Inhibitory effects of retinoic acid metabolism blocking agents (RAMBAs) on the growth of human prostate cancer cells and LNCaP prostate tumour xenografts in SCID mice

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In recent studies, we have identified several highly potent all-trans-retinoic acid (ATRA) metabolism blocking agents (RAMBAs). On the basis of previous effects of liarozole (a first-generation RAMBA) on the catabolism of ATRA and on growth of rat Dunning R3227G prostate tumours, we assessed the effects of our novel RAMBAs on human prostate tumour (PCA) cell lines. We examined three different PCA cell lines to determine their capacity to induce P450-mediated oxidation of ATRA. Among the three different cell lines, enhanced catabolism was detected in LNCaP, whereas it was not found in PC-3 and DU-145. This catabolism was strongly inhibited by our RAMBAs, the most potent being VN/14-1, VN/50-1, VN/66-1, and VN/69-1 with IC50 values of 6.5, 90.0, 62.5, and 90.0 μM, respectively. The RAMBAs inhibited the growth of LNCaP cells with IC50 values in the μM-range. In LNCaP cell proliferation assays, VN/14-1, VN/50-1, VN/66-1, and VN/69-1 also enhanced by 47-, 60-, 70-, and 65-fold, respectively, the ATRA-mediated antiproliferative activity. We then examined the molecular mechanism underlying the growth inhibitory properties of ATRA alone and in combination with RAMBAs. The mechanism appeared to involve the induction of differentiation, cell-cycle arrest, and induction of apoptosis (TUNEL), involving increase in Bad expression and decrease in Bcl-2 expression. Treatment of LNCaP tumours growing in SCID mice with VN/66-1 and VN/69-1 resulted in modest but statistically significant tumour growth inhibition of 44 and 47%, respectively, while treatment with VN/14-1 was unexpectedly ineffective. These results suggest that some of our novel RAMBAs may be useful agents for the treatment of prostate cancer.

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and CYP3A7 are ATRA 4-hydroxylases and are involved in ATRA metabolism as well, although their specificity for ATRA is low (Njar, 2002, 2006; Marill et al, 2003). PCA cells may develop resistance to ATRA using this mechanism as demonstrated by APL patients who relapse within 3–15 months after first remission with ATRA therapy (Norum, 1993).

Although research to date has concentrated on the use of exogenous retinoids, a potential new approach to the treatment and prevention of cancer is the use of retinoic acid metabolism blocking agents (RAMBAs), which increase levels of retinoic acid within tumour cells by blocking their metabolism (Wouters, 1994; Miller, 1998; Njar, 2002, 2006). Additionally, it has been shown that certain retinoids, including ATRA, are capable of directing neoplastic cells to the normal phenotype of morphological maturation and loss of proliferative capacity, thereby reversing or suppressing developing lesions and preventing cancer invasion (Sporn et al, 1976; Sporn, 1991; Lippman et al, 1994; Moon et al, 1994; Lotan, 1996).

We have designed and synthesized a number of novel RAMBAs to inhibit CYP26 and the other ATRA 4-hydroxylases with the goal of preventing in vivo metabolism and thereby increasing the endogenous levels of ATRA (Njar et al, 2000; Patel et al, 2004). These novel RAMBAs have been described as atypical, owing to their multiple biological activities especially in MCF-7 and T47D human breast cancer cells (Patel et al, 2004). The effects of the RAMBAs (VN/14-1, VN/50-1, VN/66-1, and VN/69-1; Figure 1), alone and in combination with ATRA, on ATRA metabolism, and on PCA cell viability, apoptosis, cell cycle, and differentiation, and on in vivo antitumour studies have been examined. In addition, the molecular mechanisms underlying the biological activities of these agents were also investigated. These studies are the basis of this report.

MATERIALS AND METHODS

Drug preparations

We have previously published the syntheses of the RAMBAs used in this study (Patel et al, 2004). All-trans-retinoic acid (ATRA) and the RAMBAs were dissolved in 95% ethanol and stored at 4°C. The concentrations (1 and 5 µM) of the RAMBAs were dissolved in 95% ethanol and stored at -20°C in the dark. The concentrations (1 and 5 µM) of the various agents (retinoids and RAMBAs) are typical for retinoid cancer cells inhibitory studies (Wu et al, 2001). All chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise noted.

Cell culture

Prostate cancer cell lines, LNCaP, PC3, and DU145, were incubated in the RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA, USA) preparation containing 10% FBS (HyClone, Logan, UT, USA) and 1% penicillin–streptomycin solution (Gibco-Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO₂. All cells were subcultured weekly. LNCaP cells used in the following studies were performed between passages 7–20.

Preparation of cellular CYP26 microsomes

The procedure described by Han and Choi (1996) was used. Briefly, LNCaP cells were incubated with 1 µM ATRA for 24 h to induce the CYP26 enzyme. The cells were trypsinised and rinsed with phosphate-buffered saline (PBS) (Life Technologies, Grand Island, NY, USA) by centrifugation for 5 min at 3000 x g at room temperature. The cells were resuspended in homogenate buffer (0.5 M sucrose, 10 mM Tris-Cl (pH 7.4), 1 mM ethylenediaminetetraacetate (EDTA), 1 µM phenylmethylsulfonyl-fluoride, 0.1 µg ml⁻¹ leupeptin, and 0.04 U ml⁻¹ aprotonin), homogenised, and then centrifuged at 9000 x g for 10 min at 4°C. The supernatant was then centrifuged at 100 000 x g for 45 min at 4°C. The pellet was resuspended in storage buffer (0.25 M sucrose, 10 mM Tris-Cl (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl-fluoride, 0.1 µg ml⁻¹ leupeptin, and 0.04 U ml⁻¹ aprotonin) and stored at -20°C.

Cellular microsomal CYP26 assay

The procedure for the hepatic microsomal enzyme assay (for ATRA 4-hydroxylases) was used where 100 µl of cellular CYP26 microsomes (500 µg ml⁻¹ dissolved in storage buffer) was substituted for 100 µl of hepatic microsomes. The retinoid products were extracted, processed, and analysed by HPLC as previously described (Patel et al, 2004).

Cellular CYP26 assay

As modified from a procedure described by Wouters et al (1992), approximately 10⁶ LNCaP cells were incubated with 1 µM ATRA for 24 h to induce the CYP26 enzyme. Following ATRA treatment, the LNCaP cells were incubated with 0.1 or 0.8 µM [11,12-³H]-ATRA (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA) for 5 h. Following the 5 h incubation, the medium was collected and the cells were trypsinised and collected. The retinoid products were extracted, processed, and analysed by HPLC as previously described (Patel et al, 2004).

WST-1 cell viability assay

To measure cell viability, 24-well plates were coated with a 0.05% poly-l-lysine solution for 30 min. The wells were then washed with sterilised dH₂O. LNCaP cells (1 × 10⁴) were seeded in the plates and maintained in RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA, USA). The cells were allowed to attach for 36 h. After attachment, fresh media was added and the cells were treated with a concentration range of either ATRA or each of the RAMBAs.

Figure 1 Chemical structures of RAMBAs, VN/14-1, VN/50-1, VN/66-1, and VN/69-1 and 4-HPR.
for 6 days. The media was changed every 3 days. On the day the assay was performed, the media was removed and 1 ml of a stock solution of WST-1 (4-[(3-[4-(iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) cell proliferation reagent (Boehringer Mannheim, Indianapolis, IN, USA), diluted 1:10 in RPMI 1640 medium (without FBS), was added to each well. The plates were incubated at 37°C for 3 h. The media was then removed and 500 μl of DMSO was added and agitated vigorously for 5 min. The slightly red tetrazolium salt WST-1 is reduced to a dark red, water-soluble formazan product by mitochondrial dehydrogenase from living cells, which gives absorbance at a wavelength of 450 nm.

**Differentiation assay (Western-blot analysis of cytokeratins 8/18)**

LNCaP cells were incubated in the RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA, USA) preparation containing 1 μM ATRA alone or in combination with 1 μM of each of the RAMBAs for 6 days. LNCaP cells were scraped with 1.5 ml of PBS. The cells were collected by centrifugation, resuspended in ice-cold cell lysis buffer (Boehringer Mannheim, Indianapolis, IN, USA), diluted 1:10 in RPMI 1640 medium (without FBS), was added to each well. The plates were incubated at 37°C for 3 h. The media was then removed and 500 μl of DMSO was added and agitated vigorously for 5 min. The slightly red tetrazolium salt WST-1 is reduced to a dark red, water-soluble formazan product by mitochondrial dehydrogenase from living cells, which gives absorbance at a wavelength of 450 nm.

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TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) assay

A total of 4000 LNCaP cells were plated on each well of an eight-well Lab-Tek II® chamber slide (Nalge Nunc International, Naperville, IL, USA). The LNCaP cells were then treated with either 1 or 5 μM of ATRA or RAMBA for 6 days. Cells were then fixed in 4% paraformaldehyde solution in dH2O. The cells were then processed and stained with FITC (fluorescein isothiocyanate) according to kit instructions (In situ cell death detection kit, AP, Roche Diagnostics Corp., Indianapolis, IN, USA). The FITC-stained cells are then mounted with VectorShield® mounting medium for fluorescence with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) nuclear stain (Vector Laboratories, Inc., Burlingame, CA, USA) and covered with ApoTag® plastic coverslips (Intergen Company, Purchase, NY, USA) and counted using SPOT Advance® 3.5 software (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

**Cell-cycle analysis**

LNCaP cells were treated with 1 or 5 μM of ATRA or each of the RAMBAs for 6 days. The cells were trypsinised and washed twice in PBS (Life Technologies, Grand Island, NY, USA). The cells were fixed by adding 70% ethanol and stored at −20°C until staining. The cells were washed twice in PBS. A volume of 1 ml of a propidium iodide (PI) solution (50 μg ml−1 in PBS) and 500 μl of an RNase stock solution (100 μg ml−1) were added to the cells and incubated for 1 h in the dark. Approximately 10⁶ stained cells were then analysed by flow cytometry (Becton Dickinson FACScan, Franklin Lakes, NJ, USA). The percentages of cells in G0/G1, S, and G2/M phases of the cell cycle as well as percentage of apoptotic cells (sub G0/G1) were determined using MODFIT LT software (Verity Software House, San Joe, CA, USA). The percentages of cells in G0/G1, S, and G2/M phases of the cell cycle were obtained from diploid cells in the sample and the percentage of apoptotic cells (sub G0/G1) was obtained from total cells minus the debris.

**In vivo antitumour studies (LNCaP human prostate carcinoma xenograph mice model)**

All animal studies were performed according to the guidelines and approval of the Animal Care Committee of the University of Maryland School of Medicine, and were consistent with United Kingdom Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia. The procedure was modified from Grigoryev et al (1999). Briefly, LNCaP cells were trypsinised, counted, and suspended in Matrigel (1:1000) (Fisher Scientific International, Inc., Hampton, NH, USA). Male SCID mice of 4–6 weeks of age were obtained from the National Cancer Institute (Frederick, MD, USA). Each mouse was inoculated s.c. with 0.1 ml of the cell suspension at two sites in the flank. The size of the tumours was determined by measuring the tumour volumes using calipers. Tumour volumes were calculated using the formula

\[ V = \frac{4}{3} \pi \times r_1^2 \times r_2 (r_2 < r_1) \]

The tumours were allowed to develop to approximately 100 mm³ before treatment. The mice were treated with 10 mg kg⁻¹ (which is equivalent to 0.033 mmol kg⁻¹) of ATRA or an equivalent dose to 0.033 mmol kg⁻¹ of each RAMBA (VN/14–1, VN/50–1, VN/66–1, and VN/69–1) dissolved in hydroxypropyl-β-cellulose (HPC, 0.3% in saline). Tumour volumes were measured twice weekly and weights of animals were taken weekly after the initiation of treatment. The mice were subjected to 6 weeks of treatment. The
mice were then killed and the tumours were excised, weighed, and stored at −80°C until analysis. Additionally, the plasma was collected with 50 IU of heparin and stored at −80°C until analysis.

Statistical analysis
One-way analysis of variance (ANOVA) with post test, which was the Bonferroni multiple comparisons test, was performed to compare all pairs of treatment groups using InStat3 (GraphPad Software, Inc., San Diego, CA, USA). A P<0.05 was considered significant.

RESULTS
ATRA metabolism is inducible in LNCaP cells but not in PC3 and DU145 cells
To assess the induction of ATRA metabolism in LNCaP, PC3 and DU145 prostate cells, these cells were pre-incubated with 1 μM ATRA for various time points from 4 to 48 h and then further incubated with 0.8 μM [11,12-3H]-ATRA for 5 h to measure ATRA metabolism. Our results show that LNCaP cells were induced by ATRA and were able to metabolise [11,12-3H]-ATRA to more polar products from the 4 to 48 h time points with maximum metabolism occurring at 36 h (Figure 2). PC-3 cells did not show any metabolism of [11,12-3H]-ATRA from these time points (Figure 2) and also up to 9 days (data not shown). DU145 cells were able to metabolise [11,12-3H]-ATRA from the 4 to 48 h time points (Figure 2); however, this metabolism was not enhanced despite ATRA treatment. As only the LNCaP cell line was able to metabolise ATRA, it was utilised in subsequent cellular assays.

Inhibition of ATRA metabolism in intact LNCaP cells (cellular assay)
To assess the ability of our novel RAMBA to inhibit ATRA metabolism in intact cells, we used VN/14-1, VN/50-1, VN/66-1, and VN/69-1 to evaluate their inhibitory potencies in LNCaP cells. Human LNCaP carcinoma cells cultured under control conditions are unable to metabolise ATRA into more polar metabolites (data not shown). However, after pretreatment with 1 μM ATRA for 12–15 h, the cells show extensive ATRA metabolism (Figure 3A), converting ATRA into highly polar metabolites (HPM, retention time, R₁ = 3–6 min), and prominent metabolites of medium polarity (MMP, R₂ = 8–12 min), including 4-oxo and 4-hydroxy-ATRA. ATRA metabolism is inhibited dose dependently by VN/14-1 (Figures 3B–D). Identical results were also obtained with the other three compounds tested. The IC₅₀ values for these compounds were determined from dose–response curves and are presented in Table 1. The compounds inhibited intracellular ATRA metabolism with decreasing activity in the order: VN/14-1 > VN/66-1 > VN/50-1 > VN/69-1. Compound VN/14-1 was the most active with an IC₅₀ value of 6.5 ± 1.5 nM. VN/50-1 also inhibited ATRA metabolism of microsomes prepared from T47D cells previously exposed to ATRA with an IC₅₀ value of 7.0 ± 1.2 nM, which is of about a 13-fold higher potency compared to the cellular IC₅₀ value. Although the other inhibitors were not evaluated using microsomal preparations, it is likely that they may exhibit similar higher potencies.

Effects of the RAMBAs alone and in combination with ATRA on LNCaP cell growth
Given the retinoidal nature of our RAMBAs it seemed logical to investigate their effects on the growth of LNCaP cancer cells. The antiproliferative effect of ATRA was also studied for comparison using a WST assay. Continuous exposure of LNCaP cells to various doses of the RAMBAs and ATRA for 6 days led to dose-dependent inhibition of cell growth as shown in Figure 4. The calculated IC₅₀ values (defined as the concentration of compounds required to inhibit cell growth by 50%) from these dose–response curves are presented in Table 1 and show that all RAMBAs are modest inhibitors (IC₅₀ = 4.5–10.0 μM) of LNCaP cell proliferation and comparable to the potency of ATRA (IC₅₀ = 4.3 μM).

The ability of each of these RAMBAs to enhance the antiproliferative activity of ATRA in LNCaP cells was also studied. ATRA inhibits LNCaP cell proliferation in a concentration-dependent manner (Figure 4) with a calculated IC₅₀ value of 4.3 μM (Table 1). For studies of effects of combination of RAMBAs with ATRA, we used low doses (1 μM each, doses that exhibited low (<10%) antiproliferative effects) of RAMBAs. All RAMBAs, that is, VN/14-1, VN/50-1, VN/66-1, VN/69-1 each in combination with ATRA, significantly enhance the antiproliferative activity of ATRA, by 47-, 60-, 70-, 65-fold, respectively (for VN/14-1, IC₅₀ from 10.0 to 0.092 μM; for VN/50-1, IC₅₀ from 6.0 to 0.071 μM; for VN/66-1, IC₅₀ from 4.5 to 0.061 μM; and for VN/69-1, IC₅₀ from 5.0 to 0.066 μM) (Figure 4 and Table 1). These enhancements of ATRA activity are considered synergistic because the growth inhibitory effects were each significantly greater than the predicted values.

Effects of the RAMBAs alone and in combination with ATRA on LNCaP cell differentiation
Cytokeratins 8/18 are present in the differentiated luminal epithelia in prostate and an increase in their content is considered to indicate differentiation (Owens and Lane, 2003). To determine the effects of these retinoids on cell differentiation, LNCaP cells were incubated with various concentrations of ATRA or RAMBA for 6 days; cell lysates were prepared and subjected to SDS–PAGE gel electrophoresis. Increase in cytokeratin 8/18 expression was used as the marker for differentiation (Peelh et al, 1993; Hsieh et al, 1995). There was a concentration-dependent increase in the expression of cytokeratin 8/18 in LNCaP cells treated with ATRA, VN/14-1, VN/50-1, VN/66-1, and VN/69-1 (data not shown) and also a significant increase in the cytokeratin 8/18 expression for 5
and 10 μM treatment for ATRA and all RAMBAs (P<0.05) compared to control (data not shown). This increase in cytokeratin 8/18 expression in LNCaP cells treated with ATRA and the RAMBAs is indicative of differentiation.

For combination studies, LNCaP cells were incubated with 1 μM ATRA, alone and in combination with 1 μM of RAMBA for 6 days.

Our results are summarised in Figure 5 and show significantly increase in the expression of cytokeratin 8/18 by 1.18 to 1.38-fold above control (1 ± 0.07, P<0.05). ATRA (1 μM) in combination with 1 μM of each of the RAMBAs, except for VN/69-1, also significantly increased the expression of cytokeratin 8/18 by 1.32–1.59-folds above control [1 ± 0.07, (P<0.05)]. Thus, most RAMBAs...
enhanced the prodifferentiation activity of ATRA in the LNCaP cells.

**TUNEL analysis of LNCaP cells treated with ATRA, 4-HPR, and RAMBAs**

One of the possible mechanisms underlying the observed effect of ATRA and the RAMBAs on LNCaP cell viability is the direct induction of apoptosis. To test this hypothesis, TUNEL assays were performed following treatment of cells with the various agents. LNCaP cells treated with 1 or 5 \( \mu \)M ATRA, 4-HPR, or RAMBA for 6 days were processed for TUNEL staining, and viewed under fluorescence microscopy to examine the presence of nicked DNA that are hallmarks of apoptosis. Pictures obtained from the staining of control, ATRA, and VN/69-1 are shown in Figure 6A and results from the TUNEL analysis for all the test compounds are summarised in Figure 6B. Generally, there was a concentration-dependent induction of apoptosis in LNCaP cells treated with 1 or 5 \( \mu \)M ATRA, 4-HPR, VN/14-1, VN/50-1, VN/66-1, and VN/69-1. Treatment with the most potent RAMBAs, VN/14-1 and VN/50-1 (5 \( \mu \)M), yielded a percentage of apoptotic cells of 17.03 and 17.03%, respectively (Figure 6B).

**Effects of ATRA, 4-HPR, and RAMBAs on expressions of Bad and Bcl-2**

Bcl-2 is an antiapoptotic protein and acts through inhibition of mitochondrial cytochrome c release (Murphy et al., 2000). Bad is pro-apoptotic and acts by displacing Bax from binding to Bcl-2 (Yang et al., 1995; Zha et al., 1996). Bax then dissipates the mitochondrial membrane releasing cytochrome c which leads to apoptosis (Rosse et al., 1998). 4-HPR was used as a reference compound as it was well established to induce apoptosis (Fontana and Rishi, 2002). We observed a significant increase in Bad expression for 5 and 10 \( \mu \)M treatment with ATRA and the RAMBAs compared with control (\( P<0.05 \)), with the exception of 4-HPR, where all treatment concentrations of 4-HPR (0.1, 1, and 5 \( \mu \)M) were significant (\( P<0.05 \)) and VN/69-1, where treatments with 1, 5, and 10 \( \mu \)M VN/69-1 were significant (\( P<0.05 \)) (Figure 7). Our results show that there was a concentration-dependent increase in Bad expression in LNCaP cells treated with all of the tested agents. Although there was a concentration-dependent decrease in the expression of Bcl-2 in LNCaP cells treated with ATRA and VN/14-1, treatments with 4-HPR, VN/50-1, VN/66-1, and VN/69-1, however, did not effect Bcl-2 expression (data not shown).

**Cell-cycle analysis of LNCaP cells treated with ATRA, 4-HPR, and RAMBAs**

Owing to the antiproliferative activities of these agents, it was of interest to determine their effects on cell-cycle distribution and apoptosis. LNCaP cells were treated with 1 or 5 \( \mu \)M ATRA, 4-HPR, or RAMBA for 6 days. However, only the cell-cycle analysis of LNCaP cells treated with 5 \( \mu \)M of the agents are shown in Figure 8.
and also summarised in Table 2. With these treatments, there was no change in the percentage of cells in the G₀/G₁ phase (except for ATRA treatment), a decrease in the percentage of cells in the S phase, and an increase in the percentage of cells in the G₂/M phase (a G₂/M cell arrest, Table 2). There was also an increase in the percentage of cell in the sub-G₁ phase that indicates apoptosis in LNCaP cells treated with these agents.

The effects of ATRA and RAMBAs on LNCaP xenograph SCID mouse model

We next tested the effects of our RAMBAs on growth inhibition of LNCaP tumour xenographs. Male SCID mice bearing LNCaP tumour xenographs (approximately 100 mm³) were grouped and treated once daily with 0.033 mmol kg⁻¹ each of ATRA or RAMBAs for the indicated time periods. As treatment with VN/50-1 was very toxic to the mice, experiment with this cohort was terminated. Tumour growth in the group receiving VN/14-1 was not significantly different from that observed in the vehicle control group (Figure 9). Mice receiving VN/66-1 or VN/69-1 had 44 and 47%, respectively, reduction in tumour growth compared to control (P<0.05), whereas treatment with ATRA reduced tumour growth by 75% relative to control (P<0.05) (Figure 9). During the study, all mice were weighed twice a week. Whereas the body weights of mice in the RAMBAs-treated groups were not altered, weights of mice in the ATRA-treated group were significantly (~25%) reduced compared to control (data not shown).

DISCUSSIONS

The prompt emergence of resistance to ATRA therapy in oncology and dermatology is a major concern in the development of ATRA (Miller, 1998; Njar, 2002, 2006). Agents that are able to inhibit ATRA metabolism may be used alone or in combination with low doses of ATRA or other therapeutic agents for the treatment of a variety of cancers and dermatological diseases. Interest in the development of structurally diverse agents as RAMBAs has increased over the last 5 years. We have developed several atypical RAMBAs with retinoidal scaffolds that possess unique multiple biological activities (Patel et al, 2004, 2006; Belosay et al, 2005; Njar, 2006). In this study, we have investigated the effects and mechanisms of action of several of these RAMBAs in human prostate LNCaP carcinoma cells.

Han and Choi (1996) were able to induce ATRA 4-hydroxylase in T47D cells and suggested that ATRA induces its own metabolism through a negative feedback mechanism. The enzyme activities induced by ATRA appear to be regulated at the level of transcription. Our results showed that only LNCaP cells were able to metabolise ATRA through induction of CYP26 (Figure 2). PC3 cells did not metabolise ATRA and the metabolism of ATRA did not exceed basal levels despite induction of CYP26 in DU145 cells. These results suggest that hormone-dependent cells (LNCaP) are able to metabolise ATRA following induction of CYP26 and that hormone-independent cells (PC3 and DU145) are not. To the best of our knowledge, this appears to be the first report of induction of ATRA 4-hydroxylase (CYP26) in LNCaP cells. The lack of ATRA-induced CYP26 in both PC3 and DU145 cells may be due to the loss of RARs in these cell lines. Several investigators have reported that the loss of RAR signaling in many epithelial tumours is driven by loss of expression of RARs (Campbell et al, 1998; Zhang, 2002).

Based on the IC₅₀ values of the RAMBAs in hamster hepatic microsomes that were obtained previously (Patel et al, 2004), representative RAMBAs (VN/14-1, VN/50-1, VN/66-1, and VN/69-1) (Figure 1) were studied further in LNCaP cells. In intact LNCaP cells, these compounds potently inhibit ATRA metabolism (Figure 3C–D and Table 1). The IC₅₀ values obtained for VN/14-1, VN/66-1, and VN/69-1 were comparable to the values seen in cellular MCF7 and T47D breast cancer cells that were determined previously (Patel et al, 2004). However, there was a discrepancy in the IC₅₀ value for VN/50-1 (1.0 nM in T47D cells vs 90.0 nM in LNCaP cells). This difference may be due to the ability of VN/50-1 to penetrate the cell membranes of the LNCaP PCA cells. Similar results have recently been reported for farnesol derivatives that are weak inhibitors of ATRA metabolism in human head and neck squamous cell carcinoma (AMC-HN-6) cells and their microsomal preparations (Kim et al, 2001).

In LNCaP cell proliferation experiments, each of the RAMBAs tested enhanced the antiproliferative activity of ATRA (Figure 4 and Table 1). The antiproliferative effects of ATRA was enhanced by 47- to 70-fold with the addition of 1 µM VN/14-1, VN/50-1, VN/66-1, or VN/69-1. Concentrations of RAMBAs effective in enhancing the antiproliferative activity of ATRA are themselves unable to significantly decrease LNCaP cell proliferation. Thus, these data support the hypothesis that our compounds enhance the biological activity of ATRA through inhibition of ATRA metabolism. These results are identical with those previously observed in human breast MCF-7 cancer cells (Patel et al, 2004), but are
superior to the effects observed with R116010 in human breast T47D cancer cells (Van Heusden et al., 2002). In the latter study, R116010, at a concentration of 1 μM, enhanced the antiproliferative activity of ATRA only by threefold. As expected, our RAMBAs also inhibited the growth of LNCaP cells in a dose-dependent manner, with IC50 values ranging from 4.3 to 10 μM (Figure 4 and Table 1), which correlates with their intrinsic retinoidal antiproliferative activities.

Having established the in vitro antiproliferative activities of these novel compounds alone and in combination with ATRA, we set out to investigate possible molecular mechanisms that might be involved using established procedures. On the basis of our previous studies and knowledge that these RAMBAs possess both intrinsic retinoid-like activities and also ATRA-mimetic effects (Patel et al., 2004), we assessed their effects on cell differentiation, apoptosis and cell cycle.

Indeed, this study presents evidence that our RAMBAs induce differentiation (via upregulation of cytokeratin 8/18 expression) and also significantly enhance ATRA-induced differentiation in the LNCaP cells. We have previously shown that with the exception of VN/14-1, the other three RAMBAs were weak ligands and activators of the RARα. As it is believed that only ligands that activate RARα induce cell differentiation (Bollag et al., 1997), the present data suggest that induction of differentiation can also occur via RARα-independent pathway. The ability of the RAMBAs to enhance ATRA-induced differentiation may be attributed to intracellular inhibition of ATRA metabolism with concomitant accumulation of cellular ATRA.

**Figure 8**  Cell-cycle analysis of LNCaP cells treated with 1 or 5 μM ATRA, 4-HPR or RAMBAs. LNCaP cells were incubated with either 1 or 5 μM ATRA, 4-HPR, or RAMBA for 6 days. LNCaP cells were then fixed, stained with propidium iodide, and analysed by FACScan. Histograms of the FACScan analysis from control (A), ATRA (B), 4-HPR (C), VN/14-1 (D), VN/50A-1 (E), VN/66-1 (F), and VN/69-1 (G) are shown.

**Table 2**  Cell-cycle analysis of LNCaP cells treated with 1 or 5 μM ATRA, 4-HPR, or RAMBAs

| Treatment         | G0/G1 (%) | S (%) | G2/M (%) | Sub-G1 (%) |
|-------------------|-----------|-------|----------|------------|
| Control           | 82.78     | 15.01 | 2.20     | 9.02       |
| ATRA (5 μM)       | 77.74     | 9.84  | 12.42    | 13.64      |
| 4-HPR (1 μM)      | 79.53     | 9.18  | 12.03    | 7.52       |
| VN/14-1 (5 μM)    | 82.59     | 7.26  | 10.15    | 17.88      |
| VN/50-1 (5 μM)    | 81.33     | 11.38 | 7.30     | 17.67      |
| VN/66-1 (5 μM)    | 83.80     | 11.14 | 5.07     | 14.49      |
| VN/69-1 (5 μM)    | 82.66     | 11.14 | 6.20     | 13.39      |

LNCaP cells were incubated with either 1 or 5 μM ATRA, 4-HPR, or RAMBA for 6 days. LNCaP cells were then fixed and stained with propidium iodide. 106 LNCaP cells were analysed by FACScan. The percentages of cells in the G0/G1, S, and G2/M phases of the cell cycle were determined using MODFIT LT software. aTreatment with 5 μM 4-HPR resulted in a massive amount of cell death such that no cell-cycle analysis could be performed.

Inhibitory effects of RAMBAs on the growth of human PCA cells

Translational Therapeutics
In the normal prostate, there is a balance between cell proliferation and cell death (Denmeade et al., 1996), while in PCA, the proliferative rate remains relatively low, but there is less apoptosis (Berges et al., 1995). Our results here demonstrate that RAMBAs were able to induce apoptosis in LNCaP cells as determined by the TUNEL assay. Induction of apoptosis appears to be associated with upregulation of the proapoptotic protein Bad and reduction or no change in the levels of antiapoptotic Bcl-2. Furthermore, cell-cycle analysis revealed that these agents significantly induce arrest of cell in the G2/M phase. It should be stated that there is a major checkpoint in the G2/M phase of the cell cycle where cells arrested in the G2/M phase can undergo apoptosis if optimal conditions are not met (Kastan and Bartek, 2004). This scenario may also be operational in this study. Therefore, our findings suggest that additional studies to determine the efficacy of our novel RAMBAs in the treatment of human prostate cancer are warranted.

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