DNA Damage and Cellular Stress Responses

Low-Dose Valproic Acid Enhances Radiosensitivity of Prostate Cancer through Acetylated p53-Dependent Modulation of Mitochondrial Membrane Potential and Apoptosis

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

Abstract

Histone deacetylase inhibitors (HDI) have shown promise as candidate radiosensitizers for many types of cancers, including prostate cancer. However, the mechanisms of action are not well understood. In this study, we show in prostate cancer cells that valproic acid (VPA) at low concentrations has minimal cytotoxic effects yet can significantly increase radiation-induced apoptosis. VPA seems to stabilize a specific acetyl modification (lysine 120) of the p53 tumor suppressor protein, resulting in an increase in its proapoptotic function at the mitochondrial membrane. These effects of VPA are independent of any action of the p53 protein as a transcription factor in the nucleus, since these effects were also observed in native and engineered prostate cancer cells containing mutant forms of p53 protein having no transcription factor activity. Transcription levels of p53-related or Bcl-2 family member proapoptotic proteins were not affected by VPA exposure. The results of this study suggest that, in addition to nuclear-based pathways previously reported, HDIs may also result in radiosensitization at lower concentrations via a specific p53 acetylation and its mitochondrial-based pathway(s).

Introduction

Radiation therapy is a key modality in the treatment of cancer patients. The predominant mechanism by which therapeutic irradiation kills most tumor cells is through clonogenic death. DNA double-stranded breaks (DSB) are regarded as the specific lesions that initiate this lethal response. In some cell types, ionizing radiation also triggers apoptosis, although this seems to be less significant than clonogenic cell death for many tumors. The pleiotropic nature of death pathways induced by radiation suggests that radiation resistance is likely regulated by a variety of mechanisms, each of which is associated with a specific death pathway. The molecular basis of radiation response of human tumors is complex and is likely to be multifactorial (1).

Radiation therapy has been an effective form of treatment for prostate cancer for decades, and the use of new technologies has increased the radiation doses that can be safely administered for this purpose and which has resulted in improved control rates. However, local relapses after ionizing radiation exposure can still occur (2–4). Therefore, new approaches to sensitize prostate cancer to radiotherapy are needed if clinical outcomes are to be further improved.

It is known that some drugs can act as radiosensitizers and enhance the tumor cytotoxic effects of radiotherapy. Recently, an emerging class of drugs known as histone deacetylase (HDAC) inhibitors (HDI) has shown promise in laboratory studies as candidate radiosensitizers for prostate cancer and many other types of tumors (5–11). The mechanism through which HDI seem to work in this context involves blockage of HDAC activity resulting in hyperacetylation of core histones and leading to a more relaxed chromatin structure (12). DNA in decondensed chromatin is relatively sensitive to damage induced by ionizing radiation (13). In humans, 18 HDAC enzymes have been identified and classified on the basis of homology to yeast HDACs. Class I HDACs include HDAC 1, 2, 3, and 8, which are related to yeast RPD3 deacetylase and have high homology in their catalytic sites. Class II HDACs are related to yeast Hda1 (HDAC 1) and include HDAC 4, 5, 6, 7, 9, and 10. All class I and II HDACs are zinc-dependent enzymes. Members of a third class, sirtuins, require NAD⁺ for their enzymatic activity. Class IV HDAC is represented by HDAC 11, which, like yeast Hda 1 similar 3, has...
conserved residues in the catalytic core region shared by both class I and II enzymes. The potential effect of HDI as radiosensitizer has been supported by recent studies which have shown in a variety of cancer cell types that HDIs, such as trichostatin A (TSA), sodium butyrate (NaB), FK228, and MS-275, enhance radiation-induced cell death and increase ionizing radiation-induced DNA DSBs. However, the molecular mechanisms involved in HDI-mediated radiosensitization are not clear and a better understanding of these mechanisms is needed to identify those prostate cancer patients who may benefit most from this treatment strategy.

This study investigates in detail a nonnuclear p53-based mechanism involved in the radiosensitization of prostate cancer cells exposed to the HDI valproic acid (VPA), which is an 8-carbon branched chain fatty acid and has been identified to be an effective inhibitor to HDAC classes I and II. We found that VPA at low concentrations stabilizes a specific acetyl modification of the p53 tumor suppressor protein, thereby increasing direct, proapoptotic functions of p53 at the mitochondrial membrane. Results from this study also show that p53-dependent radiosensitization with low doses of VPA is independent of any action of p53 protein in the nucleus as a transcription factor, as they take place in prostate cancer cells containing mutant forms of p53 protein having no such activity.

Methods and Materials

Reagents and antibodies

VPA was obtained from Sigma Aldrich Co., Ltd. Suberyl-anilide hydroxamic acid (SAHA) was obtained from RegulATORY Affairs Branch, Cancer Therapy Evaluation Program, National Cancer Institute, NIH. Monoclonal antibody against phospho-histone H2A.X (γ-H2A.X, Ser139), caspase 3 (4-1-18), and anti-acetyl histone H4 serum were purchased from Upstate. Polyclonal antibodies against GST (1-109), Bcl-2 (100), Bcl-xL (H-62), Bax (N-20), and a monoclonal antibody against p53 (DO-1), PCNA (proliferating cell nuclear antigen), were purchased from Santa Cruz Biotech. Inc. Monoclonal antibody against mitochondria HSP70 (MA3-028) was from Affinity Bioreagents. Monoclonal antibody against cytochrome c (SA-226) was obtained from Biomol. Monoclonal antibody against β-actin was obtained from Abcam Inc. Polyclonal antibody specific for the acetylated p53 at K120 (anti-ac-K120-p53) was a kind gift from Dr. Wei Gu (Columbia University, New York). z-VAD-fmk was purchased from Promega.

Cell culture and treatment

Human LnCaP, DU145, and PC-3 cells were obtained from the American Type Culture Collection. Human colon cancer HCT 116 cell lines (p53+/+) and (p53−/−) were kindly provided by Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD). LnCaP cells were maintained in RPMI-1640 medium with 2 mmol/L L-glutamine, 4.5 g/L glucose, 10 mmol/L HEPEES, 1.5 g/L sodium bicarbonate, and 10% FBS; DU145 cells were maintained as adherent monolayer cultures in Dulbecco’s modified Eagle’s medium containing 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, 1.5 g/L sodium bicarbonate, and 10% heat-inactivated FBS; PC-3 cells were maintained in Ham’s F12K medium with 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, and 10% heat-inactivated FBS; HCT-116 and 293 cells were maintained in McCoy’s 5A medium containing 10% heat-inactivated FBS. Cells growing as monolayers in standard 6-well plates or 60-mm tissue culture plates were irradiated using a Mark I Cs-137 Irradiator (J.L. Shepherd Association) at a dose rate between 1.43 and 1.49 Gy/min. Administered doses were validated using commercially available nanoDot optically stimulated luminescence dosimeters (Landauer, Inc.).

Clonogenic assay

To evaluate radiosensitivity, cells in log phase were plated for 8 hours and then treated with VPA at the indicated concentrations or PBS as a control; ionizing radiation was then delivered 12 hours later. Irradiated cells were maintained in VPA-containing medium for 14 to 20 days until colony counting. Colonies greater than 50 cells were counted as surviving colonies and the number of colonies was normalized to that observed for unirradiated controls. Mean inactivation doses were determined by the method of Fertil and colleagues (18), and the sensitizer enhancement ratio (SER) for HDI treatment was calculated as the ratio of mean inactivation dose_{control}/mean inactivation dose_{HDI-treated}.

Transfections

Plasmid-encoding wt-Tip60 (pcDNA3.1-HA-wt-Tip60) was a gift from Dr. Edwige Col (Université Joseph Fourier, La Tronche Cedex, France). Plasmid-encoding wild-type p53 (pCMV-Neo-Bam-p53<sup>wt</sup>) was kindly provided by Dr. Bert Vogelstein; plasmids encoding mutant p53 at codon 223 (pLPC-p53-223) and codon 274 (pPSH-p53-274) were kindly provided by Dr. Andrei V. Gudkov. Wild-type or mutant p53 fragments were reamplified by PCR and inserted into pcDNA3.1-C at BamHI and EcoR V sites. Plasmids encoding other mutations of p53 (p53<sup>223Leu</sup>, p53<sup>274Phe</sup>, and p53<sup>223Leu+K274Phe</sup>) were generated by mutagenesis PCR. All p53 plasmid constructs were then confirmed by sequencing. Electroporation were performed with Multiporator Electroporation Systems (Cole-Parmer) as per manufacturer’s instructions. Stable transfectants were then selected by G418<sup>in vitro</sup>.

Validated siRNAs targeting human p53 (target sequence: 5′-GGAAATTTGCGTGTGGAGT-3′) was obtained from Ambion; annealed siRNA oligos at final concentrations of 25 nmol/L were transiently transfection into LnCaP cells by using siPORT<sup>TM</sup> XP-1 reagent per manufacturer’s instructions.

Assessment of apoptosis

Cells were incubated with or without VPA for 12 hours, followed by irradiation at 10 Gy. After incubation in

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VPA-containing medium for additional 48 hours, cells were collected and stained with Annexin V-FITC (fluorescein isothiocyanate) as per manufacturer’s instructions (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences). The extent of apoptosis was quantified using a Becton Dickinson flow cytometer. Apoptosis was also quantified by determining the enrichment of mono- and oligonucleosomes in the cytoplasm of the apoptotic cells as per manufacturer’s instruction (Cell Death Detection ELISA PLUS; Roche Applied Science). Briefly, 1 × 10^5 cells were collected for analysis. After cell lysis, cytoplasmic fractions were subjected to an ELISA that detects apoptosis-released histones and DNA in the cytoplasm. The net absorbance of samples at 405 nm (reference wavelength at 490 nm) was then normalized to the corresponding negative controls (cells without irradiation, VPA, and/or z-VAD-fmk treatments) as the changes of apoptotic cells.

Assay of mitochondrial membrane potential

Changes in the mitochondrial membrane potential (MMP) were evaluated by staining cells with the fluorescent cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide (JC-1) by using Mt-E-Psi Apoptosis & Mitochondria Permeability Detection Kit (Biomol). Briefly, JC-1 was added directly to the cell suspension in complete medium and incubated for 15 minutes; after washing twice with prewarmed culture medium, cells were suspended in fresh medium. Fluorescence was monitored in a flow cytometer by 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 changes of MMP.

Preparation of mitochondrial and cytosolic protein fractions and whole cell lysate for immunoblotting

After the indicated treatments, approximately 2 × 10^7 cells were collected. The mitochondria and cytosolic protein fractions were separated and prepared from cell pellets, utilizing a reagent-based method per manufacturer’s instruction (Mitochondria Isolation Kit; Pierce). The mitochondria were then lysed with RIPA (radioimmunoprecipitation assay) buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 2 mmol/L EDTA, 2 mmol/L phenylmethylsulfonylflouride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 50 mmol/L NaF, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 50 μg/mL soybean trypsin inhibitor, and 20 mmol/L iodoacetamide). Ten micrograms of mitochondrial protein or 25 μg of the cytosolic fraction was then directly loaded on the SDS-PAGE gel for immunoblotting analysis.

For whole cell lysates, cells were washed twice with cold PBS and then lysed in RIPA buffer with mild sonication. To determine the acetyl histone H4 and phospho-histone H2A.X, whole cells were lysed in RIPA buffer containing 1 mmol/L TSA and 5 mmol/L nicotinamide.

RNase protection assay

Total RNA was extracted using TRIzol reagent (Invitrogen). The RNase Protection Assay was carried out as per manufacturer’s instruction (BD Biosciences). Briefly, 2 μg of RNA was incubated with α-32P-UTP–labeled single-stranded RNA probes overnight at 56°C and treated with RNase for 45 minutes at 30°C. The RNA–RNA complexes were resolved by electrophoresis in 6% denaturing polyacrylamide gel and analyzed by autoradiography. Template set for hApo-2c and custom-designed template sets for DSB-related and p53-related genes were purchased from BD Biosciences.

In vitro cytochrome c release assay

Mitochondria were purified using a differential centrifugation method from human HCT-116/p53<sup>−/−</sup> cells (19). Briefly, cells were harvested, pelleted, and resuspended in fractionation buffer A (10 mmol/L HEPES/KOH at pH 7.4, 0.1 mmol/L EDTA, 1 mmol/L EGTA, and 250 mmol/L sucrose) supplemented with protease inhibitor cocktail (Sigma). Cell disruption was carried out by passing the cells through a 23-gauge needle 3 to 5 times. The homogenates were spun at 700 × g for 10 minutes at 4°C. The supernatants were removed and spun at 3,000 × g for 15 minutes at 4°C. The mitochondrial pellets were washed with fraction buffer B (10 mmol/L HEPES/KOH at pH 7.4, 5 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 5 mmol/L succinate, and 250 mmol/L sucrose) and resuspended in fraction buffer B to a final protein concentration of 2 to 3 mg/mL.

For in vitro cytochrome c release assay, engineered PC-3 cells with wild-type or mutant p53 were treated with VPA, radiation, or the combination as described earlier. Five hours after the delivery of irradiation, 1 × 10<sup>7</sup> cells were harvested, washed with cold PBS, and resuspended in 200 μL of modified KCl buffer (15 mmol/L HEPES/NaOH, 125 mmol/L KCl, 4 mmol/L MgCl<sub>2</sub>, 5 mmol/L Na<sub>3</sub>PO<sub>4</sub>, 0.5 mmol/L EGTA, 5 μmol/L rotenone, and 5 mmol/L succinate, and 250 mmol/L sucrose at pH 7.4). Cells were swelled on ice for 2 minutes and disrupted by passing the cells through a 23-gauge needle 5 times. The homogenates were spun at 12,000 × g for 15 minutes at 4°C. The supernatants were collected as cytosolic fractions. After the determination of protein concentrations by using the Bradford reagent (Bio-Rad Laboratories, Inc.), cytosolic fractions with 150 μg protein each were incubated with 20 μg of purified mitochondria for 40 minutes at 30°C in 100 μL KCl buffer (15 mmol/L HEPES/NaOH, 125 mmol/L KCl, 4 mmol/L MgCl<sub>2</sub>, 5 mmol/L Na<sub>3</sub>PO<sub>4</sub>, 0.5 mmol/L EGTA, 5 μmol/L rotenone, and 5 mmol/L succinate at pH 7.4) and then centrifuged at 12,000 × g for 10 minutes at 4°C. The mitochondrial pellets and corresponding supernatants were immunoblotted for cytochrome c and actin antibodies to verify equal loading.

Purification of recombinant proteins

Bac-Pak-Tip60 was generously provided by Dr. Edwige Col (Université Joseph Fourier, La Tronche Cedex, France). pGEX-5×1-GST-p53<sup>wt</sup> was a gift from Dr. Jean Dorsey.
(The Wistar Institute, Philadelphia, PA). The recombinant GST-tagged proteins for mutant p53 were generated by reinsert of corresponding p53 fragments from plasmids encoding mutations of p53 in pcDNA3.1-C vector into pGEX-5×1-p53wt at PshAl/Stul sites.

The Flag/HA-Tip60 was purified from the infected insect SF-9 cells (obtained from Dr. Binghui Shen, City of Hope, Duarte, CA) to homogeneity under native conditions (Ni-NTA Spin Kit; Qiagen). The GST-p53wt, GST-p53K120R, GST-p53S23L, and GST-p53S23L+E2K120R Proteins were purified from bacterial cells BL-21 (obtained from Dr. Binghui Shen, City of Hope, Duarte, CA) and dialyzed with KCl buffer.

**In vitro acetylation assay**

One microgram of purified Tip60 protein was rebound to 20 μL Ni-NTA agarose beads (Qiagen) in modified Ni-NTA lysis buffer (50 mmol/L NaH_{2}PO_{4}, 300 mmol/L NaCl, and 10 mmol/L imidazole) and washed once with HAT buffer (50 mmol/L Tris (pH 7.9), 10% glycerol, 1 mmol/L DTT, and 10 mmol/L NaB). As a negative control, 1 μg of BSA was also incubated with Ni-NTA agarose beads at 4°C for 2 hours. After preincubation of beads with 20 μmol/L of acetyl-CoA in HAT buffer at room temperature for 30 minutes, 10 μg of p53 proteins was mixed with beads in 100 μL HAT buffer containing 20 μmol/L acetyl-CoA and incubated at 30°C for 1 hour. The mixtures were then centrifuged at 1,000 × g for 3 minutes, and the supernatant were collected for immunoblot against anti-ac-K120-p53. To investigate the role of p53 protein acetylation on cytochrome c release, 40 μL of corresponding supernatants were incubated with 30 μg of purified mitochondria at 30°C for 40 minutes in 100 μL KCl buffer and then centrifuged at 12,000 × g for 10 minutes at 4°C. The mitochondrial pellets and corresponding supernatants were immunoblotted for cytochrome c and GST antibodies to verify equal loading.

**Tumor growth assay**

DU145 cells (3 × 106/0.2 mL HBSS 1 × + 1% HSA) were inoculated subcutaneously into the right thigh of male athymic nude mice 4 to 6 weeks old. When tumor volumes reached a size of 50 to 100 mm³ (approximately day 7 postinoculation), mice were randomly grouped into 4 groups (n = 5–7) that received the following treatments: (a) saline, 0.2 mL; (b) VPA (300 mg/kg); (c) ionizing radiation 10 Gy; and (d) VPA (300 mg/kg) + ionizing radiation 10 Gy. Mice were treated with intraperitoneal injections of VPA (300 mg/kg) every 12 hours for 3 days. After the third VPA injection, the mice were irradiated locally on the right thigh with a Mark I Cs-137 irradiator, using a collimator with a 30-mm opening at a dose rate between 5.29 and 5.25 Gy/min. Doses were validated using commercially available nanoDot optically stimulated luminescence dosimeters (Landauer, Inc.). Tumors were measured biweekly, and tumor volumes were determined from caliper measurements of tumor length (L) and width (W) according to the formula (L × W³)/2.

**Results**

**VPA induced changes in histone acetylation and survival after ionizing irradiation**

Dose-response data for alteration of histone H4 acetylation levels by VPA in prostate cancer lines LnCaP, DU145, and PC-3 were obtained before investigating potential radiosensitizing effects of the drug (Supplementary Fig. S1); a minimum of 50 μmol/L VPA markedly increased histone H4 acetylation in all 3 lines. We next examined the effects of VPA on cell response to ionizing radiation by clonogenic survival assay. When exposed to relatively high concentrations of VPA (500 μmol/L and 1 mmol/L), marked radiosensitization was detected in LnCaP and DU145 cells (Fig. 1A), with observed SER₀.₁ of 1.43 and 1.62 for LnCaP and of 1.41 and 1.58 for DU145, respectively. The VPA exposures using these concentrations also caused appreciable cytotoxicity in all 3 prostate cancer lines (Supplementary Table S1). Importantly, a 10-fold lower concentrations of VPA (50 μmol/L) did not lead to any direct toxicity of the drug but significant radiosensitization was not observed for any drug concentration tested (SER₀.₁ were 1.17 for 50 μmol/L, 1.25 for 500 μmol/L, and 1.16 for 1,000 μmol/L, respectively), ionizing radiation produces DSBs in chromosomal DNA that can initiate signaling cascades and lead to cell-cycle arrest or apoptosis. One of the earliest known responses to radiation-induced DSB formation is phosphorylation of the C-terminal tails of variant H2AX (γ-H2AX) in chromatin on either side of the break (20). With prior exposure to 50 μmol/L of VPA, radiation-induced γ-H2AX formation was greater in all tested cells lines; however, the persistence of γ-H2AX at 24 hours postirradiation, which was described as corresponding well to radiosensitivity (21), was observed only in LnCaP and DU145 cells but not in PC-3 cells (Supplementary Fig. S2), suggesting that VPA exposure potentially inhibits the repair of radiation-induced DSBs in LnCaP and DU145 cells.

**VPA increases apoptotic response to irradiation in prostate cancer cells**

It has been reported that apoptosis is not a prominent mechanism of ionizing radiation-induced cell death in prostate cancer cells in culture (22). Consistently, only approximately 5% of cells undergoing apoptosis 48 hours after exposure to 10 Gy were detected in these 3 cell lines (Fig. 1B). Control exposures to 50 μmol/L of VPA alone did not induce apoptosis. However, when cells were exposed to 50 μmol/L VPA before 10 Gy of ionizing radiation, a significant increase in the percentage of cells undergoing apoptosis was observed in LnCaP (~19%) and DU145 (~17%) but not in PC-3 (Fig. 1B). These augmented apoptotic responses in LnCaP and DU145 cell were sufficient to account for the radiosensitization measured by clonogenic assay (Fig. 1A).
Previous studies have shown that HDI treatments can induce cell growth arrest at G1/G0 or G2/M phase in a number of tumor cell lines, including prostate cancer. These effects were attributed to dysregulation of cell-cycle regulators such as p21CIP/WAF1 and cyclin B1 (23, 24). It has also been reported that treatment at lower concentrations of HDI had no effect on cell-cycle distribution of prostate cancer cells (25). Results from this study showed that exposure to VPA at 50 μmol/L had no significant influence on cell-cycle distribution compared with ionizing radiation alone in all cell lines tested (data not shown), indicating that the observed VPA-enhanced radioresistance was not due to reassortment of cells into the more radiosensitive phases of the cell cycle.

**VPA enhancement of apoptosis after ionizing radiation exposure involves mitochondria and caspase pathways**

Increased mitochondrial membrane permeability and unleashing of caspase protease activity have each been shown to accompany cellular commitment to apoptosis (26, 27). To further elucidate the mechanism of low-dose VPA enhancement of apoptosis after ionizing radiation exposure, we examined the effects of VPA on MMP and caspase activation. Significant changes in MMP (Fig. 2A) and cytochrome c release into the cytosolic fraction (Fig. 2B) were detected with VPA exposure before 10 Gy ionizing radiation exposure in LnCaP and DU145 cells, but these were not observed with VPA alone or ionizing radiation alone in these cells. In contrast, VPA + ionizing radiation did not produce comparable effects in PC-3 cells. HDI treatment has been reported to enhance the ionizing radiation-induced triggering of caspase activity in DU145 cells (28). Activation of caspase 3, signaled by detection of its cleaved 17-kDa subunit, was observed in LnCaP and DU145 cells exposed to VPA + ionizing radiation, whereas ionizing radiation alone or VPA alone resulted in no cleavage products (Fig. 2C). Furthermore,
the presence of the caspase inhibitor z-VAD-fmk completely abolished VPA-enhanced apoptotic response to ionizing radiation in both LnCaP and DU145 cells (Fig. 2D). These results suggest that low-dose VPA exposure increases the susceptibility of cells to undergo apoptosis in response to ionizing radiation, in association with stereotypical losses of mitochondrial membrane integrity, and alteration of caspase 3 activity.

**Localization of p53 and Bcl-2 family proteins in mitochondria following ionizing radiation exposure is modified by VPA treatment**

Previous studies have provided evidence that p53 protein is involved in apoptosis induction through rapid, "direct" mechanisms that entail the physical interactions with regulators such as Bax, Bcl-2, and Bcl-xL at the mitochondria (29, 30). We investigated p53 protein association with the mitochondrial subcellular fraction following treatments with ionizing radiation, VPA, or both. Figure 3A shows that ionizing radiation alone or combined with VPA caused p53 protein to accumulate in mitochondrial fractions in LnCaP cells as early as 1 to 3 hours after ionizing radiation exposure. With ionizing radiation alone, the p53 mitochondrial content had decreased by 8 to 12 hours, whereas with VPA treatment before ionizing radiation exposure, the p53 in mitochondrial fractions had not returned to baseline by 24 hours. We also examined the effects of VPA on mitochondrial localization of Bcl-2 family proteins in the 3 cell lines with different p53 genotypes. As shown in Figure 3B, no changes in Bcl-2 mitochondrial accumulation were seen...
after ionizing radiation alone or after VPA followed by ionizing radiation exposure. Treatment with combination of VPA and ionizing radiation did lead to a redistribution of Bcl-xL out of the mitochondria compared with ionizing radiation alone in LnCaP and DU145 cells. In contrast, Bax mitochondrial concentrations increased with the addition of VPA to ionizing radiation in these cells. The lower observed level of Bax in DU145 extracts is likely due to a monoallelic Bax frameshift mutation and a second silent Bax allele in this line (31). Comparable changes for Bcl-xL and p53 with these treatments were not observed in p53-null mutant PC-3. Furthermore, our results suggested that the observed changes of mitochondrial Bax and Bcl-xL seemed to be correlated with the changes of mitochondrial p53 rather than the changes of protein expressions in corresponding cells.

VPA enhancement of radiation-induced apoptosis is p53 dependent

The ability of low-dose VPA to enhance apoptosis after ionizing radiation exposure seems to be correlated with the presence of p53 protein in the lines—LnCaP and DU145—that exhibit this response but not with the ability of that protein to function as a nuclear transcription factor (Supplementary Fig. S3). To establish that p53 protein is a requirement of this apoptotic response, we used specific siRNA expression to transiently downregulate the p53 protein level in LnCaP cells. As shown in Figure 4, knockdown of p53 significantly reduced the enhancement of ionizing radiation-induced apoptosis mediated by VPA pretreatment. Enhancement of Bax mitochondrial accumulation and cytochrome c release by VPA after ionizing radiation exposure was similarly impaired by the p53 knockdown (Fig. 4C).

Requirement of p53 for VPA-enhanced apoptotic response after irradiation was further confirmed by restoring wild-type p53 protein expression in engineered PC-3 cells (Fig. 5). Two different mutant alleles, p53<sup>223</sup>Leu and p53<sup>274</sup>Phe from DU145 cells (32), were also separately expressed in PC-3 cells. Stable transfectant clones were derived and propagated and then examined for apoptotic response after ionizing radiation alone or 50 μmol/L VPA pretreatment followed by ionizing radiation exposure. Compared with empty vector controls, engineered expression of
either p53wt or p53223Leu restored the ability of VPA to enhance ionizing radiation-induced apoptosis (Fig. 5B) and to sensitize cells to ionizing radiation with an SER0.1 of more than 1.3 (Supplementary Table S2 and Supplementary Fig. S4); these 2 proteins also significantly increased Bax protein levels in mitochondria and promoted cytochrome c release with combined VPA and ionizing radiation treatments (Fig. 5C). In contrast, expression of p53223Leu in PC-3 cells did not result in similar changes. These results indicate that p53 plays an essential role in VPA-mediated enhancement of apoptosis and radiosensitization and that these properties are retained by some p53 mutant alleles that are deficient in nuclear function as a transcriptional activator of target genes. Judging by relative protein levels and the degree to which apoptosis is enhanced (Fig. 5A), it seems that molecule-for-molecule, wild-type p53 may be more potent than p53223Leu in mediating these effects, however.

**Loss of K120 acetylation diminishes p53-mediated radiosensitization by VPA**

p53 is known to be a substrate for acetylation at multiple lysine residues and for deacetylation by HDACs (33). Acetylation of p53 at lysine residue 120 specifically has been shown to be important for regulation of apoptosis through its nucleus function as transcriptional factor following ionizing radiation and other genotoxic cellular insults (34, 35). We reasoned that this posttranslational modification could have similar regulatory effects at the mitochondria and so hypothesized that increasing mitochondrial levels of ac-K120-p53 with VPA + ionizing radiation treatments would amplify p53-dependent apoptotic effects there and thus produce the observed radiosensitization. We investigated the presence of K120 acetylation in mitochondrial p53 after exposure to VPA and ionizing radiation by using a specific antibody (34). As shown in Figure 6A, ac-K120-p53 was indeed present in mitochondrial fractions following ionizing radiation exposure. The use of AlphaView software (Cell Biosciences) to quantify protein levels showed that the ratio of acetylated p53 at K120 in the mitochondrial fractions between cells treated with these 2 treatments, suggesting that the increases in K120 acetylation observed after VPA and ionizing radiation treatment was not solely due to an increase in total amount of p53.

In keeping with this hypothesis, p53K120R expression in PC-3 cells did not lead to increased apoptosis or loss of MMP following ionizing radiation exposure when cells were first treated with VPA as well (Fig. 6B and Supplementary Fig. S5). To genetically dissect apart any nuclear and non-nuclear regulatory effects of p53 lysine 120 acetylation, we constructed the double-mutant p53223Leu,K120R that is defective both for nuclear transactivation and, by the model, for enhanced prosapoptotic effects at the mitochondria. When expressed in PC-3 cells, and in contrast to the p53223Leu single mutant, the double mutant did not support increased apoptosis with VPA + ionizing radiation. Correspondingly, substitutions of lysine 120 with arginine in wild-type p53 or p53223Leu eliminated radiosensitization by VPA, as assessed by clonogenic assays in engineered PC-3 cells (Supplementary Fig. S4).

Taken together, these data indicate that acetylation of the K120 residue of p53 plays an important regulatory role for the prosapoptotic effects that p53 protein mediates at the mitochondria, as has been shown to be the case for the corresponding nuclear effects, and that these mitochondrial effects are modulated by HDI.

The role of acetylated K120 was confirmed further by incubating purified mitochondria (isolated from human colon cancer HCT116/p53- cells) with cytoplasmic...
extracts from engineered PC-3 cells treated with VPA, irradiation, or the combination. As shown in Figure 7A, incubation of the mitochondria with cytoplasmic extracts from empty vector–transfected (Neo) PC-3 cells, which had been treated with VPA + ionizing radiation, or from p53wt-expressing PC-3 cells that had not been exposed to ionizing radiation, produced little cytochrome c release from mitochondria into the supernatant fraction. Extracts from irradiated PC-3/p53wt cells resulted in modest cytochrome c release, and this markedly increased with VPA treatment of the cells before ionizing radiation exposure. Mitochondria incubation with extracts prepared from p53223Leu-expressing PC-3 cells supported increased cytochrome c release after VPA + ionizing radiation treatment, whereas those from p53K120R and p53223LeuK120R did not (Fig. 7B).

Tip60, a MYST family acetyltransferase, acetylates K120 and regulates HDI-mediated apoptotic response to irradiation

Lysine 120 of p53 has been identified recently to be a substrate of Tip60, a histone acetyltransferase belonging to the MYST family (34, 36). We therefore manipulated K120 acetylation levels by incubating purified p53 protein with recombinant Tip60 in vitro. As seen in Figure 7C, Tip60 induced K120 acetylation of p53wt and p53223Leu proteins and enhanced corresponding p53-dependent cytochrome c release from mitochondria. No changes in acetylation or cytochrome c release were observed with p53 proteins with K120 mutations.

In addition, manipulation of the levels of Tip60 in irradiated cancer cells modulated apoptotic response to HDI drugs. Figure 7D shows that engineered DU145 cells with expression of wt-Tip60 presented a modest, but reproducible, increase in apoptosis after HDI + ionizing radiation compared with parental cells. DU145 cells were selected for these experiments to avoid any effects on p53 target gene transcription by Tip60 expression.

VPA induces radiosensitization of prostate cancer cells in vivo

Studies were extended to an in vivo model. We investigated the effects of VPA, ionizing radiation, or VPA combined with ionizing radiation on tumor growth of DU145 xenografts. VPA increased tumor growth suppression compared with ionizing radiation alone (Fig. 8). These results are consistent with results seen in vitro.

**Discussion**

Advances in molecular radiobiology have led to a better understanding of mechanisms involved in radiosensitivity. HDIs have shown promise as radiosensitizers in...
laboratory studies and are being actively evaluated in clinical trials. Possible mechanisms may involve cell-cycle arrest, gene expression regulation, or activation of apoptosis. Our previous study (37), as well as others (38), have also suggested that HDI-induced differential radiosensitivity in cancer cells may be influenced by p53 expression. In this study, we have shown the role of a specific acetyl modification of p53 and its mitochondrial accumulation involved in HDI-mediated apoptotic response to ionizing radiation in prostate cancer cells.

The tumor suppressor protein p53 plays a pivotal role in governing cellular responses to genotoxic damage. p53 seems to selectively activate, at the transcriptional level, networks of genes whose products support growth arrest or apoptosis (39, 40). In cells with functional p53 protein, a posttranslational increase in levels of p53 occurs in response to irradiation, which seems to be mediated through the ATM proteins (41), and ultimately results in an increase in the level of the cyclin-dependent kinase inhibitory protein p21Waf1/Cip1 (42). The role of p53 in stress-induced apoptosis was thought initially to center on its role as a transcription factor modulating gene expression. Upon activation by DNA damage signaling pathways, p53 promotes the expression of a number of genes that promote apoptosis. In cells with mutated or non-functional p53, apoptotic function is often compromised or abrogated (43). In this study, we also investigated the effects of VPA on p53 transcriptional activities in response to ionizing radiation. Our data clearly showed that VPA upregulation of p21 protein in p53-positive LnCaP cells was dose dependent (Supplementary Fig. S3B). Interestingly, while low dose of VPA enhanced p53-dependent apoptotic response and radiosensitization in prostate cancer cells, no significant effects on expression of genes known to be p53 related were observed in radiosensitized prostate cancer cells, suggesting an exclusion of p53 as transcriptional factor in this radiosensitization process. Indeed, recent studies have provided strong evidence that p53 promotes apoptosis not only through target gene activation in the cell nucleus but also through the initiation of extranuclear proapoptotic mechanisms that involve direct interaction of p53 on the mitochondria. Although the mechanisms involved remain unclear, direct physical interaction of p53 with antiapoptotic proteins such as Bcl-2 and Bcl-xL has been shown. These interactions allow Bax to oligomerize and then mediate mitochondrial membrane permeability leading to release of cytochrome c (44, 45). Cytochrome c release then triggers activity of a family of caspases, including caspases 9 and 3, that have proteolytic function and seem to be downstream effectors.

**Figure 6.** Loss of p53 acetylation at K120 reduced VPA-enhanced apoptosis response and radiosensitization. A, VPA induced acetylation of mitochondrial p53 at K120 in response to irradiation. LnCaP cells were pretreated with 50 μmol/L VPA for 12 hours and then irradiated (10 Gy). The mitochondrial fraction was isolated 8 hours later and subjected to immunoprecipitation with ac-K120-p53 antisera. The resultant proteins were analyzed by immunoblotting with a p53 antibody (DO-1). Total p53 and mt-HSP70 were included to confirm equivalent protein loading. B, mutation at K120 of p53 (K120R) diminished VPA-enhanced loss of MMP in response to irradiation on PC-3 cells engineered to express wild-type p53 or mutant p53K120R, stable transfectants of PC-3 cells with empty vector, wild-type p53, and mutant p53 (p53K120R, p53K223Leu, p53K274Phe, p53K223Leu+K120R, and p53K274Phe+K120R) were pretreated with VPA for 12 hours and then irradiated (10 Gy). Cells were collected by trypsinization 48 hours later and stained with JC-1. Loss of MMP was analyzed by flow cytometric assay. Data represent the average of 3 experiments. Error bars, 1 SD from 3 individual experiments. *, significant difference (P < 0.01).
Our current data also show that the radiosensitizing effects of low-dose VPA in prostate cancer cells were p53-dependent and involved a nonnuclear mitochondrial pathway. With VPA exposure before ionizing radiation exposure, p53 mitochondrial protein activation and mitochondrial caspase-mediated apoptosis increased. Moreover, particular p53 mutants, such as p53<sup>223Leu</sup> with a missense mutation located in DNA binding domain, also showed the capability to mediate biological effects of VPA on ionizing radiation-induced MMP, apoptosis, and radiosensitization in prostate cancer cells, further supporting the involvement of a p53-mediated extranuclear proapoptotic pathway.

Of interest is the observed difference between PC-3 cells expressing p53<sup>223Leu</sup> versus p53<sup>223R</sup>. Although these 2 mutations are located very close in the same functional domain, increased levels of apoptosis after VPA and ionizing radiation were observed only with the p53<sup>223Leu</sup> mutation. The reasons why some p53 DNA binding domain mutants can trigger apoptosis while others fail remains unexplained but may involve a critical conformational requirement for p53 activation after VPA exposure. This hypothesis is consistent with reports showing that some small molecular compounds, including SAHA, have the ability to restore the proper conformation of mutant p53 by posttranslational modification, such as acetylation, of p53 and trigger p53-dependent, transcription-independent apoptosis (46, 47).

The possibility of using low-dose HDI to restore wild-type p53 activity to some p53 mutants to enhance ionizing radiation-induced apoptosis is intriguing, particularly since it has been reported that local failures after radiation therapy are more common in prostate cancer cells with abnormal p53 (48–50). Identifying which p53 mutants are most amenable to this strategy will be clinically important.

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The precise mechanism(s) by which p53 is activated in response to cellular stress is not completely understood. However, it is generally thought to primarily involve posttranslational modifications of p53, resulting in conformational
Valproic Acid Enhances Radiation-Induced Apoptosis through a p53 Nonnuclear Pathway

Figure 8. VPA induces radiosensitization in prostate cancer in vivo. Athymic nude mice bearing isogenic DU145 xenograft tumors were treated with VPA (300 mg/kg × 6) administered intraperitoneally every 12 hours for 3 days and/or 10 Gy ionizing radiation (IR). In the combined treatment group, ionizing radiation was delivered after the third injection of VPA. The growth curves represent the average value in each group of 5 to 8 mice. Error bars, 1 SE.

changes that affect its protein levels, cellular localization, and binding specificity for both protein partners and DNA target sequences. p53 protein in vivo undergoes extensive posttranslational modifications including phosphorylation of specific serine and threonine residues and acetylation of lysine in response to irradiation. Early studies indicated that p53 is specifically acetylated at multiple lysine residues of the C-terminal regulatory domain by the HAT cofactor PCAF (51–54). Acetylated p53 can dramatically stimulate its sequence-specific DNA binding activity in vitro to modulate transcriptional proapoptotic responses in cancer cells (55–58). However, C-terminal acetylation of p53 also blocks the ε-amino group of the acetylated lysine residues for monoubiquitination (59–61). The latter promotes its nuclear export (62). Thus, C-terminal acetylation of p53 is expected to inhibit direct apoptotic p53 functions at the mitochondria following nuclear export (61). However, studies do not rule out the possibility that acetylation of other lysine residue(s) of p53 protein also regulates its mitochondrial localization and/or its interactions with Bcl-2 family proteins in response to DNA damage.

Recently, several groups have reported on the involvement of acetylated p53 both in modulation of transcription activity and in its direct impact on the mitochondria as a cell undergoes apoptosis. These posttranslational modifications include acetylation of p53 at lysine 120 and lysine 3 (34–36, 47). Studies have also shown that lysine 120 on p53 is a substrate for acetylation after DNA damage via the MYST family acetyltransferases Tip60 and hMOF (36, 63) and that acetylated p53 regulate transcription-independent apoptosis (35, 47). In contrast to other p53 acetylation sites at the C-terminus referred to earlier, K120 is within the core DNA binding domain of the protein. Conservative, nonacetylatable mutations (arginine for lysine) of these 2 residues are specifically defective for either apoptosis induction or its interaction with other proteins such as mdm2. Results from our study have also shown that Tip60 acetylated wild-type p53 and mutant p53223Leu at K120 and resulted in subsequent enhancement of p53 proteins on release of cytochrome c in vitro. However, other p53 mutants having peptide sequence changes located elsewhere within the DNA binding domain were not substrates for K120 acetylation by Tip60, suggesting the critical role of conformational structure for this modification. In addition, manipulating levels of Tip60 in irradiated DU145 cells increased the effects of HDI drugs to ionizing radiation-induced apoptosis. This was seen not only for VPA but also for SAHA (data not present), suggesting that other HDI may work through the same pathways. Of note, a very recent study has also shown that deacetylation of p53 K120 was predominately regulated by HDAC1, a potential target for both VPA and SAHA (14, 64). Whether HDAC1 or other HDACs are involved in the VPA enhancement of apoptotic response to ionizing radiation requires further investigation.

Recurrence of radiation-resistant tumors is a common problem in clinical oncology, including the treatment of prostate cancer (65, 66). An attractive solution to this problem of prostate cancer cell radioresistance would be to endow them with an increased susceptibility to undergo apoptosis in response to the DNA damage inflicted by ionizing radiation. Reducing the threshold to undergo apoptosis by interference with apoptosis resistance pathways would be expected to sensitize tumor cells to ionizing radiation by affording them an additional mechanism for induction of cell death. The fact that this may be clinically achievable with low and possibly less toxic doses of VPA, or other HDI, is theoretically attractive. Although the precise molecular mechanism(s) by which HDI potentiates radiation-induced apoptosis remains unclear, our data show that exposure to low concentrations of VPA and possibly other HDI before ionizing radiation exposure in prostate cancers with select p53 genotypes could be a novel strategy to enhance the radiosensitivity of prostate cancers in the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Xufeng Chen, Jeffrey Y.C. Wong, Patty Wong, et al.

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