation was assessed by clonogenic assays. Briefly, the human melanoma cells or the normal human lung fibroblasts were treated with the vehicle control or the HDAC-I at the indicated concentration for 24 hours and irradiated with a high dose rate  $^{137}\text{Cs}$  unit (4.5 Gy/min) at room temperature. Following treatment, cells were trypsinized and counted. Known numbers were then replated in 100-mm tissue culture dishes and returned to the incubator to allow macroscopic colony development. Colonies were counted after about 14 days, and the percent plating efficiency and fraction surviving a given treatment was calculated based on the survival of nonirradiated cells treated with the vehicle or HDAC-I.

**Western blot analysis.** Cells were harvested after treatment with various doses of NaB for 24 hours at  $37^{\circ}\text{C}$ , rinsed in ice-cold PBS, and lysed in lysis buffer containing 50 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 2  $\mu\text{g}/\text{mL}$  leupeptin, 2  $\mu\text{g}/\text{mL}$  aprotinin, 5  $\mu\text{g}/\text{mL}$  benzamide, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 1% NP40. The lysates were centrifuged at 14,000 rpm to remove any cellular debris. Protein concentrations of the lysates were determined by the Bio-Rad Dc protein assay system (Hercules, CA). Equal amounts of protein were separated by 12% SDS-PAGE, transferred to Immobilon (Millipore, Bedford, MA), and blocked with 5% nonfat dry milk in TBS/Tween 20 (0.05%, v/v) for 1 hour at room temperature. The membrane was incubated with primary antibody overnight. Antibodies for bax and bcl-xL were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), acetylated histone H4 antibody from Upstate Biotechnology (Lake Placid, NY), p21 from Calbiochem (Beverly, MA), Ku70 from Santa Cruz Biotechnology, Ku86 from Sigma Chemicals (St. Louis, MO), DNA-dependent protein kinase catalytic subunit (DNA-PKcs) from GeneTex (San Antonio, TX), and actin from Chemicon (Temecula, CA). After washing, the membrane was incubated with the appropriate horse radish peroxidase secondary antibody (diluted 1:2,000; Amersham Pharmacia Biotech, Arlington Heights, IL) for 1 hour. Following several washes, the blots were developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

**RNase protection assay.** To determine the effect of NaB on mRNA levels of the DNA repair proteins an RNase protection assay (RPA) was done using an RNase protection assay kit (BD PharMingen). In brief, total RNA was isolated from cells with TRIZOL (Life Technologies, Gaithersburg, MD). A multiprobe set, hDSBR-2, which contains the probes for the DNA repair genes *ATM*, *Ku70*, *Ku86*, DNA-PKcs, and XRCC2, XRCC3, XRCC4, XRCC9, and LIG4, and the housekeeping genes *L32* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), was labeled with [ $^{32}\text{P}$ ] ATP using T7 RNA polymerase. Total RNA and probes were hybridized at  $56^{\circ}\text{C}$  overnight, and these hybrid complexes were treated with RNase cocktail. Protected complexes were resolved on denaturing polyacrylamide sequencing gels, and the gels were then viewed on phosphorimager screens. The band intensities were normalized to the housekeeping genes and then quantitated on a STORM 860 phosphorimager using the Molecular Dynamics Imaging system (Molecular Dynamics, Hayward, CA).

**Host cell reactivation.** To test whether DNA repair capacity was suppressed following HDAC-I treatment, host cell reactivation was carried out (20). Basically, cells were plated in 6-well plates ( $1 \times 10^4$  cells per well in 3 mL of medium). Forty-eight hours after plating, cells were treated with 3 mmol/L NaB for 24 hours. Adenovirus carrying the  $\beta$ -galactosidase gene (*Ad- $\beta$ gal*) was irradiated in a centrifuge tube with a high-dose  $^{137}\text{Cs}$  unit (4000 Gy) at room temperature. Control cells were infected with irradiated *Ad- $\beta$ gal* (6,000 vector particles per cell) in 1 mL of serum-free medium. Cells treated with NaB were infected with irradiated *Ad- $\beta$ gal* (300 vector particles per cell) to take into account the ability of NaB to enhance transgene expression. After incubation for 1 hour, 2 mL of complete medium were added to the cells. Twenty-four hours after  $\beta$ -gal transduction, cells were fixed with 2% formaldehyde and 0.05% glutaraldehyde in PBS and stained with X-gal overnight. The relative capacity of the cellular repair systems was assayed by counting the positively stained cells under a microscope. The adenoviral vector required a high dose of radiation because of its small genome size compared with a mammalian cell. Our calculations indicate that 4,000 Gy should induce about 1 to 2 DSBs/vector particle (21). This is based on the target size of the vector genome ( $3.5 \times 10^4$  bp) versus that of the mammalian cell ( $3 \times 10^9$  bp) and using the value of 35 for the DSBs induced per Gy in the mammalian genome.

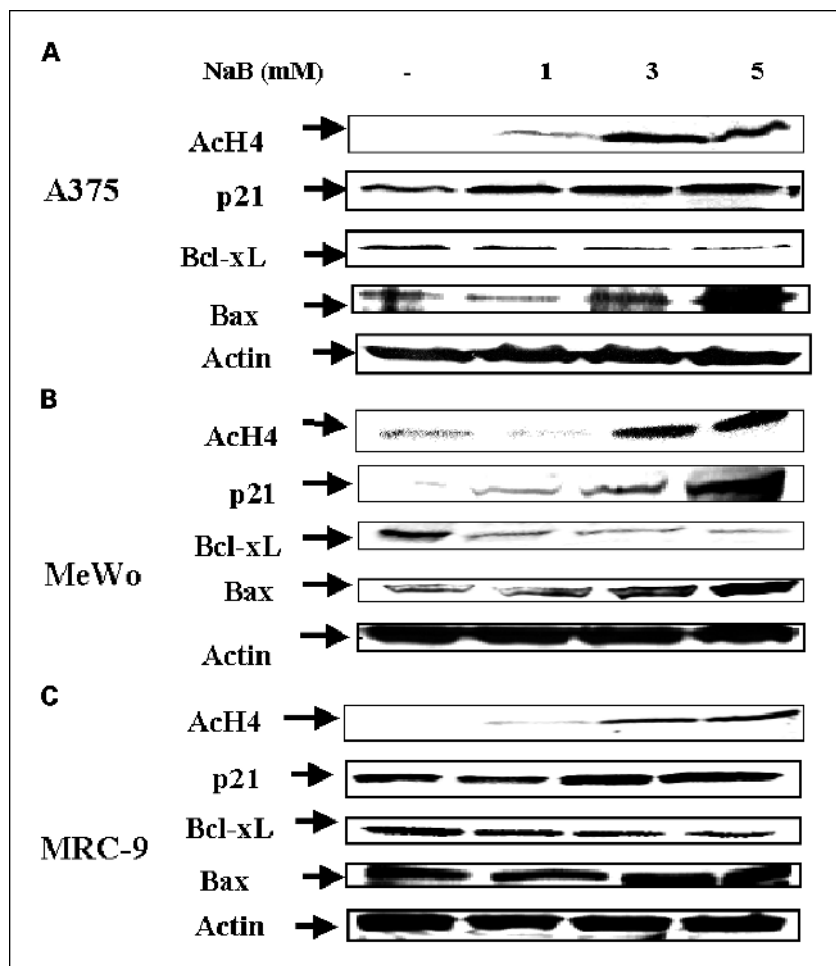
**Immunofluorescent staining for  $\gamma$ -H2AX.** Cells were grown and treated with 3 mmol/L NaB for 24 hours on coverslips placed in 35-mm dishes. At specified times, medium was aspirated and cells were fixed in 1% paraformaldehyde for 10 minutes at room temperature. Paraformaldehyde was aspirated, and the cells were fixed in 70% ethanol for 10 minutes at room temperature followed by treatment with 0.1% NP40 in PBS for 20 minutes. Cells were washed in PBS twice and

blocked with 5% bovine serum albumin in PBS for 30 minutes following which anti- $\gamma$ -H2AX antibody (Trevigen, Gaithersburg, MD) was added at a dilution of 1:300 in 5% bovine serum albumin in PBS and incubated overnight at 4°C with gentle shaking. Cells were then washed thrice in PBS before incubating in the dark with a FITC-labeled secondary antibody at a dilution of 1:300 in 5% bovine serum albumin in PBS for 30 minutes. The secondary antibody solution was then aspirated, and the cells were washed four times in PBS. Cells then were incubated in the dark with 4',6-diamidino-2-phenylindole (1  $\mu\text{g}/\text{mL}$ ) in PBS for 5 minutes and coverslips were mounted with an antifade solution (Molecular Probes, Eugene, OR). Slides were then examined on a Leica fluorescent microscope. Images were captured by a CCD camera and imported into Advanced Spot Image analysis software package for storage purposes. For each treatment condition,  $\gamma$ -H2AX foci were counted by eye in at least 50 cells from the stored images.

**Statistical analysis.** Most analyses were done using the *t* test (Sigma Plot 5.02 v, Richmond, CA) and described as mean  $\pm$  SE. At each time point examined, ANOVA was used to test whether the average number of  $\gamma$ -H2AX foci per cell after combined NaB/radiation treatment (the interaction term) was greater than expected from the sum of the foci produced by each agent alone (StataCorp. 2004. Stata Statistical Software: Release 8.0. College Station, TX). A difference was regarded as significant if  $P < 0.05$ .

## Results

**NaB treatment leads to acetylation of histones and accumulation of p21 and Bax.** Treatment with NaB induced pronounced



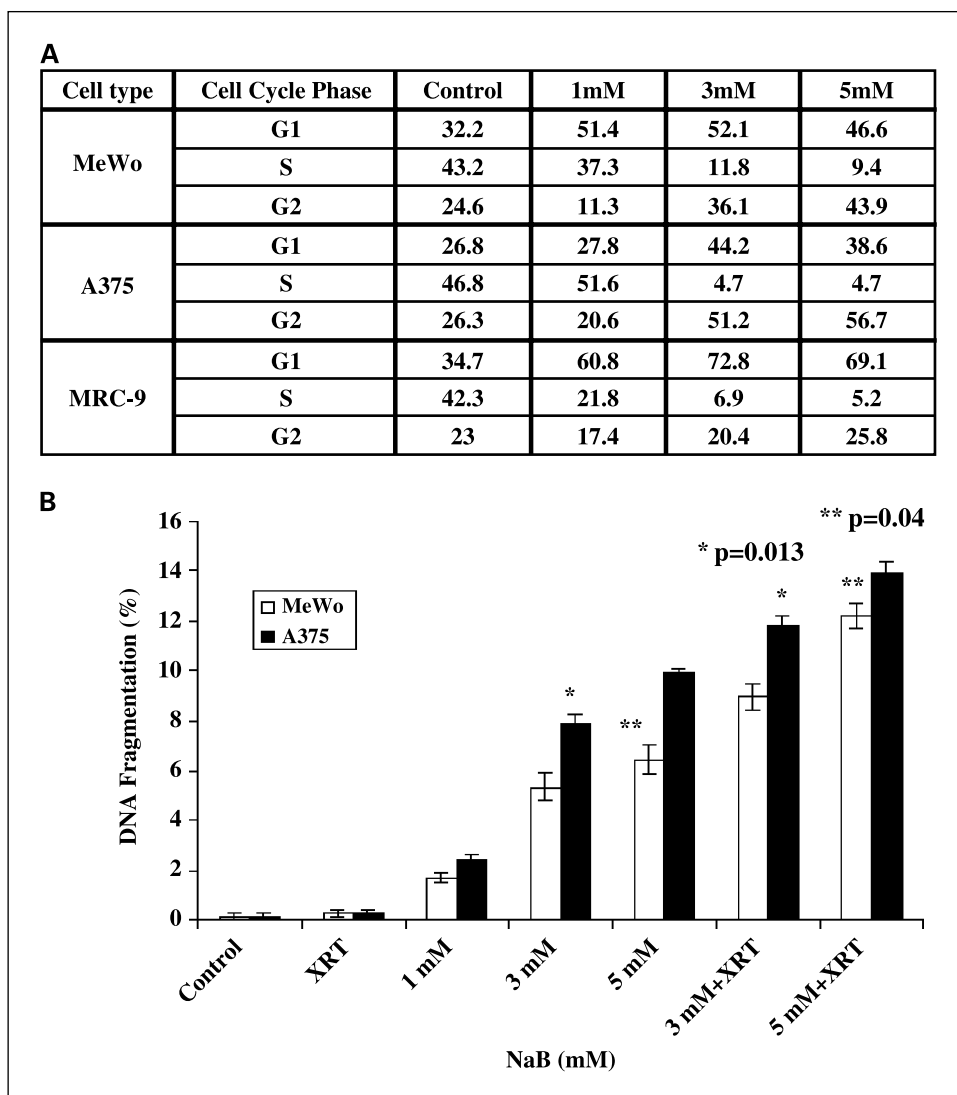
**Fig. 1.** NaB leads to accumulation of acetylated histone H4, p21 and Bax. A375 melanoma cells (A), MeWo melanoma cells (B), and MRC-9 normal human fibroblast cell lines (C) were treated with indicated doses of NaB for 24 hours. Protein was extracted and analyzed by Western blot. Actin was used as a loading control. Blots are representative of at least two independent experiments.

histone H4 hyperacetylation in both human melanoma cell lines (A375 and MeWo) and the normal human lung fibroblasts (MRC-9; Fig. 1). All three cell lines showed accumulation of acetylated histones following treatment with 3 mmol/L NaB that was not increased with 5 mmol/L. We also examined the expression of different proteins that may be involved in apoptotic pathways following treatment with NaB. Both A375 and MeWo cells showed increases in p21 and Bax levels following treatments of 1 to 5 mmol/L depending on the cell line (Fig. 1A-B). Whereas the normal lung fibroblast line, MRC-9, showed slight increases in p21 and Bax the basal levels of these proteins seemed somewhat enhanced compared with the melanoma cells (Fig. 1C). A decrease in bcl-xL levels was observed in all three cell lines.

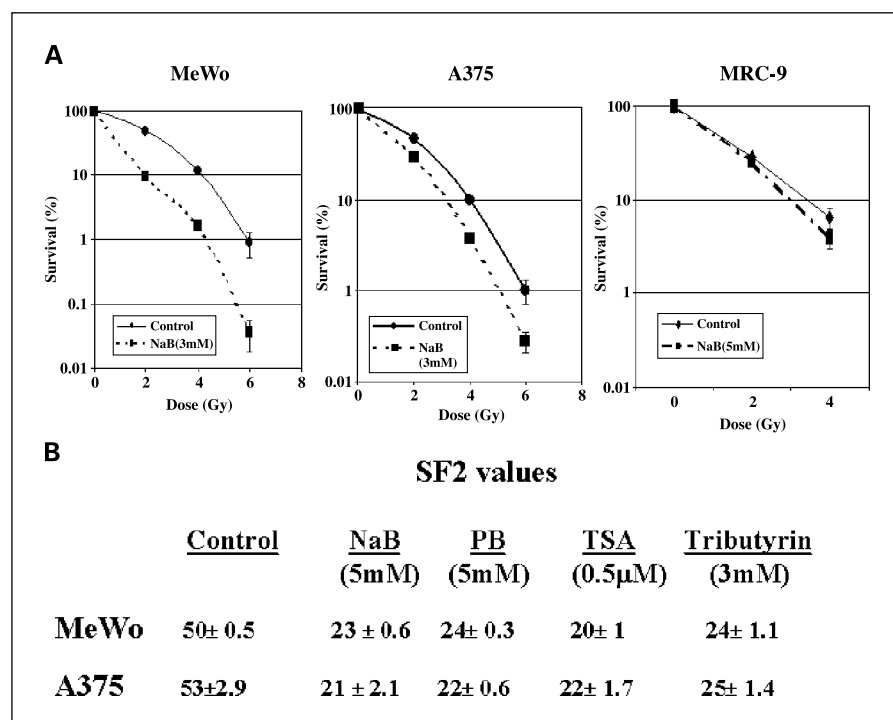
**NaB induces cell cycle arrest and apoptosis in melanoma cells.** Treatment with 1 to 5 mmol/L NaB for 24 hours arrested all three cell lines in the G<sub>1</sub> phase of the cell cycle. This was accompanied by a decrease in cells in S phase. These effects were generally maximum following exposure of 3 mmol/L as exposure to 5 mmol/L did not substantially increase these effects (Fig. 2).

Both melanoma cell lines were tested for their susceptibility to NaB-induced apoptosis using a DNA fragmentation assay. The DNA fragmentation assay showed that increasing (1-5 mmol/L) doses of NaB induced apoptosis in both the melanoma cell lines (Fig. 2B). Apoptosis in the A375 cells increased from 0.175% in the control cells to 8% and 10% following a 24-hour incubation with 3 and 5 mmol/L dose of NaB, respectively. Apoptosis in the MeWo cells increased from 0.15% in the control cells to 5.3% and 6.5% following a 24-hour incubation with 3 and 5 mmol/L dose of NaB, respectively (Fig. 2B). A 5-Gy dose of ionizing radiation did not induce apoptosis but when given at the end of the 24-hour treatment with 3 mmol/L NaB apoptosis was significantly enhanced in the A375 cells when compared with 3 mmol/L NaB alone ( $P = 0.013$ ). Similarly, ionizing radiation in combination with 5 mmol/L NaB significantly enhanced apoptosis in the MeWo cells when compared with 5 mmol/L NaB alone ( $P = 0.04$ ).

**Treatment with NaB enhances radiosensitivity in human melanoma cells in an in vitro clonogenic survival assay.** The apoptosis assays described above were carried out at a single time point following treatment and so the results may not have



**Fig. 2.** NaB alters the cell cycle profile in human melanoma cells but induces minimal apoptosis. *A*, cell cycle analysis of A375, MeWo, and MRC-9 cells treated with 1, 3, and 5 mmol/L dose of NaB for 24 hours. *B*, apoptosis induction in the A375 and MeWo cells as determined by DNA fragmentation assay.



**Fig. 3.** Radiosensitization by HDAC inhibitors. *A*, radiosensitization by NaB as indicated by clonogenic cell survival assays. A375, MeWo, and MRC-9 cells were treated with 3 mmol/L dose of NaB for 24 hours following which they were irradiated and plated for cell survival. Points, averages of two independent experiments each plated in triplicate; bars, SE. *B*, comparison of the SF2 values following treatment with various HDAC-I. Human melanoma cells, A375 and MeWo, were treated with the indicated doses of various HDAC-I, following which the cells were plated for clonogenic survival assay. Decrease in the SF2 values indicates the potent radiosensitizing effects of HDAC-I.

reflected the total toxicity over time. Therefore, we also determined the survival of human melanoma cells exposed to combinations of NaB and ionizing radiation using clonogenic assays. MeWo and A375 cells were pretreated with 3 mmol/L NaB for 24 hours following which the cells were irradiated and plated for clonogenic cell survival. Figure 3A shows that NaB suppressed the clonogenic survival of both melanoma cell lines, MeWo and A375. Survival at 2 Gy (SF2) was reduced from  $49.5 \pm 0.55\%$  in the control MeWo cells to  $9.85 \pm 0.49\%$  ( $P = 0.0016$ ) in NaB-treated MeWo cells (Fig. 3A). Similar results were obtained upon exposure of A375 cells to NaB with SF2 being reduced from  $47.7 \pm 1.1\%$  in the control cells to  $29 \pm 1\%$  ( $P = 0.049$ ; Fig. 3A). Survival enhancement ratios were calculated at 10% cell survival by dividing radiation dose of the radiation only survival curve with that of the corresponding NaB plus radiation curve. Survival enhancement ratio for the MeWo cells was 2.1 and that for A375 was 1.3. Normal cells were not radiosensitized after treatment with same concentration of NaB (Fig. 3A). The 3 mmol/L treatment of NaB alone was more toxic to the melanoma cell lines reducing plating efficiencies from 48% to 20% and 54% to 25% for MeWo and A375 cells, respectively, compared with the normal cells where plating efficiency was reduced from 38% to 36%. In addition we also examined the ability of more specific HDAC inhibitors to radiosensitize both melanoma cell lines. All three inhibitors, phenylbutyrate, trichostatin A and tributyrin radiosensitized the melanoma cell lines. Data is summarized in Fig. 3B where SF2 values for NaB given at 5 mmol/L are also provided to make a direct comparison with phenylbutyrate. Interestingly, in the case of the MeWo cells treated with NaB, 5 mmol/L seemed slightly less radiosensitizing than was 3 mmol/L (Fig. 3A). All of these agents produced roughly similar toxicities to the melanoma cells lowering plating efficiencies from about 50% in the untreated controls to about 18% to 25% for drug alone.

#### Modulation of DNA repair gene expression by NaB may be responsible for mediating the radiosensitivity of melanoma cells.

As an initial investigation into the mechanism responsible for NaB-mediated radiosensitization, we examined the effect of NaB treatment on the expression of proteins known to be involved in the repair of radiation-induced DSBs. It has been reported that suppressed levels of these proteins enhances the radiosensitivity of human tumor cells. Western blot analysis for Ku70, Ku86, and DNA-PKcs was done on whole cell extracts harvested from NaB-treated cells (Fig. 4). The levels of all three proteins decreased following NaB treatment in the two melanoma cell lines. The degree of this decreased protein expression depended to some extent on the dose of NaB and the specific cell line. In the A375 cells, this effect was evident at 3 and 5 mmol/L. However, in the MeWo cells, the effect was greatest at 5 mmol/L for Ku70 and Ku86. DNA-PKcs was decreased in the MeWo cells at 1 and 3 mmol/L but not at 5 mmol/L (Fig. 4B). We observed no change in the levels of Ku70 and Ku86 in the normal fibroblasts, MRC-9 (Fig. 4C), whereas DNA-PKcs seemed to increase slightly.

To test whether these changes in protein levels reflected changes at the transcriptional level, a RNase Protection Assay was carried out using a template specific for the genes that encode these and other DNA repair proteins (Fig. 5). We observed reductions in the mRNA level in the melanoma cells, A375 and MeWo, (Fig. 5B) for several of these genes following treatment with NaB for 24 hours. However, similar reductions were not observed at the mRNA level in the normal cells treated with NaB for either Ku70, Ku86, or DNA-PKcs (Fig. 5B).

**NaB suppresses DNA repair detected using a host cell reactivation assay.** We did host cell reactivation to determine if the radiosensitizing effect of NaB could be explained as a suppression of the DNA repair capacity of the melanoma cells. For this, A375 cells, mock treated or pretreated with NaB,

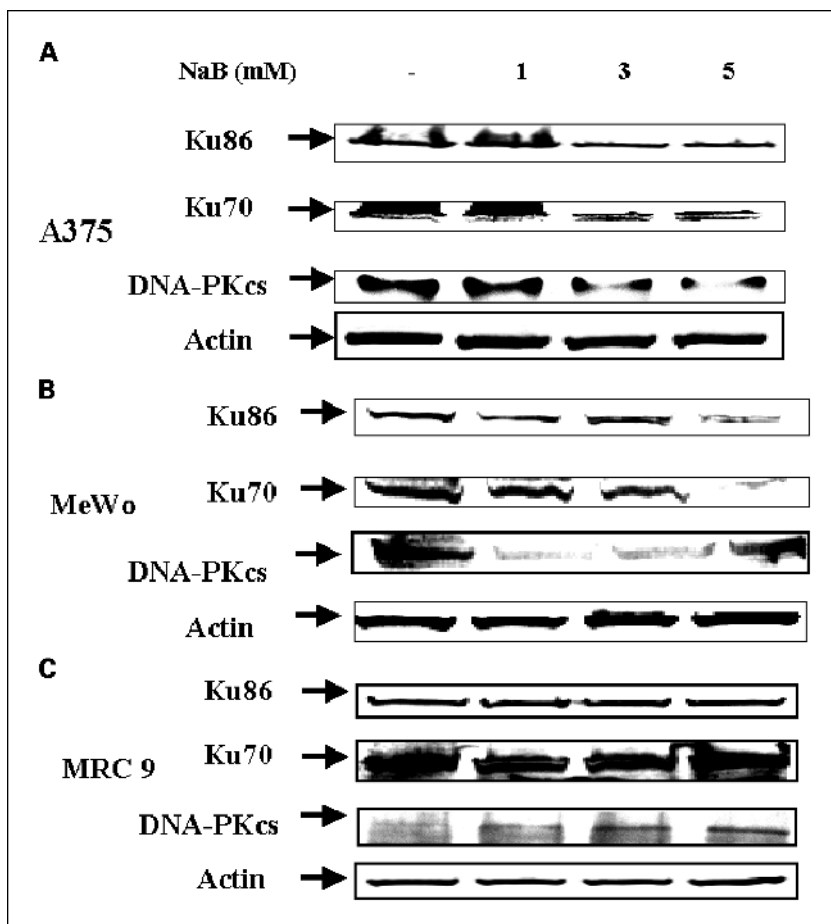
were subsequently infected with Ad- $\beta$ gal that had been either unirradiated or irradiated with 4,000 Gy of  $\gamma$ -radiation. The ability of the melanoma cells to reactivate the irradiated Ad- $\beta$ gal based on  $\beta$ gal expression was assessed 24 hours later. NaB-pretreated A375 cells had a significantly ( $P = 0.019$ ) lower capacity to reactivate irradiated Ad- $\beta$ gal compared with A375 cells that received no treatment (Fig. 6). This was most evident after the 3 mmol/L dose of NaB, at which host cell reactivation was suppressed to 40% of control. A dose of 5 mmol/L suppressed host cell reactivation to 72% of control (Fig. 6).

**NaB prolongs the expression of  $\gamma$ -H2AX foci.** As an additional test of our hypothesis that NaB impairs the repair of damaged DNA,  $\gamma$ -H2AX foci were assessed as indicators of DNA damage. As shown by the micrographs in Fig. 7A,  $\gamma$ -H2AX foci could be clearly distinguished after irradiation (2 Gy) of A375 cells. The average number of  $\gamma$ -H2AX foci per cell were counted in the micrographs and the results are presented in Fig. 7B. The average number of  $\gamma$ -H2AX foci per cell in cultures receiving the combined NaB/radiation treatment was significantly greater compared with the radiation-only group at the 30 minutes, 1, and 2 hours time points (Fig. 7B) with  $P = 0.005$ ,  $P = 0.019$ , and  $P = 0.0008$ , respectively. Treatment with NaB alone had a slight effect on  $\gamma$ -H2AX foci that was not statistically significant ( $P = 0.22$ ) compared with untreated controls. However, we tested the significance of the increase in foci per cell seen for the NaB plus radiation time points compared with the sum of the foci produced by radiation and NaB when used as single agents. These tests indicated that the foci per cell for the NaB plus

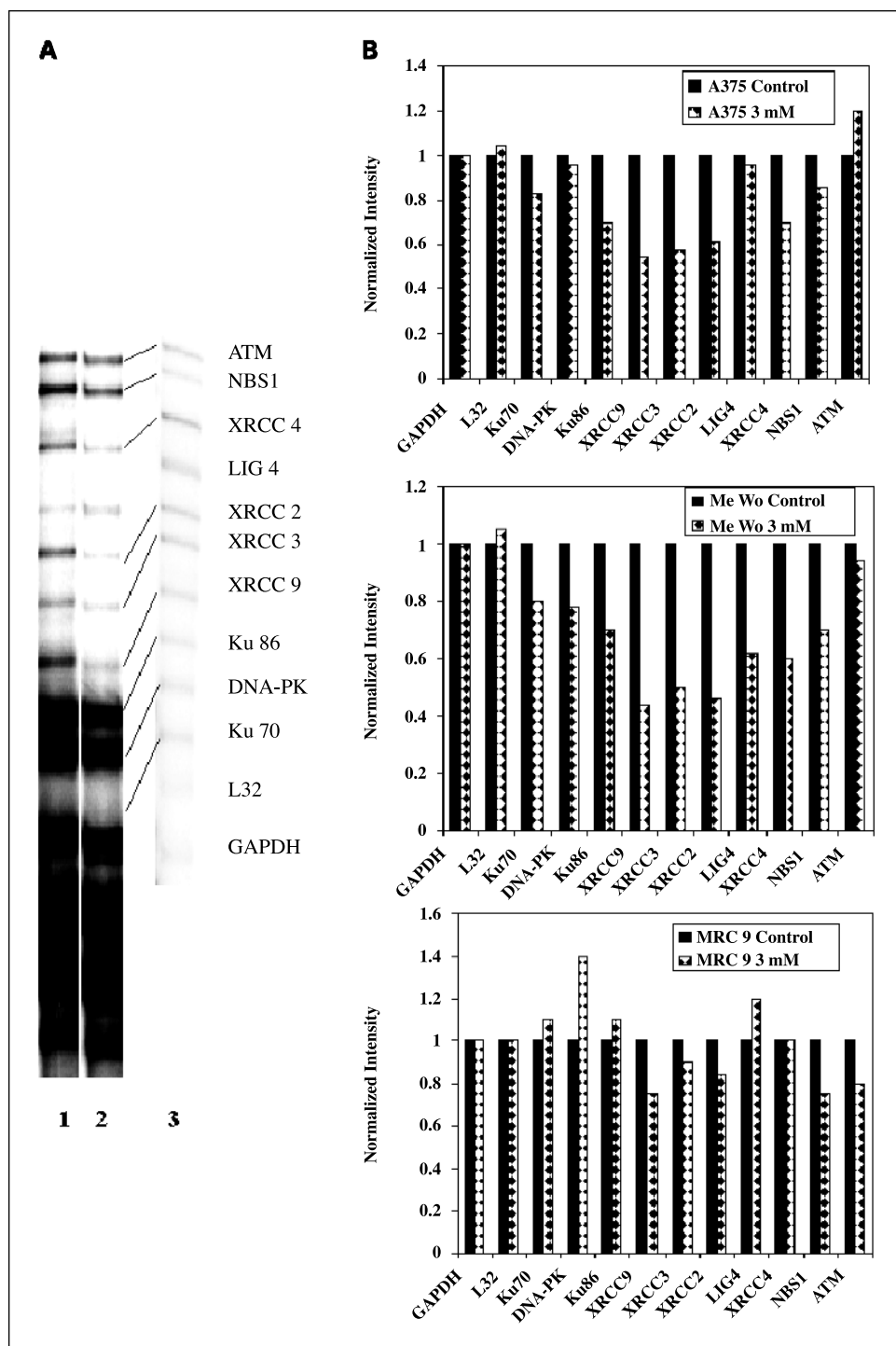
radiation were significantly increased at 30 minutes ( $P = 0.0003$ ) and approached significance at 1 hour ( $P = 0.057$ ) compared with the sum of the foci produced by the agents alone. This prolongation of  $\gamma$ -H2AX foci levels following the combination compared with controls suggests that NaB-mediated radiosensitization involves an inhibition of the repair of DNA damage.

## Discussion

In this study, we have shown that the combination of HDAC inhibitors and ionizing radiation enhanced radiosensitivity of two human melanoma cell lines. Although it was originally reported several years ago that sodium butyrate enhances radiosensitivity, the exact mechanism behind this effect has not been uncovered (16–18). This unexplored phenomenon was the stimulus for the present study, in which we assessed the effect of the HDAC-I, NaB, on the radiosensitivity of two relatively radioresistant human melanoma cell lines, A375 and MeWo. To verify the HDAC-I activity of NaB on these cell lines, the acetylation status of histone H4 was determined as a function of dose after NaB addition for 24 hours. We observed that NaB caused an accumulation of acetylated histone H4 in both human melanoma cell lines and the normal human fibroblasts. As shown in Fig. 3, NaB significantly enhanced the radiosensitivity of the human melanoma cells MeWo and A375, based on a clonogenic cell survival assay but not the normal human lung fibroblasts, indicating that the radiosensitizing



**Fig. 4.** Involvement of DNA repair proteins in NaB-mediated radiosensitization process. Effect of NaB treatment on DNA repair proteins Ku70, Ku86, and DNA-PKcs was analyzed by Western blot analysis in A375 (A), MeWo (B), and MRC-9 (C) cell lines. Actin was used as a loading control.

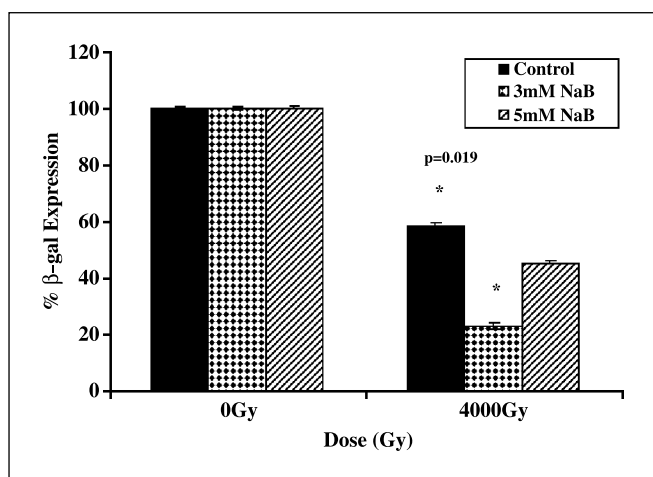


**Fig. 5.** Analysis of mRNA levels by ribonuclease protection assay for DNA repair genes following NaB treatment. Total RNA was isolated, and ribonuclease protection assay was done as described in Materials and Methods. *A*, gel photograph of DNA repair genes at the mRNA levels. Lane 1, untreated control; lane 2, treated with NaB (3 mmol/L for 24 hours); lane 3, labeled template. *B*, quantitative analysis of the ribonuclease protection assay comparing control untreated and NaB-treated cells for expression of various DNA repair genes.

effect of HDAC inhibitors was not mediated by the mere accumulation of acetylated histones. Although histone hyperacetylation is presumed to be involved, the downstream events responsible for the NaB-mediated enhancement in radiosensitivity are not defined by this measurement.

Therefore, we also examined the effect of NaB on the distribution of the cells in various phases of the cell cycle in both melanoma cell lines and observed a dose-dependent suppression of cells in the S phase with concomitant increase in the population of cells in the G<sub>1</sub> phase. However, although

normal human fibroblasts were not radiosensitized by NaB, they also showed a substantial suppression of cells in the S phase and an accumulation of cells in G<sub>1</sub>. Thus, the radiosensitizing effects of NaB are not likely due to changes in cell cycle distribution. Similar to what has been shown for a number of HDAC inhibitors, p21 protein expression was induced in both the melanoma cell lines and the normal fibroblasts treated with NaB, irrespective of the p53 status of the cells (MeWo cells express mutant p53 protein and A375 cells express wild type p53). This increase in p21 expression did not



**Fig. 6.** Host cell reactivation. NaB suppresses host cell reactivation in A375 cells. Cells ( $2 \times 10^4$ ) were seeded in each well of 6-well plates and were either left untreated or were treated with 3 mmol/L NaB for 24 hours. They were then infected with unirradiated or irradiated (4,000 Gy) Ad- $\beta$ gal for another 24 hours. The cells were then stained for  $\beta$ -gal and the  $\beta$ -gal-positive cells were counted and recorded. The percent positive cells were normalized to controls for comparison. Columns, averages of at least four independent experiments; bars, SE.

correlate with NaB's radiosensitizing effect either. The anti-apoptotic protein bcl-xL showed a dose-dependent down-regulation, whereas Bax was up-regulated in the A375 and MeWo melanoma cells following treatment with NaB for 24 hours. A similar decrease in bcl-xL following treatment with sodium butyrate and phenylbutyrate has been described earlier (22–24). Enhanced radiosensitivity of A375 and MeWo cells could be associated with induction of Bax protein because normal human fibroblasts did not show marked up-regulation of Bax following treatment with NaB. Thus, NaB may enhance radiosensitivity by altering the ratio of proapoptotic to antiapoptotic proteins in these cell lines. NaB also restored radiation-induced apoptosis to a small extent in both the A375 and MeWo cells as shown in Fig. 2. However, this small effect could not account for the much greater loss of clonogenic survival. Besides inducing apoptosis, ionizing radiation may also kill mammalian cells through senescence and mitotic catastrophe. Both of these modes may result from unrepaired or misrepaired DNA damage; therefore, we assessed whether cellular DNA repair processes were affected in the context of NaB treatment.

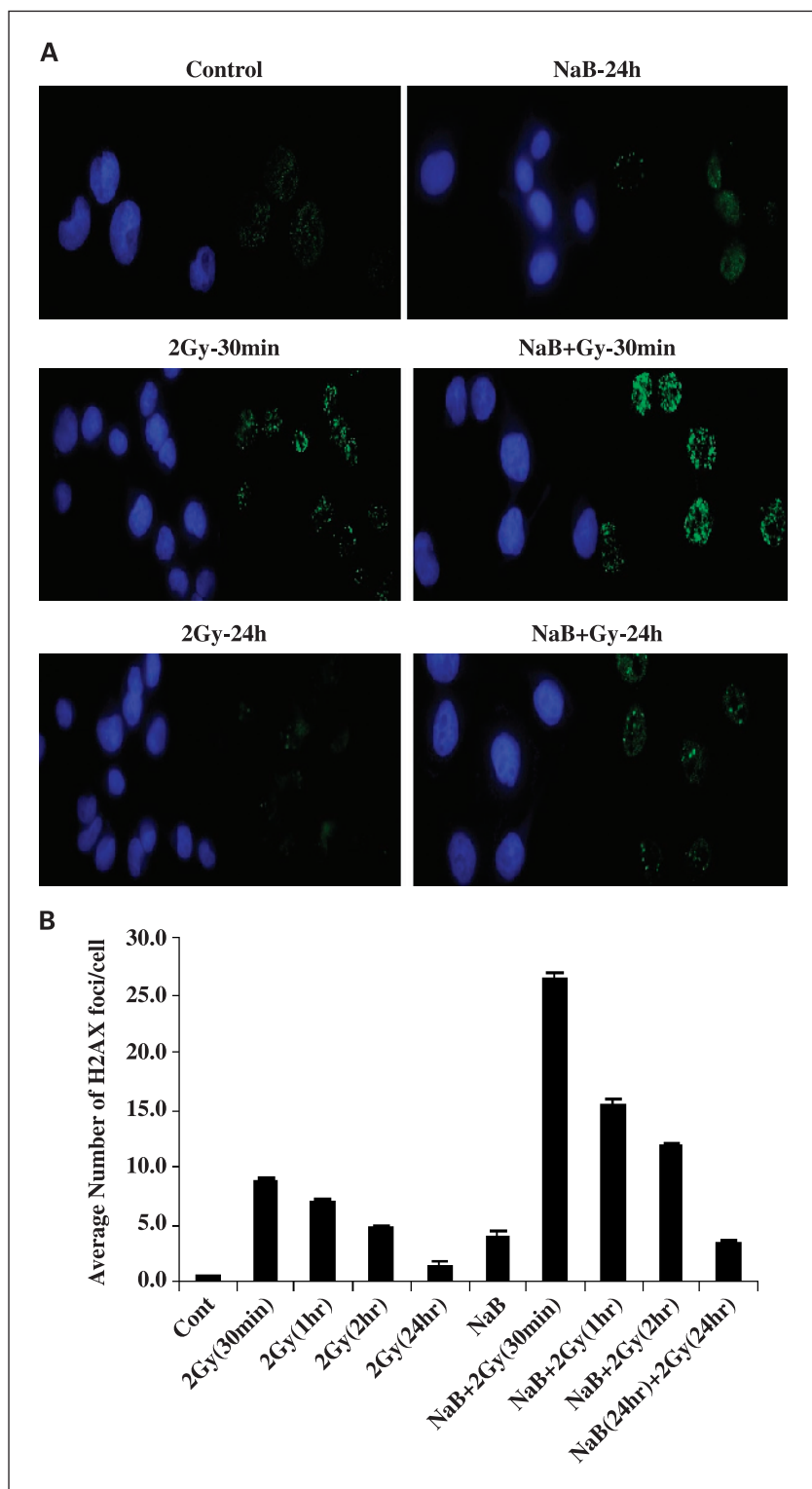
Genetic insults, such as radiation, induce DNA DSBs that are repaired by interchromosomal and intrachromosomal homologous recombination and nonhomologous end joining (25–27). The nonhomologous end joining pathway is especially important for repairing radiation-induced DSBs that are responsible for loss of clonogenic survival (28). Proteins that participate in nonhomologous end joining include components of DNA-PK comprising Ku70, Ku86, and DNA-PKcs, X-ray-sensitive complementation group 4 (XRCC4), and DNA ligase IV. A deficiency in any of these proteins also results in defects in V(D)J recombination, leading to severe combined immunodeficiency (29–31). Mutations in any of the three subunits of DNA-PK can lead to extreme radiosensitivity and DSB repair deficiency. There are several reports linking down-regulation of DNA repair proteins with an enhanced radio-response. Small interfering RNA molecules targeting DNA-PKcs

have been explored in two reports (32, 33). In both, modest radiosensitizing effects were observed in cell systems where protein levels of DNA-PKcs were suppressed to varying degrees. Omori et al. evaluated an antisense construct to Ku70 and showed a substantial suppression of Ku70 protein but only a modest radiosensitizing effect (34). In another study, using adenoviral-mediated, heat-activated antisense Ku70, the SF2 values were reduced from about 0.80 to 0.50 in the context of an almost complete suppression of Ku70 protein expression (35). Therefore, down-regulation of the expression of any of these DNA repair genes could explain an interaction between HDAC-I and radiation.

Because Ku70, Ku86, and DNA-PKcs are key elements of the nonhomologous end joining pathway, we examined the effect of NaB on the levels of these proteins by Western blot analysis. Ku70, Ku86, and DNA-PKcs protein levels were suppressed in melanoma cells treated with NaB. Similar effects on DNA-PK were reported by Goh et al. (22) in prostate cancer cells treated with phenylbutyrate. In our study, NaB treatment only partially suppressed gene/protein expression, raising the question of whether this partial suppression is sufficient to affect DNA repair capacity. Therefore, we tested the ability of NaB to affect DNA repair using two independent methods. Although the repair of radiation-induced DSBs can be detected using pulsed-field gel electrophoresis techniques, this approach is insensitive to small deletions and other types of misrepaired lesions. The first method we chose to assess DNA repair in our investigation was using host cell reactivation. We updated this relatively old approach (20) to incorporate expression of a reporter gene as the readout; radiation-induced DNA lesions in the reporter gene must be repaired by the host cell with complete fidelity in order for functional gene expression to be restored. We have shown previously that this modified assay detects deficiencies in cellular DSB repair capacity (21). The results of our host cell reactivation assays (Fig. 6) indicate that pretreating the A375 cells with 3 mmol/L NaB suppresses their ability to restore reporter gene expression using irradiated Ad- $\beta$ Gal vector by >50%. Thus, the ability of NaB to radiosensitize human cells as determined based on clonogenic survival correlated with suppressed capacity for host cell reactivation. Interestingly, the treatment with 5 mmol/L NaB was less effective in suppressing host cell reactivation compared with the 3 mmol/L treatment and this generally correlated with some of the other end points, where we observed that in some cases 5 mmol/L was less effective than 3 mmol/L including the effects on the cell cycle. This was especially true for the MeWo cells where 3 mmol/L was more effective in producing a radiosensitizing effect on cell survival compared with 5 mmol/L (Fig. 3); an effect that correlated with the NaB-induced suppression of DNA-PKcs expression (Fig. 4B). This result suggests that, at the higher dose, NaB may alter the expression of other factors that counterbalance the radiosensitizing effect seen at 3 mmol/L.

A second approach that we used to assess involvement of DNA repair in NaB-mediated radiosensitization was to see if NaB treatment caused a prolonged expression of phosphorylated H2AX ( $\gamma$ -H2AX) foci indicating a decrease in the rate of repair of radiation-induced DNA DSBs.  $\gamma$ -H2AX expression has been established recently as a sensitive indicator of DSBs induced by clinically relevant doses of ionizing radiation (36). At sites of radiation-induced DNA DSBs, the histone H2AX becomes phosphorylated rapidly ( $\gamma$ -H2AX), forming nuclear





**Fig. 7.** Influence of NaB on radiation-induced  $\gamma$ -H2AX foci. A375 cells growing on slides in 35-mm dishes were exposed to NaB (3 mmol/L) for 24 hours, irradiated (2 Gy), and fixed at the specified times for immunocytochemical analysis of nuclear  $\gamma$ -H2AX foci. *A*, micrographs obtained from cells exposed to NaB. *B*, quantitative analysis of foci present in the cells following various treatments. *Columns*, means of three independent experiments.

foci that can be visualized by immunofluorescence microscopy (36, 37). Although the specific role of  $\gamma$ -H2AX in the repair of DSBs has not been defined, recent reports indicate the dephosphorylation of  $\gamma$ -H2AX and dispersal of  $\gamma$ -H2AX foci in irradiated cells correlates with the repair of DNA DSBs (38–40) and cellular radiosensitivity (41–43). Moreover, Macphail et al. (43) in their study of 10 cell lines reported that the rate of loss

of  $\gamma$ -H2AX foci correlated with the respective line's SF2 value. We chose the times of 30 minutes, and 1, 2, and 24 hours for foci analysis based on reports illustrating that the decay of  $\gamma$ -H2AX foci takes place over a protracted time period following irradiation (38, 41–43). Recently, however, Riballo et al. have reported that the maximum number of foci may form as early as 3 minutes post-irradiation and decay exponentially with

biphasic kinetics thereafter (44). Based on these new findings, we conclude that our results depicted in Fig. 7B showing an increase in radiation-induced  $\gamma$ -H2AX foci in NaB treated cells compared with controls at all time points examined is consistent with an inhibition of the early phase of focus decay for NaB.

These results are consistent with several previous reports in which HDAC-I radiosensitized other types of human tumor cells (45–50). Several combinations of HDAC-I with ionizing radiation have been tested by others, including phenylbutyrate, trichostatin A, and more recently MS-275. In a recent study, Kim et al. (46) found that treatment of human glioblastoma cells with trichostatin A at nanomolar concentrations sensitized human glioblastoma cells to radiation-induced cell killing. Recent studies have shown the ability of HDAC-I such as phenylbutyrate and trichostatin A to enhance the radiosensitivity of human tumors cells *in vitro*. In addition, Zhang et al. (50) observed that several potent HDAC-I, including trichostatin A, suberoylanilide hydroxamic acid, M344 (an analogue of hydroxamic acid), and depsipeptide (FR90228), modulate cellular responses to ionizing radiation in human squamous carcinoma cells. They implicated a G<sub>1</sub> arrest and inhibition of DNA synthesis as the underlying mechanism of radiosensitization induced by trichostatin A. Evidence has also shown that phenylbutyrate, in addition to its ability to induce tumor growth inhibition *in vitro* and *in vivo*, can potentiate the efficacy of ionizing radiation in human tumor cells. Ferrandina et al. (51) showed that phenylbutyrate can enhance the sensitivity of human cervical cancer cells to radiation. The effects of phenylbutyrate on radiation response were also investigated in human prostate, colon, breast cancer, and glioblastoma cell lines (52). This latter study showed that short exposures to phenylbutyrate decreased radiation sensitivity, whereas longer ones enhanced radiation sensitivity. The authors attributed the changes in radiosensitivity to changes in antioxidant capacity.

Our data are also consistent with a recent report by Camphausen et al. (48), who investigated the effects of the HDAC-I, MS-275, on the radiosensitivity of two human prostate cancer cell lines. As an initial investigation into the mechanism responsible for MS-275-mediated radiosensitization, they used  $\gamma$ -H2AX foci as an indicator of DNA damage. Their results suggested that enhanced radiosensitivity induced by MS-275 involves an inhibition of the repair of DNA damage and is associated with a prolonged expression of  $\gamma$ -H2AX foci, suggesting a decrease in the repair of radiation-induced DNA DSBs. In a similar study Stoilov et al. (53) showed that sodium butyrate inhibited the repair of chromosome breaks as detected by the premature chromosome condensation technique, suggestive of an inhibition of DNA DSBs.

In summary, we have shown that HDAC-I radiosensitize human tumor cells by suppressing the cellular DNA repair capacity. Although radiation-induced apoptosis is also restored to some extent, it may be a less important factor. Inhibition of DNA repair by NaB may be due to a general suppression of the levels of proteins required for this process. Because normal human fibroblasts are not radiosensitized, DNA repair may be differentially governed in normal cells versus tumor cells. It is important to understand the molecular mechanisms involved in mediating the radiosensitization response of HDAC-I. Future studies on changes in gene expression induced by HDAC-I may reveal genes whose expression is affected by altered transcription factors in various human tumors. The data presented here indicate that NaB enhances the radiosensitivity of two human melanoma cell lines and that HDAC inhibition may serve as a general strategy for enhancing tumor cell radiosensitivity. A complete understanding of these effects must await future studies. However, our observations reported here underscore the need for continued development of strategies for sensitizing human tumor cells to cancer therapies that kill cells by inducing DNA damage.

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