

Enhancement of radiotherapy by oleandrin is a caspase-3 dependent process

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Abstract

Cardiac glycosides such as digitoxin and ouabain have previously been shown to be selectively cytotoxic to tumor as opposed to normal cells. Moreover, this class of agents has also been shown to act as potent radiosensitizers. In the present study we explored the relative radiosensitization potential of oleandrin, a cardiac glycoside contained within the plant extract known as Anvirzel™ that recently underwent a Phase I trial as a novel drug for anticancer therapy. The data show that oleandrin produces an enhancement of sensitivity of PC-3 human prostate cells to radiation; at a cell survival of 0.1, the enhancement factor was 1.32. The magnitude of radiosensitization depended on duration of exposure of cells to drug prior to radiation treatment. While a radiosensitizing effect of oleandrin was evident with only 1 h of cell exposure to drug, the effect greatly increased with 24 h oleandrin pretreatment. Susceptibility of PC-3 cells to oleandrin and radiation-induced apoptosis was dependent on activation of caspase-3. Activation was greatest when cells were exposed simultaneously to oleandrin and radiation. Inhibition of caspase-3 activation with Z-DEVD-FMK abrogated the oleandrin-induced enhancement of radiation response suggesting that both oleandrin and radiation share a caspase-3 dependent mechanism of apoptosis in the PC-3 cell line. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Cardiac glycosides are a class of natural products that have been traditionally used to increase cardiac contractile force in patients suffering from congestive heart failure. These agents have the ability to inhibit Na⁺, K⁺-ATPase, which results in modification of Na⁺, K⁺ and Ca⁺⁺ ion fluxes in cells. Specifically, cardiac glycosides maintain elevated intracellular K⁺

and a decreased intracellular Na⁺ compared to extracellular fluid. There are, however, suggestions that cardiac glycosides may also play a role in the treatment of cancer [1,2]. In a recent publication, Stenkvist [2] noted that breast carcinoma patients who were on digitalis medication at the time of cancer diagnosis had significantly better response to anticancer therapy and better overall survival than breast cancer patients who were not taking digitalis. The antitumor efficacy exhibited by the glycosides is supported by studies showing that these agents can be selectively cytotoxic to tumor cells in vitro [3]. Among these agents is an extract from *Nerium oleander*, designated Anvirzel™.

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It contains oleandrin, a cardiac glycoside whose structure is similar to that of other cardiac glycosides. We observed that oleandrin induces apoptosis in human but not in murine tumor cell lines [4], inhibits activation of NF- κ B [5], and mediates cell death through a calcium-mediated release of cytochrome *c* [6]. A Phase I trial of Anvirzel™ has been completed recently [7] and plans are being made for Phase II studies.

In addition to being selectively cytotoxic for tumor cells, cardiac glycosides may also enhance cell response to cytotoxic actions of ionizing radiation. The glycoside ouabain was reported to enhance in vitro radiosensitivity of A549 human lung adenocarcinoma cells but was ineffective in modifying radioresponse of normal human lung fibroblasts [8]. Ouabain was subsequently shown to radiosensitize human tumor cells of different histology types including squamous cell carcinoma and melanoma [9]. Although the mechanisms of ouabain-induced radiosensitization are still not fully explained, inhibition of repair from sublethal radiation damage and an increase in radiation-induced apoptosis have been advanced as possibilities [8–10].

Because oleandrin is a potent inducer of apoptosis in human cancer cells as reported by others [8,9] and by our group [6], and because other cardiac glycosides were reported to enhance the response of tumor cells to irradiation [8–11], the present study was undertaken to investigate whether oleandrin possesses the ability to induce radiosensitization in human tumor cells. In addition, we sought to determine if specific components of apoptosis pathways were involved in the radiosensitization effect.

2. Experimental

2.1. Cell culture

The human prostate carcinoma cell line PC-3 was obtained from the American Type Culture Collection and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 10,000 U/ml of penicillin–streptomycin, and 2 mM L-glutamine. Cells were grown as monolayers in 75-cm² flasks and maintained in a humidified 5% CO₂/95% air atmosphere at 37°C.

2.2. Clonogenic survival

Cells in culture were exposed to oleandrin (0.05 and 0.025 μ g/ml) for 24 h and in some experiments, the cells were pretreated with an inhibitor of caspase-3, Z-DEVD-FMK (10.0 μ M, 2 h) (Calbiochem, San Diego, CA) before oleandrin was added to the culture. The cells were then irradiated with graded doses (2, 4 or 6 Gy) of γ -rays using a ¹³⁷Cs source (3.7 Gy/min) and assayed for colony forming ability by replating them in specified numbers into 100-mm dishes containing drug-free media. After 12 days of incubation, cells were stained with 0.5% crystal violet in absolute ethanol, and colonies with >50 cells were counted. Radiation survival curves were plotted after normalizing for cytotoxicity induced by oleandrin alone. Clonogenic survival curves were constructed from at least three independent experiments. The average survival levels were fitted by least squares regression using a linear quadratic model [12].

2.3. Apoptosis assay by flow cytometry

The terminal deoxynucleotidyltransferase (TdT) dUTP nick end labeling (TUNEL) assay to identify the DNA fragmentation (APO-DIRECT kit, Pharmingen, San Diego, CA) was performed according to the manufacturer's instructions. Briefly, cells (2×10^6) were fixed in 1% paraformaldehyde and washed in PBS. Cells were suspended in 70% ethanol and stored at –20°C until use. Re-suspended cells were stained in a solution containing TdT and FITC-dUTP and incubated overnight at room temperature in the dark. They were then rinsed and re-suspended in 0.5 ml propidium iodide/RNase A solution and analyzed by flow cytometry.

2.4. Fluorescent staining of apoptotic cells

Cells were plated in chamber slides. After the cells were treated with irradiation (2 Gy) or oleandrin (0.05 μ g/ml) or both, they were fixed in 4% paraformaldehyde, washed with PBS and stained with 10 μ M Hoechst 33258 (Sigma, St. Louis, MO) for 10 min. Stain was then removed and the cells were covered with mounting media (Slowfade, Molecular Probes, Eugene, OR) prior to observation using fluorescence microscopy.

2.5. Western blot analysis

After experimental treatment, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8), 450 mM NaCl, 1% triton X-100, 5 mM EDTA, 1% (v/v) of protease inhibitor cocktail, and 1% (v/v) phosphatase inhibitor cocktails I and II (Sigma, St. Louis, MO). Protein (30–40 mg per lane) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked by 5% non-fat dry milk in Tris-buffered saline and 0.1% Tween-20 (TBS-T) and before incubation with a designated primary antibody (Pharminogen, San Diego, CA). After washing the membrane with TBS-T, it was incubated in secondary antibody and the immunoreaction was visualized using an ECL western blotting kit (Amersham Corp., Arlington Heights, IL).

3. Results

PC-3 prostate carcinoma cells, shown to be sensitive to the cytotoxic action of oleandrin [8], were used to investigate whether oleandrin enhances cell response to radiation. Cells in monolayer cultures were exposed to 0.05 $\mu\text{g/ml}$ oleandrin for 24 h and then irradiated using 2–6 Gy of radiation. They were then assayed for colony forming ability, and survival curves were generated and corrected for cell killing induced by the drug alone. As shown in Fig. 1, treatment with oleandrin resulted in an increased response of PC-3 cells to radiation. The enhancement factor at the cell survival fraction of 0.1 was 1.32. Oleandrin alone reduced colony forming efficiency of PC-3 cells from 95 to 21%. The concentration of oleandrin at 0.025 $\mu\text{g/ml}$, which by itself was non-cytotoxic, had no significant influence on radiosensitization of PC-3 cells (data not presented).

The magnitude of radiosensitization depended on duration of exposure of cells to oleandrin prior to radiation. PC-3 cells were incubated in the presence of 0.05 $\mu\text{g/ml}$ oleandrin for 1–24 h and then exposed to 3 Gy radiation. As shown in Fig. 2, cell exposure to oleandrin for only 1 h enhanced radiation-induced cell death: cell survival after 3 Gy radiation only was 50%

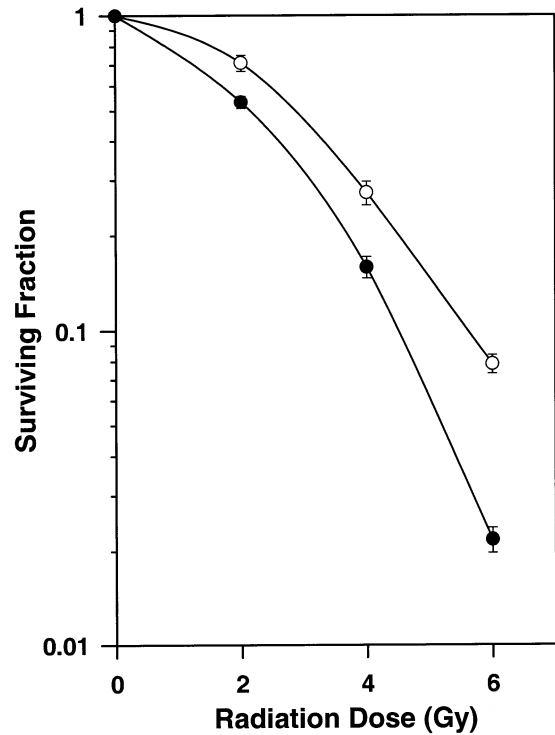


Fig. 1. Effect of oleandrin on radiosensitivity of PC-3 cells in culture. Cells were treated with oleandrin (0.05 $\mu\text{g/ml}$) for 24 h before irradiation then trypsinized and plated in specified numbers. Twelve days later, colonies were counted and survival curves were constructed with normalized values for the cytotoxicity induced by oleandrin alone. (Oleandrin by itself reduced plating efficiency from the 95% control value to 21%.) untreated control (○); Oleandrin treated (●). Values shown are the means \pm SE for three independent experiments.

compared to 30% after 3 Gy radiation plus 0.05 $\mu\text{g/ml}$ oleandrin. The radiosensitizing effect of oleandrin increased as the duration of exposure to oleandrin increased up to 24 h observation period.

Previously, we reported [6] that cytotoxicity after treatment with oleandrin was mediated by apoptosis. To test whether oleandrin enhanced susceptibility to radiation-induced apoptosis, PC-3 cells were incubated with 0.05 $\mu\text{g/ml}$ oleandrin for 24 h and then exposed to 2 Gy. The medium containing oleandrin was removed, fresh medium added and 24 h later, the apoptotic index determined. The controls were untreated cells, and the experimental cells were exposed to oleandrin or irradiation or both. Fig. 3 shows that 2 Gy increased the percentage of apoptotic

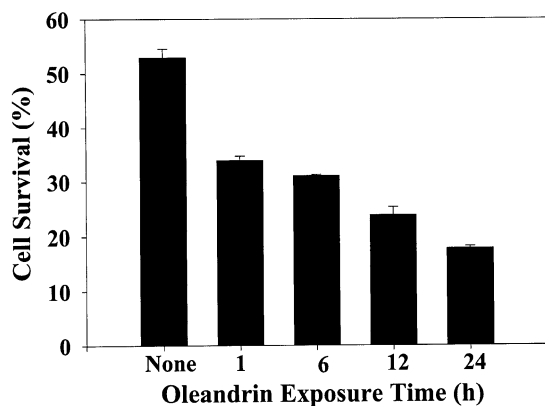


Fig. 2. Time dependent cytotoxic effect of oleandrin on the PC-3 cells. Cells were exposed to oleandrin (0.05 $\mu\text{g/ml}$) for a specified time ranging from 1 to 24 h. After oleandrin treatment, cells were irradiated (3 Gy) and plated for clonogenic survival assay. Colonies were counted after 12 days and percent surviving fractions were plotted. Values shown are means \pm SE for three independent experiments.

cells from the control value of 2.1 ± 0.4 to $8.2 \pm 3.4\%$, oleandrin increased it to $63.5 \pm 6.1\%$, and the combination of oleandrin and 2 Gy increased it to $85.8 \pm 5.3\%$. The latter was more than the additive effects of oleandrin and 2 Gy when given as individual treatments. In addition, the control cells and the experimental cells were stained with a fluorescent

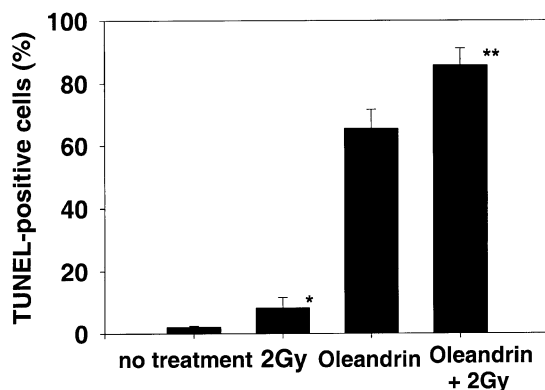


Fig. 3. Induction of apoptosis by oleandrin in PC-3 cells. Cells were exposed to oleandrin (0.05 $\mu\text{g/ml}$) for 24 h or irradiation (2 Gy) or both. Apoptosis was determined by flow cytometry analysis of TUNEL-stained cells. The data shown are mean \pm SE for three independent experiments. *Radiation (2 Gy) alone compound compared to no treatment; $P \leq 0.005$; **Oleandrin + 2 Gy compared to oleandrin alone; $P \leq 0.005$.

dye, Hoechst 33258, that binds to fragmented DNA. As shown in Fig. 4 apoptotic cells were induced by the above treatments with more apoptotic cells appearing in the oleandrin plus radiation group than in any other treated or control cell population.

To determine whether caspase-3 activation was involved in the oleandrin-induced enhancement of PC-3 cell radioresponse, cells were treated with 5 Gy or 0.05 $\mu\text{g/ml}$ oleandrin or a combination of both treatments for 24 h. Cells were assayed for caspase-3 activation 24 h after treatment using western blotting. As shown in Fig. 5, radiation (5 Gy) alone was ineffective whereas oleandrin was effective in activating caspase-3. However, the level of activation was higher when the two agents were combined, indicating that oleandrin made PC-3 cells susceptible to radiation-induced activation of caspase-3. If caspase-3 activation was responsible for the oleandrin-induced enhanced radiosensitivity of PC-3 cells, we hypothesized that treatment with caspase-3 inhibitors should reduce or abolish the radioenhancement. To test this, PC-3 cells were incubated with 0.05 $\mu\text{g/ml}$ oleandrin for 24 h before being irradiated with 2 or 6 Gy. Z-DEVD-FMK, an inhibitor of caspase-3, at a dose of 100 μM was added 2 h before oleandrin. Clonogenic cell survival was then determined. The results in Table 1 show that the inhibitor of caspase-3 abolished the oleandrin-induced enhancement of radiation response. However, the inhibitor alone did not significantly affect the radioresponse of PC-3 cells.

4. Discussion

The results presented here show that oleandrin, in addition to being cytotoxic to PC-3 human prostate carcinoma cells, has the ability to enhance the sensitivity of these cells to cytotoxic action by ionizing radiation. The enhancement of radiosensitivity was observed over a range of doses of 2–6 Gy γ -radiation. The magnitude of the enhancement was by a factor of 1.32, calculated at a 0.1 level of the cell survival (Fig. 1), and was similar to enhancement values reported for ouabain [8]. Oleandrin-induced radiosensitization was dependent on the duration of drug exposure to cells prior to irradiation. Radiosensitization was achieved by incubating cells with the drug for only 1 h but increased with an increase in incubation time during

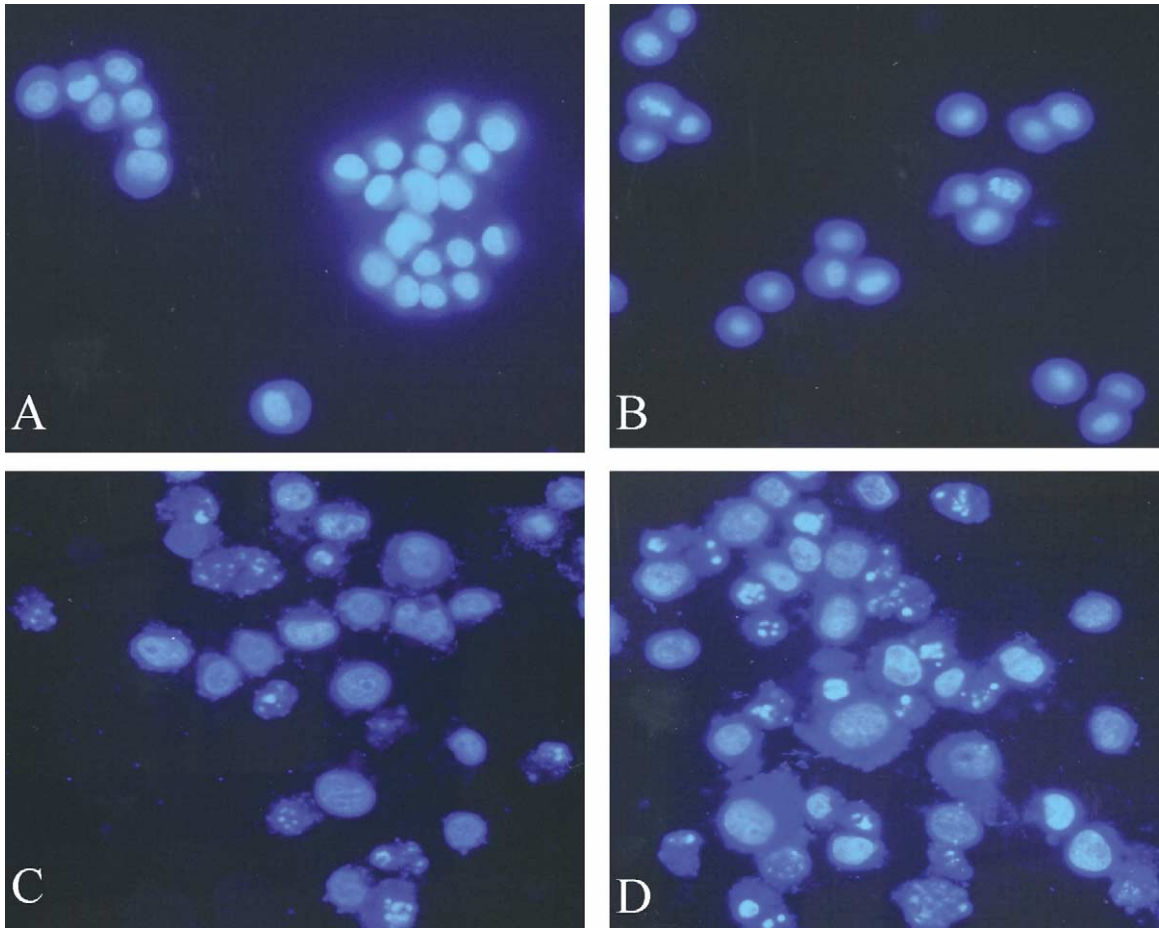


Fig. 4. Visualization of apoptosis induced by oleandrin. PC-3 cells were plated on chamber slides and exposed to oleandrin (0.05 $\mu\text{g/ml}$) for 24 h or irradiation (5 Gy) or both. Cells were stained with Hoechst 338258 and observed under a fluorescent microscope. (A) Untreated control, (B) irradiated (5 Gy), (C) oleandrin (0.05 $\mu\text{g/ml}$, 24 h) and (D) irradiated after oleandrin exposure.

the 24-h test period. In studies of ouabain-induced radiosensitization, enhancement was achieved when the drug was given either before or shortly after irradiation [8–11].

Our study also addressed mechanistic interactions between oleandrin and radiation that might underlie the observed radioenhancement. Because we previously found that oleandrin kills PC-3 tumor cells via apoptosis [4,6], we specifically investigated whether oleandrin treatment makes tumor cells more susceptible to radiation-induced apoptosis. We observed that pretreatment of PC-3 cells with oleandrin significantly increased their sensitivity to apoptosis induction by radiation (Fig. 3). Our earlier study

showed that oleandrin-mediated apoptosis in PC-3 cells was associated with early release of cytochrome *c* from mitochondria, followed by proteolytic processing of caspase-3. In concordance with those findings, results of the present study showed that treatment with oleandrin decreased procaspase-3 expression and this was associated with a concomitant increase in activated caspase-3 expression (Fig. 5). Treatment with radiation alone did not induce any measurable amount of activated caspase-3 suggesting that other caspases may be involved in the effect of radiation on the survival of these cells. Or, the effect of radiation may be mediated by non-apoptotic cell death mechanisms. However, co-treatment of cells with both oleandrin

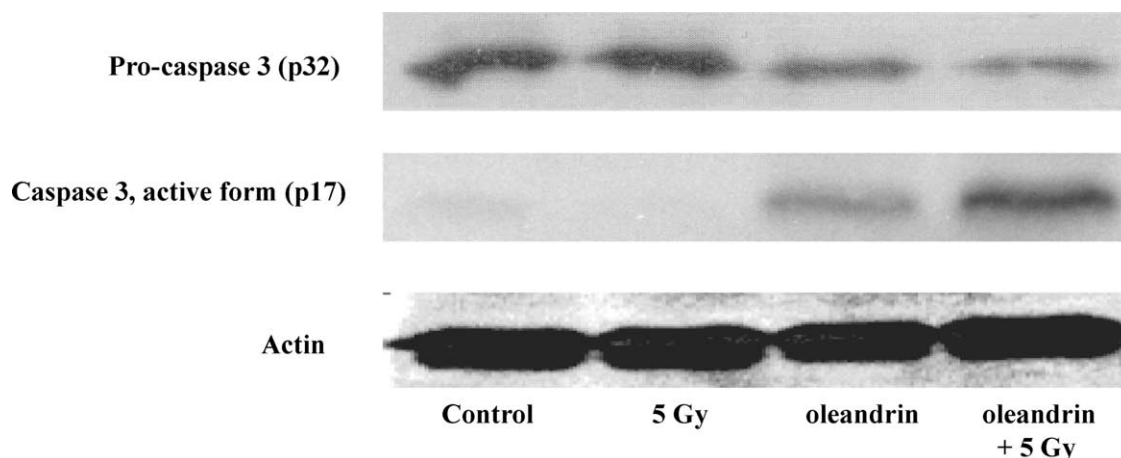


Fig. 5. Expression of procaspase-3 and caspase 3 proteins by Western blot analysis. After oleandrin (0.05 $\mu\text{g/ml}$, 24 h) or irradiation (5 Gy) treatments or both, cells were lysed and immunoblot analysis was performed as described in Section 2.

and radiation produced an even greater formation of caspase-3, suggesting that this particular component of apoptosis may be common to both types of treatment. Further supportive evidence that caspase-3 activation plays a crucial role in oleandrin-induced enhancement of cell radiosensitivity was provided through the use of a caspase-3 inhibitor (Table 1) that abolished oleandrin-induced enhancement of PC-3 cell radiosensitivity. Taken together, the data suggest that oleandrin enhances radioresponse of tumor cells through rendering cells more sensitive to radiation-induced apoptosis.

At the molecular level, the cell death process involved activation of caspase-3, the inhibition of which abrogated the radiosensitizing ability of oleandrin. Induction of caspase-3 activity by cardiac glycosides has been previously shown to be associated with

inhibition of the exchange of Na^+ and K^+ though binding to the enzyme Na^+ , K^+ -ATPase. Of interest is the fact that tamoxifen which has also been shown to inhibit the enzyme Na^+ , K^+ -ATPase (albeit at a different site on the enzyme than cardiac glycosides) has also been shown to act as a radiosensitizer to human tumor cells [13,14]. Inhibition of this important enzyme therefore appears to represent not only an uncommon means of inducing apoptosis not typically shared by established cytotoxic agents used for the treatment of malignant disease, but also a means of selectively sensitizing cancer cells to irradiation.

Induction of apoptosis was also found to play a mechanistic role in ouabain-induced enhancement of radiosensitization of a number of human tumor cell lines [11]. Interestingly, this radioenhancement was independent of the mutational status of p53, an onco-

Table 1
Effect of caspase 3 inhibitor on oleandrin-induced radiosensitivity^a

Treatment	Survival of cells (%) following radiation of		
	0 Gy	2 Gy	6 Gy
Control	100	74.6 \pm 3.4	7.8 \pm 0.6
Oleandrin	88.9 \pm 2.9	55.5 \pm 1.9*	3.1 \pm 0.8*
Z-DEVD-FMK	97.4 \pm 1.7	77.8 \pm 3.2	8.6 \pm 2.1
Z-DEVD-FMK + oleandrin	104 \pm 1.6	70.4 \pm 2.2	6.2 \pm 0.8

^a PC-3 cells were exposed to oleandrin (0.05 $\mu\text{g/ml}$, 24 h) with or without pretreatment with the caspase-3 inhibitor, Z-DEVD-FMK. They were then exposed to 2 or 6 Gy of radiation and replated for assessment of clonogenic survival. Colonies were counted and the survival of cells (percent control) was calculated. Data are presented as mean \pm SE from three experiments. * $P < 0.05$ compared to control cells.

gene involved in induction of apoptosis. Other studies showed that the ouabain-mediated radiosensitization was associated with suppression of cell repair capacity [8,9].

To be of therapeutic value in cancer radiotherapy, any radioenhancing agent must enhance tumor cell radioresponse more than in normal cells. Although studies that would provide information on normal cell radioresponse are still non-existent for oleandrin, data are available for the structurally similar cardiac glycoside ouabain, showing that this compound selectively radiosensitizes tumor cells [8,11]. Thus, the observation that cardiac glycosides produce a differential cytotoxic response through injury to malignant but not normal cells [3,8] increases the rationale for combining selected cardiac glycoside therapy with radiation treatment.

Acknowledgements

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