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Synergistic inhibition of prostate cancer cell lines by a 19-*nor* hexafluoride vitamin D_3 analogue and anti-activator protein 1 retinoid

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Summary The secosteroid hormones, all-*trans*- and 9-*cis*-retinoic acid and vitamin D_3, have demonstrated significant capacity to control proliferation in vitro of many solid tumour cell lines. Cooperative synergistic effects by these two ligands have been reported, and it is, therefore, possible that greater therapeutic effects could be achieved if these compounds were administered together. The role of retinoid-dependent anti-activator protein 1 (anti-AP-1) effects in controlling cancer cell proliferation appears significant. We have utilized an anti-AP-1 retinoid [2-(4,4-dimethyl-3,4-dihydro-2H-1 benzopyran-6-yl)carbonyl-2-(4-carboxyphenyl)-1,3,-dithiane; SR11238], which does not transactivate through a retinoic acid response element (RARE), and a potent vitamin D_3 analogue [1α,25$(OH)_2$-16-ene-23-yne-26,27-F_6-19-*nor* -D_3, code name LH] together at low, physiologically safer doses against a panel of prostate cancer cell lines that represent progressively more transformed phenotypes. The LNCaP (least transformed) and PC-3 (intermediately transformed) cell lines were synergistically inhibited in their clonal growth by the combination of LH and SR11238, whereas SR11238 alone was essentially inactive. DU-145 cells (most transformed) were completely insensitive to these analogues. LNCaP cells, but neither PC-3 nor DU-145, underwent apoptosis in the presence of LH and SR11238. Transactivation of the human osteocalcin vitamin D response element (VDRE) by LH was not enhanced in the presence of SR11238, although the expression of E-cadherin in these cells was additively up-regulated in the presence of both compounds. These data suggest the anti-AP-1 retinoid and the vitamin D_3 analogue may naturally act synergistically to control cell proliferation, a process that is interrupted during transformation, and that this combination may form the basis for treatment of some androgen-independent prostate cancer.

Keywords: vitamin D_3; retinoids; activator protein 1; growth inhibition; prostate cancer

The use of vitamin D_3 analogues in combination with either naturally occurring or synthetic retinoids may form an attractive therapy for androgen-independent metastatic prostate cancer. To investigate this potential, we have examined the effect of a potent vitamin D_3 analogue and a synthetic anti-activator protein 1 (anti-AP-1) retinoid on clonal proliferation and investigated the possible mechanism of the observed cooperative effects.

Prostate cancer has become the most frequently diagnosed, non-skin cancer among American men and the second leading cause of cancer mortality among this group (Parker et al, 1997). Despite the increase in the incidence of the disease, no successful long-term therapies exist if the cancer progresses beyond the prostate capsule. Metastatic growth of prostate cancer tissue may be controlled, and even a long-term remission induced, by blockade of endogenous androgen stimulation. Unfortunately, the disease often re-emerges within a few years in a lethal, poorly differentiated and androgen-independent form. The shortage of curative therapies for such a widespread disease has resulted in a large impetus to develop alternatives therapies. Drugs that have been intensively studied in recent years are biological modifiers of growth, either alone or in combinations to retard cell proliferation (Novichenko et al, 1995; Campbell et al, 1998*a*), promote cell death (Danesi et al, 1994; Welsh, 1994; Li et al, 1995; Planchon et al, 1995) and/or induce differentiation of cells to a terminally mature, non-dividing stage (Samid et al, 1993; Liu et al, 1994; Hseih et al, 1995).

Various potential biological modifiers have been investigated, including the physiologically active metabolites of both vitamins A and D (Niles, 1995; Kantarjian et al, 1996) namely all-*trans*-retinoic acid (ATRA) and its isomer 9-*cis*-retinoic acid (9cRA) and 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$]. We and others have shown that these compounds, at relatively high doses, can inhibit the in vitro growth of cancer cells from several different tissues, including prostate (Norman et al, 1990; Bollag, 1994; Lotan, 1994; Peehl et al, 1994; Feldman et al, 1995; Saunders et al, 1995; Elstner et al, 1996; Campbell et al, 1997*a*).

One route by which vitamin D_3 compounds and retinoids mediate their activity is by binding with specific nuclear receptors; for example 1 α,25$(OH)_2D_3$ interacts exclusively with the vitamin D_3 receptor (VDR); ATRA and 9cRA with the retinoic acid receptors (RAR), and 9cRA also with the retinoid X receptor (RXR) (Mangelsdorf et al, 1995). These nuclear receptors are part of the steroid hormone superfamily and act as ligand-inducible transcription factors. They exert specific cellular effects by binding to hexameric response elements within the promoter/enhancer regions and, thereby, regulating transcription of target genes, some

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of which are associated with inhibition of proliferation, induction of differentiation or apoptosis. Evidence has also emerged that these compounds work together, for example, the promoter of the cyclin-dependent kinase inhibitor (CDKI) ($p21^{waf1}$), which appears to be important for many of the antiproliferative actions of these compounds, has response elements for both $1\alpha,25(OH)_2D_3$ [vitamin D response element (VDRE)] and ATRA [retinoic acid response element (RARE)] (Liu et al, 1996*a*, 1996*b*). Similarly, we (Elstner et al, 1996) and others (Brown et al, 1994; Blutt et al, 1997) have demonstrated synergistic inhibition and differentiation of various cancer cell lines by these drugs. Separate from the transcriptional activation profile of $1\alpha,25(OH)_2D_3$ and ATRA are other effects that appear to be associated with post-transcriptional regulation of protein expression. For example, $1\alpha,25(OH)_2D_3$ has been shown to increase expression of the CDKI $p27^{kipl}$ in HL-60 leukaemic cells by increasing the rate of mRNA translation and extending protein half-life, but not by altering the mRNA level (Hengst and Reed, 1996; Wang et al, 1996). Similar post-transcriptional effects have been attributed to retinoids in the regulation of another CDKI ($p21^{waf1}$) (Schwaller et al, 1993; Li XS et al, 1996).

Steroid hormone receptors also appear to regulate indirectly other transcriptional pathways via a mechanism known as AP-1 repression. Mitogens, such as cytokines, induce dimerization of the AP-1 proteins, for example c-Jun and c-Fos, into an active form which transactivates genes associated with growth. The potential of this pathway to trigger unregulated growth has been realized in cellular transformation involving Fos and Jun oncoproteins. The thyroid and RAR-α receptors participate in the normal regulation of the AP-1 complex by down-regulating c-*fos* expression (Perez et al, 1993). The relationship between these two opposite regulatory pathways allows for fine-tuning of responses with the possibility of synergistic positive and negative effects (Simonson, 1994; Alroy, 1995; Liu et al, 1995). Both sets of transcriptional elements converge on the regulation of key growth factors, thereby allowing their spatial and temporal expression. For example, interleukin 2 and transforming growth factor β_1 are positively regulated by AP-1 proteins and negatively controlled by ATRA (Salbert et al, 1993; De Grazi et al, 1994). Correspondingly, deregulation of this process occurs in cancer cells, for example retinoid-resistant breast cancer cell lines (e.g. BT-20) have a high level of endogenous AP-1 activity (Burg et al, 1995), and osteosarcomas may have abnormally high expression of osteocalcin as a result of deregulated AP-1 activity (Jaaskelainen et al, 1994).

Clinical applications of $1\alpha,25(OH)_2D_3$ and ATRA alone at doses that are pharmacologically active have been curtailed because of side-effects. Vitamin D_3 is limited to topical application, most notably for the treatment of psoriasis, and although retinoids have achieved remarkable success in the treatment of acute promyelocytic leukaemia (APL) and the prevention of secondary lesions in individuals with head and neck cancers (Huang et al, 1988; Hong et al, 1990), both vitamin analogues have toxicities. For example, many potent vitamin D_3 analogues when injected into mice at doses that are active in vitro produce lethal hypercalcaemia (Pakkala et al, 1995). ATRA can produce skin, mucous membrane and hepatic toxicities as well as a high teratogenicity (Morosetti and Koeffler, 1996). Furthermore, over several months of ATRA therapy, APL cells develop insensitivity to the differentiating effects of ATRA. This resistance is multifactorial, which can include abnormal ligand binding and altered transcriptional activation (Rosenauer et al, 1996).

We have previously discovered that the potent analogue of vitamin D_3 [1α,25-$(OH)_2$-16-ene-23-yne-26,27-F_6-19*nor*-D_3 (code name LH)] inhibits clonal proliferation of LNCaP, PC-3 and DU-145 prostate cancer cells (Campbell et al, 1997*a*). Unfortunately, this analogue also causes hypercalcaemia at high doses (Pakkala et al, 1995). In a preliminary study to enhance the action of this compound, we investigated combinations of LH with various naturally occurring and receptor-selective retinoids that transactivate through either RARE or RXRE (Campbell et al, 1998*b*) and demonstrated that the capacity for synergistic effects was reduced with increased cellular transformation. We have now investigated a synthetic retinoid that has predominately anti-AP-1 effects, as few anti-cancer studies have focused on the contribution of AP-1 repression to retinoid-mediated inhibitory effects. One such retinoid is SR11238, which has approximately 100% of the anti-AP-1 effect of ATRA but has minimal ability to transactivate through a RARE (Fanjul et al, 1994). In this study, we show that combined low doses of LH with SR11238 have prominent antiproliferative effects. We also examined the mechanism of this inhibition by considering various target proteins, such as $p21^{(waf1)}$ and E-cadherin, which we and others have previously shown to be modulated by vitamin D_3 and retinoids in cancer cells (Liu et al, 1996; Munker et al, 1996; Campbell et al, 1997*a*,*b*). Their effects on the induction of apoptosis and transactivation of the vitamin D response element (VDRE) present in the upstream region of the human osteocalcin gene were also examined.

MATERIALS AND METHODS

Cells

Human prostate cancer cell lines were obtained from ATCC (Rockville, MD, USA) and maintained as recommended; LNCaP was established from a metastatic lesion in the supraclavicular lymph node of a patient with prostate cancer; PC-3 was derived from a primary adenocarcinoma of the prostate; and DU-145 was established from prostate cancer metastatic to the brain. LNCaP was maintained in RPMI with 10% fetal calf serum (FCS); PC-3 and DU-145 were grown in Dulbecco's modified Eagle medium (DMEM) with 10% FCS.

Vitamin D_3 analogues and retinoids

The vitamin D_3 analogue [$1\alpha,25(OH)_2$-16-ene-23-yne-26,27-F_6-19-*nor*- D_3, code name LH], ATRA, and the synthetic anti-AP-1 retinoid [2-(4,4-dimethyl-3,4-dihydro-2H-1benzopyran-6-yl)-carbonyl-2-(4-carboxyphenyl)-1,3,-dithiane] (SR11238) were kept in stock vials at 10^{-3} M in ethanol at –20°C in the dark. For experimental use, these solutions were diluted in normal medium immediately before use.

Colony formation in soft agar

The antiproliferative potency of LH and retinoids was determined by single- and combination-dose (10^{-9} M) studies in soft agar. Trypsinized and washed single-cell suspensions of LNCaP, PC-3 and DU-145 prostate cancer cells from 80% confluent cultures were enumerated and plated into 24-well, flat-bottomed plates using a two-layer soft agar system with 1×10^3 cells in 400 µl of media per well, as described previously (Munker et al, 1986). The cells were grown in either RPMI or DMEM. Both layers were prepared with agar (1%) equilibrated at 42°C. Before addition of

the bottom layer to the plate, the LH and either ATRA or SR11238 (final concentration 10^{-9} M) were pipetted into the wells and then, after this layer had set, the top layer containing cells was pipetted into the wells. Stock solutions of LH, ATRA and SR11238 and the experimental plates were kept in the dark to minimize UV-catalysed degradation. After 14 days of incubation, the colonies (≥ 50 cells) were counted using an inverted microscope. All experiments were done at least three times in triplicate dishes per experimental point. Dose–response studies with SR11238 were undertaken in the same assay system.

Measurement of apoptosis

Combinations of LH and SR11238 were investigated for their capacity to induce apoptosis of LNCaP, PC-3 and DU-145 cells. These cells were exposed to either LH or SR11238 alone or a combination of each at 10^{-7} M for 4 days, with fresh analogues added at day 2. Total cells, both in the media and those adhering to the plastic, were harvested and fixed in 1% methanol-free formaldehyde for 15 min and washed in phosphate-buffered saline (PBS) (Li and Daryzynkiewcz, 1995). The cell concentration was corrected to 1×10^6 cells ml^{-1} and fixed in 5 ml of 70% ethanol. Single- and double-strand DNA breaks were labelled with bromodeoxyuridine triphosphate (BrD-UTP) for 40 min at 37°C with terminal transferase (Boehringer Mannheim, Indianapolis, IN, USA). The cells were permeabilized with a 0.3% solution of Triton-X 100 in 0.5% bovine serum albumin (BSA) in PBS, DNA breaks were tagged by the incorporation of BrDU and then identified using a fluorescein isothiocyanate (FITC) conjugated anti-BrDU antibody. Cells were stained with propidium iodide (PI) for 30 min, and green fluorescence was measured by FACS analysis at 510–550 nm.

Transactivation assays

PC-3 cells (1×10^7) were plated in normal media in culture dishes to give 70% confluent cultures. The cells were washed and maintained in DMEM with 10% charcoal-stripped fetal calf serum (CS-FCS) for 24 h before transfection, and then transfected overnight with 3 μg of pBLCAT2 plasmid with the VDRE-containing region of the human osteocalcin promoter, as described by Kuno et al (1994), and 3 μg of cytomegalovirus (CMV) promoter luciferase plasmid (internal standard) in the presence of 18 μg of lipofectamine (Gibco, Grand Island, NY, USA). After transfection, the cells were trypsinized, washed and divided into groups which were cultured in DMEM with 10% CS-FCS with either LH or SR11238 separately, each at 1×10^{-7} M, or together at a combined concentration of 1×10^{-7} M (5×10^{-8} M each). Treated and non-treated cultures were harvested after 48 h for chloramphenicol acetyltransferase (CAT) reporter assays. After measurement of light production from luciferase and normalization of lysate volume, CAT assays were performed as described previously (Rosenthal, 1987). Relative intensities of acetylated and non-acetylated chloramphenicol were measured on an AMBIS 4000 image analyser (Ambis, San Diego, CA, USA), and the percentage of conversion was calculated. Increases in CAT activity were compared with reporter gene activities in the absence of either retinoid or vitamin D_3 analogue. As controls for determination of the linear region of CAT activity, two standards were used. As a negative control, pBLCAT2 plasmid without the human osteocalcin VDRE promotor region was used; and, as a positive control, a CAT plasmid containing a CMV promoter region was used.

Analysis of protein expression

The effects of LH and SR11238 on the expression of $p21^{(waf\ 1)}$ and E-cadherin were examined. We previously found that significant increases in expression of $p21^{(waf\ 1)}$ and E-cadherin occurred after 96 h exposure of LNCaP cells to LH (Campbell et al, 1997*b*). We, therefore, examined the effects on subconfluent cultures of LNCaP when exposed to LH and SR11238, either alone (at 1×10^{-7} M each) or in combination (at 5×10^{-8} M each) for 4 days. Lysates were compared with non-treated cells by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Zhang et al, 1995). Briefly, extracts from 1.5×10^6 cells were boiled in sample buffer for 5 min and loaded onto a 12.5% ($p21^{(waf\ 1)}$) or 7.5% (E-cadherin) SDS-polyacrylamide gel. After electrophoresis at 150 V, the proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA), blocked with Tris-buffered saline Tween 20 (0.1%) (pH 7.5), 1% gelatin for 1 h, then incubated with antibodies to either $p21^{(waf\ 1)}$ (Oncogene Research Products, Cambridge, MA, USA) or E-cadherin (Transduction Laboratories, Lexington, KY, USA). The proteins were then detected using an enhanced chemiluminescence (ECL) system (Amersham Life Sciences, Little Chalfont, Bucks, UK). Comparison of non-specific, background, protein bands and staining of the membrane with Ponceau S were used to adjust the level of lysate added to ensure even loading of protein. Densitometry was performed on bands to quantify the changes in the expression of target proteins.

Statistical analysis

The interactions of two compounds were assessed by measuring the mean effect of either LH or retinoid acting alone (± s.e.m.). The combination of the mean clonal inhibition for each compound acting alone was the predicted combined effect. The mean observed combined clonal inhibition was then compared with this value using the Student's *t*-test. Classification of the inhibitory effects were as follows: synergistic effects were those with an experimental value significantly greater than the predicted value, additive effects were those in which the experimental value did not significantly differ from the predicted value, subadditive effects were those in which the experimental value was significantly less than the predicated value and 'squelching' effects were those in which the experimental value was significantly lower than either agent acting alone.

Other statistical analyses were performed using the Student's *t*-test.

RESULTS

Effects of vitamin D_3 analogue LH, ATRA and anti-AP-1 retinoid SR11238 on clonal proliferation of human prostate cancer cells

Figure 1 shows the effects of these compounds either alone or in combination at the relatively low dose of 10^{-9} M on the clonal growth of prostate cancer cells. LH (10^{-9} M) inhibited 24 ± 2.6% (mean ± s.e.) clonal growth of LNCaP cells, and ATRA and SR11238 (10^{-9} M) alone resulted in a mean colony inhibition of 12 ± 5% and 11 ± 3.6% respectively. Together, LH and either ATRA or SR11238 (10^{-9} M) synergistically inhibited growth by a

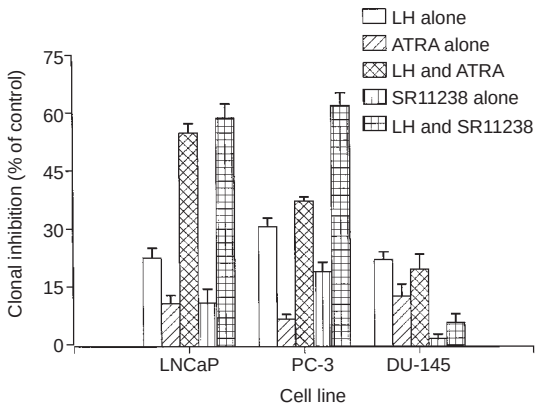

Figure 1 Inhibition of clonal growth of prostate cancer cells exposed to LH, ATRA and SR11238 alone or in combination (10^{-9} M). Results expressed as per cent (mean ± s.e.m.) of non-treated control cells. Each point represents the mean of at least three experiments with triplicate dishes. Cells were treated with analogue LH, ATRA and SR11238 either separately at 10^{-9} M each or together at 5×10^{-9} M each (total analogue concentration 10^{-9} M)

Table 1 Summary of clonal inhibition by the combinations of LH and various retinoids

Combination	Clonal inhibition LNCaP	PC-3	DU-145
ATRA + LH[a]	Syn*	Add	Sque***
SR11238 + LH	Syn*	Syn**	Sque*

[a]The interactions of two compounds were assessed by measuring the means of either LH or retinoid acting alone (± s.e.m.). The combination of the mean clonal inhibition for each compound acting alone was the predicted combined effect. The mean observed combined clonal inhibition was then compared with this value using the Student's *t*-test. Classification of the inhibitory effects were as follows: synergistic effects were those with an experimental value significantly greater than the predicted value, additive effects were those in which the experimental value did not significantly differ from the predicted value, subadditive effects were those in which the experimental value was significantly less than the predicted value and squelching effects were those in which the experimental value was significantly lower than either agent acting alone. Sque, squelching effects; Add, additive effects; Syn, synergistic effects. *$P < 0.001$; **$P < 0.05$; ***$P < 0.01$.

mean 56 ± 4% ($P < 0.05$) and 59 ± 3.6% ($P < 0.001$) compared with the non-treated control.

The PC-3 cells were less sensitive than LNCaP cells to the combined effects of LH plus ATRA (mean inhibition was 38% ± 1.5%). However, the combination of LH plus SR11238 retained significant potency resulting in a synergistic inhibition of clonal growth (62 ± 3.2%) ($P < 0.05$). In contrast, DU-145 cells were insensitive to both of these combinations and, indeed, displayed squelching effects with either combination. These results are summarized in Table 1.

Separate dose–response studies of SR11238 alone revealed this compound to be inactive against all three cell lines with less than 30% colony inhibition even at 10^{-6} M (data not shown).

Induction of apoptosis

Of the three prostate cancer cell lines, only LNCaP cells underwent significant apoptosis in the presence of either LH or SR11238. LNCaP cells underwent a small, but constant, level of apoptosis under normal growth conditions (approximately 3%). In the presence of LH, a small but statistically significant ($P < 0.01$) increase in apoptotis occurred (mean 9.4 ± 0.6%). SR11238 alone also significantly ($P < 0.01$) increased the adoptotic cells (11.5 ± 1.5%). The combination of LH and SR11238 did not significantly differ from the predicted combined value and was considered an additive effect.

Transduction through a VDRE

The human osteocalcin promoter contains a well-characterized VDRE, which has a RXR and VDR half-sites, and a closely associated AP-1 site. Studies have suggested that the *Jun/Fos* AP-1 complex is able to inhibit VDR binding to the VDRE, thereby down-regulating gene expression (Jaaskelainen et al, 1994). Therefore, we hypothesized that the synergistic inhibition by LH and SR11238 may be due to the reduction in AP-1's antagonism to transactivation of the VDRE by VDR. Transient transfection assays in PC-3 cells showed that LH and SR11238 alone (10^{-7} M) resulted in a mean increase in CAT activity of 3.5-fold ± 0.25-fold and 0.5-fold ± 0.06-fold respectively; whereas, the combination of the two compounds did not significantly alter the CAT activity from that of LH alone (mean 3.6-fold ± 0.12-fold increase over control cells not exposed to ligand) (Figure 2).

Transactivation through VDRE-containing plasmid, in the absence of ligand was not significantly greater than that achieved through a minimal promoter-containing plasmid that lacked a VDRE (data not shown).

p21$^{(wafl)}$ and E-cadherin protein expression

Analysis of the p21$^{(wafl)}$ protein expression revealed that only LH but not SR11238 modulated it, and the combination of both compounds was not greater than LH alone (data not shown). E-cadherin expression was enhanced by both LH and SR11238

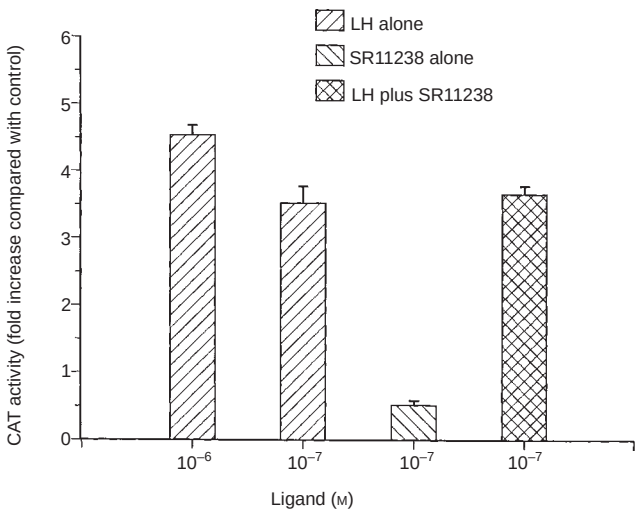

Figure 2 Transactivation of a VDRE-CAT reporter construct. PC-3 cells transiently transfected with the human osteocalcin CAT reporter construct were exposed separately to analogue LH (10^{-6} or 10^{-7} M) and SR11238 (10^{-7} M) or together at 5×10^{-8} M each (total analogue concentration 10^{-7} M). CAT activity was measured as described in the Materials and methods section

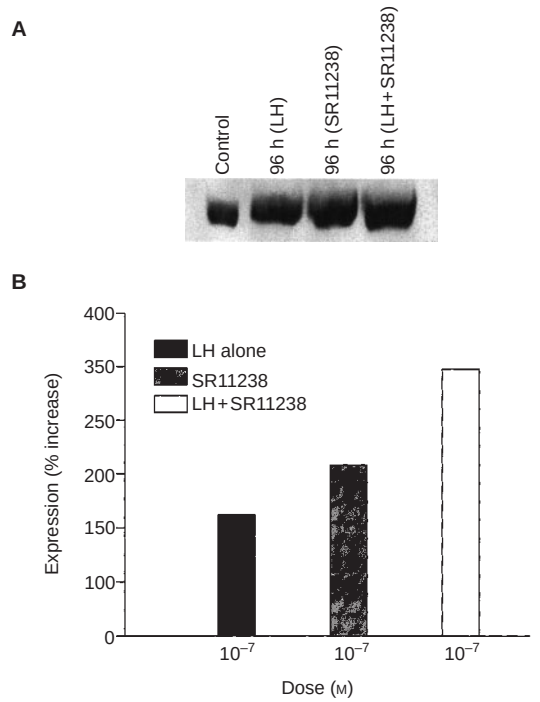

Figure 3 Modulation of E-cadherin by LH and/or SR11238. Expression of E-cadherin by LNCaP cells was compared with untreated control after treatment with analogue LH and SR11238 either separately at 10^{-7} M each, or together at 5×10^{-8} M each (total analogue concentration 10^{-7} M). (**A**) Cell lysates were resolved by SDS-PAGE, and E-cadherin was detected by Western blot analysis using the antibody described in Materials and methods. (**B**) Densitometry was performed on the bands and expressed as a percentage increase compared with non-treated control

separately at 10^{-7} M (210% and 250% increased expression respectively, compared with untreated control). The combination of both together at a total reagent concentration of 10^{-7} M (each at 5×10^{-8} M) produced a greater increase (350% increased levels compared with untreated controls) (Figure 3).

DISCUSSION

In the present study, we attempted to enhance the pharmaceutical index of the potent vitamin D_3 analogue LH by combination treatment with a conformationally restricted retinoid SR11238 that has been reported to have the AP-1 repressive capacities of ATRA, but not its ability to transactivate through the RARE (Fanjul et al, 1994). One of the more interesting findings of the present study was the observation that LH plus SR11238 produced a dramatic, synergistic inhibition of the LNCaP and PC-3 cells. Previously, we have shown LH to be a potent growth inhibitor of prostate cancer cells (Campbell et al, 1997*a*). In the current study, we found that, although the retinoid SR11238 alone had very low inhibitory activity, its combination with LH caused synergistic inhibition of proliferation. A similar finding was reported in a recent study which showed that anti-AP-1 retinoids could be as potent as ATRA at inhibiting transformation of murine JB6 cell (Li JJ et al, 1996). The current study is of special note because the LNCaP and PC-3 cells were insensitive to SR11238 alone, but were significantly inhibited by SR11238 in combination with LH.

AP-1 repression has been investigated intensively and the inhibition of *Jun/Fos*-mediated gene transcription by steroid hormone receptors has been demonstrated in several cell types (Simonson, 1994; Alroy, 1995; Liu et al, 1995). Similarly, synthetic anti-AP-1 retinoids and vitamin D_3 analogues have been shown to repress synergistically the TPA-induced AP-1 activity in MCF-7 breast cancer cells (Chen et al, 1995). We explored how LH and SR11238 might be mediating their synergistic clonal growth inhibition of LNCaP and PC-3 cells. To investigate this, we utilized a human osteocalcin promoter with the VDRE and AP-1 sites in close proximity attached to the CAT reporter gene. We hypothesized that if LH and SR11238 acted synergistically to enhance VDRE-mediated growth inhibition, we may observe a corresponding synergistic increase in VDRE-mediated CAT activity. However, the LH and SR11238 combination did not increase transactivation of the VDRE-containing reporter. Similarly, we did not observe a synergistic up-regulation, in the presence of both compounds, of $p21^{(waf1)}$ expression, a protein whose gene has multiple VDRE sites within its promoter region. Therefore, although the effects of LH and SR11238 converge to inhibit clonal proliferation, these compounds probably do not affect the same genomic targets. Thus, we do not know, as yet, the mechanism by which SR11238 potentiated the effect of LH.

The expression of E-cadherin was additively up-regulated in the presence of LH and SR11238. This observation is of interest because the loss or down-regulation of E-cadherin protein is observed in many metastatic cancers of epithelial origin. In prostate cancer, 50% of metastases have either low or no expression of E-cadherin (Umbas et al, 1992; Cheng et al, 1996); whereas normal, differentiated prostate cells display high levels of this protein. It has been suggested that the E-cadherin gene may play a role in preventing metastasis and behave as a tumour suppressor (Umbas et al, 1994). Taken together, E-cadherin is a good marker of functional differentiation (Otto et al, 1993; Campbell et al, 1997*a,b*). In the current study, up-regulation of E-cadherin may involve more than exclusively VDRE-mediated pathways because SR11238 did not appear to affect a VDRE reporter construct. Vitamin D_3 has been shown to play a physiological role with testosterone to regulate normal prostate cells (Konety et al, 1996). Perhaps retinoids, acting in an anti-AP-1 capacity, also have a physiological role in the control of growth of normal prostate cells.

The extent to which the anti-tumorigenic effects of ATRA are mediated by an anti-AP-1 pathway is undetermined. Previously, we have shown synergistic inhibition of prostate cancer cell proliferation by LH and certain novel and naturally occurring retinoids, and that a loss of synergistic inhibition correlated with loss of expression of RAR-β (Campbell et al, 1998*b*). However, this previous study was not able to assess to what extent the anti-AP-1 effects of retinoids contributed to the inhibitory behaviour. We have now shown, using a similar experimental design, that the contribution of these retinoid effects appears to be highly significant because the effect of LH plus SR11238 was potent in LNCaP and PC-3 cells, whereas that of LH and ATRA was only synergistically inhibitory only in LNCaP cells. This is intriguing because PC-3 cells are more transformed than LNCaP cells (Carrol et al, 1993; Gaddipati et al, 1994; Issacs et al, 1994; Ewing et al, 1995; Tamimi et al, 1996) and, in particular, do not express RAR-β, whereas LNCaP cells do. This is possibly reflected by the reduced sensitivity of PC-3 cells to LH and various retinoids as found in the present study, and more comprehensively in the previous one (Campbell et al, 1998*b*). Strikingly, in PC-3 cells, antiproliferative potency appears to be retained by anti-AP-1

retinoids in combination with LH. This finding suggests that anti-AP-1 effects may contribute significantly to the anti-cancer effects of certain retinoids and may be retained by androgen-independent prostate cancer cells.

In summary, we have demonstrated a significant enhancement of the anti-proliferative effects against androgen-sensitive (LNCaP) and androgen-insensitive (PC-3) prostate cancer cells by the combination, at low and more physiological doses, of a potent vitamin D_3 analogue and a synthetic anti-AP-1 retinoid. The mechanism of inhibition did not appear to be increased by up-regulation of purely VDRE-mediated events, but included other cellular events that may represent a convergence between several signalling pathways, including, for example, increased expression of E-cadherin.

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