Dichloroacetate (DCA) Sensitizes Both Wild-Type and Over Expressing *Bcl-2* Prostate Cancer Cells In Vitro to Radiation

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BACKGROUND. *Bcl-2* protects cells from apoptosis and provides a survival advantage to cells over-expressing this oncogene. In addition, over expression of *Bcl-2* renders cell resistant to radiation therapy. Recently, dichloroacetate (DCA) was proven to potentiate the apoptotic machinery by interacting with *Bcl-2*. In this study, we investigated whether treating human prostate cancer cells with DCA could modulate *Bcl-2* expression and if the modulation in *Bcl-2* expression could render the *Bcl-2* over expressing cells more susceptible to cytotoxicity effects of radiation.
METHODS. PC-3-*Bcl-2* and PC-3-Neo human prostate cancer cells treated with DCA in addition to irradiation were analyzed in vitro for changes in proliferation, clonogenic survival, apoptosis, cell cycle phase distribution, mitochondrial membrane potential, and expression of *Bcl-2*, *Bcl-xL*, *Bax*, or *Bak* proteins.
RESULTS. DCA alone produced significant cytotoxic effects and was associated with G1 cell cycle arrest. Furthermore, DCA was associated with an increased rate of apoptosis. The combination of DCA with irradiation sensitized both cell lines to radiation's killing effects. Treatment of PC-3-*Bcl-2* or PC-3-Neo with DCA and irradiation resulted in marked changes in various members of the *Bcl-2* family. In addition, DCA therapy resulted in a significant change in mitochondria membrane potential, thus supporting the notion that DCAs effect is on the mitochondria.
CONCLUSIONS. This is the first study to demonstrate DCA can effectively sensitize wild-type and over expressing *Bcl-2* human prostate cancer cells to radiation by modulating the expression of key members of the *Bcl-2* family. Together, these findings warrant further evaluation of the combination of DCA and irradiation. *Prostate* © 2008 Wiley-Liss, Inc.

KEY WORDS: **dichloracetate; radiation; prostate cancer; *Bcl-2***

INTRODUCTION

Recurrence after definitive radiation therapy for localized prostate cancer is a common phenomenon occurring in 33–56% of men [1]. Recently, radiation dose escalation has resulted in improved prostate cancer control outcomes [2,3]. Since there is an increased risk of complications in nearby critical structures, the amount of radiation that can be delivered is limited and thus dose escalation is likely not the ultimate solution to overcome radiation resistance. Instead, investigators have turned to strategies for

Abbreviations: DCA, dichloroacetate; PDK, pyruvate dehydrogenase kinase; PBS, phosphate buffered saline; CTL, control; IC_{25}, inhibitor concentration$_{25}$; Gy, gray; PARP, poly(ADP-ribose) polymerase; $\Delta\Psi_m$, mitochondrial membrane potential.

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Received 28 February 2008; Accepted 1 April 2008
DOI 10.1002/pros.20788
Published online in Wiley InterScience (www.interscience.wiley.com).

sensitizing prostate tumors to the effects of irradiation [4–7]. However, all such strategies tested over the past 20 years have involved systemic administration of agents whose own unique side effect profiles almost always limit pharmacologic doses to levels below those needed to actually sensitize tumors to irradiation. Moreover, none of the sensitizing strategies tested to date are available for widespread use.

Multiple studies have consistently implicated two genes related to apoptosis, *p53* and *Bcl-2*, as being important in post radiation therapy prostate cancer recurrence [8–13]. Specifically, aberration of these genes can induce faulty mitochondria and apoptotic pathways [14]. Recently, researchers have reported that dichloroacetate (DCA), a known inhibitor of mitochondrial pyruvate dehydrogenase kinase (PDK) and drug utilized for hereditary lactic acidosis disorders, can shift cellular metabolism from glycolysis to glucose oxidation. Cancer cells, and specifically cancer cells known to be resistant to chemotherapy and radiation therapy, are recognized to possess aberrant apoptotic signaling [15]. Researchers have demonstrated that the administration of DCA is associated with correction in glucose utilization and restoration of apoptotic pathways in cancer cells [16]. Thus, we hypothesize that treatment of radiation resistant prostate cancer cells that overexpress *Bcl-2* with DCA prior to radiation therapy may restore functional apoptotic function and render the cells more susceptible to the cytotoxic effects of radiation.

MATERIALS AND METHODS

Human Prostate Cancer Cell Lines and Reagents

PC-3-*Bcl-2* cells (characterized by *Bcl-2* overexpression, deleted *PTEN*, and mutant *p53*) and PC-3-Neo cells (characterized by wild-type *Bcl-2* expression, deleted *PTEN*, and mutant *p53*) were generous gifts from Dr. Timothy McDonnell (University of Texas MD Anderson Cancer Center, Houston, TX). The cells were maintained in Dulbecco's modified Eagle's medium supplement with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, and 400 μg/ml G418. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

DCA (Sigma–Aldrich, St. Louis, MO) was dissolved in phosphate buffered saline (PBS) at a concentration of 100 mM. For further experiments, DCA was diluted in PBS, which served as a vehicle control for all experiments. JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide) (Calbiochem, San Diego) was dissolved in DMSO at a concentration of 2 mg/ml. For further experiments, JC-1 was diluted in culture medium.

In Vitro Cytotoxicity Assay

PC-3-*Bcl-2* and PC-3-Neo were seeded in 96-well plates at a density of 2.5×10^3 cells per well and treated with DCA or PBS. Cells were treated with DCA at concentrations ranging from 0.01 mM to 100 mM. After 1–4 days, 100 μl of 1 mg/ml MTT (Sigma–Aldrich) solution was added to appropriate plates and allowed to incubate at 37°C for 2.5 hr. Each reaction was stopped with lysis buffer (200 mg/ml SDS, 50% *N*,*N*-dimethylformamide, pH 4) at room temperature for 1 hr, and the optical density was read on a microplate autoreader (Bio-Tek Instruments, Winooski, VT) at 560 nM. Absorbance values were normalized to the values obtained for the control treated cells to determine survival percentage. Each assay was performed in triplicate, and the mean of the three assays was calculated. Cellular viability was confirmed by means of the crystal violet exclusion test.

Clonogenic Survival

Clonogenic survival was assayed using a technique previously employed in our laboratory [17]. Briefly, 5×10^5 PC-3-*Bcl-2* or PC-3-Neo prostate cancer cells were plated into sterile T 25 flasks and allowed to attach overnight. The next day, cells were treated with DCA at the concentration that would inhibit the growth of 25% of the cells (IC_{25}) or with PBS (control). Twenty-four hours later, flasks were irradiated with Gamma 40 (0.7 Gy/min) to a total of dose of 2, 4, or 6 Gy or left unirradiated as a control. Immediately after irradiation, cells were trypsinized, serially diluted, replated onto 10-cm dishes, and incubated for 14 days. Next, colonies were stained with 0.2% crystal violet and counted. The surviving fraction (SF) was calculated relative to the unirradiated (control) cells. Each experiment was performed in triplicate, and the mean SF for each set of three experiments was calculated.

Cell Cycle Analysis

For the analysis of cell-cycle distribution, PC-3-*Bcl-2* or PC-3-Neo prostate cancer cells were seeded at 5×10^5 cells in 10-cm tissue culture dishes and incubated overnight. Cells were then treated with DCA at their IC_{25} or PBS (control) and then maintained in supplemented medium. After 12 hr, some cells were irradiated with 2 Gy. After another 12 hr, cells were trypsinized, washed with 1× PBS, fixed in 1% paraformaldehyde, and stored at 4°C in 70% ethanol. Following incubation in 70% ethanol, cells were treated with RNase A and incubated in propidium iodide solution. Cell-cycle distribution was determined by flow cytometry of at least 10,000 gated cells using FACScan flow cytometer (Becton Dickinson, Franklin

Lakes, NJ). All cell-cycle analyses were performed in triplicate.

Western Blot Analysis

PC-3-*Bcl-2* and PC-3-Neo cells were seeded in 10-cm plates at 4×10^5 cells/well and treated with DCA at the IC_{25} or PBS (control) for 1 hr; some cells were then irradiated with 2 Gy. After 24 hr, cells were incubated in lysis buffer [250 mM Tris–HCl (pH 6.8), 2% SDS, and 10% glycerol] and protein inhibitor cocktail (Sigma–Aldrich). Then, cells were subjected to a standard protein assay using the DC Protein Assay kit (Bio-Rad, Hercules, CA) and Western blot analysis was completed as described previously [18]. Immunoblotting was performed by first incubating the proteins with primary antibodies against *Bcl-2*, *Bcl-xl*, total *PARP*, *Bax* and γ-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), *Bak* (BD Pharmingen, San Jose, CA) and then with secondary antibody (Bio-Rad). Protein antibody complexes were detected by means of chemiluminescence (Amersham, Arlington Heights, IL).

Membrane Potential Analysis

For the analysis of mitochondrial membrane potential ($\Delta\Psi_m$), PC-3-*Bcl-2* or PC-3-Neo prostate cancer cells were seeded at 5×10^5 cells in 10-cm tissue culture dishes and incubated overnight. Cells were then treated with DCA at their IC_{25} or PBS (control) and then maintained in supplemented medium. After 12 hr, some cells were irradiated with 2 Gy. After another 12 hr, cells were trypsinized, washed with 1× PBS, incubated with medium containing JC-1 dye (10 μg/ml) for 20 min at 37°C. In normal cells, due to the electrochemical potential gradient, the dye concentrates in the mitochondrial matrix, where it forms orange fluorescent aggregates. A reaction that affects the mitochondrial membrane potential prevents the accumulation of the JC-1 dye in the mitochondria and, thus, the dye is dispersed throughout the entire cell leading to a shift from orange to green fluorescence. Lastly, the cells were washed and resuspended in 1 ml PBS for fluorescent flow cytometry analysis using FACScan flow cytometer (Becton Dickinson) measuring at least 10,000 gated cells. Mitochondrial depolarization is indicated by a decrease in orange/green fluorescence ratio. All mitochondrial membrane potential analyses were performed in triplicate.

Statistical Analysis

Differences between experimental groups were analyzed for statistical significance using Student's *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS

Treatment With DCA is Associated With Decrease Rates of Cellular Proliferation and Sensitization to Irradiation

Treatment with increasing concentrations of DCA inhibited the proliferation of PC-3-*Bcl-2* and PC-3-Neo cells in a dose dependent manner. The IC_{25} values of DCA were 1 and 0.5 mM, respectively, for PC-3-*Bcl-2* and PC-3-Neo cells (Fig. 1A,B). These results were

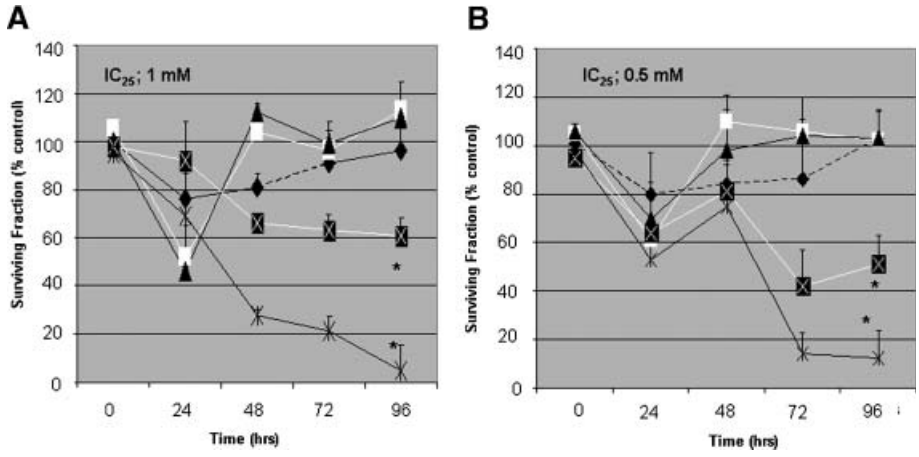

Fig. 1. DCA inhibits prostate cancer cell proliferation. PC-3-*Bcl-2* and PC-3-Neo cells were treated with DCA (0.01–100 mM) or phosphate-buffered saline (PBS) (control). DCA inhibited cellular proliferation in a dose-dependent manner in both cell lines (□, 0.01 mM; Δ, 0.1 mm; ◇, 1 mM; ×, 10 mM; *, 100 mM). Ninety-six hours after DCA, 10 (×) and 100 (*) mM concentrations in both PC-3-*Bcl-2* and PC-3-Neo cells significantly inhibited cellular proliferation ($P < 0.05$). The concentrations of DCA that produced 25% inhibition of cell growth (IC_{25}) were calculated to be 1 mM in PC-3-*Bcl-2* and 0.5 mM in PC-3-Neo prostate cancer cells. Shown here are the representative dose-response curves for DCA in each cell line. Each assay was performed in triplicate for two experiments. Results were confirmed with the crystal violet exclusion test.

confirmed by crystal violet exclusion test. The IC_{25} concentrations determined above for DCA were then used to evaluate possible radiosensitizing effects in a clonogenic survival assay. In four experiments, the clonogenic response of PC-3-*Bcl-2* and PC-3-Neo cells was next evaluated after the treatment of DCA or PBS (control) followed by irradiation. Both cell lines were relatively resistant to radiation alone; however, after controlling for plating efficiencies, PC-3-*Bcl-2* proved to be more resistant to irradiation. In experiments in which cells were irradiated to a total dose of 2 Gy alone (Control), the surviving fraction (SF) was 75% of PC-3-*Bcl-2* compared with 64% of PC-3-Neo cells (Fig. 2). In experiments in which cells were pretreated with DCA and then irradiated to 2 Gy, relatively more PC-3-*Bcl-2* cells survived (65%, reduction of 10% compared to no DCA pretreatment, $P = 0.02$) than did PC-3-Neo cells (27%, reduction of 37% compared to no DCA pretreatment, $P = 0.001$) (Fig 2A,B). Thus, DCA sensitizes previously radiation resistant cells to the killing effects of irradiation.

DCA Treatment Affects Cell-Cycle Distribution

When compared with PBS treatment (control), irradiation alone caused a G2M phase arrest in both PC-3-*Bcl-2* and PC-3-Neo cells. In comparison, DCA alone produced no significant change in cell cycle in either in PC-3-*Bcl-2* or PC-3-Neo cells. Treatment with DCA in combination with irradiation did not produce a significant change in cell-cycle over irradiation alone (Fig. 3A,B).

Bcl-2 Family and Apoptotic Marker Are Affected by DCA Treatment

In PC-3-*Bcl-2* cells, DCA did not alter *Bcl-2* or *Bcl-xl* expression, whereas irradiation resulted in diminished expression of both *Bcl-2* and *Bcl-xl*. Combination therapy with DCA and irradiation did not reduce expression of *Bcl-2* and *Bcl-xl*. *Bak* expression was virtually unchanged in PC-3-*Bcl-2* cells treated with DCA, radiation or combination. In PC-3-*Bcl-2* cells, *Bax* expression was reduced by DCA treatment; interestingly, combination therapy increased *Bax* protein levels. Of note, total *PARP* expression was not changed in cells treated with DCA alone or irradiation alone. Though combinational therapy of DCA and radiation was associated with radiation sensitization as seen on clonogenic assay (Fig. 2) and increased expression of *Bax*, total PARP level was also increased (Fig. 4). Thus it is feasible that the cells succumbing to irradiation are undergoing a cellular death not associated with apoptosis.

In PC-3-Neo cells, DCA decreased the expression of *Bcl-2* and *Bcl-xl* expression, whereas irradiation resulted in an increase expression of *Bcl-2* and *Bcl-xl*. Compared to radiation alone, combination therapy with DCA and irradiation reduced expression of *Bcl-2* and *Bcl-xl*. *Bak* expression was slightly increased in PC-3-Neo cells treated with DCA alone, irradiation alone or combination. *Bax* expression was unchanged in cells treated with DCA alone, and irradiation alone, however in cells treated with both DCA and irradiation, *Bax* expression was increased. *PARP* expression

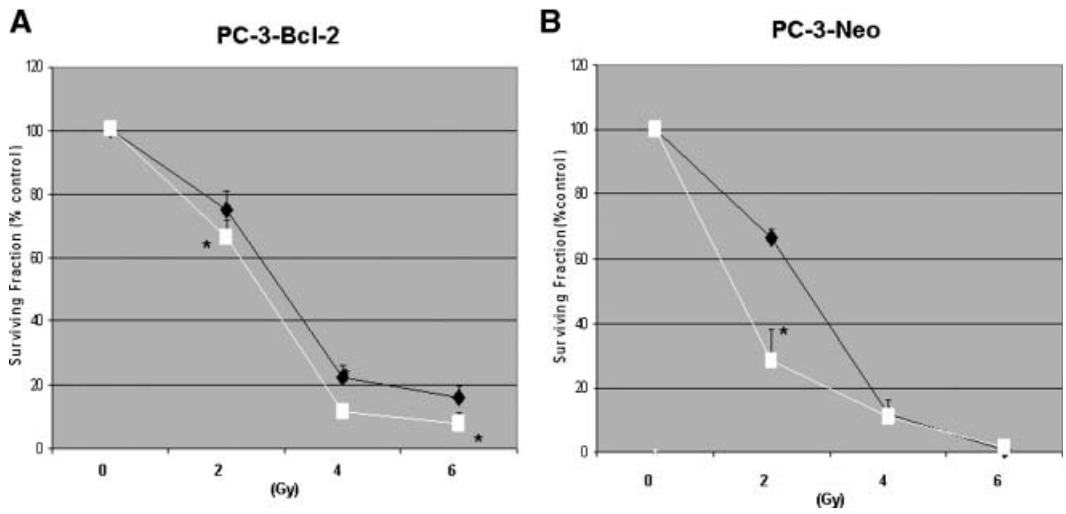

Fig. 2. Clonogenic survival of prostate cancer cells treated with DCA and irradiation. PC-3-*Bcl-2* (**A**) and PC-3-Neo (**B**) prostate cancer cells were treated first with DCA (solid white line) or PBS (control) (solid black line). Twenty-four hours later flasks were irradiated to a total of 0, 2, 4, or 6 Gy, and then replated for a clonogenic survival assay. Each experiment was performed in triplicate, and the mean SF for all three experiments was calculated. Error bars represent ± SEM. After controlling for plating efficiencies, PC-3-*Bcl-2* cells proved to be more radiation resistant than PC-3-Neo cells. Treatment with DCA sensitized both PC-3-*Bcl-2* and Pc-3-Neo cells to irradiation. *$P < 0.05$ compared with PBS (control).

was increased in PC-3-Neo cells after exposure to DCA or irradiation; whereas, combination therapy with DCA and irradiation decreased total *PARP* protein levels, signifying total *PARP* was undergoing cleavage resulting in apoptosis (Fig. 4).

DCA Alters Mitochondrial Membrane Potential

Next, we studied mitochondrial membrane potential ($\Delta\Psi_m$) in both PC-3-*Bcl-2* and PC-3-Neo prostate cancer cell lines (Fig. 5). PC-3-*Bcl-2* cells proved to possess a significantly higher $\Delta\Psi_m$ of the two lines ($P = 0.009$). When compared with PBS treatment (control), DCA alone lowered $\Delta\Psi_m$ in both PC-3-*Bcl-2*, $P < 0.05$. The DCA effects on mitochondrial $\Delta\Psi_m$ occurred within 10 min after exposure in both cell lines and were dose dependent (data not shown). In comparison, irradiation alone increased $\Delta\Psi_m$ in PC-3-*Bcl-2* cells ($P = 0.02$) whereas irradiation alone decreased $\Delta\Psi_m$ in PC-3-Neo cells ($P = 0.04$). Interestingly combinational therapy of DCA and irradiation was associated with a reduction of $\Delta\Psi_m$ compared to irradiation alone in PC-3-*Bcl-2* and PC-3-Neo.

DISCUSSION

DCA is one of many organohalides to which humans have been chronically exposed. Environmental sources of DCA include chlorinated drinking water [19–21] and groundwater contamination by certain industrial solvents and other chlorinated precursors [22]. Evidence suggests that DCA is a potential health hazard since rodents administered DCA at supratherapeutic concentrations developed hepatotoxicity and neoplasia [23], common side effects of our current chemotherapeutic agents. Interestingly,

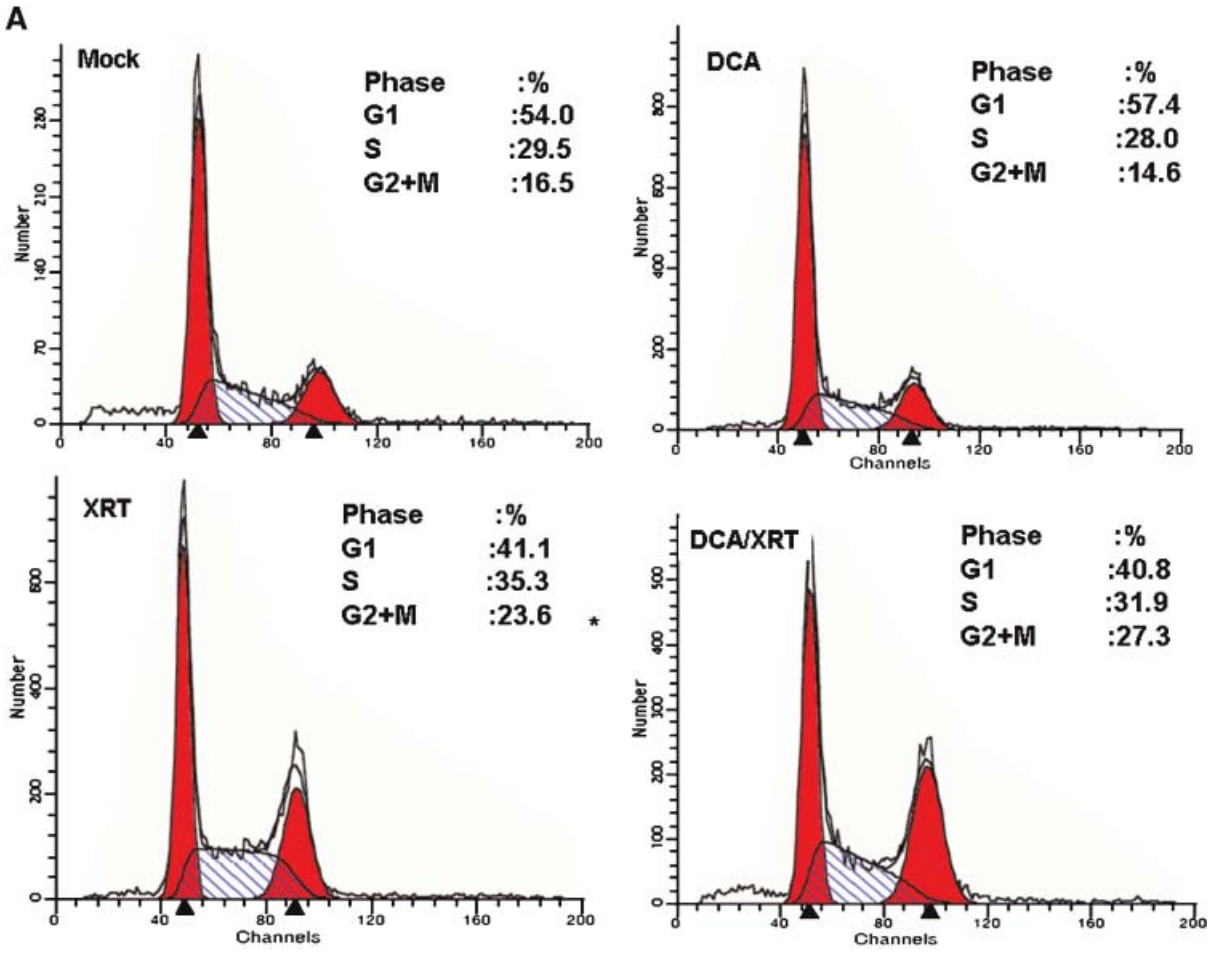

Fig. 3. Flow cytometric analysis of PC-3-*Bcl-2* and PC-3-Neo cells stained for DNA content. Cells were treated with DCA (**A**, 1 mM in PC-3-*Bcl-2* and **B**, 0.5 mM in PC-3-Neo) or PBS (control) for 12 hr followed by irradiation with 0 or 2 Gy. Twelve hours later cells were harvested, processed and subjected to flow cytometry. Experiments were performed in triplicate. The figure depicts the results of one experiment. Exposure of PC-3-*Bcl-2* and PC-3-Neo cells to DCA was not associated with a significant change in cell cycle distribution while radiation was associated with a significant G2M block in both cell lines. (* denotes significant change in phase of cell cycle compared to CTL, where $P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

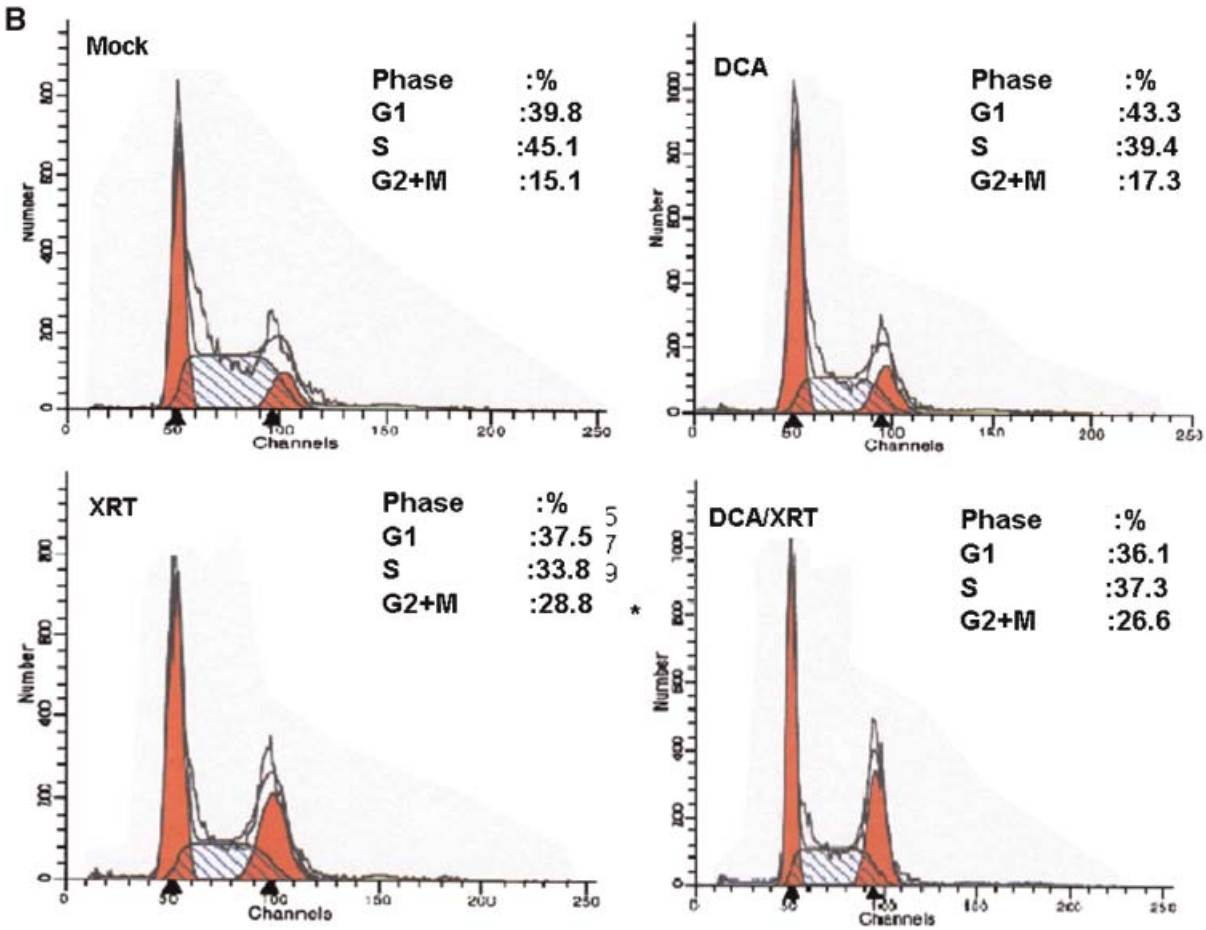

Fig. 3. (*Continued*)

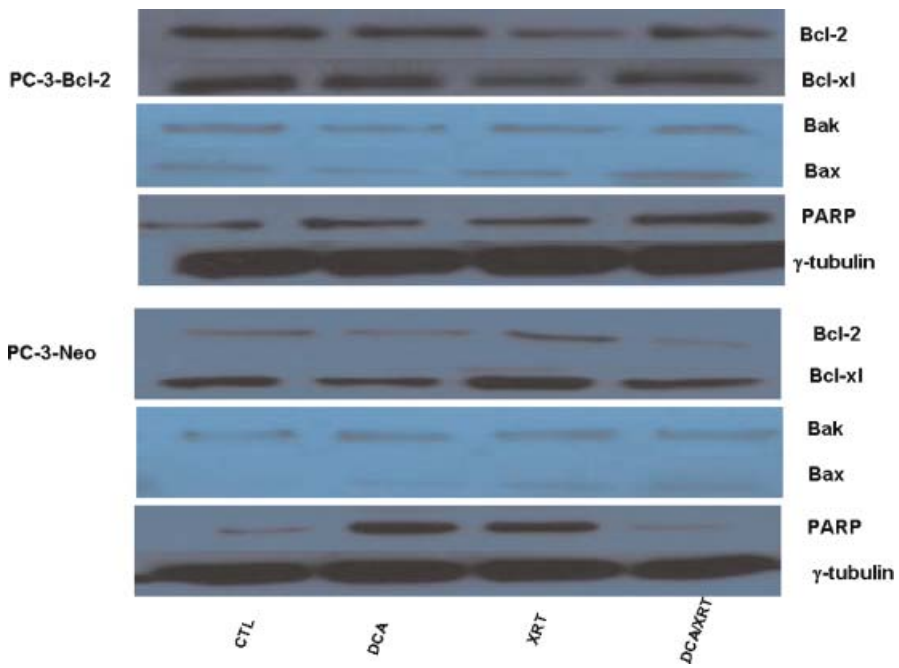

Fig. 4. Western blot analysis of *Bcl-2*, *Bcl-xl*, *Bak*, *Bax*, and *PARP* expression in prostate cancer cell lines treated with DCA. PC-3-*Bcl-2* and PC-3-Neo prostate cancer cells were treated with DCA and irradiation (2 Gy) as per Materials and Methods Section. In both cell lines, DCA alone was not associated with changes in *Bcl-2* or *Bcl-xl* expression, while *Bak* expression was increased in PC-3-Neo. On the other hand, radiation was associated with increased *Bcl-2* and up regulated Bax expression in both PC-3-*Bcl-2* and PC-3-Neo reduction in total *PARP* was evident only in PC-3-Neo treated with the combination of DCA and irradiation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

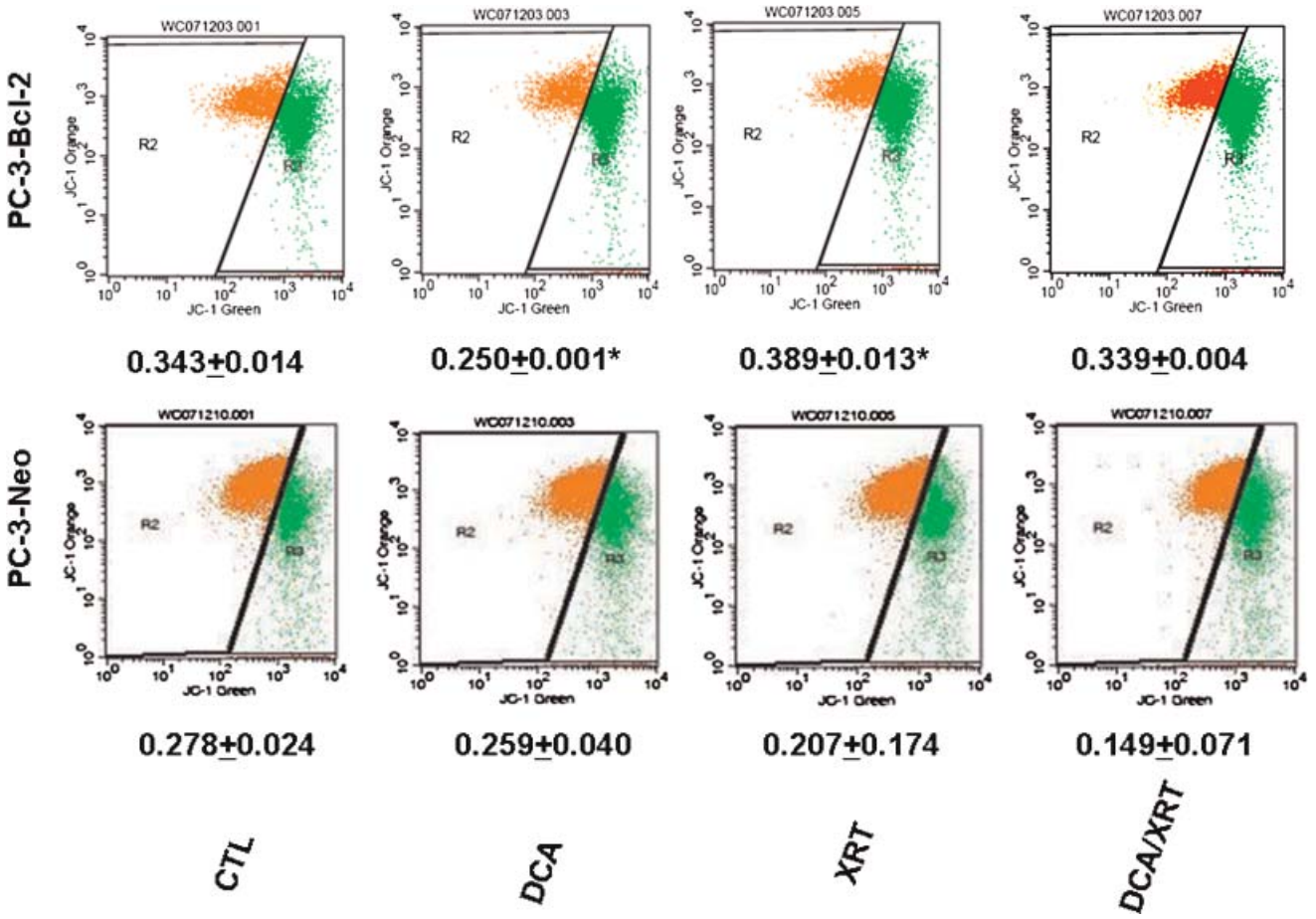

Fig. 5. Flow cytometric analysis of changes of mitochondrial membrane potential ($\Delta\Psi_m$) in prostate cancer cells treated with DCA. Cells were treated with DCA or PBS (control) for 12 hr followed by irradiation with 0 or 2 Gy. Twelve hours later, cells were subjected to media containing JC-1 dye for 20 min at 37°C. Next cells were harvested, washed and suspended in PBS for fluorescent flow cytometry analysis. Experiments were performed in triplicate. The figure depicts the results of one experiment. Exposure of PC-3-*Bcl-2* cells to DCA was associated with a significant reduction in $\Delta\Psi_m$ compared to CTL. (* denotes significant change in $\Delta\Psi_m$ compared to CTL, where $P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DCA has been administered orally and parenterally for decades as an investigational drug for the treatment of numerous cardiovascular and metabolic disorders. However, several randomized controlled trials utilizing DCA in adults or children with lactic acidosis either demonstrated no clinical benefit or was stopped early do to significant neurotoxicity [24–26].

The interest of DCA in cancer therapeutics hinges on that fact that cancer cells generally utilize glycolysis rather than oxidation for energy (the Warburg effect) [15]. Glycolysis leads to tumor hypoxia which in turn stimulates a panel of cell survival genes that enable the tumor to grow and thrive [15]. However, limited studies have addressed the usage of DCA in cancer therapeutics. Compared to normal cells, several human cancer cell lines have high $\Delta\Psi_m$ and low expression of the K + channel Kv1.5, both thought to contribute to apoptosis resistance in cancer cells. DCA can inhibit mitochondrial PDK thus shifting metabolism from glycolysis to glucose oxidation which increases mitochondrial H_2O_2 leading to release of cytochrome *c* preferentially in cancer cells. Thus, DCA induces apoptosis, decreases proliferation, and inhibits tumor growth, with minimal toxicity. Molecular inhibition of PDK by siRNA strategy produced effects similar to DCA administration [16].

We report here the first study using DCA in combination with irradiation for the treatment of prostate cancer. We demonstrate that both human prostate cancer cells with or without overexpression of *Bcl-2*,could be rendered sensitive to the killing effects of irradiation by DCA. The molecular mechanism thought to be responsible for this sensitization to radiation is related to the *Bcl-2* family. In this regard, the *Bcl-2* family can induce (pro-apoptotic members, e.g., *Bax*, *Bak*, *Bad*) or inhibit (anti-apoptotic members, e.g., *Bcl-2*, *Bcl-xl*, *Mcl-1*) the release of cytochrome *c* into the cytosol, which subsequently activates caspase-9 and

caspase-3, leading to apoptosis (programmed cell death) [27,28]. In this study, we demonstrated that the combination of DCA and irradiation led to increase expression of *Bax*, which in PC-3-Neo cells resulted in increased rates of apoptosis. Lastly to confirm the results of Bonnett and others [16], we demonstrated that DCA resulted in a change in $\Delta\Psi_m$ which correlated to the induction of apoptosis in PC-3-Neo prostate cancer cells. Though PC-3-*Bcl-2* prostate cancer cells were sensitize to irradiation by DCA, this was not associated with increased rates of apoptosis. Thus we speculate that the *Bcl-2* expressing tumor cells may succumb to the effects of radiation by a mechanism other than apoptosis.

Previously our group demonstrated the importance of *Bcl-2* overexpression in human prostate cancer cells. Specifically cells that were engineered to overexpress *Bcl-2* were more resistant to chemotherapy and to radiation therapy [17,29–31]. However therapy aimed to down regulate *Bcl-2* proved to sensitize these cancer cells to these conventional therapies. In addition, human prostatic tumors that overexpressed *Bcl-2* were more prone to fail radiation therapy [32]. This concept was definitively addressed when Pollack and others reported that men with high *Bcl-2* expression or low *Bax* expression on prostate biopsies had a lower rates of biochemical disease free survival [33]. Thus the importance of targeting *Bcl-2* family is obvious. To date however, limited neoadjuvant chemotherapy or molecular targeted trials have been conducted in humans.

With the recent success of such targeted agents as sorafenib, sunitinib, Bevacizumab, and Imatinib Mesylate in advanced human tumors, it is time to utilize these and similar agents earlier in the disease process. Even with androgen deprivation therapy and external beam radiation therapy, a high risk prostate cancer has a 5-year biochemical failure rate of $>30\%$ [34,35]. To further improve survival results, a multimodality approach that combines systemic chemotherapy or targeted therapy with local therapy seems warranted. Such a multimodality approach could take the form of neoadjuvant therapy (i.e., therapy before surgical resection) or adjuvant therapy (i.e., therapy after surgical resection), which over the past several decades has been studied extensively in many types of cancer. Molecular markers may be assessed in tumors treated with neoadjuvant therapies. Future regimens may be formulated based on changes of these molecular parameters.

Radiotherapy is a popular treatment modality for localized prostate cancer. However molecular signatures exist that render the cell radiation resistant. In PC-3 prostate cancer cells, we demonstrated that DCA can significantly reduce cellular proliferation and sensitize cells to the killing effects of radiation. Furthermore, cells not expressing *Bcl-2* were demonstrated to undergo marked apoptosis when treated with radiation and DCA. DCA may prove to be a promising selective anticancer agent. Obviously the usage of DCA in cancer therapeutics is still in its infancy and requires methodical preclinical and clinical evaluation.

ACKNOWLEDGMENT

Support from American Cancer Society student scholar program (S.Y.) is gratefully acknowledged.

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