

# HDAC Inhibitor, Valproic Acid, Induces p53-Dependent Radiosensitization of Colon Cancer Cells

Xufeng Chen, Patty Wong, Eric Radany, and Jeffrey Y.C. Wong

## Abstract

Agents that inhibit histone deacetylases (HDAC inhibitors) have been shown to enhance radiation response. The aim of this study was to evaluate the effects of low, minimally cytotoxic concentrations of the HDAC inhibitor, valproic acid (VPA), on radiation response of colorectal cancer cells. Cell lines LS174T and an isogenic pair of HCT116, which differed only for the presence of wild-type p53, were exposed to ionizing radiation (IR) alone, VPA alone, or the combination. Clonogenic survival,  $\gamma$ -H2AX induction, apoptosis, changes in mitochondrial membrane potential, and mitochondrial levels of p53 and Bcl-2 family proteins were assessed. *In vivo* studies monitored tumor growth suppression after therapy in mice bearing HCT116/p53<sup>+/+</sup> and HCT116/p53<sup>-/-</sup> tumor xenografts. VPA led to radiosensitization, which was dependent on p53 status. A decrease in clonogenic survival, an increase in apoptosis, and an increase in levels of  $\gamma$ -H2AX were observed after VPA+IR, compared to IR alone, in wild-type p53 cells (LS174T and HCT116/p53<sup>+/+</sup>), as opposed to p53 null cells (HCT116/p53<sup>-/-</sup>). Exposure to VPA resulted in enhancement of IR-induced mitochondrial localizations of Bax and Bcl-xL, mitochondrial membrane potential, and cytochrome c release only in wild-type p53 cell lines. VPA also enhanced tumor growth suppression after IR only in wild-type p53 xenografts. These data suggest that VPA may have an important role in enhancing radiotherapy response in colorectal cancer, particularly in tumors with the wild-type p53 genotype.

**Key words:** histone deacetylase inhibitors, apoptosis, colorectal cancer, p53, radiation therapy

## Introduction

Although the molecular basis of radiation response is a complex and multifactorial,<sup>1,2</sup> the predominant mechanism by which therapeutic irradiation kills most tumor cells is through clonogenic death. DNA double-stranded breaks (DSBs) are regarded as the specific lesions that initiate this lethal response,<sup>3,4</sup> and the repair of DNA DSBs is critical in determining radiosensitivity.<sup>5</sup> Therefore, agents such as histone deacetylase inhibitors (HDIs), targeting DNA and abrogating the responses of the DNA/protein complex to ionizing radiation (IR)-induced DSBs, may serve as a potential radiosensitizing strategy.

Reversible, post-translational acetyl modifications of proteins are increasingly being recognized as critical modulators of protein-protein and protein-nucleic acid interactions in eukaryotic cells. Acetylation has been shown to mediate cer-

tain intracellular signaling events by governing such macromolecular interactions, much like the perhaps better-known phosphorylation modifications.<sup>6</sup> Histone acetyltransferases (HATs) were among the first activities capable of mediating protein acetylation to be described, and they were originally defined as enzymes that can acetylate core histones, with resulting regulatory effects on chromatin structure and gene expression.<sup>6</sup> The counterpart-regulated removal of acetyl modifications is mediated by histone deacetylase activity (HDAC).<sup>7</sup> HDACs play important roles in many diverse and essential biologic processes, including intracellular signaling.<sup>7</sup> These activities have been categorized into four groups termed classes I through IV.<sup>7</sup> The enzymes in classes I, II, and IV contain a structurally conserved catalytic domain, and for this reason, they are quite broadly and commonly susceptible to inhibitory drugs ("HDAC inhibitors"; here, "HDI") that chelate an essential active-site zinc cation.<sup>8,9</sup> Commonly used

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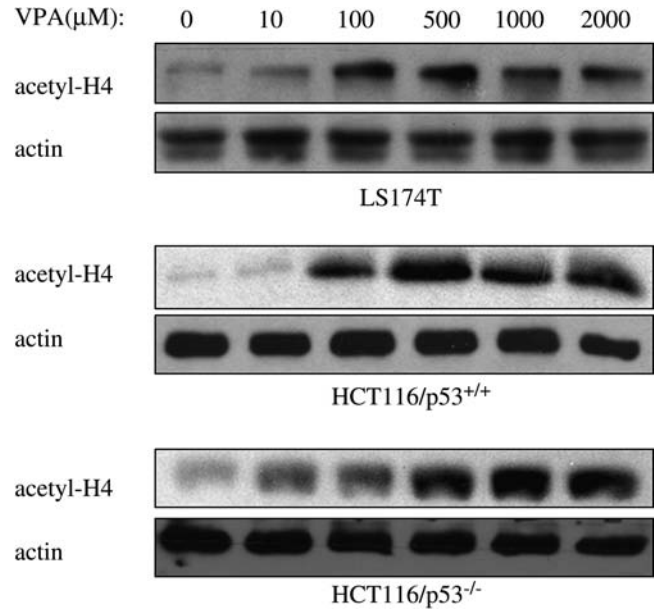
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agents of this kind include valproic acid (VPA), trichostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA).<sup>8,9</sup> The mammalian class III enzymes (homologs of the yeast Sir2 protein; hence, the names “Sirtuins” or “SIRTs”) are structurally distinct and have an NAD-dependent mechanism; for this reason, they are insensitive to conventional HDI (but they are inhibited by distinct drug classes).<sup>10</sup>

HDI are currently showing considerable promise as therapeutic agents for human malignant diseases.<sup>8,9</sup> The use of these drugs is largely empirical at this point in time, and there is little information regarding the structure-function relationship of HDIs, their targeting specificity (if any) for individual HDACs, or the specific biologic processes they impact in mediating anticancer actions.<sup>8,9,11</sup>

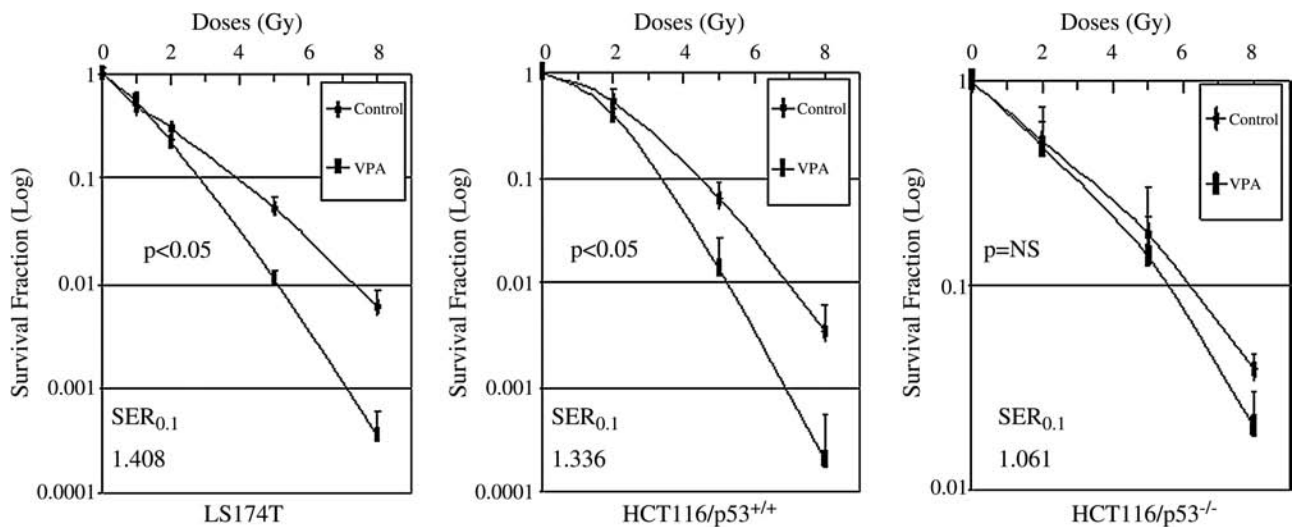
HDI also show promise in laboratory studies as candidate radiosensitizers in many types of tumors. The initial idea for HDI as a radiosensitizer is that the blockage of HDAC activity by HDI drugs promotes hyperacetylation in core histones and leads to a more relaxed chromatin structure<sup>12,13</sup>; DNA in decondensed chromatin is relatively sensitive to DNA damage induced by IR.<sup>14,15</sup> This model is supported by recent studies that have demonstrated that HDI, such as trichostatin A (TSA), sodium butyrate (NaB), FK228, and MS-275, enhance radiation-induced cell death and increase IR-induced DNA DSBs in a variety of cancer cells.<sup>16-19</sup> However, details of molecular mechanisms mediating radiosensitization by HDI are, at present, not clear. Therefore, more information is needed to be able to identify cancer patients for whom HDI treatment with radiation might be helpful.

In the present study, we examined the effects of the HDAC inhibitor, VPA, on radiosensitivity in human colorectal cancer cells lines *in vitro* and *in vivo*. The results of this study demonstrate that exposure to VPA leads to a decrease in clonogenic survival and an increase in radiation-induced apoptosis in wild-type p53 cell lines (LS174T and HCT116/p53<sup>+/+</sup>), but not in the p53 knock-out cell line (HCT116/p53<sup>-/-</sup>).



**FIG. 1.** Changes in histone acetylation after valproic acid (VPA) exposure. LS174T and HCT116 cells were exposed to varying concentrations of VPA for 16 hours. Cellular protein extracts were prepared, as described in Materials and Methods, and analyzed by immunoblot assay with antibody against acetylated histone H4 (acetyl-H4).  $\beta$ -actin was included as a control to show equivalent protein loading.

p53<sup>-/-</sup>). VPA also results in modulation of the radiation induced by p53-dependent mitochondrial localization of Bax and Bcl-xL, as well as mitochondrial membrane potential and cytochrome c release. Taken together, these data indicate an important role of p53 in HDAC inhibitor-mediated radiosensitization.



**FIG. 2.** Clonogenic survival after valproic acid (VPA) and ionizing radiation (IR) exposure. Log-phase cells were trypsinized and plated as single cells. After 6 hours of incubation to allow for cell attachment, cells were pretreated with 500  $\mu$ M of VPA for 16 hours and then exposed to different doses of IR. Colony survival was determined 14–20 days later. Values represent the mean from three to four independent experiments. Error bars indicate one standard deviation. There was a significant reduction in clonogenic survival with the addition of VPA for LS174T and HCT116/p53<sup>+/+</sup>, but not for HCT116/p53<sup>-/-</sup>.

## Materials and Methods

### Cell lines and external beam radiotherapy

The human colorectal cancer cell line, LS174T, was obtained from the American Type Culture Collection (Manassas, VA). Isogenic HCT116 cell lines (p53<sup>+/+</sup> and p53<sup>-/-</sup>) were kindly provided by Doctor Bert Vogelstein (John Hopkins, Baltimore, MD). LS174T cells were maintained in minimum essential medium with 2 mM of L-glutamine, 0.1 mM of non-essential amino acids, 1.0 mM of sodium pyruvate, 1.5 g/L of sodium bicarbonate, and 10% fetal bovine serum (FBS). HCT-116 cells were maintained in McCoy's 5A medium containing 10% heat-inactivated FBS. Cells were irradiated by using a Mark I Cs-137 Irradiator (J.L. Shepherd Association, San Fernando, CA) at a dose rate of 1.49 Gy/min.

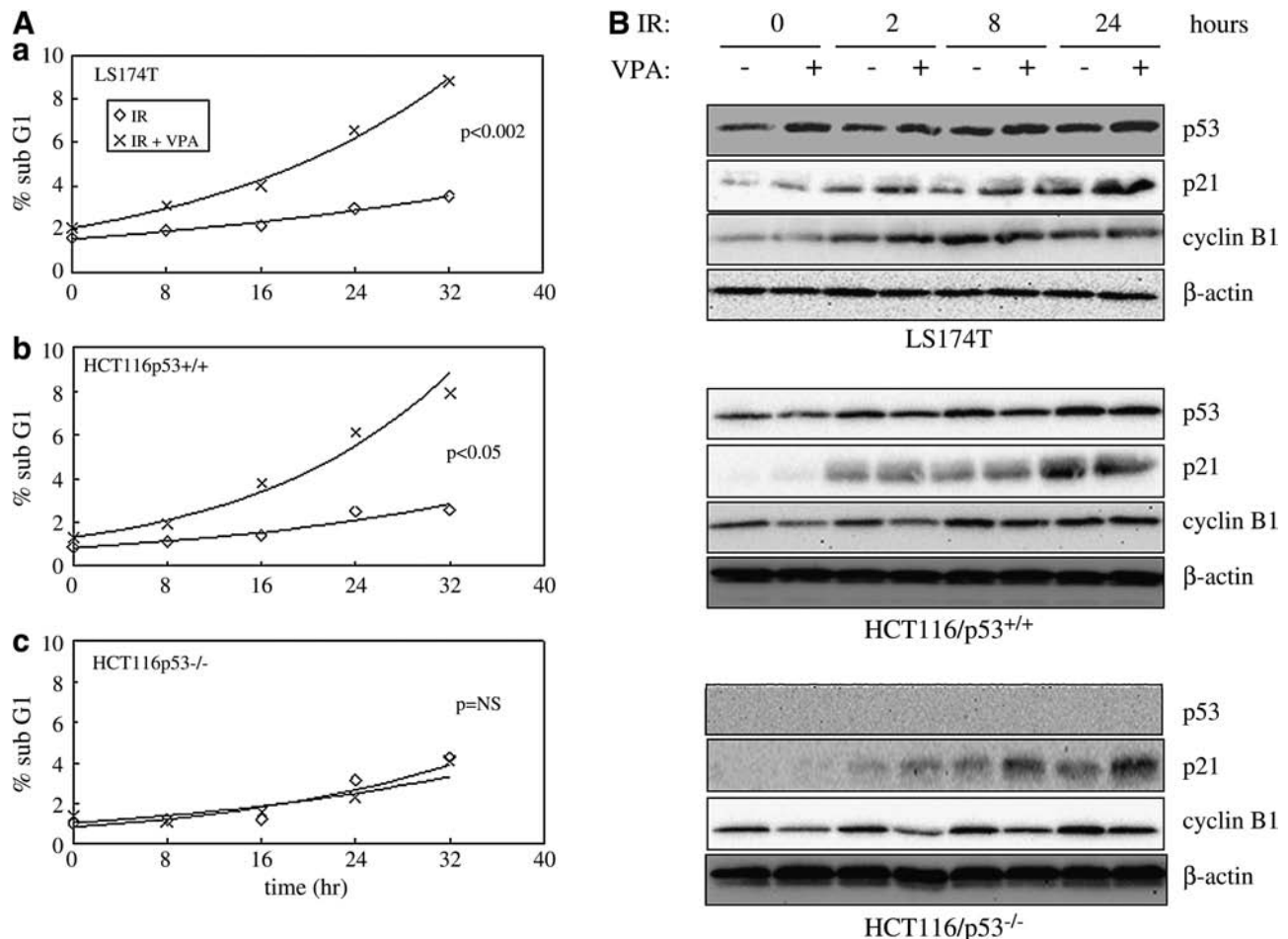
### Reagents and antibodies

VPA was obtained from Sigma Aldrich Co., Ltd. (St. Louis, MO). Monoclonal antibody (mAb) with specificity for

phospho-histone H2A.X and DNA-PK, and polyclonal antibodies with specificity for acetyl-H4 and Rad51, were purchased from Upstate (Charlottesville, VA). Polyclonal antibodies against cyclin B1, Bcl-xL (H-62), Bax (N-20), Ku-86 (H-300), and a mAb against p53 (DO-1), p21 (F-5), and Ku-70 (E-5) were from Santa Cruz Biotech, Inc. (Santa Cruz, CA). The mAb against mitochondria Hsp-70 (MA3-028) was from Affinity Bioreagents (Golden, CO). The mAb against cytochrome c (SA-226) was obtained from BIOMOL (Plymouth Meeting, PA). The mAb against beta-actin was obtained from Abcam, Inc. (Cambridge, MA).

### Clonogenic assay

To evaluate radiosensitivity, cells in the log phase were plated into individual wells of six-well plates or 60-mm dishes. Six (6) hours later, cells were treated with VPA, or phosphate-buffered saline (PBS) as a control, for 16 hours, followed by irradiation to various doses. Colonies were allowed to form



**FIG. 3.** Effects of valproic acid (VPA) on ionizing radiation (IR)-induced sub-G<sub>1</sub> cell-cycle distribution and expression of p53, p21, and cyclin B1. Cells were pretreated with or without 500 μM of VPA for 16 hours before γ-radiation (4 Gy). (A) Cells were then stained with propidium iodide and cell-cycle distribution analyzed by flow cytometry at 0, 8, 16, 24, and 32 hours after IR. The data from the two separate experiments were averaged and plotted. Because the percentage of cells in sub-G<sub>1</sub> followed more closely an exponential curve than a linear one, a linear regression was performed on the logarithm of the percentages. The slopes of the curves for IR were compared to that of IR+VPA, using a *t* statistic, and the pooled variances for the pair-wise regressions. (B) Total protein extracts were also prepared at 0, 2, 8, and 24 hours after IR and analyzed by immunoblot assay with antibodies against p53, p21, and cyclin B1. β-actin was included as a control to show equivalent protein loading.

over the next 14–20 days in complete medium with or without VPA. Colonies greater than 50 cells were counted and the number of colonies normalized to that observed in unirradiated controls. Mean inactivation doses were determined by the method of Fertl et al.,<sup>20</sup> and the sensitizer enhancement ratio for VPA was calculated as the ratio of mean inactivation dose control/mean inactivation dose of VPA.

#### Flow cytometry assay and cell-cycle phase analysis

Cells in log phase were treated with or without VPA for 16 hours, followed by exposure to 4 Gy IR. Cells were then fixed at indicated time intervals with 80% ethanol, stained with propidium iodide (50  $\mu\text{g}/\text{mL}$ ), and analyzed by flow cytometry with  $5 \times 10^4$  events counting per run, as described previously.<sup>21</sup> The percentage of cells in the sub- $G_1$ ,  $G_0/G_1$ , S, and  $G_2/M$  phases of the cell cycle were determined by using ModFitLT software (Verity Software, Topsham, ME).

#### Assessment of apoptosis

Cells were treated with or without VPA for 16 hours and then irradiated. After incubation in drug-containing medium for additional 48 hours,  $5 \times 10^5$  cells were collected and analyzed by Annexin V-FITC (fluorescein isocyanate) staining, as per the manufacturer's instructions (MBL Co. LTD., Woburn, MA). The extent of apoptosis was quantified by using a Becton-Dickinson (Franklin Lakes, NJ) flow cytometer.

#### Assay of mitochondrial membrane potential

Changes in mitochondrial membrane potential (MMP) were evaluated by staining cells with the fluorescent cationic dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzamidazolocarboxy-

nin iodide (JC-1), as per the manufacturer's instructions (MitoProbe™ JC-1 Assay Kit for Flow Cytometry; Molecular Probes, Eugene, OR). Briefly,  $1 \times 10^6$  cells were collected and suspended in fresh medium and stained with 2  $\mu\text{M}$  of JC-1 for 15 minutes. Fluorescence was monitored by using flow cytometry, measuring both the monomer (527-nm emission; green) and J-aggregate (590-nm emission; red) forms of JC-1 following 488-nm excitation. The percentage of monomeric form or fluorescence green was then quantified as the MMP.

#### Preparation of mitochondrial and cytosolic protein fractions and whole-cell lysates for immunoblotting

After treatment, approximately  $2 \times 10^7$  cells were collected. The mitochondria and cytosolic protein fractions were prepared from cell pellets and separated by utilizing a reagent-based method per the manufacturer's instructions (Mitochondria Isolation Kit; Pierce, Rockford, IL). Mitochondria were lysed with RIPA buffer [150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM ethylene diamine tetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu\text{g}/\text{mL}$  aprotinin, 10  $\mu\text{g}/\text{mL}$  leupeptin, 50 mM NaF, 1 mM  $\text{Na}_2\text{VO}_4$ , 50  $\mu\text{g}/\text{mL}$  soybean trypsin inhibitor, and 20 mM iodoacetamide]. Then, 10  $\mu\text{g}$  of mitochondrial protein or 20  $\mu\text{g}$  of the cytosolic fraction were directly loaded onto SDS-PAGE polyarylamide gel electrophoresis gels for immunoblotting analysis. Anti-PCNA (Santa Cruz, CA), anti-mtHSP70, and anti-cytochrome *c* antibodies were utilized to test the purity of the preparation. For whole-cell lysates, cells were washed with cold PBS twice and lysed in RIPA buffer with mild sonication. To determine the acetylation status of histone H4, cells were washed twice with cold PBS and resuspended in lysis buffer containing Tris

TABLE 1. EFFECTS OF VPA ON IR-INDUCED CELL-CYCLE DISTRIBUTION

Cell lines	Cell cycles	Times after IR (hours)	0	8	16	24
LS174T	Sub- $G_1$	Control	1.56	1.93	2.15	2.97
		VPA	2.04	3.07	3.99	6.55
	$G_1/G_0$	Control	70.06	53.76	59.39	71.79
		VPA	70.08	53.90	53.63	64.96
	S	Control	16.29	15.08	15.96	9.80
		VPA	15.42	15.98	11.37	8.33
	$G_2/M$	Control	12.04	29.05	23.23	15.20
		VPA	12.34	26.98	30.91	19.98
HCT116-p53 <sup>+/+</sup>	Sub- $G_1$	Control	0.85	1.13	1.14	2.51
		VPA	1.21	1.95	3.80	6.12
	$G_1/G_0$	Control	45.63	40.58	19.71	41.47
		VPA	44.19	39.81	16.40	30.08
	S	Control	29.91	19.32	11.77	17.06
		VPA	26.56	21.04	8.36	15.04
	$G_2/M$	Control	27.21	39.37	66.85	38.46
		VPA	28.68	37.93	70.86	48.71
HCT116-p53 <sup>-/-</sup>	Sub- $G_1$	Control	1.03	1.22	1.28	3.14
		VPA	1.43	1.11	1.60	2.31
	$G_1/G_0$	Control	41.26	9.39	6.53	23.98
		VPA	38.80	10.65	5.73	20.25
	S	Control	28.93	36.54	27.13	33.53
		VPA	25.93	32.88	32.65	14.66
	$G_2/M$	Control	28.81	52.79	65.07	40.30
		VPA	33.84	55.33	60.31	62.63

Cells were pretreated with or without 500  $\mu\text{M}$  of VPA for 16 hours before  $\gamma$ -radiation (4 Gy). Cells were then harvested at different time points, stained with propidium iodide, and analyzed by flow-cytometry. Data are presented as an average from two experiments. IR, ionizing radiation; VPA, valproic acid.

(0.02 M, pH 7.4), 1% Triton X-100, 0.02% 2-mercaptoethanol, and 2 ng/mL of aprotinin.

#### Tumor growth assay

HCT116/p53<sup>+/+</sup> and HCT116/p53<sup>-/-</sup> cells ( $3 \times 10^6/0.2$  mL HBSS 1x + 1% HSA) were inoculated subcutaneously (s.c.) into the right leg of 4–6-week-old female athymic nude mice (Charles River/NCI, Frederick, MD). When tumor volumes reached a size of 50–100 mm<sup>3</sup> (approximation day 7 after inoculation), mice were randomly grouped into four groups, each with 5–7 mice that received the following: (1) saline (0.2 mL); (2) VPA (300 mg/kg); (3) IR (10 Gy) and (4) VPA (300 mg/kg)+IR (10 Gy). Mice were treated with intraperitoneal (i.p.) injections of VPA (300 mg/kg) every 12 hours for 3 days. Localized irradiation of 10 Gy was delivered after the third VPA injection. Tumors were measured biweekly and tumor volumes were determined from caliper measurements of tumor length (L) and width (W), according to the following formula:  $(L \times W^2)/2$ .

#### Results

Effects of VPA on histone acetylation were first examined by exposing the human colorectal cancer cell lines, LS174T, HCT116/p53<sup>+/+</sup>, and HCT116/p53<sup>-/-</sup>, to different concentrations of VPA for 16 hours. As shown in Figure 1, acetylation of histone H4 increased in a dose-dependent manner. In all cell lines tested, an increase in the level of acetylated histone H4 was detected after the addition of VPA at concentrations ranging from 100 to 500  $\mu$ M, with no further increase up to a maximum of 2 mM.

#### VPA differentially reduces clonogenic survival in irradiated colorectal cancer cells

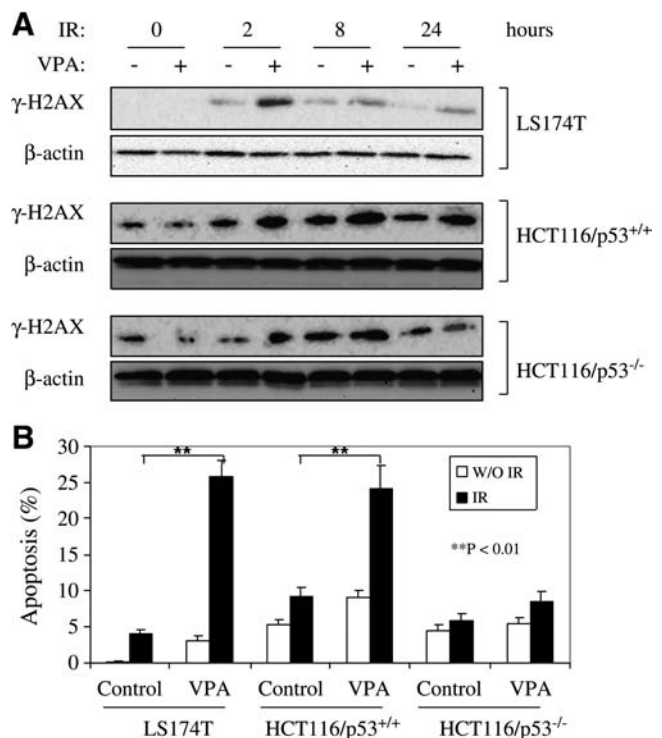
We next determined the survival of colorectal cancer cells exposed to the combination of VPA and IR by clonogenic assay. Although exposure to VPA increased the levels of acetylated histone proteins in all cell lines, only LS174T and HCT/p53<sup>+/+</sup> cells that express wild-type p53 showed significant reduction in IR-induced clonogenic survival when exposed to VPA (Fig. 2). VPA alone had no significant cytotoxic effects, compared to untreated controls, in all tested cell lines. The plating efficiencies in untreated control cells, compared to cells treated with VPA alone, were  $28.67 \pm 0.96$  and  $26.67 \pm 1.41$  in LS174T cells,  $36.6 \pm 0.73$  and  $32.7 \pm 1.28$  in HCT116/p53<sup>+/+</sup> cells, and  $36.93 \pm 0.58$  and  $30.8 \pm 1.05$  in HCT116/p53<sup>-/-</sup> cells, respectively. The sensitizer enhancement ratio (SER) for VPA-treated cells at 0.1 and 0.01 isosurvival, compared to controls, were 1.408 and 1.480 for LS174T and 1.336 and 1.327 for HCT116/p53<sup>+/+</sup>, respectively. No obvious decrease ( $SER_{0.1}:1.061$ ) in clonogenic survival with the combination of VPA and IR, compared to IR alone, was observed in HCT116/p53<sup>-/-</sup> cells in which the p53 gene had been removed through genetic engineering.<sup>22</sup> Therefore, our results suggest that p53 likely plays an important role in VPA-enhanced radiosensitization.

#### Effects of VPA on cell-cycle phase redistribution after IR

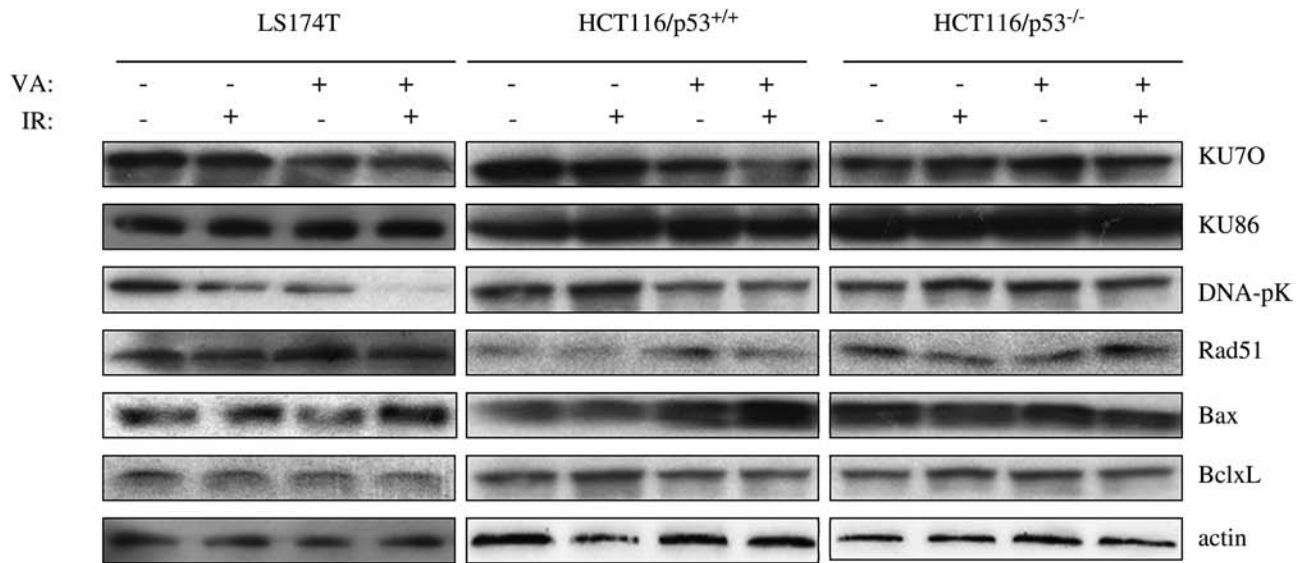
Cells were analyzed by flow cytometry after VPA and IR exposure to determine whether cell-cycle phase redistribution

contributed to the observed VPA-induced radiosensitization. VPA pretreatment resulted in a significant increase in the sub-G<sub>1</sub> population after irradiation in LS174T and HCT116/p53<sup>+/+</sup>, but not in HCT116/p53<sup>-/-</sup> cells (Fig. 3A). Since the sub-G<sub>1</sub> population represents cells undergoing apoptosis, these data suggest that the increase in radiosensitivity seen with VPA on colony formation assay may be due to VPA enhancement of IR-induced apoptosis in the p53-positive lines, LS174T and HCT116/p53<sup>+/+</sup>. All cell lines exhibited some degree of G<sub>2</sub>/M delay, after IR alone. Pretreatment with VPA led to a modest increase in G<sub>2</sub>/M delay, compared to IR alone, which was not significant (Table 1). There were no major differences seen for the other cell-cycle subpopulations with VPA and IR, compared to IR alone.

We also examined the effects of VPA on the levels of cell-cycle-related proteins. IR alone resulted in an upregulation of p21<sup>CIP/WAF1</sup> and cyclin B1 within 24 hours in all three cell lines (Fig. 3B). IR alone also increased p53 stabilization in LS174T and HCT116/p53<sup>+/+</sup>. The addition of VPA to IR had



**FIG. 4.** Effects of valproic acid (VPA) on ionizing radiation (IR)-induced  $\gamma$ -H2AX duration and apoptosis. (A) LS174T, HCT116/p53<sup>+/+</sup>, and HCT116/p53<sup>-/-</sup> cells were pretreated with 500  $\mu$ M of VPA or no VPA for 16 hours prior to 4-Gy irradiation. Cells were then harvested at indicated times, and cell lysates were prepared for immunoblot analysis of  $\gamma$ -H2AX.  $\beta$ -actin was included to show equivalent protein loading. The data are representative of three independent experiments. (B) Apoptosis after IR and VPA. Cells were first exposed to 500  $\mu$ M of VPA or left untreated for 16 hours before irradiation (4 Gy). Cells were then collected 48 hours after irradiation and apoptosis assayed by Annexin-V-fluorescein isothiocyanate (FITC) staining and flow cytometric analysis, as described in Materials and Methods. Data represent the average of three experiments. Error bars represent one standard deviation.



**FIG. 5.** Changes in expression of apoptosis-related proteins after valproic acid (VPA) and ionizing radiation (IR). LS174T, HCT116/p53<sup>+/+</sup> and HCT116/p53<sup>-/-</sup> cells were pretreated with 500  $\mu$ M of VPA or left untreated for 16 hours, followed by 4-Gy irradiation. Proteins were extracted and lysed in RIPA buffer 24 hours later. Immunoblot assays were performed to detect the expression of DSB-related proteins (KU70, KU86, DNA-pK, and Rad51) and Bcl-2 family proteins (Bax and Bcl-xL) in cells after VPA and IR.  $\beta$ -actin was included to show equivalent protein loading. DSB, double-strand break.

no major observable effect on the level of these proteins, although a modest increase in p21<sup>CIP/WAF1</sup> protein levels at 24 hours was observed in HCT116/p53<sup>-/-</sup> cells. Although p53 is primarily considered a G<sub>1</sub>-phase regulatory protein,<sup>23,24</sup> studies indicate that p53 can also regulate G<sub>2</sub> arrest through p21<sup>CIP/WAF1</sup> after DNA damage,<sup>22</sup> which may, in part, explain the changes in G<sub>2</sub>/M subpopulations observed at 24 hours with the addition of VPA to IR.

These results indicate that the observed differences in VPA-enhanced radioresponse are not fully explained by changes in cell-cycle redistribution or arrest of cells in more sensitive phases of the cell cycle.

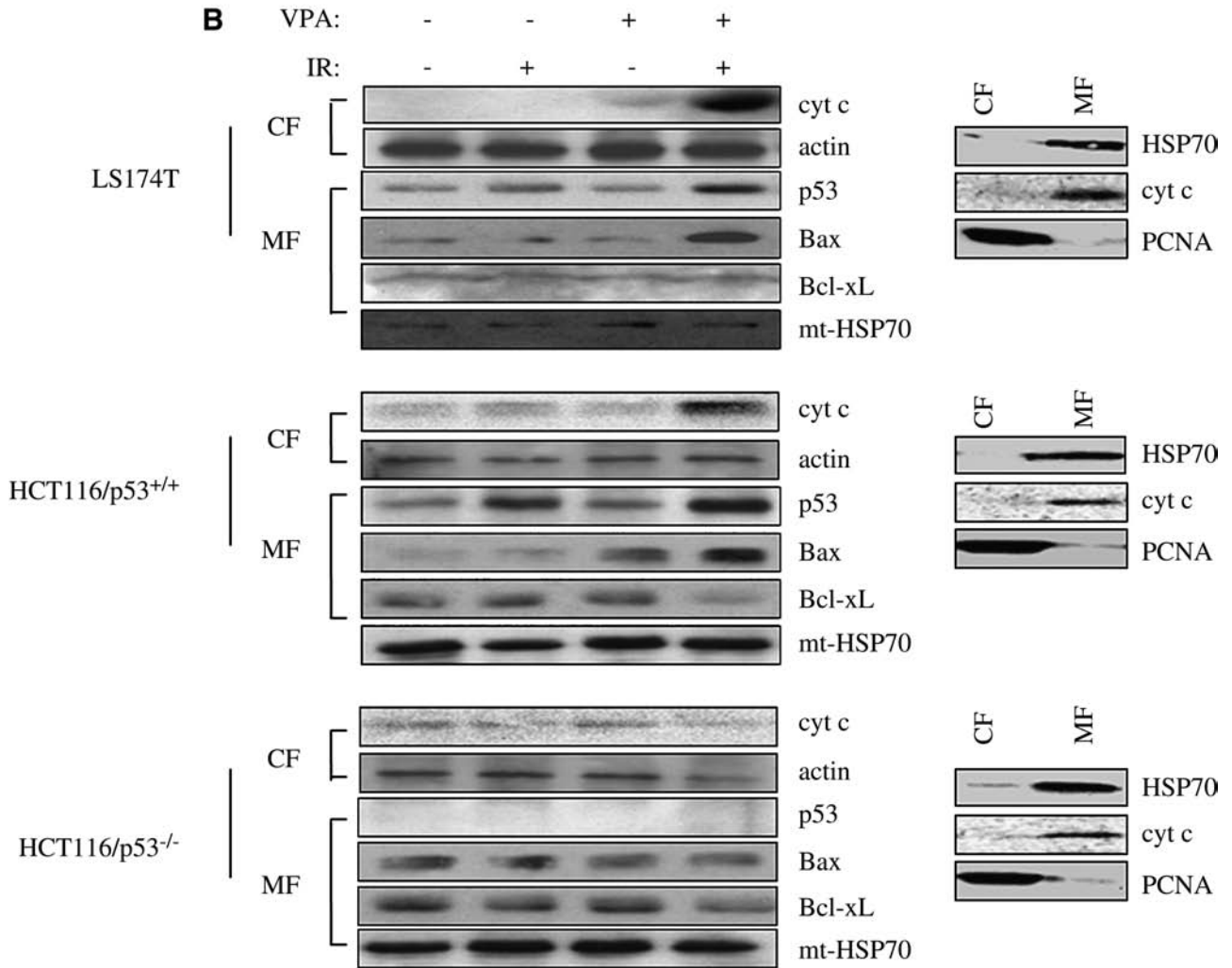
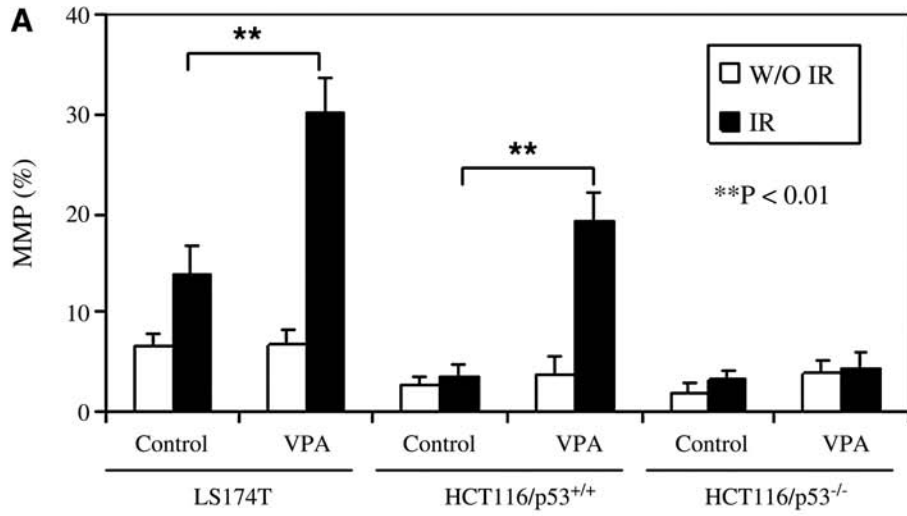
#### VPA increases DNA double-strand breaks and apoptotic response to irradiation

The occurrence of DNA DSBs after IR represents a potentially lethal lesion to the cell. One of the earliest known responses to radiation-induced DSB formation is phosphorylation of the C-terminal tail of variant H2AX ( $\gamma$ -H2AX) in nucleosomes located in the vicinity of the break.<sup>25</sup> Exposure

to VPA prior to IR resulted in an increase in  $\gamma$ -H2AX, compared to IR alone, in LS174T and HCT116p53<sup>+/+</sup>. This difference peaked at 2 hours and remained detectable up to 24 hours. For HCT116/p53<sup>-/-</sup>, VPA enhancement of IR-induced  $\gamma$ -H2AX induction was seen at 2 hours, but was not observed at 8 and 24 hours (Fig. 4A). These data suggest that exposure to VPA increased the formation of irradiation-induced DNA damage, the magnitude of which may be dependent on p53 status.

Next, we assessed the apoptotic response to IR or the combination of VPA and IR *in vitro*. Exposure to 4-Gy IR or 500- $\mu$ M VPA alone did not significantly induce apoptotic death in all three colorectal cancer cell lines evaluated. However, when cells were first exposed to 500- $\mu$ M VPA followed by IR, a significant increase in the percentage of cells undergoing apoptosis was detected in LS174T (from 4.03  $\pm$  0.61 to 25.90  $\pm$  2.17;  $p$  < 0.01) and HCT116/p53<sup>+/+</sup> (from 9.25  $\pm$  1.27 to 24.21  $\pm$  3.11;  $p$  < 0.01), but not in HCT116/p53<sup>-/-</sup> cells (from 5.79  $\pm$  1.03 to 8.52  $\pm$  1.33;  $p$  > 0.05) (Fig. 4B). These data demonstrate that VPA not only enhances IR-induced  $\gamma$ -H2AX, but also augments apoptotic

**FIG. 6.** Effects of valproic acid (VPA) exposure on ionizing radiation (IR)-induced changes in mitochondrial membrane potential and mitochondrial accumulation of p53 and Bcl-2 family membrane proteins. (A) Cells were pretreated with or without 500  $\mu$ M of VPA for 16 hours before 4-Gy IR. Cells were then collected and stained with JC-1, as described in Materials and Methods. Mitochondrial membrane potential was quantified by the measurement of JC-1 fluorescence intensity. Data represent the average of three independent assays. Error bars represent one standard deviation. (B) Cytochrome c release and mitochondrial accumulation of p53 and Bcl-2 family proteins in response to VPA and IR exposure. Cells were pretreated with or without 500  $\mu$ M of VPA for 16 hours before 4-Gy IR. Cytosolic (CF) and mitochondrial (MF) fractions were prepared 24 hours after irradiation. Immunoblot assay with antibodies against cytochrome c, p53, Bax, and Bcl-xL was then performed.  $\beta$ -actin and mt-Hsp70 were included to show equivalent protein loading. The data are representative of three independent experiments. To assess the integrity of purified CFs and MFs, equal amounts of proteins (10  $\mu$ g) were loaded, and mitochondrial proteins cytochrome c (cyto c), HSP70, and the cytosolic/nuclear protein proliferating cell nuclear antigen (PCNA) were assessed by immunoblotting (right panel).



response to IR in a p53-dependent way, which seems to account for the radiosensitization observed by clonogenic assay.

*Effects of VPA exposure on modulation of DNA-repair and Bcl-2 family member gene expression after irradiation*

We next examined the effect of VPA exposure on the expression of proteins known to be involved in the repair of radiation-induced DSBs (e.g., KU70, KU86, and DNA-pK abd Rad51) and in apoptotic response (e.g., Bax and Bcl-xL). As shown in Figure 5, VPA exposure decreased the expressions of KU70 and DNA-pKin LS174T and HCT116/p53<sup>+/+</sup>, but not in HCT116/p53<sup>-/-</sup> cells, whereas VPA also induced Bax expression in HCT116/p53<sup>+/+</sup> cells. The combination of VPA and IR significantly reduced DNA-pK protein levels only in LS174T cells and increased Bax protein level in HCT116/p53<sup>+/+</sup> cells. Therefore, the changes in expression of these apoptotic-related proteins in response to VPA seem to play some role in enhanced apoptosis; however, they do not easily explain the differences in VPA enhancement of radiation-induced apoptotic response observed in the various colorectal cancer cell lines.

*Exposure to VPA increases IR-induced mitochondrial membrane depolarization*

Recent studies have demonstrated that mitochondrial dysfunction plays a critical role in HDIs-induced apoptosis,

as well as in HDIs-enhanced apoptotic response to IR.<sup>26-28</sup> To further understand the mechanism of VPA-mediated enhancement of apoptosis after IR, we examined the effects of VPA on mitochondrial membrane potential and the modulation of IR-induced mitochondrial localization of Bcl-2 family proteins.

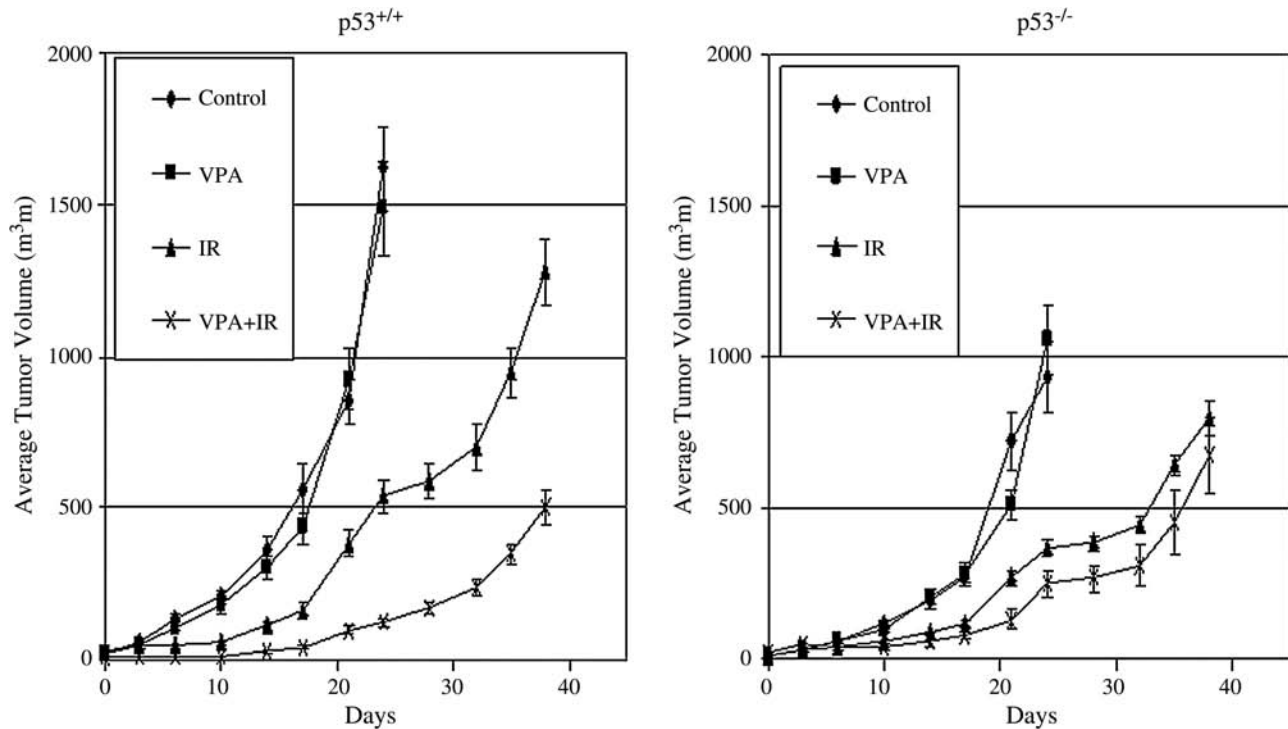
Significant changes in MMP were detected after exposure to the combination of VPA and IR, compared to IR or VPA alone, in LS174T and HCT116/p53<sup>+/+</sup> cells (Fig. 6A). This was not observed in HCT116/p53<sup>-/-</sup> cells. Consistent with this was an observed increase in cytochrome c release from mitochondria detected only in LS174T and HCT116/p53<sup>+/+</sup> cells treated with the combination of VPA and IR (Fig. 6B).

Figure 6B shows that the combination of IR and VPA increased p53 protein level in mitochondria in LS174T and HCT116/p53<sup>+/+</sup> cells, compared to IR or VPA alone. The combination of VPA and IR also increased mitochondrial levels of Bax over IR and VPA alone in LS174T and HCT116/p53<sup>+/+</sup>, but not in HCT116/p53<sup>-/-</sup>. No significant changes were observed in Bcl-xL mitochondrial levels.

Taken together, these results suggest that p53 and its localization at the mitochondria may play a role in VPA enhancement of apoptosis after IR and radiosensitivity of colorectal cancer cells.

*p53-dependent radiosensitization by VPA in vivo*

Studies were extended to an *in vivo* model. We investigated the effects of VPA, IR, or VPA combined with IR on tumor growth of HCT116 xenografts. VPA significantly in-



**FIG. 7.** Tumor-growth delay after valproic acid (VPA) and ionizing radiation (IR) exposure *in vivo*. Athymic nude mice bearing isogenic HCT 116/p53<sup>+/+</sup> and HCT 116/p53<sup>-/-</sup> xenograft tumors were treated with VPA (300 mg/kg×6) administered intraperitoneally every 12 hours for 3 days and/or 10-Gy irradiation. In the combined treatment group, IR was delivered after the third injection of VPA. The growth curves represent the average value in each group of 5–8 mice. Error bars represent one standard error.



creased tumor growth suppression, compared to IR alone, in HCT116/p53<sup>+/+</sup>, but not in isogenic HCT116/p53<sup>-/-</sup> (Fig. 7). These results are consistent with results seen *in vitro*.

## Discussion

Colorectal cancer is the second leading cause of cancer mortality in the United States. Radiation therapy plays an important role in the treatment of this disease, as adjuvant therapy after surgery for locally advanced rectal cancer or as neoadjuvant therapy often combined with chemotherapy.<sup>29-31</sup> When utilized in the preoperative setting, radiotherapy, in many cases, can convert an initially unresectable cancer to one resectable by surgery or result in reduction of tumor size to permit sphincter preservation and avoidance of a permanent colostomy. Therefore, strategies that can further sensitize tumors to IR have potential significant clinical importance.

While tumor cells of hematopoietic or lymphatic origin undergo significant apoptosis after irradiation *in vitro*, and are often highly radioresponsive clinically,<sup>32,33</sup> solid tumor cells tend to be relatively refractory to the induction of apoptosis by radiation. The initial response to irradiation in these cells is, instead, growth arrest.<sup>34</sup> When cell death does occur, this is usually subsequent to one or more divisions (i.e., so-called reproductive cell death or mitotic catastrophe).<sup>35,36</sup>

Results from the present study demonstrate that VPA blocked HDAC activity in a dose-dependent manner in all colorectal cell lines studied (LS174T and HCT116). However, in cells exposed to VPA prior to IR, clonogenic survival decreased and radiation-induced DSBs and apoptosis increased, compared to IR exposure alone, only in those cells that expressed wild-type p53 (LS174T and HCT116/p53<sup>+/+</sup>), but not in cells with the absence of wild-type p53 lines (HCT116/p53<sup>-/-</sup>). Mice bearing HCT116 wild-type p53 tumor xenografts demonstrated increased tumor growth suppression with the combination of VPA and IR, compared to IR alone, which was not observed in p53 null isogenic tumors. Taken together, these data strongly suggest that VPA may enhance radiation-induced apoptosis and serve as radiosensitizer in a p53-dependent manner in colorectal cancer cells.

The growth-arrest and cell-death pathways, which are activated in response to DNA-damaging agents, such as IR, appear to be closely intertwined, in that both are p53 dependent. The results from this study indicate that augmented apoptotic cell death seems to account for most of the observed radioenhancement secondary to VPA.

The tumor-suppressor protein, p53, is one of the most commonly altered genes in human cancer and plays an important role in cellular response to genotoxic stress.<sup>37,38</sup> The role of p53 in apoptosis induced by a variety of stress stimuli was thought, initially, to center on its role as a transcription factor modulating gene expression. In cells with functional p53 protein, a post-translational increase in levels of p53<sup>39,40</sup> occurs in response to irradiation, which appears to be mediated through the ATM proteins,<sup>41,42</sup> and, ultimately, results in an increase in the level of the cyclin-dependent kinase inhibitory protein, p21<sup>Waf1/cip1</sup>, and the proapoptotic protein, Bax.<sup>39,43,44</sup>

Recent work has also provided evidence that p53 can contribute to the induction of apoptosis via non-nuclear pathways through the initiation of proapoptotic mechanisms that involve the physical interaction of the core DNA-

binding domain of p53 with apoptosis regulators, such as Bcl-2 and Bcl-X<sub>L</sub>, at the mitochondria.<sup>45-48</sup> Bcl-2 protein binds to other proteins with which it shares amino-acid sequence homology, including Bax, Bcl-X<sub>L</sub>, Bcl-X<sub>S</sub>, Mcl-1, Bik, and Bad. Bax (and Bad) and Bcl-2 (and Bcl-xL) are, respectively, pro- and antiapoptotic proteins that influence mitochondrial integrity and membrane potential. Studies have demonstrated that mitochondrial translocation of p53 in response to DNA damage triggers cell death. This appears to involve the localization of p53 to the mitochondria and its interaction with Bcl-xl and Bcl-2, and activation of Bax to increase mitochondrial membrane permeability.<sup>46,49,50</sup>

Results from the present study show that VPA exposure also leads to significant accumulation of mitochondrial p53 and Bax proteins in response to IR, which occurred in parallel with observed enhancement of mitochondrial membrane potential, and correlated with the observed radiosensitization, in LS174T and HCT116/p53<sup>+/+</sup> cells. In addition, the pattern of mitochondrial Bax accumulation did not seem to correlate well with overall expression level, but rather more with the degree of p53 translocating to the mitochondria. This suggests that p53 might directly or indirectly interact and enhance Bax protein accumulation in mitochondria in response to the combination of IR and VPA.

## Conclusions

In summary, radiation-induced DNA DSBs, apoptosis, and clonogenic death increased in colorectal cancer lines exposed to VPA, a known HDAC inhibitor. This effect was p53 dependent and observed only in cell lines with wild-type p53. Results also suggest that p53 and its localization at the mitochondria may play a critical role in the VPA enhancement of apoptosis after IR and are consistent with the findings of others, which suggest that localization of p53 to the mitochondria and its interaction with Bcl-xl and Bcl-2, and activation of Bax to increase mitochondrial membrane permeability, may be involved in apoptosis.<sup>46,49,50</sup> The observed VPA enhancement of radiation-induced tumor growth suppression *in vivo* further supports the fact VPA, and possibly other HDAC inhibitors, may be potentially useful in improving tumor response to radiotherapy in the clinical setting, especially for cancers with the wild-type p53 genotype.

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## Disclosure Statement

No competing financial interests exist.

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