Interactive effects of 9-*cis*-retinoic acid and androgen on proliferation, differentiation, and apoptosis of LNCaP prostate cancer cells

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9-*cis*-Retinoic acid (9cRA), which binds to both retinoic acid receptors and retinoic X receptors, inhibits prostate cancer induction in rats and reduces growth of prostate cancer cells. However, the nature of this growth inhibition and the interactive influence of androgens are not well defined and are the subject of this report. LNCaP and PC-3 cells were cultured and treated with a range of 9cRA concentrations for 3–6 days in the absence or presence of 5α-dehydrotestosterone. 9cRA inhibited cell proliferation in a dose-dependent manner, plateauing at 10^{-7} mol/l. Treatment of cells with 10^{-6} mol/l 9cRA inhibited 5α-dihydroxytestosterone (DHT)-stimulated proliferation, the effect of which was maximal at 10^{-9} mol/l DHT. Treatment of DHT (10^{-9} mol/l)-exposed cells with 9cRA caused a dose-dependent increase in prostate-specific antigen in the medium after 6 days, but not 3 days. 9cRA caused a dose-dependent increase in apoptotic cells stained with H33258 after 3 days, but not 6 days; however, on using flow cytometry, apoptosis was apparent at both 3 and 6 days. Flow cytometry also revealed interference of G0/G1 to S phase transition by 9cRA. Inhibition by 9cRA of anchorage-independent growth of PC-3 cells was also found; LNCaP cells did not grow colonies in soft agar. 9cRA inhibited growth and induced differentiation of human LNCaP prostate cancer cells *in vitro* and inhibited anchorage-independent growth of PC-3 cells. Because 9cRA and 13-*cis*-retinoic acid, which is retinoic acid receptor-selective, prevent prostate carcinogenesis in rats, and 13-*cis*-retinoic acid also inhibits growth of human prostate cancer cells, the RAR is a potential molecular target for prostate cancer prevention and therapy. *European Journal of Cancer Prevention* 26:71–77

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Introduction

Prostate cancer continues to be a major health problem in the USA (Siegel *et al.*, 2012). Beyond surgery and radiotherapy, androgen ablation therapy remains the standard therapy for prostate cancer patients, but nearly all cases will progress to become castration-resistant (Rubin, 2008) and lack curative treatment options (Wei *et al.*, 2002). Thus, prevention would be preferable, but despite a considerable amount of effort, effective chemopreventive treatments for prostate cancer have remained elusive (Hamilton and Freedland, 2011). Consequently, there remains urgent need to identify new agents capable of preventing development and progression of prostate cancer. A range of candidate chemoprevention agents have been tested in preclinical models (Ozten-Kandas and Bosland, 2011), and we identified the retinoic acid metabolite 9-*cis*-retinoic acid (9cRA) as the most active in preventing induction of prostate cancer in a rat model (McCormick *et al.*, 1999), even with delayed treatment (McCormick *et al.*, 2007).

The idea of using retinoids in cancer prevention and treatment is based on the major role of biologically active metabolites of vitamin A in growth and differentiation of normal, premalignant, and malignant epithelial cells (Fields *et al.*, 2007). Retinoids inhibit tumor cell growth, suppress the development of epithelial tumors in rodents, induce cell differentiation and apoptosis, and inhibit angiogenesis (Sporn and Roberts, 1983; Gudas, 1992; Sun and Lotan, 2002). These pleiotropic effects are most likely associated with the complexity of metabolism and signaling of retinoids, which include various retinol isomers, cellular retinoic acid binding proteins, and the nuclear retinoic acid receptors (RARs) and retinoic X receptors (RXRs; Leid *et al.*, 1992; Mongan and Gudas, 2007). 9cRA can bind to both RARs and RXRs, whereas all-*trans* retinoic acid and 13-*cis*-retinoic acid (13cRA) bind only to RARs, suggesting that 9cRA may elicit a

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broader range of biological responses (Heyman *et al.*, 1992; Mangelsdorf *et al.*, 1992). 9cRA inhibited cell proliferation and induced differentiation and apoptosis in various cell lines as well as in cancer models (Hayashi *et al.*, 2000; Szabó *et al.*, 2013), but few studies have addressed the effect of 9cRA on prostate cancer cells, indicating growth inhibition (Esquenet *et al.*, 1996; de Vos *et al.*, 1997), although growth simulation has also been reported (Blutt *et al.*, 1997).

In view of our abovementioned in-vivo data (McCormick *et al.*, 1999), we examined the effects of 9cRA on LNCaP human prostate cancer cells to further define this growth inhibition in relation to the presence of androgen because of studies indicating interactions between RXRα and the androgen receptor (AR; Chuang *et al.*, 2005b). The LNCaP cell line is androgen-dependent and reflective of early prostate cancers that should be the target of chemoprevention in middle-aged men, who have a 30–40% prevalence of small prostate carcinomas (Sakr *et al.*, 1994).

Materials and methods

Materials and cell culture

9cRA, obtained from Ligand Pharmaceuticals (San Diego, California, USA) or Sigma (St Louis, Missouri, USA), was dissolved in 100% ethanol and stored at −20°C protected from light. LNCaP cells and the androgen-independent PC-3 cells were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Propidium iodide (PI; Sigma Aldrich) was protected from light and freshly dissolved in PBS. Crystalline 5α-dihydroxytestosterone (DHT, Sigma Aldrich) was dissolved in 95% ethanol and reconstituted in culture medium at varying concentrations. The final ethanol concentration never exceeded 0.1%. ^{3}H-Thymidine was purchased from NENTM (Boston, Massachusetts, USA). All culture media, Hoechst 33258, and charcoal were purchased from Sigma. Penicillin and streptomycin were purchased from Life Technologies Inc. (Gaithersburg, Maryland, USA), and fetal bovine serum (FBS) was purchased from Life Technologies Inc. or Hyclone (South Logan, Utah, USA).

LNCaP and PC-3 cells were cultured (37°C, 5% CO_2) in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). Where indicated, FBS stripped by pretreatment with dextran-coated charcoal (DCC-FBS) was used.

Cell proliferation assays

Proliferation of LNCaP cells was assessed by manual counting after trypan blue staining. LNCaP cells were seeded in six-well plates at 5×10^{4} cells/well in 2 ml of phenol-red-free RPMI with DCC-FBS. One day later, the cells were incubated with fresh medium containing 9cRA and/or DHT for 3 and 6 days; the medium was replaced once on day 3, and the cells were removed from the plate with trypsin-EDTA. For [^{3}H]-thymidine incorporation, LNCaP cells were seeded at 2×10^{4} cells/well in 1 ml of medium in 24-well plates, and after 24 h, fresh medium was added containing vehicle or 9cRA and/or DHT. After 3 days, the cells were incubated with ^{3}H-thymidine (2 μCi/well) for 1 h. After aspiration of ^{3}H-thymidine, the wells were washed with cold PBS and rinsed with 1.0 ml of 5% trichloroacetic acid. The cells were collected in polypropylene tubes, solubilized overnight in 2.0 ml of 0.1 mol/l sodium hydroxide, and radioactivity was counted in a liquid scintillation counter (Model LS 3800; Beckman Instruments, Columbia, Maryland, USA).

PSA secretion analysis

Cells were grown as described for proliferation assays, except that 1×10^{5} cells were plated in 60 mm dishes. Cells and medium were collected, centrifuged (4000 rpm, 5 min), and the supernatant was stored at −20°C. Prostate-specific antigen (PSA) levels were determined with an AIA-600 enzyme immunoassay analyzer, using the Hybritech PSA antibody (TOSOH Medics Inc., Foster City, California, USA).

Apoptosis assays

Cells were stained with Annexin V–fluorescein isothiocyanate and PI and then evaluated for apoptosis by flow cytometry. For each experiment, 20 000 cells were analyzed using a CyAn flow cytometer (Beckman Coulter, Brea, California, USA). For microscopic identification of cells in apoptosis, cells were grown on collagen-coated microscopy slides, and on days 3 and 6, they were fixed with 10% formalin. After washing with PBS and water, the cells were stained in the dark with Hoechst 33 258 for 15 min, and the slides were air dried and coverslipped. Using a fluorescence microscope, 200 cells were counted and the percentage of apoptotic cells was determined. A positive control was included by adding 100 μl of 1 mmol/l H_2O_2 to untreated cells for 2 h before staining.

Cell cycle analysis

Cells were harvested using trypsin-EDTA, washed in PBS, fixed in ice-cold 70% ethanol, centrifuged (2000 rpm, 5 min), and washed with PBS. The cells were then treated with 100 ml of 100 mg/ml ribonuclease A for 5 min at room temperature, suspended in 400 ml PI (50 mg/ml), and analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, California, USA).

Soft agar colony formation assay

Anchorage-independent growth of PC-3 cells was assessed by a soft agar colony formation assay. The bottom of each well of a 12-well culture dish was coated with 1 ml noble agar mixture (RPMI, 10% FBS, 0.6% agar) and left to solidify, and then 1 ml top agar medium mixture (RPMI, 10% FBS, 0.3% agar) containing 5×10^{3} cells with different concentrations of 9cRA was added and incubated at 37°C for 3 weeks. Thereafter, colonies were

stained with crystal violet, and the number of colonies was counted using GelCount equipment and software (Oxford Optronix, Abingdon, UK).

Statistical analysis

All data are presented as mean ± SEM of triplicate experiments and comparisons between treatments were analyzed with one-way analysis of variance followed by Dunnett's test or a test for linear trend, unless indicated otherwise. A *P*-value of less than 0.05 was considered significant. All statistical analyses were carried out using GraphPad Software (GraphPad Inc., La Jolla, California, USA).

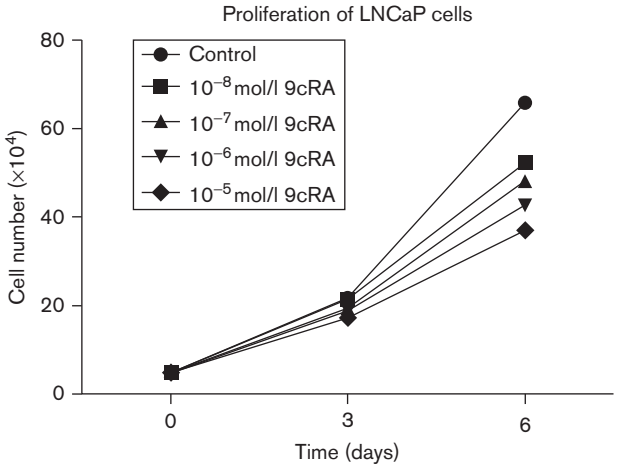
Results

Inhibitory effects of 9cRA on growth of LNCaP cells

9cRA inhibited the proliferation of LNCaP cells without added DHT, assessed by hemocytometer cell counting, in a dose-dependent manner at all concentrations tested, ranging from 10^{-5} to 10^{-8} mol/l (Fig. 1). Growth inhibition was not apparent after 3 days, but it became profound after 6 days. There was no evidence of cytotoxicity induced by 9cRA at these concentrations.

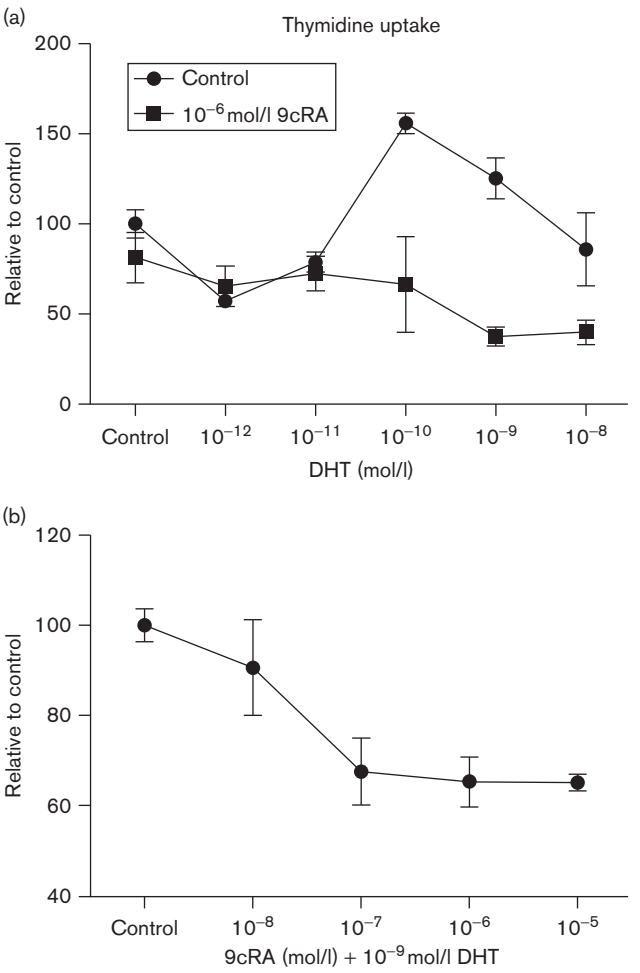
To determine whether 9cRA interferes with the effects of androgens on proliferation, LNCaP cells were exposed to 10^{-6} mol/l 9cRA and DHT at concentrations ranging from 10^{-8} to 10^{-12} mol/l for 3 days (Fig. 2a). Compared with control cells, a very low concentration of DHT (10^{-12} mol/l), in the absence of 9cRA, had a slightly inhibitory effect on ^{3}H-thymidine incorporation ($P = 0.008$; two-sided *t*-test), whereas a concentration of 10^{-10} mol/l had a stimulatory effect ($P = 0.004$; two-sided *t*-test). Growth stimulation was reduced in cells treated with 10^{-9} mol/l DHT in the absence of 9cRA and disappeared at 10^{-8} mol/l DHT. Cotreatment with 10^{-6} mol/l 9cRA inhibited DHT-stimulated proliferation, as indicated by thymidine incorporation, at concentrations of 10^{-10} mol/l DHT and, maximally, 10^{-9} mol/l. We used the latter concentration of DHT in subsequent experiments, as we expected that any cellular effect would be most profound at this androgen concentration. We next cotreated cells with a range of

Fig. 1



Effects of 9cRA on proliferation of LNCaP cells. LNCaP cells were incubated in medium with stripped serum containing increasing concentrations of 9cRA and counted after 3 and 6 days, respectively. Cell counting was performed with a hemocytometer following trypan blue staining. Values are expressed as mean values (error bars not shown). Growth was inhibited in a dose-dependent manner ($P = 0.05$ for trend). 9cRA, 9-*cis*-retinoic acid.

Fig. 2



^{3}H-Thymidine incorporation in LNCaP cells cotreated with increasing concentrations of 9cRA and DHT. LNCaP cells were grown in 24-well plates at a density of 2×10^{4} cells/well for 3 days and exposed to ^{3}H-thymidine for 1 h. Values are expressed as mean percentage of control values (± SEM). (a) Effects of androgen concentration on the proliferation of LNCaP cells in the presence or absence of 9cRA. ^{3}H-thymidine uptake was reduced by 10^{-6} mol/l 9cRA at DHT concentrations of 10^{-10} mol/l ($P = 0.006$) and 10^{-9} mol/l ($P = 0.002$; two-sided *t*-test). (b) Effects of increasing 9cRA concentrations on proliferation of cells incubated with 10^{-9} mol/l DHT. ^{3}H-Thymidine uptake was reduced by 9cRA at concentrations of 10^{-7} mol/l ($P = 0.017$), 10^{-6} mol/l ($P = 0.008$), and 10^{-5} mol/l ($P = 0.001$; two-sided *t*-test). 9cRA, 9-*cis*-retinoic acid; DHT, 5α-dihydroxytestosterone.

9cRA concentrations and 10^{-9} mol/l DHT (Fig. 2b). 9cRA treatment resulted in a dose-dependent decrease in proliferation, plateauing at 10^{-7} mol/l.

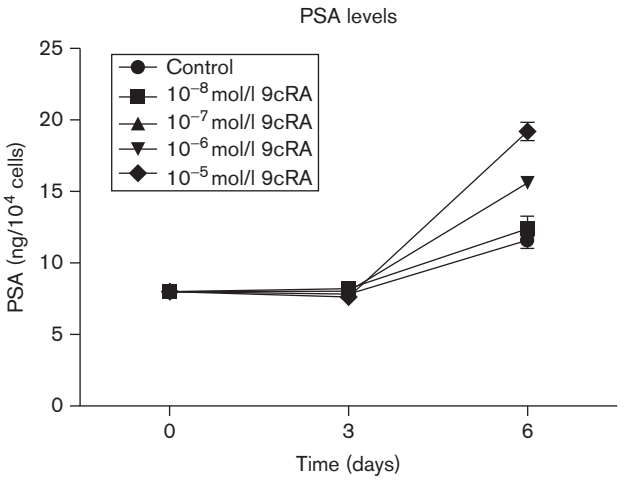
Effects of 9cRA on PSA production by LNCAP cells

PSA secretion by LNCaP cells was measured to determine whether 9cRA affects cell differentiation. After 3 days of treatment with 9cRA and 10^{-9} mol/l DHT, there were no differences in PSA levels in the medium (Fig. 3). After 6 days, however, compared with control-treated cells, the PSA level was significantly elevated at the two highest doses (10^{-6} and 10^{-5} mol/l 9cRA) in a dose-dependent manner. DHT treatment alone also stimulated PSA production after 6 days, compared with day 0 ($P = 0.03$; two-sided t-test).

Induction by 9cRA of apoptosis in LNCaP cells

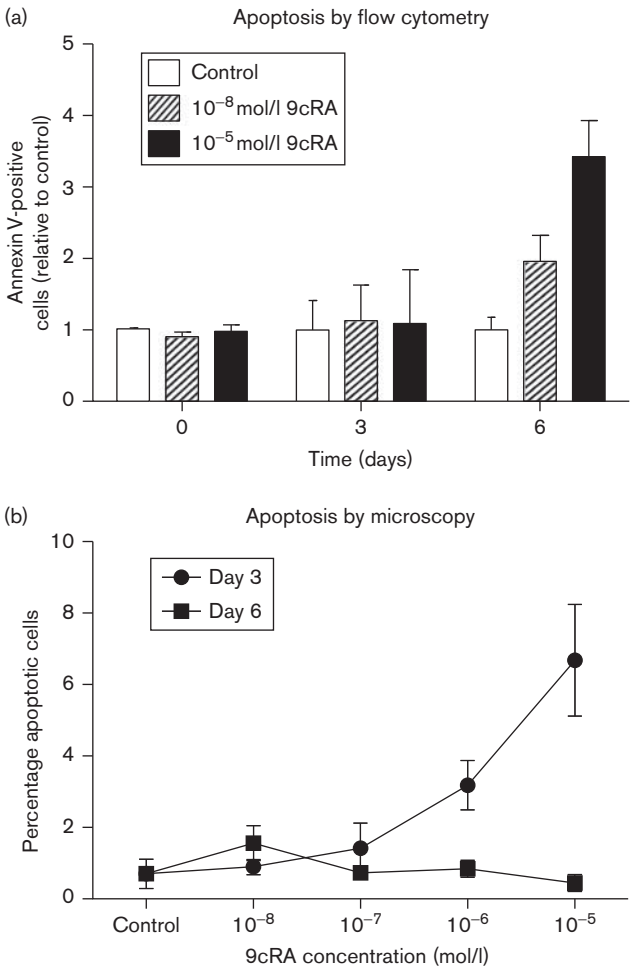
Effects of 9cRA on apoptosis were evaluated by flow cytometry after staining of cells with PI and Annexin V; cells positive for both were considered apoptotic. At concentrations of 10^{-8} and 10^{-5} mol/l, there was a 1.9-fold and 3.2-fold ($P < 0.01$) increase in PI-positive and Annexin V-positive apoptotic cells on day 6, but no difference was observed on day 3 (Fig. 4a). Apoptosis was also evaluated by staining LNCaP cells with H33258 and microscopically counting the number of apoptotic cells. 9cRA treatment caused a dose-dependent increase in apoptotic cells after 3 days, which was significant only at 10^{-5} mol/l 9cRA. No signs of apoptosis induced by 9cRA were observed on day 6 (Fig. 4b). The dose-dependent increase in apoptotic cells on day 3 paralleled the pattern of growth inhibition seen on day 6.

Fig. 3



Effects of 9cRA on PSA secretion by LNCaP cells treated with increasing concentrations of 9cRA and 10^{-9} mol/l DHT for 3 or 6 days. Media were harvested and PSA levels were determined, while hemocytometry cell counts were performed simultaneously to assess levels of PSA secretion on a per cell basis. Values are expressed as nanograms SPSA per 10^4 cells (means ± SEM). PSA levels compared with vehicle-treated cells were significantly ($P < 0.01$) elevated at the two highest doses (10^{-6} and 10^{-5} mol/l 9cRA) in a dose-dependent manner ($P < 0.0001$ for trend). 9cRA, 9-*cis*-retinoic acid; DHT, 5α-dihydroxytestosterone; PSA, prostate-specific antigen.

Fig. 4



Apoptosis-inducing effects of 9cRA on LNCaP cells. (a) Cells were cultured for 3 and 6 days with 10^{-8} and 10^{-5} mol/l 9cRA, with 10^{-9} mol/l DHT. The percentage of Annexin V-positive cells was measured by flow cytometry. Data are expressed as mean values relative to control (± SEM). On day 6, PI-positive and Annexin V-positive apoptotic cells were increased on treatment with 10^{-5} mol/l 9cRA ($P < 0.01$), and the increase was dose-related ($P = 0.004$ for linear trend). (b) Cells were grown on collagen-coated microscopy slides in the presence of increasing concentrations of 9cRA with 10^{-9} mol/l DHT, fixed, and stained. The percentage of apoptotic cells was determined using fluorescence microscopy on day 3 and day 6 and increased on day 3 in a dose-dependent manner ($P = 0.0003$ for linear trend); the increase was significant at 10^{-5} mol/l 9cRA ($P < 0.01$). 9cRA, 9-*cis*-retinoic acid; DHT, 5α-dihydroxytestosterone.

Effects of 9cRA treatment on cell cycle progression of LNCaP cells

After 6 days of incubation with 9cRA in the presence of 10^{-9} mol/l DHT, there was shift in the percentage of cells in the S phase toward the G0/G1 phase, as measured with flow cytometry. The reduction in cells in the S

Table 1 The effects of 9-*cis*-retinoic acid on cell cycle distribution of LNCaP cells after 3 and 6 days of cotreatment with 10^{-9} mol/l DHT

	3 days			6 days		
	G0/G1	S	G2/M	G0/G1	S	G2/M
Control	67.32 ± 0.28	19.27 ± 0.24	12.08 ± 0.07	82.21 ± 0.19	9.28 ± 0.24	8.14 ± 0.13
10^{-8} mol/l 9cRA	62.93 ± 0.06*	23.85 ± 0.13*	13.25 ± 0.22*	84.18 ± 0.16*	9.01 ± 0.02	6.81 ± 0.17*
10^{-7} mol/l 9cRA	63.32 ± 0.28*	23.81 ± 0.17*	12.88 ± 0.11*	84.81 ± 0.17*	8.12 ± 0.11*	7.07 ± 0.06*
10^{-6} mol/l 9cRA	64.32 ± 0.28*	22.06 ± 0.05*	13.32 ± 0.28*	88.92 ± 0.07*	5.86 ± 0.13*	5.25 ± 0.22*
10^{-5} mol/l 9cRA	67.26 ± 0.23	19.86 ± 0.12*	12.88 ± 0.11*	90.12 ± 0.10*	4.33 ± 0.28*	5.26 ± 0.23*

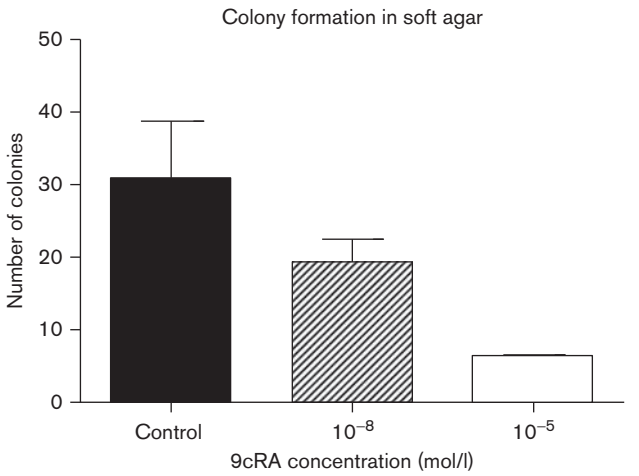
Cells were plated in T25 flasks 24 h before treatment. Cells (1×10^6 cells) were harvested using trypsin-EDTA, washed twice with cold PBS, and stained with 5 µl propidium iodide (5 µg/ml) in 1 × binding buffer [10 mmol/l HEPES (pH 7.4), 140 mmol/l NaOH, 2.5 mmol/l $CaCl_2$] for 15 min at room temperature in the dark. Results in this table represent the mean ± SD of three experiments.
9cRA, 9-*cis*-retinoic acid.
*$P < 0.01$ compared with the vehicle control group.

phase and the increase in cells in the G0/G1 phase were dose-related ($P < 0.0001$ for linear trend) and most noticeable at higher 9cRA concentrations (Table 1). The percentage of cells in the G2/M phase was also reduced in a dose-related manner ($P < 0.0001$ for linear trend). At 10^{-5} mol/l 9cRA, the percentage of cells in the G0/G1 phase was increased by 10%, that in the S phase was decreased by 53%, and that in the G2/M phase was decreased by 35%, compared with the control group. After 3 days, we observed a slight, but statistically significant, accumulation of cells in the S phase among cells treated with low doses (10^{-8} to 10^{-6} mol/l) of 9cRA, but overall there were no marked differences in number of cells in the three phases of the cell cycle in response to 9cRA.

Inhibitory effects of 9cRA on anchorage-independent growth of PC-3 cells

Because LNCaP cells do not grow well in soft agar, PC-3 cells were used to evaluate effects of 9cRA treatment on growth in soft agar for 3 weeks. There was a significant dose-dependent reduction in the number of colonies formed in the 9cRA-treated groups compared with the vehicle-treated group for cells treated with 10^{-8} and 10^{-5} mol/l 9cRA (Fig. 5).

Fig. 5



Effects of 9cRA on colony formation by PC-3 cells. Cells were grown in 0.3% agar with 10^{-8} and 10^{-5} mol/l 9cRA for 3 weeks, and the number of colonies was counted. Data are expressed as mean values ± SEM. The number of colonies was reduced compared with vehicle-treated cells by 10^{-5} mol/l 9cRA ($P = 0.03$) in a dose-dependent manner ($P = 0.012$ for linear trend). 9cRA, 9-*cis*-retinoic acid.

Discussion

Our previous in-vivo studies demonstrated a strong inhibitory effect of 9cRA on carcinogen-initiated, testosterone-promoted prostate carcinogenesis in a rat model (McCormick *et al.*, 1999), and the in-vitro findings of the present study support those observations. 9cRA inhibited cell growth and induced apoptosis in LNCaP cells in a dose-dependent manner. 9cRA also modified cell cycle progression, inhibited colony formation, and increased secretion of PSA, which is used as a biomarker of prostate cancer and indicator of the effects of treatment.

The effects of DHT alone and 9cRA in combination with DHT were studied because proliferation of LNCaP cells is responsive to androgens. The growth response of LNCaP cells to DHT was biphasic, with maximal growth stimulation occurring at 10^{-10} and 10^{-9} mol/l. This observation is similar to a previously reported maximal response of LNCaP cells on treatment with 10^{-10} mol/l R1881, a synthetic androgen (Esquenet *et al.*, 1996). In our study, addition of 10^{-6} mol/l 9cRA entirely eliminated the stimulatory effects on thymidine incorporation across a range of DHT concentrations, indicating that one way that 9cRA may act to inhibit proliferation is through interference with androgen signaling pathways. Although in most other studies 9cRA inhibited LNCaP growth, in one study 10^{-8} mol/l 9cRA slightly stimulated cell proliferation in the presence of serum that was not stripped (Blutt *et al.*, 1997); we used stripped serum supplemented with controlled amounts of androgen. Discrepancies between experiments may also be related to the LNCaP passage number used as indicated by results from a study with all-*trans* retinoic acid (Esquenet *et al.*, 1997).

Our observation that PSA secretion increases in response to 9cRA has also been found by others (Esquenet *et al.*, 1996) and is consistent with the idea that 9cRA is involved in androgen signaling. PSA secretion is regulated by activation of the AR, which interacts with both RARs and RXRs, forming dimers and modulating gene transcription (Rivera-Gonzalez *et al.*, 2012), and the presence of 9cRA can alter the expression of AR target genes (Chuang *et al.*, 2005a). Increased PSA secretion is also consistent with the observed reduction in proliferation, suggesting that cells entered a state of growth arrest, accompanied by an increased production of PSA, as expression of cellular differentiation increased. Induction of expression of the homeobox gene *NKX3.1* by 9cRA has been observed and was interpreted as a differentiating affect as well (Jiang *et al.*, 2006).

The prodifferentiating effects of 9cRA may also provide an explanation for the observation that treatment of PC-3 cells capable of anchorage-independent growth with 9cRA reduced their ability to form colonies. LNCaP cells were not used because they do not perform well in this assay. In contrast to LNCaP cells, PC-3 cells are part of an androgen-independent cell line that does not express AR or PSA. The action of 9cRA on colony formation in this cell line renders it likely that its effects are not exclusively mediated by androgen pathways and suggests that 9cRA may act through multiple mechanisms.

Apoptosis was induced by 9cRA in a dose-dependent, time-dependent, and assay-dependent manner at concentrations that were also proliferation-inhibitory. In previous studies with breast cancer cells, 9cRA interfered with cell cycle progression by inducing a G1–S block, resulting in decreased numbers of S-phase cells (Tang and Gudas, 2011). We found that LNCaP cells also accumulated in the G0/G1 phase, with a more than 50% decrease in cells in the S phase on day 6, indicating interference of 9cRA with entry into the S phase. This is consistent with the reduction in thymidine incorporation observed on day 3, even though differences in cell cycle distribution in response to 9cRA were not yet substantial on day 3. However, others did not find a decrease in LNCaP cells in the S phase after 9cRA treatment (Jiang *et al.*, 2006). Our findings support the notion that 9cRA induces arrest in the G0/G1 phase, slowing proliferation, in addition to induction of apoptosis. Others have demonstrated that 9cRA alters the expression levels of genes involved in cell cycle regulation and checkpoints (Szabó *et al.*, 2013), such as upregulation of GADD45A responsible for signaling growth arrest at the G2/M checkpoint following exposure to stressful stimuli (Yang *et al.*, 2000). Thus, 9cRA may elicit multiple effects on the regulation of cell cycle progression, which may be dependent on cell type and environment.

9cRA can bind to both RXRs and RARs, and LNCaP cells express both types of retinoic acid receptor (Blutt *et al.*, 1997). We previously found that 9cRA and the RAR-selective retinoid 13cRA inhibited prostate carcinogenesis in a rat model, whereas the RXR-selective agonist targretin (bexarotene) was completely inactive (McCormick *et al.*, 1999; McCormick *et al.*, 2007). Dahiya *et al.* (1994) showed that 13cRA also inhibited LNCaP cell growth and slowed the growth of these cells xenografted in nude mice. They also found inhibition of LNCaP cell growth in soft agar by 13cRA, as we found for 9cRA, suggesting that this effect is also mediated by RARs, not RXRs. However, 9cRA may have additional effects on prostate cancer that are mediated by RXRs, not RARs, involving its interactions with the AR and likely adding an additional layer of regulation (Chuang *et al.*, 2005b). This notion is supported by the striking difference observed by us in PSA release by LNCaP cells in response to 9cRA (increased) and in response to treatment with 13cRA (decreased) (reported by Dahiya *et al.*, 1994). These data and the marked effects of 9cRA and 13cRA on prostate carcinogenesis in animal models and on growth of human prostate cancer cells identify RARs as a potential molecular target for prostate cancer prevention and therapy.

In conclusion, 9cRA inhibited growth in an androgen concentration-dependent manner and induced differentiation and apoptosis, altered cell cycle distribution, and inhibited anchorage-independent growth of human prostate cancer cells *in vitro*. Although 9cRA appears to be a promising agent for prevention or treatment of prostate cancer on the basis of these results and animal data, clinical studies with 9cRA have demonstrated that oral intake is associated with dose-limiting side effects (Lawrence *et al.*, 2001; Bissonnette *et al.*, 2010). As a therapeutic agent, these side effects could be acceptable, but they render 9cRA unacceptable for prevention, particularly because the effects of retinoids are reversible (Esquenet *et al.*, 1996) and sustained consumption would be necessary to maintain the protective effect. Agents with considerably less human toxicity that selectively target RARs may provide a better approach for prostate cancer chemoprevention and could also have therapeutic potential.

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Conflicts of interest

There are no conflicts of interest.

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