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Review paper

The prospects of retinoids in the treatment of prostate cancer

Lisette A Hammond,[1] Geoffrey Brown,[2] Richard G Keedwell,[2] Jennifer Durham[2] and Roshantha AS Chandraratna[3]

Divisions of [1]Cancer Studies, and [2]Immunity and Infection, University of Birmingham Medical School, Edgbaston, Birmingham B15 2TT, UK. [3]Retinoid Research, Departments of Chemistry and Biology, Allergan Inc, Irvine, CA 92612, USA.

Prostate cancer is the most prevalent cancer amongst males and accounts for 13% of cancer deaths in this population in the US. Aggressive, androgen-independent, metastatic prostate cancer is incurable, and the search for new therapies has been directed towards identifying agents that block proliferation and induce differentiation and/or apoptosis of prostate cancer cells. Retinoid receptor agonists, such as all-*trans* retinoic acid, can induce apoptosis of prostate cancer cells, but clinical studies have demonstrated only mild to moderate efficacy. Retinoic acid receptor antagonists are a new class of retinoids, and pre-clinical studies have shown that they potently inhibit the growth of prostate cancer cells and induce apoptosis. Here, we review whether retinoids have a role in the fight against prostate cancer. [© 2002 Lippincott Williams & Wilkins.]

Key words: Apoptosis, differentiation therapy, prostate cancer, retinoids.

Introduction

The most prevalent cancer amongst males is prostate cancer, accounting for 29% of cancer cases diagnosed in males in the US in 1998, while the next most prevalent, cancer of the lung and bronchus, accounted for 15%.[1] Probably as a result of early detection, mortality rates associated with prostate cancer declined by about 0.5% per year between 1990 and 1994.[2] However, prostate cancer still accounts for 13% of cancer deaths in males in the US, and was the third leading cause of cancer deaths in males in the UK between 1995 and 1999, with a death rate of 17 per 100 000.[1] In early-stage disease, surgery can be curative and anti-androgen therapy can be of significant benefit. Later-stage disease, although initially responsive to androgen ablation, invariably reappears in an androgen-insensitive form which cannot be effectively treated and is incurable after metastasis.[3,4] Unfortunately, greater than 60% of cases of prostate cancer are in later-stage disease at diagnosis.[5] Because of the aggressive nature of androgen-independent, metastatic prostate cancer, newer therapies for treatment, as well as for prevention or delay of progression, are urgently needed.

Since prostate cancer develops and initially progresses relatively slowly, early diagnosis coupled with effective chemopreventive therapies are likely to be of significant benefit in this area. A large body of research has been directed towards identifying agents that inhibit the proliferation and/or induce differentiation of prostate cancer cells. Compounds that have shown promise as chemopreventive agents include retinoids, vitamin D receptor agonists and selective estrogen receptor modulators.[5,6] Of these, retinoids induce apoptosis of prostate cancer cells and are potentially useful in the chemotherapy of advanced disease in combination with other cytotoxic agents.[7,8]

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Correspondence to LA Hammond, Division of Cancer Studies, University of Birmingham Medical School, Edgbaston, Birmingham B15 2TT, UK.
Fax: (+44) 121 414 4019;
E-mail: hammondla@cancer.bham.ac.uk

Retinoids

Retinoids are a group of small, lipophilic molecules that are naturally occurring and synthetic derivatives of vitamin A. Apart from the well-characterized and critical functions of vitamin A aldehydes in vision,

most of the biological functions of retinoids are believed to be mediated by two families of nuclear receptors, the retinoic acid receptors (RARα, RARβ and RARγ) and the retinoid X receptors (RXRα, RXRβ and RXRγ).[9] These are ligand-activated transcription factors belonging to the steroid/thyroid superfamily of nuclear receptors. Activated retinoid receptors directly regulate gene transcription by binding to response elements in the promoters of target genes or they can indirectly effect transcription by antagonizing the activity of transcription factors such as AP1 and NF-IL6.[10,11] However, all of the non-vision-related activities of retinoids may not necessarily be mediated by the nuclear retinoid receptors. All-*trans* retinoic acid (ATRA), the carboxylic acid metabolite of vitamin A and the natural hormone for the RARs, binds to and regulates the activity of the mannose–6-phosphate receptor.[11,12] Vitamin A and the 14-hydroxy-4,14-retro-retinol (14-HRR) metabolite mediate normal lymphocyte proliferation by a non-RAR mechanism and the ZiF domains of certain serine/theronine kinases, such as cRaf and specific protein kinase C isoforms, are the likely targets of action.[13,14]

Retinoids have been extensively studied in a variety of pre-clinical models of cancer as well as in human clinical studies.[15] Three retinoids have been approved in the US for the treatment of malignancies: ATRA for acute promyelocytic leukemia, bexarotene, a RXR-selective agonist, for cutaneous T cell lymphoma and 9-*cis*-retinoic acid, a pan RAR/RXR agonist, for Kaposi's sarcoma.[16–18] However, newer retinoids, with more specific and robust anti-proliferation or pro-differentiation or pro-apoptotic effects and fewer systemic toxic effects, are required before the full potential of retinoids in oncology is realized. Recent medicinal chemistry advances have resulted in the design of agonists that are specific for the RAR or RXR families of receptors and the individual RAR subtypes.[19] Receptor antagonists and inverse agonists have also been developed. These compounds elicit biological activities that are quite distinct from the natural hormones and might have novel applications in cancer.[20–22] Here, we review the effects of retinoids on prostate cancer, with particular emphasis on the potential therapeutic use of RAR antagonists.

Expression of retinoid receptors in prostate cancer

Immunohistochemical examination of primary prostate carcinoma revealed a correlation between RARα expression and tumor grade.[23] In addition, higher levels of RARα expression were found in more proliferative tumors. In another study, ***in situ*** hybridization was used to compare expression levels of all RARs and RXRs in normal and cancerous human prostate tissues.[24] While RARα, RARγ, RXRα and RXRγ were expressed in both normal and cancerous prostates, RARβ and RXRβ expression was selectively reduced in prostate cancers.

Clinical studies of retinoids in prostate cancer

Many studies in pre-clinical models have indicated the utility of retinoids in prostate cancer, though clinical studies have been limited and have demonstrated only mild to moderate efficacy of retinoids in the treatment of prostate cancer. Prostate-specific antigen (PSA) levels were used as surrogate efficacy end points in patients with rising PSA after radical prostatectomy and treated with 1 mg/kg/day of 13-*cis* retinoic acid (isotretinoin).[25] Only modest effects on reduction or stabilization of PSA levels were observed. In two sequential clinical trials, androgen-independent prostate cancer patients were treated with ATRA, and both androgen-independent and androgen-dependent patients were treated with a combination of isotretinoin and interferon (IFN)-α.[26] Only modest anti-tumor effects were observed with either of the two treatment protocols. In a phase I/II trial of isotretinoin and IFN-α in patients with recurrent prostate cancer, a decrease of PSA was observed in 26% of patients.[27] The same investigators then conducted a phase I study in which escalating doses of paclitaxel were added to the combination of isotretinoin and IFN-α, and two patients achieved partial responses. Based on these results, a phase II study of isotretinoin, IFN-α and paclitaxel has been initiated. In an alternative approach, liarazole, a cytochrome P450 inhibitor, which prevents catabolism of ATRA and thereby increases endogenous ATRA levels, has been used to treat hormone-resistant, progressive prostate cancer.[28] The pain score was decreased in over 50% of the patients and 41% showed a PSA decrease of 50% or more. These clinical results and promising pre-clinical results with newer compounds argue for the continued search for retinoid drugs for prostate cancer.

Pre-clinical studies of retinoids in prostate cancer

Prostate adenocarcinoma lines are commonly used to evaluate the potential of retinoids in the treatment

of prostate cancer: these include LNCaP, DU-145 and PC-3. LNCaP cells are androgen-responsive, whereas DU-145 and PC-3 are androgen-insensitive. LNCaP cells express RARα, RARβ and RARγ, and DU-145 and PC-3 cells express RARα and RARγ.

High concentrations of ATRA inhibit the growth and induce apoptosis of androgen-dependent, and derived, androgen-independent, LNCaP cells.[29] However, ATRA, at 2 μM, is ineffective in inhibiting colony formation of DU-145 and PC-3 cells.[30] In another study, ATRA, in the presence or absence of androgens, induced differentiation of LNCaP cells, as measured by PSA secretion.[31] In this study a growth inhibitory effect of ATRA against LNCaP cells was only observed in the presence of androgen. The chemopreventive value of ATRA has also been evaluated in a family of human prostate epithelial cell lines which represent various stages of prostate cancer progression.[32] ATRA inhibited both the anchorage-independent and anchorage-dependent growth of WPEI-NB14 and WPEI-NB11 tumor cells. ATRA also inhibited *in vitro* invasion by these cells. The combination of a differentiating agent, phenybutyrate, and 13-*cis*-retinoic acid gave additive inhibition of proliferation and increased apoptosis in rodent prostate cancer cell lines.[33] The same combination inhibited prostate tumor growth *in vivo* and decreased angiogenesis. The administration of 13-*cis*-retinoic acid to athymic nude mice xenografted with LNCaP cells resulted in a significant reduction in the size of established tumors, with 50% of the animals showing complete regression of tumors.[34] A synergistic reduction in tumor size was observed when xenografts were treated with 13-*cis*-retinoic acid together with androgen ablation.

A number of synthetic retinoids have been tested for activity against prostate carcinoma cells. A RARγ-selective agonist, CD 271, was much more effective than ATRA in inhibiting growth and colony formation, and in inducing apoptosis of DU-145 cells.[30] SR11262, a RARβ/γ-selective retinoid, gave a synergistic inhibition of clonal growth of LNCaP cells when combined with a vitamin D analog.[35] This synergistic effect was not found in PC-3 and DU-145 cells which do not express RARβ. Stable expression of RARβ into the PC-3 cells resulted in synergistic inhibition of growth by SR11262 and the vitamin D analog. These studies indicate roles for RARγ and RARβ in controlling the growth of prostate cancer cells.

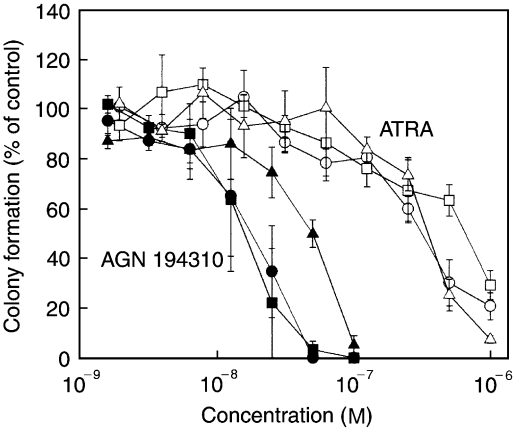
The pan RAR and RXR activator, 9-*cis*-retinoic acid, induced differentiation of LNCaP cells.[31] Also, 9-*cis*-retinoic acid effectively inhibited colony formation of LNCaP cells and gave synergistic inhibition with a vitamin D analog.[36,37] In a carcinogen-induced prostate carcinogenesis model in rats, 9-*cis*-retinoic acid was effective in the chemoprevention of prostate cancer at doses which did not cause overt toxicity.[38] However, since 9-*cis*-retinoic acid is a pan-activator of RARs and RXRs, the actual potential of RXR activators in prostate cancer cannot be determined by the above studies. A potential role for RXR ligands is confirmed by the observation that synthetic RXR-selective retinoids, such as SR11246, are effective in inhibiting the clonal growth of LNCaP and PC-3 cells.[37,39]

A novel retinoid analog, *N*-(4-hydroxyphenyl) retinamide (4-HPR), was approximately 10 times more potent than ATRA in reversing the malignant phenotype and in inducing apoptosis in DU-145 cells.[46] 4-HPR was also significantly more potent than ATRA in inhibiting growth and *in vitro* invasion of WPEI-NB14 and WPEI-NB11 cells.[32] LNCaP cells treated with 4-HPR showed growth arrest, high levels of apoptosis, and a significant decrease in intracellular and secreted PSA.[41] In an *in vivo* study, tumor incidence and mass were significantly decreased by 4-HPR in the *ras* + *myc*-induced mouse prostate reconstitution model system.[42] In a similar study, in the mouse prostate reconstitution model using heterozygous (+/−) or homozygous (−/−) p53 mutant epithelial cells, 4-HPR did not have an effect on primary tumor weight but significantly suppressed the development of bone metastases.[43] While 4-HPR activates traditional RAR/RXR pathways, there are studies indicating that 4-HPR induces apoptosis by generating radical oxygen species by a RAR/RXR-independent pathway.[44] CD437, another RARγ-selective synthetic retinoid, significantly inhibited the proliferation of LNCaP and PC-3 cells and concomitant S phase arrest was followed by apoptosis.[45] A dual mechanism of action has been proposed for CD437 in squamous cell carcinoma cells: a RAR/RXR-mediated suppression of squamous differentiation and a RAR/RXR-independent induction of apoptosis.[46] In another study, CD437 induced rapid apoptosis of DU-145, PC-3 and LNCaP cells, and this correlated with the increased expression of certain oncogenes (c-Myc and c-Jun) and death receptors (DR4, DR5 and Fas).[47] A study using human leukemia cells has provided evidence for a lysosomal pathway for CD437 involving release of a protease, cathepsin D, into the cytosol resulting in increased oxidative stress and apoptosis.[48] Thus, whilst retinoids can effect prostate cancer cell proliferation and apoptosis by classical RAR/RXR-mediated pathways, other pathways may involve RARs in non-classical settings or may not involve RARs at all.

RAR antagonists in prostate cancer

RAR antagonists are a new class of retinoids that bind to RARs but do not activate gene transcription. Instead, they inhibit the transcriptional activity of agonists such as ATRA.[20,50] Additionally, antagonists entirely block ATRA-induced neutrophil differentiation of the promyeloid cell line HL60.[49,51] Interestingly, some antagonists can suppress the basal transcriptional activity of a retinoid-responsive promoter by recruiting co-repressor molecules to the receptor, and hence function as inverse agonists.[21,22] Table 1 shows the structures of RAR antagonists and their binding properties.

We have shown that RAR antagonists are significantly more potent than agonists in inhibiting the growth of prostate cancer cell lines as well as cultures of primary prostate cancer cells derived from patient biopsies.[49] We screened a panel of retinoid agonists and antagonists (at 100 nM) for their ability to block colony formation in a plate assay by LNCaP, PC-3 and DU-145 cells. We used fetal bovine serum (FBS)-grown cells and sub-lines that had been grown long term under serum-free conditions (ITS^+ supplement): the latter cells were used to prevent naturally occurring retinoids from masking the effects of added antagonists. We compared activities of a RAR pan-antagonist (AGN 194310, K_d=2–5 nM for all three RARs), a RARα-specific antagonist (AGN 194301) and synthetic RAR agonists with selectivity for RARα (AGN 194078 and AGN 195153) or RARβ/γ (AGN 190299). Agonists, ATRA and those with RARα and RARβ/γ selectivity, had very little effect on serum and serum-free grown cells. In striking contrast, the RAR pan-antagonist (AGN 194310) nearly completely inhibited colony formation (by 93–97%) by serum-free grown cells of all three lines. The RARα-specific antagonist was also effective against serum-free grown cells (42–74% inhibition). The inhibitory effects of both the antagonists were much lower



Cell line	Receptors expressed	AGN 194310 $\alpha \sim \beta \sim \gamma$	AGN 194431 $\beta > \gamma >> \alpha$	AGN 194301 $\alpha >> \beta / \gamma$
LNCaP	RARα,β,γ	IC_{50} = 16 nM	IC_{50} = 99 nM	IC_{50} = 203 nM
PC-3	RARα,γ	IC_{50} = 18 nM	IC_{50} = 104 nM	IC_{50} = 235 nM
DU145	RARα,γ	IC_{50} = 34 nM	IC_{50} = 88 nM	IC_{50} = 201 nM

Figure 1. RAR antagonists are potent inhibitors of colony formation by serum-free grown prostate cancer cell lines. The figure shows that a pan-antagonist of RAR (AGN 194310, closed symbols) is more potent than ATRA (open symbols) in inhibiting colony formation in a plate assay by LNCaP (circles), DU-145 (triangles) and PC-3 (squares) cells. The table shows IC_{50} values obtained from full dose–response curves for the activities of pan-, α-selective and β/γ-selective antagonists against the three lines.

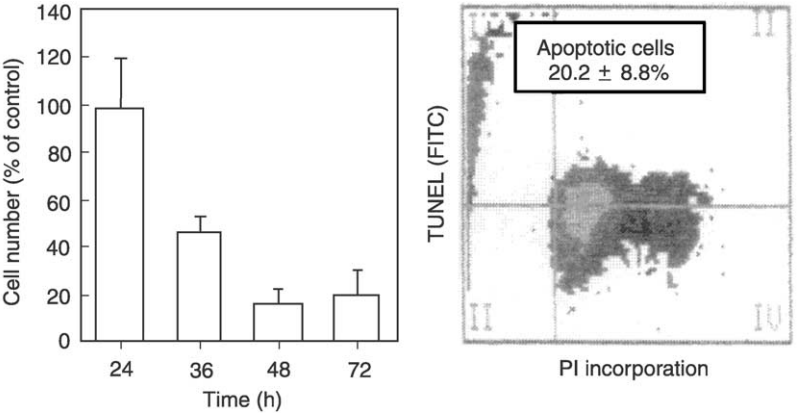


Figure 2. Antagonists induce apoptosis of prostate cancer cells. Treatment of flask cultures of LNCaP cells with 1 μM AGN 194310 for 3 days resulted in high levels of inhibition of cell growth (left panel) and of apoptotic cells (right panel). Apoptotic cells were identified by TdT end-labeling and flow cytometry.

against FBS-grown cells (11–23% inhibition by AGN 194310), suggesting the presence of a serum 'reversal factor' (see below).

Full dose–response curves obtained for inhibition of colony formation of the three serum-free grown lines demonstrated clearly the increased potency of RAR antagonists (pan-, α-selective and β/γ-selective) versus agonists, such as ATRA (see Figure 1). The pan-antagonist AGN 194310 was the most potent compound, with IC_{50} values ranging from 16 to 34 nM (see table in Figure 1). Selective α (AGN 194301) and β/γ (AGN 194431) antagonists were less potent, though they completely inhibited colony formation of all three lines. ATRA did not completely inhibit colony formation even at 1 μM. To ascertain the generality of the potency of RAR pan-antagonists, we have shown that another compound (AGN 193109) is as effective as AGN 194310. RAR antagonists inhibit colony formation of prostate cancer cells by causing an initial accumulation of cells in the G_1 phase followed by an induction of apoptosis.[49] Treatment of flask cultures of serum-free grown LNCaP cells with 1 μM AGN 194310 caused an 80% inhibition of cell growth and a high level of apoptotic cells (20 versus 3% in controls, see Figure 2) by 3 days. At day one, there was reduced viability (to 50%) and a transient increase in the proportion of G_1 cells (from 62 to 71%).

Our initial screening data suggested that a serum component interferes with the activity of antagonists. Indeed, adding FBS to serum-free grown cells treated with 100 nM of the pan and RARα antagonists caused a partial reversal of inhibitory effects.[49] Similarly, a full dose–response curve for AGN 194310 against serum grown cells was shifted to the right of that obtained for serum-free grown cells. However, at higher concentrations, AGN 194310 still essentially completely inhibited the growth of serum grown cells and was more effective than ATRA (compare the titrations in Figure 3). Additionally, LNCaP cells produce a factor that interferes with the growth inhibitory effect of RAR antagonists. A higher concentration of AGN 194310 was required to inhibit the growth of a large number of cells seeded in flask cultures (1 μM) than when fewer cells were plated and treated in dishes (100 nM). Strikingly, conditioned medium from exponentially growing flask cultures of LNCaP cells completely reversed the growth inhibitory effect of 100 nM AGN 194310 when tested against LNCaP cells in the plate colony formation assay.[49] Conditioned medium also stimulated colony formation of serum-free grown cells. Thus, an autocrine growth-stimulatory loop can override the effect of antagonists. The most likely candidates, insulin-like growth factor-1 and epidermal growth factor, did not block the effect of antagonists.[49] Autocrine/serum factor(s) may limit the therapeutic effectiveness of RAR antagonists and identifying these factors is important.

Of crucial importance is: are RAR antagonists potent growth inhibitors of primary prostate cancer cells derived from patient biopsies and, moreover, more effective than ATRA? We tested the pan-antagonist AGN 194310 and ATRA against cancer cells derived from malignant biopsies from eight patients. Dose–response curves were obtained

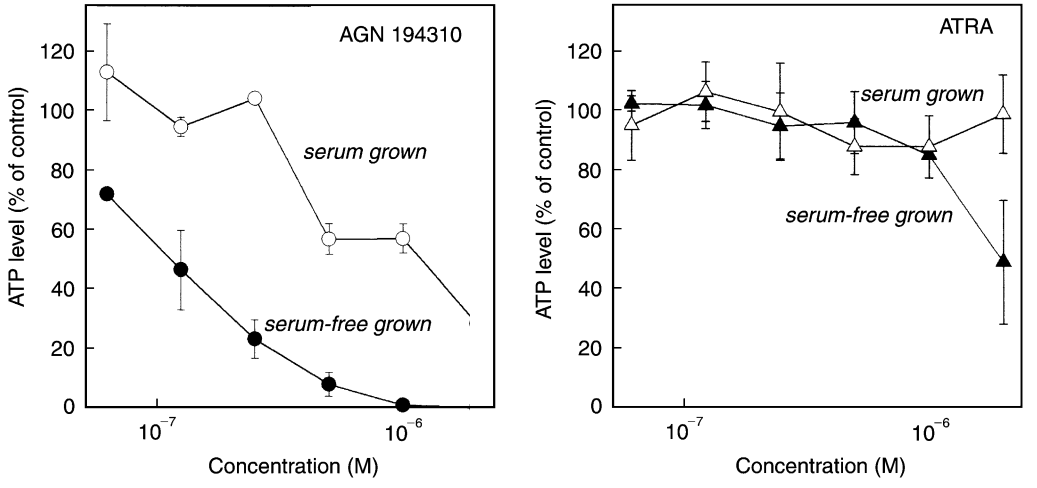
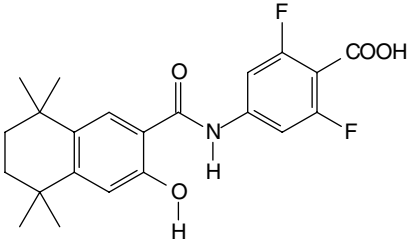
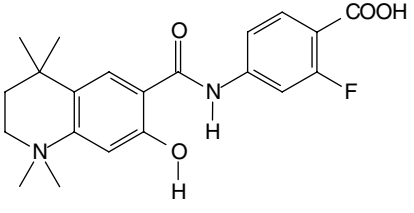
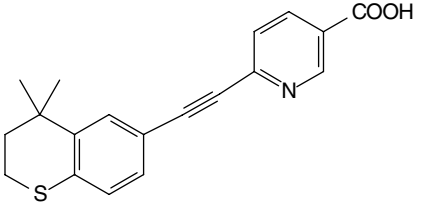
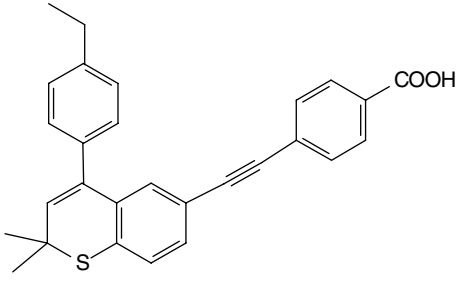
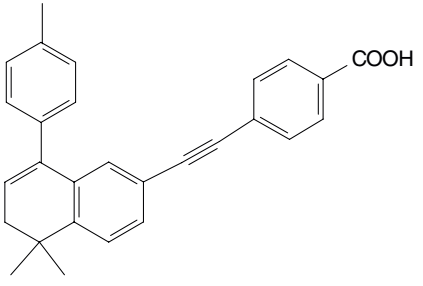
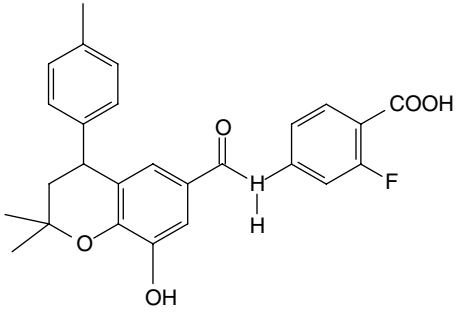
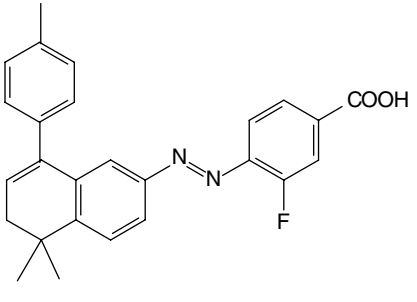


Figure 3. Antagonists are more effective against serum-free grown than serum grown prostate cancer cells. The left panel shows the activity of the pan-antagonist AGN 194310 against serum-free grown (closed symbols) and serum grown (open symbols) LNCaP cells. The right panel shows the activity of ATRA against these two populations of cells (serum-free, closed symbols; serum, open symbols). Cells were treated in liquid culture in microtiter wells and cell growth/survival was measured using a fluorometric assay of ATP levels.

Table 1. Novel retinoid analogs, and their receptor binding and transactivation properties

Compound no. (AFN)	Structure	Receptor specificity[a]	RARα		RARβ		RARγ	
			K_d[b] (nM)	EC_{50}[c] (nM)	K_d[b] (nM)	EC_{50}[c] (nM)	K_d[b] (nM)	EC_{50}[c] (nM)
194078		RARα agonist	4	140	> 5000	WA	> 5000	NA[d]
195153		RARα agonist	40	130	> 5000	WA	> 5000	WA[d]
190299		RARβγ agonist	616	> 1000	41	18	57	42
194310		RARαβγ agonist	3	NA[d]	2	NA[d]	5	NA[d]

193109		RARαβγ agonist	2	NA[d]	2	NA[d]	3	NA[d]
194301		RARα agonist	3	NA[d]	320	NA[d]	7250	NA[d]
194431		RARβγ agonist	300	NA[d]	6	NA[d]	70	NA[d]

[a]None of the compounds bound to (K_d values $> 10\ \mu M$) or activated any of the RXR subtypes.
[b]Receptor binding was determined with full-length, baculovirus-expressed receptors in competitive binding assays using radiolabeled ligands.
[c]Functional activity of the compounds was determined in CV-1 cells transiently transfected with an appropriate RAR/RXR- or RXR/RXR-responsive reporter gene together with an expression vector for a specific receptor subtype.
[d]Abbreviations: NA, inactive; WA, weak partial agonist.

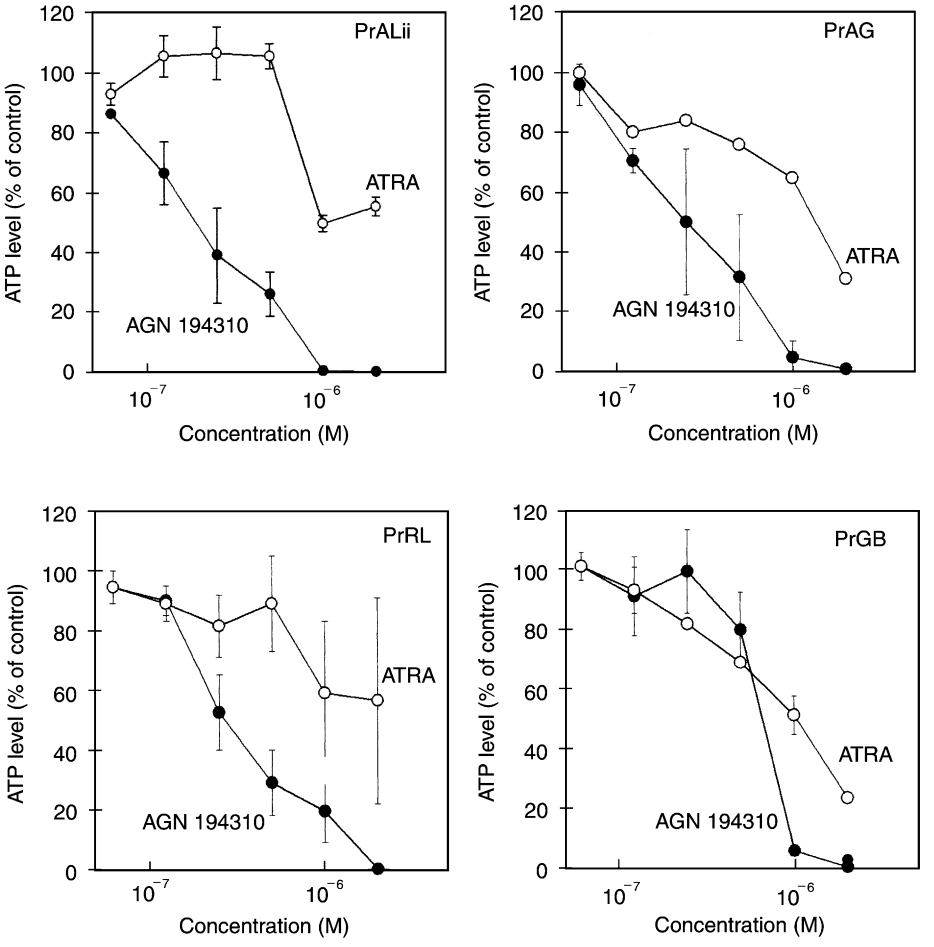


Figure 4. A pan RAR antagonist is a potent growth inhibitor of primary prostate cancer cells. Primary prostate cancer cells were derived from malignant biopsies from patients. The figure compares the activities of the pan-antagonist AGN194310 (closed circles) and ATRA (open circles) against cells from six patients. Cells were treated in liquid culture in microtiter wells and cell growth/survival was measured using a fluorometric assay of ATP levels.

against cells grown in liquid culture in microtiter wells and cell survival was measured using a fluorometric assay of ATP levels. AGN 194310 effectively and completely inhibited the growth of prostate cancer cells derived from all eight patients (Figure 4).[49] By contrast, ATRA, at 2 μM, only partially inhibited (around 50%) the growth of patients' cells. The titrations shown in Figure 4 demonstrate the robust anti-proliferative effect of the pan-antagonist versus the moderate efficacy of ATRA.

Prospects

Limited clinical studies, and studies in pre-clinical models, have demonstrated a moderate efficacy of ATRA in the treatment of prostate cancer. However, advances in medicinal chemistry have led to the development of RAR antagonists that are considerably more potent than ATRA in inhibiting the growth of patients' prostate cancer cells. Information as to the effectiveness of antagonists in *in vivo* mouse model systems of prostate carcinoma is important to the clinical development of these compounds. These studies should also resolve whether a serum 'reversal factor' interferes with the effectiveness of antagonists. Identification of this factor(s) is important in determining which other therapies should be combined with antagonists in prostate cancer clinical trials.

Are there prospects for developing retinoids that are even more specific and more potent? A focus of attention in retinoid research has been the classical

RAR/RXR-mediated pathways, although, as mentioned in this review, retinoids can exert effects via pathways that do not involve RARs or which may involve RARs in non-classical settings. For estrogen receptors, ligands of different sizes and shapes induce a spectrum of receptor conformational changes.[52,53] In addition, whether these different ligand–receptor complexes are bound to DNA directly via the estrogen receptor or tethered via another moiety and the availability and recruitment of specific coactivators (SRC1) appear to determine whether selective estrogen receptor modulators, such as tamoxifen and raloxifene, act as agonists in some tissues (bone, liver and cardiovascular), antagonists in others (breast and brain) and as mixed agonists/antagonists (uterus).[54,55] Such levels of complexity, of conformation, context and co-regulators, are likely to be exploitable in the context of retinoid receptors. A greater understanding of the breadth and mechanisms of action of retinoids may allow the development of newer and more powerful synthetic retinoids to assist in the fight against prostate cancer.

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