British Journal of Cancer (2003) 89, 746 – 753
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1α,25-Dihydroxyvitamin D_3 (calcitriol) and its analogue, 19-nor-1α,25$(OH)_2D_2$, potentiate the effects of ionising radiation on human prostate cancer cells

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Radiotherapy with external beam radiation or brachytherapy is an established therapeutic modality for prostate cancer. Approximately 30% of patients with localised prostate cancer relapse at the irradiated site. Secondary effects of ionising radiation (IR), for example, bowel and bladder complications, are common. Thus, the search for biological response modifiers that could potentiate the therapeutic effects of radiation and limit the occurrence of serious side effects is an important task in prostate cancer therapy. 1α,25-Dihydroxyvitamin D_3 (calcitriol), the active metabolite of vitamin D, and its analogues are under investigation for the treatment of several malignancies including prostate cancer. Here, we report that 1α,25-dihydroxyvitamin D_3 and its less calcaemic analogue 19-nor-1α,25-$(OH)_2D_2$ (Zemplar®) act synergistically with IR to inhibit the growth of the human prostate cancer cells *in vitro*. 1α,25-dihydroxyvitamin D_3 potentiated IR-induced apoptosis of LNCaP cells, and nanomolar doses of 1α,25-dihydroxyvitamin D_3 and 19-nor-1α,25-$(OH)_2D_2$ showed synergistic inhibition of growth of LNCaP cells at radiobiologically relevant doses of IR (1–2 Gy). At higher doses of IR, the combination of 1α,25-dihydroxyvitamin D_3 and IR or 19-nor-1α,25-$(OH)_2D_2$ and IR resulted in moderate antagonism. The synergistic effect at radiobiologically relevant doses of radiation suggests that a combination of 1α,25-dihydroxyvitamin D_3 or 19-nor-1α,25-$(OH)_2D_2$ with IR could permit a reduction in the dose of radiation given clinically and thus potentially reduce treatment-related morbidity.

British Journal of Cancer (2003) **89,** 746 – 753. doi:10.1038/sj.bjc.6601161 www.bjcancer.com
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Keywords: Zemplar; vitamin D; radiosensitizer; synergistic

Adenocarcinoma of the prostate is the most commonly diagnosed nonskin cancer and the second leading cause of cancer death in men in the United States (Keely and Gomella, 1998; Garnik and Fair, 1998; Jemal *et al*, 2003). Most men will present with clinically localised disease but a significant minority will present with locally advanced disease. In patients with locally advanced disease (T3 – 4) the rates of local control with radiation therapy alone have been reported to range from 60 to 77% (Shipley *et al*, 1995). In a recent Phase III trial in men with locally advanced prostate cancer, the rate of local regional control in the radiation-alone arm was 83% (Bolla *et al*, 2002). Since this trial did not require prostate biopsies following treatment, the rate of loco-regional control is likely an overestimate.

It is clear that in men with locally advanced disease, optimal local control with radiation therapy has not been achieved. There is some evidence that higher radiation doses may improve local control, but higher doses lead to increased morbidity, especially rectal bleeding (Pollack *et al*, 2002). Whether used for cure or palliation, side effects of RT are common and include adverse effects on urinary, bowel and sexual function (Hamilton *et al*, 2001). Evidence also exists suggesting that the cells that survive the initial RT are the ones most likely to repopulate the irradiated area and metastasise (Fuks *et al*, 1991). Thus, agents or methods that could potentiate the effects of ionising radiation would be very desirable. Ideally, such agents would permit a reduction in the dose of radiation administered and thereby, a potential reduction in the incidence and/or severity of side effects.

The steroid hormone 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), also known as calcitriol, the active metabolite of vitamin D, and its synthetic analogues are currently under intensive investigation for several malignancies, including prostate cancer (Campbell and Koeffler, 1997). Based on the observation that the descriptive epidemiology of prostate cancer resembles that of vitamin D insufficiency, Schwartz and Hulka (1990) proposed that vitamin D maintained the differentiated phenotype of prostate cells and that low levels of vitamin D increase the risk for prostate cancer. Many subsequent epidemiologic (Ahonen *et al*, 2000; Luscombe *et al*, 2001) and laboratory studies (Feldman *et al*, 2000) have supported this hypothesis. For example, 1α,25$(OH)_2D_3$ and its

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Received 3 March 2003; revised 22 May 2003; accepted 4 June 2003

analogues exhibit significant growth-inhibitory, proapoptotic, anti-invasive and antimetastatic effects on prostate tumour cell lines *in* vitro and *in vivo* (Miller *et al*, 1992; Peehl *et al*, 1994; Schwartz *et al*, 1995, 1997; Skowronski *et al*, 1995; Campbell *et al*, 1997; Asou *et al*, 1998; Lokeshwar *et al*, 1999; Blutt *et al*, 2000; Seol *et al*, 2000). These studies support the potential use of $1\alpha,25(OH)_2D_3$ and its analogues as therapeutic agents for prostate cancer (reviewed in Blutt and Weigel, 1999).

The principal limitation of $1,25(OH)_2D_3$ administration is the risk of hypercalcaemia. Consequently, many synthetic analogues of $1,25(OH)_2D_3$ have been developed with the goal of developing analogues that exhibit similar or enhanced growth-inhibitory and antimetastatic properties with reduced calcaemic effects (Schwartz and Hulka, 1990) (see also Hansen *et al* (2001) for a review). Prominent vitamin D analogues include EB1089 (Seocalcitol), which is currently in Phase III trials in Europe for hepatocellular carcinoma, 1-α-vitamin D_2 and 19-nor-$1\alpha,25$-$(OH)_2D_2$ (Zemplar), a $1,25(OH)_2D_3$-analogue that is FDA-approved for the treatment of secondary hyperparathyroidism. Laboratory studies have demonstrated that the antiproliferative effects of 19-nor-$1\alpha,25$-$(OH)_2D_2$ on prostate cancer cells are indistinguishable from those of $1\alpha,25(OH)_2D_3$ (Chen *et al*, 2000).

In addition to its use as monotherapy, calcitriol and analogues may prove useful in conjunction with other treatment modalities, notably, ionising radiation. Observations that support combining $1,25(OH)_2D_3$ with ionising radiation include the following: (a) both IR and $1,25(OH)_2D_3$ induce apoptosis in prostate carcinoma cells by apparently distinct pathways (Welsh *et al*, 1995; Billis *et al*, 1998; James *et al*, 1998; Pirianov *et al*, 1999; Ding and Fisher, 2001; McGuire *et al*, 2001; Sheard, 2001; Liu *et al*, 2002); (b) other agents that induce cellular differentiation, for example, phenylacetate, platelet-derived growth factor and retinoic acid, are known to enhance the cytotoxic effects of IR (Bill *et al*, 1992b; Miller *et al*, 1997; Hoffmann *et al*, 1999); (c) EB 1089 has been shown to enhance the radiation sensitivity of breast carcinoma cells (Sundaram and Gewirtz, 1999). These findings strongly suggest that $1,25(OH)_2D_3$ may enhance the cytotoxic effects of IR on prostate cancer cells. In this report, we investigated the effects of combined treatments of $1\alpha,25(OH)_2D_3$ and 19-nor-$1\alpha,25$-$(OH)_2D_2$ with IR on the apoptosis and growth of the human prostate cancer cell line LNCaP, as well as on the growth of a human primary prostatic cell strain obtained from patients who had undergone radical prostatectomy.

MATERIALS AND METHODS

Reagents

$1\alpha,25(OH)_2D_3$ was purchased from Biomol, Inc. (Plymouth Meeting, PA, USA). 19-nor-$1\alpha,25(OH)_2D_2$ was a generous gift from Abbott Laboratories (Chicago, IL, USA). Both were dissolved in 100% ethanol and kept at -80°C until use. Final concentration of EtOH in the media was 0.1% ($V\,V^{-1}$).

Cell culture

The LNCaP cell line was purchased from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 supplemented with 10% foetal calf serum (FCS). Primary human epithelial cell cultures from histologically normal prostatic peripheral zones or prostate cancer were obtained from radical prostatectomies performed at Wake Forest University School of Medicine as previously described (Peehl and Stamey, 1986; Peehl *et al*, 1988; Barreto *et al*, 2000). Briefly, a small piece of tissue from each specimen is removed and minced. The tissue is digested with collagenase overnight. To remove the collagenase and the majority of the stromal cells, the tissue is rinsed and centrifuged. The collagenase-digested tissue is inoculated into a 60-mm tissue culture dish coated with collagen type I (Collagen Corporation, Palo Alto, CA, USA) and grown in medium PFMR-4A supplemented with growth factors and hormones (Peehl *et al*, 1988). Previous studies have demonstrated that prostatic stromal cells do not grow in the serum-free conditions used in this study, yet these conditions maintain the growth and differentiation of prostatic epithelial cells (Peehl and Stamey, 1986; Peehl *et al*, 1988). The cells that grew out from the tissue were aliquoted and stored in liquid nitrogen. The histology of each specimen was verified by inking and fixing the prostate after dissection and serially sectioning the marked area. The histology of sections immediately adjacent to the area of the dissection was reviewed and classified as histologically normal or cancerous by a trained pathologist. The frozen aliquots were thawed to produce secondary cultures, which were grown in medium MCDB 105 (Sigma, St Louis, MO, USA) supplemented with growth factors and hormones (Peehl and Stamey, 1986).

Irradiation

LNCaP cells were plated in 24-well plates at densities of 5000 cells per well. Primary cells were plated onto 35 mm dishes at a cell density of $1 \times 10^4\,\text{dish}^{-1}$. At the end of preincubation with $1\alpha,25(OH)_2D_3$ or 19-nor-$1\alpha,25(OH)_2D_2$, cells were irradiated in a ^{137}Caesium irradiator (Shepherd and Associates) at a dose rate of $472\,\text{rad}\,\text{min}^{-1}$. Following IR, the cells were returned to the incubator and were allowed to grow for another 12–14 days, before assessment of viability as described below.

Apoptosis assay

Cells were plated in 60 mm plastic dishes at a density of 2×10^5 cells per plate 24 h prior to treatment. Following treatments, determination of the number of apoptotic cells was assayed. Cells with apoptotic morphology are determined by incubating cells with Bisbenzimide (Hoechst 33342) dye and propidium iodide (PI), both at a concentration of $2\,\mu\text{g}\,\text{ml}^{-1}$ for 10 min. Cells with apoptotic morphology (fragmented nuclei and PI-positive cells) were counted in each of four fields randomly selected in the dish and expressed as a ratio to total number of cells. Both attached and floating cells were counted. The results represent the average of three independent experiments ($\pm$s.e.m.).

Cellular proliferation assays

Two types of cellular proliferation assays were employed. For assessing human prostatic cell strain proliferation, trypan-blue exclusion was used as a marker of viable cells 9–12 days after treatments. The WFU10Ca strain cells were plated onto 35 mm dishes and treated with $1\alpha,25(OH)_2D_3$ or ethanol, € EtOH). After 24 h later, cells were irradiated as described above. To assess the effects of $1\alpha,25(OH)_2D_3$ or 19-nor-$1\alpha,25(OH)_2D_2$ on LNCaP cell proliferation, 5000 cells were plated onto 24-well plates and treated with $1\alpha,25(OH)_2D_3$ or 19-nor-$1\alpha,25(OH)_2D_2$. Cells were irradiated 24 h later. After 10–12 days, cell viability was assessed by the MTT assay according to the manufacturer's protocol. A volume of 200 μl aliquots from each reaction was transferred into a 96-well plate and absorbance values were measured with an automated plate reader (Molecular Dynamics, Sunnyvale, CA, USA). Each experiment was performed in triplicate.

Isobologram analysis of combination treatments

The isobologram analysis was performed using the CalcuSyn software (Biosoft, Cambridge, UK). The analysis is based on the median-effect principle and the median effect equation by Chou (1974): $f_a/f_u = (D/D_m)^m$, where D is the dose of the drug; D_m is the median-effect dose; f_a is the fraction affected by the dose, f_u the

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Table 1 Combination Index (CI) values and Dose Reduction Index (DRI) for combined treatments of vitamin D and IR (**A**) or Zemplar and IR (**B**)

	Mutually nonexclusive CI for experimental values				Dose Reduction Index (DRI)	
	IR (Gy)	f_a	CI (point #)	Degree of syn./antag.	Vitamin D	IR
(A) Vitamin D (nM)×						
0.1	1	0.34	0.85 (1)	+	3.77	2.18
1	2	0.60	0.57 (2)	+++	9.46	2.36
10	4	0.74	0.76 (3)	++	6.70	1.89
100	4	0.75	2.43 (4)	−−−	0.79	1.96
(B) Zemplar (nM)					Zemplar	
0.1	1	0.45	1.27 (1)	−−	2.08	1.88
1	2	0.79	0.45 (2)	+++	10.81	3.05
10	4	0.89	0.54 (3)	+++	7.85	2.75
100	4	0.86	4.22 (4)	−−−−	0.39	2.23

CI values for each combination treatment at doses shown on the left were calculated with the CalCuSyn software as described in Materials and Methods. CI values correspond to points shown in the isobolograms depicted in Figure 3. The DRI for each drug:IR combination is a measure of how much (-fold) the dose of a drug or agent (e.g. IR), in synergistic combination may be reduced at a given effect level compared with the dose of each drug alone. The DRI is another mathematical interpretation of the CI, and $CI = 1/(DRI)_1 + 1/(DRI)_2$. +++++, very strong agonism; −−−−− very strong antagonism.

fraction unaffected by the dose, $f_u = 1 - f_a$; and m is an exponent signifying the sigmoidy (shape) of the dose–effect plot.

The Combination Index (CI) (Table 1) for multiple drug combinations is calculated based on the equation and plot by Chou and Talalay:

$$CI = \sum_{j=1}^{n} \frac{(f_a)_j}{(f_u)_j}$$

where n = number of drugs.

In the simplest form, a CI < 1, = 1 and > 1 indicate synergism, additive effect and antagonism, respectively. Chou and Talalay (1984) also recommend a scale based on the value of CI and using symbols ranging from + + + + + for very strong agonism to −−−−− for very strong antagonism. The CI values calculated from our experiments follow this scale.

RESULTS

To test whether $1\alpha,25(OH)_2D_3$ affects the apoptotic response of LNCaP cells to IR, cells were treated with vehicle (0.1% EtOH) or with 100 nM $1\alpha,25(OH)_2D_3$. After 48 h, the cells were either irradiated with 4 Gy IR or were mock-irradiated. Cells with apoptotic morphology (fragmented nuclei and PI-positive cells) were counted. Figure 1 shows photographs from representative populations at ×100 and ×250 magnifications. Treatment with $1\alpha,25(OH)_2D_3$ or IR alone did not induce substantial apoptosis of LNCaP cells, as is evident from the absence of cells with fragmented and/or condensed nuclei in this representative field. However, pretreatment with $1\alpha,25(OH)_2D_3$ for 48 h followed by IR resulted in a significant increase in the percentage of cells with apoptotic morphology (indicated by white arrows). Figure 1B shows the cumulative results obtained by counting cells with apoptotic *vs* normal morphology in four randomly selected fields from three independent experiments. Although $1\alpha,25(OH)_2D_3$ and IR induced only modest levels of apoptosis (13 and 17%, respectively) compared to basal levels (5%), pretreatment of LNCaP cells with $1\alpha,25(OH)_2D_3$ for 48 h prior to IR resulted in 48% apoptosis. These results indicate that pretreatment of LNCaP cells with $1\alpha,25(OH)_2D_3$ prior to IR sensitises these cells to IR-induced apoptosis.

While apoptotic assays can be useful in determining the short-term effects of agents on cells, they may not reflect the long-term effects of these agents on cellular proliferation. To investigate the effects of $1\alpha,25(OH)_2D_3$ and 19-nor-$1\alpha,25(OH)_2D_2$ on long-term proliferation of prostate cells, we treated LNCaP cells with different doses of the hormones and IR, and assessed cell viability 8 days later using the MTT assay. As seen in Figure 2A and B, both agents induced a dose-dependent decrease in the survival of LNCaP cells. An isobologram analysis of the data revealed that at lower doses of drug and IR, a significant but modest synergistic effect is observed (Figure 3A and B). At increased concentrations of drug (100 nM) and IR (4 Gy) (point 4 in isobolograms), the two agents exert antagonistic effects on proliferation. These results suggest that the combination of low nanomolar doses of $1\alpha,25(OH)_2D_3$ or 19-nor-$1\alpha,25(OH)_2D_2$ with low doses of IR may have beneficial effects *in vivo* for the treatment of prostate cancer.

We also wanted to test the effects of $1\alpha,25(OH)_2D_3$ on the response of primary prostate tumour cells to IR. Previously, it was shown that $1\alpha,25(OH)_2D_3$ inhibited the growth of primary prostatic cells in a dose-dependent manner (Peehl *et al*, 1994; Barreto *et al*, 2000). We first tested a range of doses of $1\alpha,25(OH)_2D_3$ for its ability to inhibit cell growth in a variety of normal and cancer-derived cell clones (data not shown). A dose of 1 nM had only a minimal effect on the growth of all strains tested. We next tested the effects of combined treatments of 1 nM $1\alpha,25(OH)_2D_3$ with increasing doses of IR. As shown in Figure 4 for a cancer-derived strain (WFU10Ca), IR treatments caused a dose-dependent decrease in cell proliferation. Pretreatment of these cells with 1 nM $1\alpha,25(OH)_2D_3$ caused a significant reduction in cell proliferation at 1 and 2 Gy, but it failed to further increase the inhibitory effect of $1\alpha,25(OH)_2D_3$ at a dose of 4 Gy. Therefore, similar to the results obtained with LNCaP cells, radiation and $1\alpha,25(OH)_2D_3$ act synergistically to inhibit cell proliferation at low doses of drug and IR.

A desired effect of radiation sensitisers/enhancers is a differential cytotoxic profile between tumour and normal cells. Thus, we also investigated the effects of the combined treatments of $1\alpha,25(OH)_2D_3$ and IR on the proliferative capacity of normal prostatic stromal cells, which are of fibroblastic origin and thus provide a good model for normal tissue injury. These cells were pretreated with various doses of $1\alpha,25(OH)_2D_3$ for 48 h, after which they were irradiated with doses of 1 and 2 Gy. Table 2 shows the results of selected treatment combinations for these primary normal cells and the corresponding data for LNCaP cells. These data show that with the exception of the combined dose of 1 nM $1\alpha,25(OH)_2D_3$ and 2 Gy, which produced the highest levels of synergy for both the normal and LNCaP cells, the stromal cells were substantially more resistant to combined treatments than LNCaP cells. Furthermore, even at the synergistic combination of 1 nM $1\alpha,25(OH)_2D_3$ and 2 Gy, the overall inhibitory effect on

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stromal cell proliferation was lower than that observed for LnCaP cells, as evident by the affected fraction (F_a) values (0.4 *vs* 0.6, respectively).

DISCUSSION

$1\alpha,25(OH)_2D_3$ and its analogues have shown synergistic activities with a variety of chemotherapeutic agents, including cisplatin and doxorubicin. In breast cancer cells, $1\alpha,25(OH)_2D_3$ and EB1089 were reported to enhance the effects of ionising radiation, although the lack of isobologram analysis in that study does not permit an assessment of a synergistic or additive effect (Sundaram and Gewirtz, 1999). Here, we demonstrate for the first time that in a prostate tumour cell line and primary tumour cells, $1\alpha,25(OH)_2D_3$ and 19-nor-$1\alpha,25(OH)_2D_2$ act synergistically with IR to inhibit the growth of these tumour cells.

The synergistic effects of these agents with IR are manifested as an increase in apoptotic index of treated LNCaP cells, and as a decreased proliferation in longer-term assays in LNCaP and primary prostate cells. However, since the synergistic effect on apoptosis was observed with high doses of IR and $1\alpha,25(OH)_2D_3$, a combination that was antagonistic in longer-term assays, our results suggest that apoptosis is probably not involved in any synergistic long-term antiproliferative effects of these agents. One possible explanation for these results is the following: inhibition of proliferation by $1\alpha,25(OH)_2D_3$ and its analogues and IR involves multiple mechanisms, of which apoptosis is only one component. Terminal differentiation (or permanent cell cycle arrest) and mitotic (necrotic) death due to unrepaired DNA strand breaks also contribute substantially to decreased proliferation. It is possible that $1\alpha,25(OH)_2D_3$ and IR induce apoptosis via distinct mechanisms and therefore exhibit synergistic activation of apoptosis, but that differentiation and/or necrosis work by mechanisms that are mutually inhibited at higher drug or IR doses. This result is not surprising, since similar drug combinations (e.g., cisplatin and $1\alpha,25(OH)_2D_3$) also appear to produce antagonistic effects at high doses.

Although short-term apoptotic assays do not always demonstrate a good correlation with overall cell survival in *in vitro* assays, apoptosis may play a role *in vivo*. For example, increased apoptosis in a tumour as a result of treatment with a cytotoxic agent may contribute to reoxygenation of previously hypoxic tumour cells, and thus contribute to cytotoxicity of subsequent IR treatments (Brown and Wouters, 1999; 2001). In LNCaP cells, the increased apoptotic index after a 48 h incubation suggests that pretreatment of cells with $1\alpha,25(OH)_2D_3$ lowers the apoptotic threshold of the cells to the subsequent stress of IR. A potential mechanism for this finding involves downregulation of the antiapoptotic protein bcl-2, which has been reported to occur as a result of treatment with $1\alpha,25(OH)_2D_3$ or its analogues (Welsh *et al*, 1995; Simboli-Campbell *et al*, 1997; Narvaez *et al*, 2001).

If an increase in apoptosis does not play a major role in the long-term antiproliferative synergistic interaction between $1\alpha,25(OH)_2D_3$ and its analogues, what other potential mechanisms may be involved in this process? First, calcitriol and its analogues induce cellular differentiation, a process in which a precursor cell acquires morphological and biochemical characteristics of specialised lineage (Studzinski *et al*, 1993). In the processes of tumorigenesis, cells reverse this process and become *less* differentiated (i.e., they dedifferentiate). Cells undergoing differentiation appear to be more sensitive to ionising radiation than

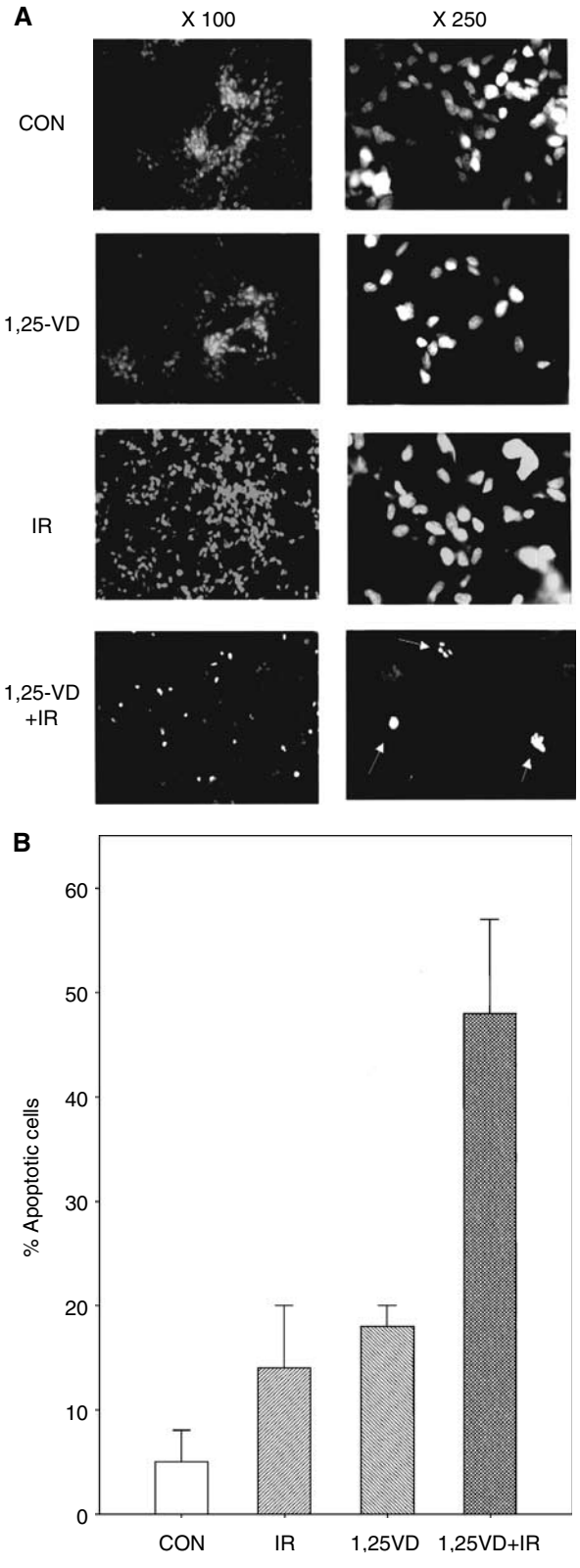

Figure 1 Pretreatment of LNCaP cells with $1\alpha,25(OH)_2D_3$ sensitises LNCaP cells to IR-induced apoptosis. (**A**) Photographs depict representative populations from the different treatment groups at × 100 and × 250 magnifications. Cells with apoptotic morphology appear with brighter stained nuclei (× 100 magnification). In the × 250 magnification, apoptotic cells are indicated by white arrows in the 1,25VD + IR treated group. (**B**) Cells were treated with ethanol (control) or 100 nM $1\alpha,25(OH)_2D_3$ for 48 h prior to treatment with 4 Gy IR and apoptotic cells were counted 12 h after IR. The apoptotic cells from four randomly selected fields were counted (between 50 and 150 cells field^{-1}). Three independent experiments were performed. Error bars represent ± s.e. values.

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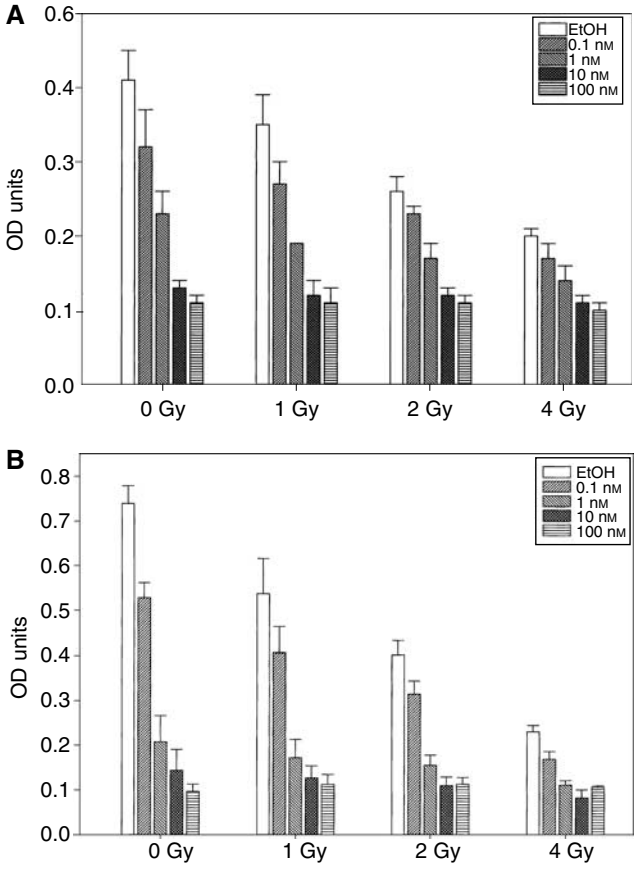

Figure 2 Dose–response effects of $1\alpha,25(OH)_2D_3$ (**A**) or 19-nor-$1\alpha,25(OH)_2D_2$ (**B**) alone, or combined with IR on the growth of LNCaP prostate tumour cells. Cells were plated in medium and drugs were added 24 h later. Cells were irradiated 24 h following addition of $1\alpha,25(OH)_2D_3$ or 19-nor-$1\alpha,25(OH)_2D_2$ and allowed to grow for an additional 7 days, at which time MTT assay was performed as described in Materials and Methods. Optical density (OD) values represent the average of four readings per experiment. Each experiment was performed three times. Error bars represent s.e. values.

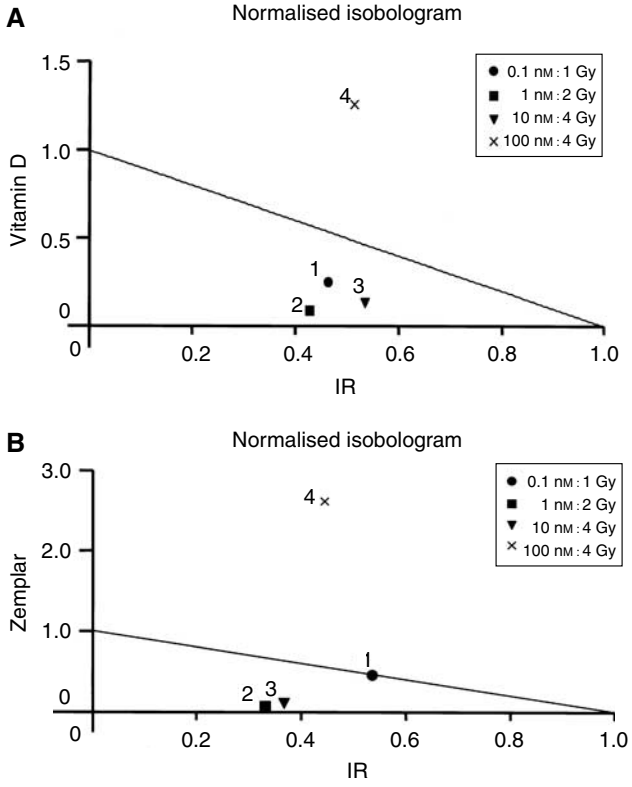

Figure 3 Isobologram plots for vitamin D and IR (**A**) or Zemplar and IR (**B**) combinations. Shown are normalised isobolograms indicating the equipotent combinations of the drugs at IC_{50} doses. As nonconstant ratios of drug : IR doses were used, the drug and IR concentrations on the isobol are normalised by the corresponding IC_{50} doses. Individual points refer to the specific dose ratios of combinations of drug : IR, indicated in the legend. If a combination data point for $f_a = 0.5$ falls on the diagonal, an additive effect is indicated; on the lower left, synergy is indicated; on the upper right, antagonism is indicated. For actual CI values and degrees of synergism or antagonism, see Tables 1 and 2.

cells that have a more undifferentiated phenotype. For instance, using a murine 3T3-T proadipocyte cell line, Bill *et al* have demonstrated that although both undifferentiated and differentiated cells sustain similar levels of DNA damage when irradiated, undifferentiated cells exhibit higher levels of DNA repair (Bill *et al*, 1992a, b). Similar mechanisms may explain the increased sensitivity of cells treated with the prodifferentiating agents phenylacetate and phenylbutyrate (Miller *et al*, 1997). Indeed, calcitriol has been shown in earlier studies to potentiate the cytotoxic effects of chemotherapeutic agents by inducing cell differentiation (Ravid *et al*, 1999). Second, treatment of the human prostate cells LNCaP and PC-3 with calcitriol results in cell cycle arrest, primarily at the G_1/S interphase, while a G_2/M arrest has also been reported (Eisman *et al*, 1989; Godyn *et al*, 1994; Zhuang and Burnstein, 1998). As shown by work with synchronised cell cultures, with the exception of the M phase of the cell cycle (where cells in culture are the most radiosensitive), the majority of cell lines show the greatest sensitivity to ionising radiation in late G_1/early S phase and during the G_2/M transition (Nias, 1988), and are the most radioresistant during early G_1 and S phases. Therefore, it is reasonable to expect that pretreatment of prostate cancer cells with $1\alpha,25(OH)_2D_3$ and 19-nor-$1\alpha,25(OH)_2D_2$ will increase their sensitivity to IR by inducing the entry of the majority of the cells in these two radiosensitive phases.

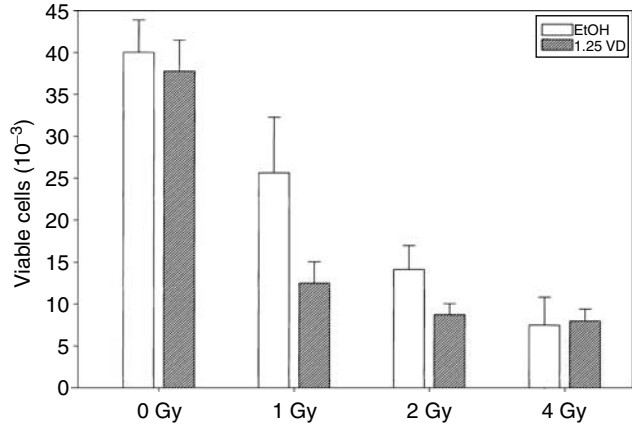

Figure 4 Effects of combined treatments of $1\alpha,25(OH)_2D_3$ and IR on primary prostate cells. Cells were isolated from patients who underwent radical prostatectomy at WFU as described in Materials and Methods. Cells were plated into 35 mm dishes and treated with 1 nM $1\alpha,25(OH)_2D_3$. After 24 h, cells were irradiated in a caesium irradiator with the indicated doses and returned to 37°C for growth for an additional 10–12 days. At the end of treatment, cells were trypsinised and counted. Control cells received only 0.1% ethanol. Experiments were performed in triplicate. The example shown is from the WFU10Ca, a cancer-derived primary strain. Similar results were obtained with two other cancer-derived and three normal prostate-derived strains.

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Table 2 Combination Index (CI) values, for combined treatments of vitamin D and IR in LNCap cells and normal prostatic stromal cells

	Mutually nonexclusive CI for experimental values				
	Vitamin D (nM)	**IR (Gy)**	f_a	**CI**	**Degree of syn.antag.**
LNCaP	0.1	2	0.45	0.77	++
	1	1	0.54	0.53	+++
	1	2	0.6	0.58	+++
	10	1	0.70	0.47	+++
Normal	0.1	2	0.27	1.135	−−
stromal cells	1	1	0.3	1.60	−−
	1	2	0.4	0.527	+++
	10	1	0.39	0.925	+

CI values for each combination treatment at doses shown on the left were calculated with the CalcuSyn software as described in Materials and methods. +++++, very strong agonism; −−−−− very strong antagonism.

Regardless of the mechanism(s) of synergy, our data show that $1\alpha,25(OH)_2D_3$ and 19-nor-$1\alpha,25(OH)_2D_2$ (which are both currently in clinical trials for the treatment of advanced prostate cancer) exhibit significant levels of synergy with one of the most widely used therapeutic modalities for prostate cancer, IR. Importantly, this potentiation occurs at low doses of both drug and IR, which fall well within the range of clinically relevant doses. For example, radiotherapy protocols for prostate cancer patients routinely employ multiple daily fractions of IR at 1.5–2 Gy per treatment for a total dose to the prostate between 64 and 72 Gy (Perez, 1997). As shown in our isobolograms, greatest synergism occurs at the IR doses of 2 Gy. Furthermore, the doses of $1\alpha,25(OH)_2D_3$ and 19-nor-$1\alpha,25(OH)_2D_2$ that exhibit synergism are in the low nanomolar range. Intracellular levels of $1\alpha,25(OH)_2D_3$ in the prostate have not been characterised. However, it is now clear that these levels likely exceed by several fold the levels of $1\alpha,25(OH)_2D_3$ in the systemic circulation (picomolar levels (Platz *et al*, 2000)). This is because both normal and cancerous prostate cancer cells express 1-α-hydroxylase, which enables these cells to convert the prohormone 25-OHD_3, which circulates at nanomolar concentrations, into $1\alpha,25(OH)_2D_3$, intraprostatically (Schwartz *et al*, 1998). Prostate cells that express 1-α-hydroxylase are growth inhibited by 25-OHD_3 at nanomolar concentrations (Barreto *et al*, 2000). Therefore, both $1\alpha,25(OH)_2D_3$ and 19-nor-$1\alpha,25(OH)_2D_2$ are capable of acting synergistically with IR at physiological doses, at least *in vitro*. Animal experiments will be required in order to validate these concepts *in vivo*.

Another conclusion of our study is that at higher doses of $1\alpha,25(OH)_2D_3$ and 19-nor-$1\alpha,25(OH)_2D_2$ and IR, antagonism occurs. This finding is in agreement with similar studies using $1\alpha,25(OH)_2D_3$, cisplatin and carboplatin (Moffatt *et al*, 1999). Although the mechanism(s) for this antagonistic effect is unknown, it is well established that the synergy or antagonism between two drugs with different modes of action is largely dependent upon the drug concentrations and ratios. If these findings extend to *in vivo* models, they would suggest that careful dosage and patient monitoring must be employed if a combination of $1\alpha,25(OH)_2D_3$ or 19-nor-$1\alpha,25(OH)_2D_2$ with IR is administered clinically.

The experiments with the primary cell cultures indicate that 1 nM $1\alpha,25(OH)_2D_3$ can also sensitise primary prostatic cells (both tumour and normal) to IR treatments. This finding supports the potential use of $1\alpha,25(OH)_2D_3$ and Zemplar as IR-response modifiers *in vivo*, since these cell strains retain most of the morphological and biochemical features of human prostatic tissue. Conversely, the ability of certain combinations of these agents to sensitise normal prostatic tissue to the effects of IR raises the possibility of increased radiation-induced adverse effects (i.e., prostatitis) in surrounding normal prostatic tissue. However, the data also suggest that at least *in vitro*, combinations of $1\alpha,25(OH)_2D_3$ and IR exist that might preferentially sensitise tumour but not normal prostate cells. Ultimately, such effective drug combinations will need to be established experimentally in either animal tumour models of prostate cancer before these agents are administered for therapeutic purposes in the clinic. Finally, it is possible that in a clinical setting, potential damage to normal tissue might be mitigated by brachytherapy in which radioactive 'seeds' are delivered locally to the tumour tissue, which would minimise the exposure of surrounding normal tissue to radiation.

The isobologram analysis and DRI of the effects of these agents on LNCaP cells also suggest that it may be possible to decrease the dose of IR and/or $1\alpha,25(OH)_2D_3$ and 19-nor-$1\alpha,25(OH)_2D_2$ in combined modalities, without sacrificing efficacy. This finding has important clinical implications because the incidence of morbidity caused by RT increases with radiation dose (Perez, 1997). For instance, the DRI value of 2.4 for IR in the combination of $1\alpha,25(OH)_2D_3$ (1 nM) and IR (2 Gy) indicates that to achieve the same efficacy level in the absence of $1\alpha,25(OH)_2D_3$, one would have to give 2.4 times higher dose of IR. In other words, the presence of $1\alpha,25(OH)_2D_3$ allows for a 2.4 times lower dose of IR to obtain the same therapeutic effect. Conversely, the 9.5 DRI value for $1\alpha,25(OH)_2D_3$ indicates that at this combination of doses, the presence of IR allows for almost a 10-fold decrease in the level of $1\alpha,25(OH)_2D_3$ to achieve the same therapeutic effect. As noted above, one of the limiting factors of administration of $1\alpha,25(OH)_2D_3$ is hypercalcaemia. Our results show that by combining the two modalities it may be possible to reduce the levels of $1\alpha,25(OH)_2D_3$ or19-nor-$1\alpha,25(OH)_2D_2$ without a significant compromise in efficacy, which would minimise the risk of hypercalcaemia.

In summary, we have shown that $1\alpha,25(OH)_2D_3$ and its less calcaemic analogue 19-nor-$1\alpha,25(OH)_2D_2$ show synergistic effects when combined with radiation treatments. Both these drugs are approved for clinical use and both are presently under clinical investigation as monotherapy in prostate cancer. These findings have implications for limiting the disability (e.g., rectal and bladder injury) induced by radiation therapy. Future studies will focus on the *in vivo* interactions between these treatment modalities (using LNCaP xenograft tumour models) for the treatment of prostate cancer.

ACKNOWLEDGEMENTS

We thank Christine Naczki for excellent technical assistance, Dr Robert Lee (Wake Forest University School of Medicine) and Dr David Gewirtz (Medical College of Virginia, Richmond, VA, USA) for helpful discussions and Abbott Laboratories (Chicago, IL, USA) for their generous supply of Zemplar®. This work was supported in part by Developmental funds (PUSH Grant) from the Comprehensive Cancer Center of Wake Forest University.

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