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14. ABSTRACT

This is the final report of this project. We have made excellent progress and have obtained strong data that has enable licensing of our technologies by University of Maryland, Baltimore to Cancer Research UK, London, UK and Chesapeake BioDiscovery, Baltimore, MD, USA. Our studies show that the anti-tumor activity of our lead compound, VN/14-1 is dependent on the presence of ERα and/or RARα receptors in the breast cancer cells. We have also determined that VN/14-1 is a potent inhibitor of aromatase, and importantly, it is an exquisite inhibitor of the growth of human breast cancer cells and tumors that have become resistant to clinically used aromatase inhibitors (AI) or anti-estrogens, such as tamoxifen and faslodex. The agent could be developed for treatments of both endocrine-sensitive and endocrine-resistant breast cancers. In addition, we have also discovered that the combinations of retinoids/RAMBAs with histone deacetylase inhibitors (HDACi) or their corresponding mutual pro-drugs lead to additive/synergistic inhibition of growth of a variety of human breast cancer cells. We have also determined that although VN/14-1 doses not bind to ERα, it behaves as a potent antiestrogen with potent anti-uterotrophic activity. We also show that VN/14-1 is highly orally bioavailable and orally active. We have also synthesized novel mutual prodrugs of all-trans retinoic acid (ATRA) and histone deacetylase inhibitors (HDIs) with enhanced anticancer activities in a variety of breast cancer cell lines. Studies supported by this grant funds has also generated data that has led to other grant funds and technologies in prostate cancer. The data has also enabled us to apply for new grant funding to tackle challenges in the development of new treatments in neuroblastoma from PRMRP. This final report summarizes these exciting and promising data that may lead to the next generation of anti-cancer drugs that would be beneficial to the US military, their immediate families and the world in general.

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Title: RAMBAs for the prevention and Treatment of Breast Cancer

INTRODUCTION:

All-*trans*-retinoic acid (ATRA) is a potent inhibitor of cell proliferation and inducer of differentiation and apoptosis [1]. However, the clinical use of ATRA in the treatment of cancer is significantly hampered by the prompt emergence of resistance, believed to be caused by increased (auto-induced) ATRA metabolism [2]. Potent and selective inhibitors of ATRA [also referred to retinoic acid metabolism blocking agents (RAMBAs)] may prove valuable for cancer therapy, including breast cancer [3-5].

The goal of this project is to develop compounds with characteristics that are likely to provide effective antitumor activity against breast cancer. Our overall strategy is to identify compounds that are potent and inhibitors of CYP26, the enzyme responsible for ATRA metabolism. However, we have now determined that our RAMBAs are atypical because in addition to blocking ATRA metabolism, they also possess other multiple desirable anticancer activities [6-14]. Their unique multi-functional properties appear to be the reason(s) why they are effective against endocrine-sensitive and -resistant breast cancers. Indeed, our novel atypical RAMBAs act on multiple pathways that are essential for tumor growth.

The specific aims of the proposal were: 1) To synthesize rationally designed inhibitors of CYP26. 2) To evaluate compounds as inhibitors of CYP26 and to determine the selectivity of the potent inhibitors by assessing their inhibitory potency of other cytochrome P450 enzymes. 3) To determine the effects of inhibitors on mammary cancer growth. These studies would be carried out in cell cultures of human breast cancer. 4) To study the effects of the RAMBAs in animal models in vivo: Normal adult mice/rats would be treated with doses of inhibitors and endogenous plasma and tissue concentrations of ATRA measured; in addition their pharmacokinetic parameters would be studied. Growth of human breast (MCF-7Ca) tumors in nude mice treated with RAMBAs would be determined. 5) To investigate proliferation, differentiation and apoptosis of human breast tumors from nude mice treated with inhibitors.

A. BODY:

A.1. FINAL REPORT *(Work accomplished between 12/15/2003 – 01/14/2009)***:**

We have successfully completed all the aims proposed in our grant proposal but with other related aims that arose during the entire funding period. We will present our final report in a format that show how our research evolved as demonstrated by our journal articles.

A.2. Novel Retinoic Acid Metabolism Blocking Agents (RAMBAs) Endowed with Multiple Biological Activities Are Efficient Growth Inhibitors of Human Breast and Prostate Cancer Cells In Vitro and a Human Breast Tumor Xenograft in Nude Mice. (Patel et al., *Journal of Medicinal Chemistry*, **2004**, 47: 6716-6729 – ***Appendix 1***).

Novel retinoic acid metabolism blocking agents (RAMBAs) have been synthesized and characterized. The synthetic features include introduction of nucleophilic ligands at C-4 of all-*trans* retinoic acid (ATRA) and 13-*cis* retinoic acid, and modification of terminal carboxylic acid group. Most of our compounds are powerful inhibitors of hamster liver microsomal ATRA metabolism enzyme(s). The most potent compound is methyl (2E,4E,6E,8E)-9-(3-imidazolyl-2,6,6-trimethylcyclohex-1-enyl)-3,7-dimethylnona-2,4,6,8-tetraenoate (5) with an IC_{50} value of

0.009 nM, and is *666,667 time*s more potent than the well-known RAMBA, liarozole (Liazal™, IC_{50} = 6000 nM). Quite unexpectedly, there was essentially no difference between the enzyme inhibitory activities of the two enantiomers of compound 5. In MCF-7 cell proliferation assays, they RAMBAs also enhance the ATRA-mediated antiproliferative activity in a concentration dependent manner. The novel atypical RAMBAs, in addition to being highly potent inhibitors of ATRA metabolism in microsomal preparations and in intact human cancer cells (MCF-7, T47D and LNCaP) also exhibit multiple biological activities, including induction of apoptosis and differentiation, retinoic acid receptor binding and potent antiproliferative activity on a number of human cancer cells. Following subcutaneous administration to mice bearing human breast MCF-7 tumor xenografts, 6 (VN/14-1, the free carboxylic acid of 5) was well-tolerated and caused significant tumor growth suppression (~ 85.2% *vs.* control, p = 0.022). Our RAMBAs represent novel anticancer agents with unique multiple mechanisms of action. The most potent compounds are strong candidates for development as therapeutic agents for the treatment of a variety of cancers.

A.3. Quantification of a novel retinoic acid metabolism inhibitor, 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA) and other retinoids in rat plasma by liquid chromatography with diode-array detection. (Wu C et al., *Journal of Chromatography B*, **2004**, 810: 203-208 – ***Appendix 2***).

A simple reverse phase high performance liquid chromatography (HPLC) method was developed for the separation of a novel retinoic acid metabolism inhibitors, 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA) and other retinoids in rat plasma. VN/14-1RA alone or in combination with ATRA is effective at inhibiting the proliferation of prosate and breast cancer cell lines in vitro. Aliquots of rat plasma were spiked with the retinoids followed by addition of acetonitrile for precipitation of plasma proteins. The decanted supernatant was evaporated under a stream of nitrogen and reconstituted in acetonitrile. Analysis was accomplished by injection of an aliquot of the reconstituted sample into an HPLC system consisting of a Zorbax Rx-C18 column and a diode array detector. A mobile phase composed of ammonium acetate (0.1 M), acetic acid solution (2% (v/v)) and methol at a flow rate of 1.0 mL/min was used for gradient elution. The recoveries for all compounds ranged from 65 to 85% regardless of the concentration examined. The HPLC assay was linear over the range 0.10-5.0 μg/mL (CV < 10%) with a limit of quantitation of 100 ng/mL for VN/14-1RA. A one-compartment model wit apparent first-order elimination was used to describe the plasma concentration-time profile for VN/14-1RA after intravenous administration. The mean terminal elimination half-life (t1/2) was 19.0 ± 3.2 min. This HPLC method is useful for the analysis and evaluation of the pharmacokinetics of VN/14-1RA in rats.

A.4. Retinoic Acid Metabolism Blocking Agents (RAMBAs) for Treatment of Cancer and Dermatological Diseases (Njar et al., *Bioorganic & Medicinal Chemistry*, **2006**, 14: 4323-4340, Review - ***Appendix 3***)

The naturally occurring retinoids and their synthetic analogs play a key role in differentiation, proliferation and apoptosis, and their use/potential in oncology, dermatology and a variety of diseases are well documented. This review focuses on the role of all-*trans*-retinoic acid (ATRA), the principal endogenous metabolite of vitamin A (retinol) and its metabolism in oncology and dermatology. ATRA has been used successfully in differentiated therapy of acute promyelocytic leukemia, skin cancer, Kaposi's sarcoma and cutaneous T cell lymphoma and also in the treatment of acne and psoriasis. However, its usefulness is limited by the rapid

emergence of acquired ATRA resistance involving multifactoral mechanisms. A key mechanism of resistance involves ATRA-induced catabolism of ATRA. Thus, a novel strategy to overcome the limitation associated with exogenous ATRA therapy has been to modulate and/or increase the levels of endogenous ATRA by inhibiting the cytochrome P450-dependent ATRA-4-hydroxylase enzymes (particularly CYP26s) responsible for ATRA metabolism. These inhibitors are also referred to as retinoic acid metabolism blocking agents (RAMBAs). This review highlights development in the design, synthesis and evaluation of RAMBAs. Major emphasis is given to liarozole, the most studied and only RAMBA in clinical use and also the new RAMBAs in development and with clinical potential.

A.5. Retinoids in clinical use (Njar et al., *Medicinal Chemistry*, **2006**, 2: 431-438, Review - ***Appendix 4***)

Retinoids have been investigated for their therapeutic potential for the past 3 decades. They have a reputation for being both beneficial in the treatment of several diseases and detrimental due to toxic and/or teratogenic side effects. The purpose of this review is to highlight retinoids that are currently used in the clinic. We also discuss their mechanisms of action and research strategies to develop new and safer retinoid-based therapies.

A.6. Effects of Novel Retinoic Acid Metabolism Blocking Agent (VN/14-1) on Letrozole Insensitive Breast Cancer Cells (Belosay A., et al., *Cancer Research*, **2006**, 66: 11485-11493 - ***Appendix 5***)

Aromatase inhibitors (AIs) are proving to be more effective than tamoxifen for postmenopausal estrogen receptor positive breast cancer. However, inevitable development of resistance to treatment is a concern. We investigated the effects of novel retinoic acid metabolism-blocking agent (RAMBA), VN/14-1 in overcoming letrozole resistance in **L**ong **T**erm **L**etrozole **C**ultured cells (LTLC). Compared to MCF-7 cells stably transfected with aromatase (MCF-7Ca), LTLC cells were no longer sensitive to growth inhibition by AIs. The HER-2/pMAPK growth factor signaling pathway was activated and ER□ and coactivator AIB1 upregulated almost 3-fold in LTLC cells. VN/14-1 inhibited aromatase activity (AA) and growth in MCF-7Ca cells with IC_{50} of 8.5 nM and 10.5 nM respectively. In human placental microsomes, AA was inhibited with IC_{50} of 8.0 pM. The IC_{50} in LTLC cells was 0.83 nM, similar to letrozole (IC_{50} 0.3 nM) in MCF-7Ca cells. LTLC cells were 10-fold more sensitive to growth inhibition by VN/14-1 than MCF-7Ca cells. VN/14-1 treatment effectively down-regulated ER□, AIB1, pMAPK, HER-2, cyclin-D1, cdk4, Bcl2, and up-regulated cytokeratins 8/18, BAD, and BAX. Tumor growth of LTLC cells in ovariectomized nude mice were independent of estrogens but inhibited by VN/14-1 (20 mg/kg/day ($p < 0.002$). Decreases in ER□, cyclin-D1, cdk4, pMAPK and up-regulation of cytokeratins, BAD and BAX with VN/14-1 in tumor samples may be responsible for the efficacy of this compound in inhibiting LTLC cell growth *in vitro* and *in vivo*.

A.7. Murine Toxicology and Pharmacokinetics of Novel Retinoic Acid Metabolism Blocking Agents (RAMBAs) (Patel JB et al., *Cancer Chemotherapy & Pharmacology*, **2007**, 60: 899-905 - ***Appendix 6***)

Purpose: Novel potent C-4 azolyl retinoic acid metabolism blocking agents (RAMBAs) – VN/14-1, VN/50-1, VN/66-1, VN/67-1 and VN/69-1, have been synthesized and investigated for their *in vitro* and *in vivo* effects against breast and prostate cancers. These RAMBAs, in addition

to being potent inhibitors of all-*trans*-retinoic acid (ATRA) metabolism have potent anti-cancer properties and *in vivo* anti-tumor efficacies as characterized in breast and prostate cancer models. Here we determined the toxicity and pharmacokinetics of these various RAMBAs. *Methods:* Preliminary acute toxicity studies of these RAMBAs were carried out using Swiss NIH mice. The toxicity profile of the RAMBAs was evaluated relative to ATRA. Three different doses (8.3, 33 and 100 μmol/kg/day) of ATRA and RAMBAs were administered on a daily basis subcutaneously for 14 days to the mice. Clinical signs of toxicity alopecia, scaly skin, and loss of body weight in the mice were observed during the study and the maximum tolerated dose was determined. Pharmacokinetics (PK) of selected agents (VN/14-1, VN/50-1, and VN/66-1) was studied in Balb/C mice after a single dose subcutaneous administration. Plasma concentrations of the agents were quantitatively determined using a high performance liquid chromatographic (HPLC) method with ultraviolet detection. Plasma concentration versus time profiles were fit to various PK structural models and relevant PK parameters were estimated. *Results:* VN/66-1 and VN/69-1 were found to be the least toxic even at the highest doses when compared to the other RAMBAs and ATRA. VN/66-1 had the longest half-life, the slowest clearance, and the greatest exposure. *Conclusions:* Based on PK characteristics and toxicity studies, VN/66-1 appeared to be the most favorable agent. However, both VN/14-1 and VN/66-1 are our leads based on the fact that VN/14-1 has been found to be highly effective in endocrine-sensitive and -resistant breast cancer cells and tumors with little toxicity. Our findings provide valuable information that will be used to select RAMBAs and establish therapeutic regimens that provide optimal efficacy with minimal toxicity.

A.8. Novel Retinoic Acid Metabolism Blocking Agents (RAMBAs) Have Potent Inhibitory Activities on Human Breast Cancer Cells and Tumor Growth (Patel JB et al., *British Journal of Cancer*, **2007**, 96: 1204-1215 - ***Appendix 7***)

Anti-tumor effects of retinoids are attributed to their influence on cell proliferation, differentiation, apoptosis, and angiogenesis. In our effort to develop useful agents for breast cancer therapy, we evaluated the effects of four representative retinoic acid metabolism blocking agents (RAMBAs, VN/14-1, VN/50-1, VN/66-1 and VN/69-1) on growth inhibition of estrogen receptor positive (ER +ve, MCF-7 and T-47D) and estrogen receptor negative (ER –ve, MDA-MB-231) human breast cancer cells. Additionally, we investigated the biological effects/molecular mechanism(s) underlying their growth inhibitory properties as well as their anti-tumor efficacies against MCF-7 and MCF-7Ca tumor xenografts in nude mice. We also assessed the effect of combinatingVN/14-1 and ATRA on MCF-7 tumor xenografts. The ER (+ve) cell lines were more sensitive (IC_{50} values between 3.0 and 609 nM) to the RAMBAs than the ER (-ve) MDA-MB-231 cell line (IC_{50} = 5.6 – 24.0 μM). RAMBAs induced cell differentiation as determined by increased expression of cytokeratin 8/18 and ERα. Similar to ATRA, they also induced apoptosis via activation of caspase 9. Cell cycle analysis indicated that RAMBAs arrested cells in the G1 and G2/M phases and caused significant down-regulation (> 80%) of cyclin D1 protein. *In vivo*, the growth of MCF-7 mammary tumors was dose-dependently and significantly inhibited (92.6%, $p < 0.0005$) by VN/14-1. The combination of VN/14-1 and ATRA also inhibited MCF-7 breast tumor growth in vivo (up to 120%) as compared with single agents ($p < 0.025$). VN/14-1 was also very effective in preventing the formation of MCF-7Ca tumors and it significantly inhibited the growth of established MCF-7Ca tumors, being as effective as the clinically used aromatase inhibitors, anastrozole and letrozole. Decrease in cyclin D1 and up-regulation of cytokeratins, Bad Bax with VN/14-1 may be responsible for the efficacy of this

compound in inhibiting breast cancer cell growth in vitro and in vivo. Our results suggest that our RAMBAs, especially VN/14-1 may be useful novel therapy for breast cancer.

A.9. Targeting Cytochrome P450 Enzymes: A New Approach in Anti-cancer Drug Development (Bruno RD and Njar VCO, *Bioorganic & Medicinal Chemistry*, **2007**, 15: 5047-5060, Review - ***Appendix 8***)

Cytochrome P450s (CYPs) represent a large class of heme-containing enzymes that catalyze the metabolism of multitudes of substrates both endogenous and exogenous. Until recently, however, CYPs have been largely overlooked in cancer drug development, acknowledged only for their role in Phase I metabolism of chemotherapeutics. The first successful strategy targeting CYP enzymes in cancer therapy was the development of potent inhibitors of CYP19 (aromatase) for the treatment of breast cancer. Aromatase inhibitors ushered in a new era in hormone ablation therapy for estrogen dependent cancers, and have paved the way for similar strategies (i.e. inhibition of CYP17) that combat androgen dependent prostate cancer. Identification of CYPs involved in the inactivation of anti-cancer metabolites of Vitamin D_3 and Vitamin A has triggered development of agents that target these enzymes as well. The discovery of the over-expression of exogenous metabolizing CYPs, such as CYP1B1, in cancer cells has roused interest in the development of inhibitors for chemoprevention and of prodrugs designed to be activated by CYPs only in cancer cells. Finally, the expression of CYPs within tumors has been utilized in the development of bioreductive molecules that are activated by CYPs only under hypoxic conditions. This review offers the first comprehensive analysis of strategies in drug development that either inhibit or exploit CYP enzymes for the treatment of cancer.

A.10. Improved Synthesis of Histone Deacetylase Inhibitors (HDIs) (MS-275 and CI-994) and Inhibitory Effects of HDIs Alone or in Combination with RAMBAs or Retinoids on Growth of Human LNCaP Prostate Cancer Cells and Tumor Xenografts (Gediya JK et al., *Bioorganic & Medicinal Chemistry*, **2008**, 16: 3352-3360 - ***Appendix 9***).

We have developed new, simple and efficient procedures for the synthesis of two promising histone deacetylase inhibitors ((HDIs), CI-994, (*N*-(2aminophenyl)-4-acetylaminobenamide), and MS-275 (*N*-(2-aminophenyl)4-[*N*-(pyridine-3-ylmethoxycarbonyl)aminomethyl]benzamide) from commercially available acetamidobenzoic acid and 3-(hydroxymethyl)pyridine, respectively. The procedures provide CI-994 and MS-275 in 80 and 72% overall yields, respectively. We found that the combination of four HDIs (CI-994, MS-275, SAHA and TSA) with retinoids all-*trans*-retinoic acid (ATRA) or 13-*cis*-retinoic acid (13-CRA) or our atypical retinoic acid metabolism blocking agents (RAMBAs) **1** (VN/14-1) or **2** (VN/66-1) produced synergistic anti-neoplastic activity on human LNCaP prostate cancer cells. The combination of **2** and SAHA induced G1 and G2/M cell cycle arrest and decrease in the S phase in LNCaP cells. **2** + SAHA treatment effectively down-regulated cyclin D1 and cdk4 and up-regulated pro-differentiation cytokeratin 8/18 and pro-apoptotic Bad and Bax. Following subcutaneous administration, **2**, SAHA or **2** + SAHA were well tolerated and caused significant suppression/regression of tumor growth compared with control. These results demonstrate that compound **2** and its combination with SAHA are potentially useful agents that warrant further preclinical development for treatment of prostate cancer.

A.11. Design, Synthesis and Evaluation of Novel Mutual Prodrugs (Hybrid Drugs) of All-*trans* retinoic acid and Histone Deacetylase Inhibitors with Enhanced Anticancer Activities in Breast and Prostate Cancer Cells *In Vitro* (Gediya LK et al., *Journal of Medicinal Chemistry*, **2008**, 51: 3895-3904 - ***Appendix 10***)

Novel mutual prodrugs (MPs) of ATRA (all-*trans*-retinoic acid) and HDIs (histone deacetylase inhibitors) (**10**, **13**, **17**-**19**) connected *via* glycine acyloxyalkyl carbamate linker (AC linker) or through a benzyl ester linker (1, 6-elimination linker) were rationally designed and synthesized. Most of our novel MPs were potent inhibitors of growth of several hormone-insensitive/drug resistant breast cancer cell lines and the hormone-insensitive PC-3 prostate cancer cell line. The novel MPs exhibited differential antiproliferative potencies in both MDA-MB-231 and PC-3 cell lines. Whereas **19** (VNLG/124) [4-(butanoyloxymethyl)phenyl(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate] with a GI_{50} of 10 nM was the most potent MP *versus* the MDA-MB-231 cells, **13** (VNLG/66) [{*N*-[*N*-{2-[4-{[3-pyridylmethoxy)carbonyamino]methyl}phenyl) carbonylamino]phenyl} carbamoylcarbamoyloxy}methyl(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethyl cyclohex-1-enyl)nona-2,4,6,8-tetraenoate] with a GI_{50} = 40 nM was the most potent *versus* the PC-3 cells. MP **19** exhibited the most benefit because its GI_{50} of 10 nM *versus* MDA-MB-231 cells was remarkably 1085-fold lower than that of parent ATRA and over 100,000-fold lower than butyric acid (BA).

A.12. Two manuscripts are currently in preparation: 1**) Preclinical pharmacokinetics, absolute oral bioavailability and anti-cancer activity of VN/14-1 in human breast and prostate models. & 2) Effects of novel retinoic acid metabolism blocking agent (VN/14-1) on N-methyl-N-nitrosourea (MNU)-induced mammary carcinoma and uterus in the rat model**

A.13. Talks on RAMBAs and Retinoids: *(see Njar in C1 below)*

Because of the enormous potential of VN/14-1 and mutual prodrugs of retinoids/RAMBAs with HDIs as novel therapies for breast cancer, we were invited to present our work at major universities and research centers.

B. KEY RESEARCH ACCOMPLISHMENTS:

- Completed studies on murine toxicology and pharmacokinetics of lead RAMBAs, including VN/14-1 and VN/66-1.

- Established that lead VN/14-1 is highly orally bioavailable. This property is significant and desirable for a drug candidate because the ultimate route of administration of any drug oral.

- We have shown that VN/14-1 is a potent antiestrogen with potent anti-uterotrophic activity.

- VN/14-1 exhibits potent anti-proliferarive activity in breast cancer cells that express the ERα and/or RARα receptors.

- We have synthesized novel mutual prodrugs of ATRA and HDIs that exhibit enhance activity against MDA-MB-231 and endocrine-resistant breast cancer cells that are resistant to most therapeutic agents.

- Our results suggest that **VN/14-1**, a multi-target agent, may be a useful, novel therapy for endocrine-sensitive and resistant breast cancer.

- On the basis of research from this grant we are currently developing novel mutual prodrugs of RAMBAs and HDIs.

- We are also developing noel 2nd generation RAMBAs.

C. REPORTABLE OUTCOMES:

C.1. Publications/presentations/patents:

Abstracts & Talks:

1. **Njar, V. C. O.** Novel atypical RAMBAs endowed with potent anti-cancer activities. *Invited Lecture at Massachusetts General Hospital Cancer Center, Harvard Medical School, Boston, MA, USA, March 29, **2007**.*
2. **Njar, V. C. O**. Development of VN/14-1 for the treatment of endocrine-sensitive and –insensitive breast cancer. *Invited Lecture at Cancer Research, UK, Inaugural Meeting for Further Development of VN/14-1 Project London UK, July 26, **2007.***
3. **Njar, V. C. O**. Novel atypical RAMBAs for the treatment of endocrine-sensitive and –insensitive breast cancer. *Invited Lecture at Johns Hopkins University, School of Medicine, Cancer Center, Baltimore, MD, USA, October 23, **2007**.*
4. **Njar, V. C. O**. Novel Atypical RAMBA (CYP26 Inhibitor), VN/14-1 for the Treatment of Endocrine-Sensitive & -Insensitive Breast Cancer. *Invited Lecture at Sequoia Pharmaceutical Inc., Gaithersburg, MD, USA, December 20, **2007.***
5. Lalji K. Gediya, Akanksha Khandelwal, Jhalak Mehta, Puranik Purushottamachar and **Vincent C. O. Njar**. Mutual prodrugs of all-trans retinoic acid and Histone deacetylase inhibitors: Potent anticancer agents. *232st American Chemical Society (ACS) National Meeting, March 25-29, **2007**, Chicago, IL, USA. (Abstract #" MEDI 286)*
6. Khandelwal A, Gediya L, Upreti V, and **Njar V. C. O.** Preclinical pharmacokinetics, absolute oral bioavailability and anti-cancer activity of VN/14-1 in human prostate cancer PC-3 cells. *Centennial Conference: Translational Cancer Medicine, November 4-8, **2007**, Singapore.*
7. Goss PE, Njar VCO, Qi S, Hu H, Gediya LK, Purushottamachar P and Khandelwal A. Effects of novel retinoic acid metabolism blocking agent (VN/14-1) on N-methyl-N-nitrosourea (MNU)-induced mammary carcinoma and uterus in the rat model. San Antonio Breast Cancer Meeting, December

Manuscripts & Book Chapter:

1. Patel, J., Huynh, C., Handratta, V. D., Gediya, L. K., Brodie, A. M. H., Goloubeva, O. G., Clement, O. O., Nnane, I. P., Soprano, D. R., and **Njar, V. C. O.,** Novel Retinoic Acid Metabolism Blocking Agents (RAMBAs) Endowed with Multiple Biological Activities Are

Efficient Growth Inhibitors of Human Breast and Prostate Cancer Cells In Vitro and a Human Breast Tumor Xenograft in Nude Mice. *J. Med. Chem.,* **2004**, 47: 6716-6729.

2. Wu, C. K., **Njar, V. C. O**., Brodie, A. M. H., Nnane, I. P. Simultaneous quantification of 4-(1H-Imidazol-1-yl)retinoic acid and Three Retinoids in Rat Plasma by Reverse Phase Liquid Chromatography with Diode-array Detection. *J. Chromatography B,* **2004**, *810*: 203-208.
3. **Njar, V. C. O.**, Gediya, L., Purushottamachar, P., Chopra, P., Vasaitis, T. S., Khandelwal, A., Mehta, J., Huynh, C., Belosay, A., Patel, J. Retinoic acid metabolism blocking agents (RAMBAs) for treatment of cancer and dermatological diseases. *Bioorg. Med. Chem.*, **2006**, 14: 4323-4340. **(1. This review article that appeared on July 1, 2006, was recognized as a **"top-25 most downloaded"** article from *Bioorganic & Medicinal Chemistry (BMC)* on ScienceDirect during 2006. 2. Recently, this article has been recognized in the **"top-50 most cited article"** as published in *Bioorganic & Medicinal Chemistry 2005-2008*).
4. **Njar, V. C. O.**, Gediya, L., Purushottamachar, P., Chopra, P., Belosay, A., Patel, J. Retinoids in clinical use. *Med. Chem.*, **2006**, 2: 431-438.
5. Belosay, A., Brodie, A. M. H., **Njar, V. C. O.**, Effects of novel retinoic acid metabolism blocking agent (VN/14-1) letrozole insensitive breast cancer cells. *Cancer Res.*, **2006**, 66: 11485-11493.
6. Patel, J. B., Khandelwal, A., Handratta, V. D., Chopra, P., **Njar, V. C. O.** Murine toxicology and pharmacokinetics of novel retinoic acid metabolism blocking agents (RAMBAs). *Cancer Chemother. Pharmacol.,* **2007**, 60: 899-905.
7. Patel, J. B., Mehta, J., Belosay, A., Sabnis, G., Khandelwal, A., Brodie, A. M. H., Soprano, D. R., **Njar, V. C. O.** Novel retinoic acid metabolism blocking agents have potent inhibitory activities on human breast cancer cell and tumor growth. *Br. J. Cancer*, **2007**, 96: 1204-1215.
8. Bruno, R. D., **Njar, V. C. O**. Targeting cytochrome P450 enzymes: A new approach in anti-cancer drug development. *Bioorg. Med. Chem*., **2007**, 15: 5047-5060.
9. Gediya, L. K., Khandelwal, A., Patel, J. B., Belosay, A., Sabnis, G., Mehta, J., Purushottamachar, P., **Njar, V. C. O**. Design, synthesis and evaluation of novel mutual prodrugs (hybrid drugs) of all trans retinoic acid and histone deacetylase inhibitors with enhanced anticancer activities in breast and prostate cancer cells *in vitro*. *J. Med. Chem.*, **2008**, 51: 3895-3904
10. Gediya, L. K., Belosay, A., Khandelwal, A., Purushottamachar, P., **Njar, V. C. O**. Improved synthesis of histone deacetylase inhibitors (HDIs) (MS-275 and CI-994) and inhibitory effects of HDIs alone or in combination with RAMBAs or retinoids on growth of human LNCaP prostate cancer cells and tumor xenografts. *Bioog. Med. Chem.,* **2008**, 16: 3352-3360.
11. **Njar, V. C. O.** Retinoids in clinical use. In "Nuclear Receptors as Drug Targets", Ottow, Weinmann (Eds.), WILEY-VCH Veriag & Co. KGaA, Weinheim, **2008**, chapter 12, pp. 389-407.
12. Two manuscripts are currently in preparation: 1) Preclinical pharmacokinetics, absolute oral bioavailability and anti-cancer activity of VN/14-1 in human breast and prostate models. & 2) Effects of novel retinoic acid metabolism blocking agent (VN/14-1) on N-methyl-N-nitrosourea (MNU)-induced mammary carcinoma and uterus in the rat model.

Intellectual Property:

- Two patents awarded:
- **Njar, V. C. O.**, Nnane, I. P. and Brodie, A. M. H. Novel C-4 substituted retinoids useful in oncology and dermatology. *Australian Patent. 2001271265 B2* (January 6, **2005**) (*Appendix*).
- **Njar, V. C. O.**, Brodie, A. M. H. and Nnane, I. P. Novel C-4 substituted retinoids useful in oncology and dermatology. *United States Patent, 7,265,143* (Issued September 4, **2007**)
- Several others are pending – new and anticipated discoveries will be covered by patent expansions *(Appendix)*.

- RAMBAs Technology:
- This technology "RAMBAs for Cancer Therapy" Licensed to with Cancer Research UK, London, UK.
- On going talks with Chesapeake BioDiscovery, Baltimore, MD, USA to license part of RAMBAs technology for both breast and prostate cancer therapies.

C.2. Funding Granted/Applied for:

Granted:

1. As a result of studies with funds provided by PRMRP we were awarded an R21 grant by NIH (01/06/05 – 31/05/08) - **"Retinoids, RAMBAs, and Histone Deacetylase Inhibitors for Prostate Cancer"**
2. Institutional grant of from University of Maryland Marlene and Stewart Greenebaum Cancer Center. **"Oral pharmacokinetics and bioavailability studies on VN/14-1"**.
3. Industry Sponsored Research: Syndax Pharmaceuticals, Inc. **"Synthesis and Evaluation of MS-275 analogs as inhibitors of histone deacetylase"**.

Pending:

1. **"Retinoids, RAMBAs and Histone Deacetylase Inhibitors for Neuroblastoma"** Submitted (for April 23, 2009 deadline) to DOD Peer Reviewed Medical Research Program Investigator Initiated Research Award Funding Opportunity).
2. **"Development of VN/14-1, A Novel Atypical Chemotherapeutic for Breast Cancer"** from National Institutes of Health *(for resubmission for November 5, 2009 deadline)*.

C3. Research Opportunities/Training Support:

Jyoti Patel, Jhalak Mehta, Aakakshaw Khandelwal, Abhijit Godbole (graduate students) and Drs. Jyoti Patel, Aashvini Belosay, Aakakshaw Khandelwal, Lalji Gediya, and Puranik Purushottamachar (Postdoctoral fellows) were supported by this grant.

CONCLUSIONS:

We thank DOD Peer Reviewed Medical Research Program (PRMRP) for their support. The results obtained during this grant period have been published and presented at several national and international meetings and one manuscript is in review. We were awarded an Australian and USA patents. Other related patents in several countries are pending. Our technology based on

this project has been licensed to Cancer Research UK for development of breast cancer therapeutics. Other related technologies will soon be licensed to Chesapeake BioDiscovery, Baltimore, MD, USA. Some data obtained in the study have been used as preliminary results to apply for two new grants. Without doubt, the investment by DOD has paid off.

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10. **Njar VCO**, Purushottamachar P, Chopra P, Belosay A, Patel J. Retinoids in clinical use. *Med. Chem*., 2: 431-438, 2006**.**
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potent inhibitory activities on human breast cancer cell and tumor growth. *Br. J. Cancer*, 96: 1204-1215, 2007.

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Appendices:

This contains front pages and abstracts of manuscripts for journal publications and presentation at meetings. – Published articles and patent are available on line at PubMed.

1. Patel, J., Huynh, C., Handratta, V. D., Gediya, L. K., Brodie, A. M. H., Goloubeva, O. G., Clement, O. O., Nnane, I. P., Soprano, D. R., and **Njar, V. C. O.,** Novel Retinoic Acid Metabolism Blocking Agents (RAMBAs) Endowed with Multiple Biological Activities Are Efficient Growth Inhibitors of Human Breast and Prostate Cancer Cells In Vitro and a Human Breast Tumor Xenograft in Nude Mice. *J. Med. Chem.,* **2004**, 47: 6716-6729.
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3. **Njar, V. C. O.**, Gediya, L., Purushottamachar, P., Chopra, P., Vasaitis, T. S., Khandelwal, A., Mehta, J., Huynh, C., Belosay, A., Patel, J. Retinoic acid metabolism blocking agents (RAMBAs) for treatment of cancer and dermatological diseases. *Bioorg. Med. Chem.*, **2006**, 14: 4323-4340. **(1. This review article that appeared on July 1, 2006, was recognized as a **"top-25 most downloaded"** article from *Bioorganic & Medicinal Chemistry (BMC)* on ScienceDirect during 2006. 2. Recently, this article has been recognized in the **"top-50 most cited article"** as published in *Bioorganic & Medicinal Chemistry 2005-2008*).
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10. Gediya, L. K., Belosay, A., Khandelwal, A., Purushottamachar, P., **Njar, V. C. O**. Improved synthesis of histone deacetylase inhibitors (HDIs) (MS-275 and CI-994) and inhibitory effects of HDIs alone or in combination with RAMBAs or retinoids on growth of human LNCaP prostate cancer cells and tumor xenografts. *Bioog. Med. Chem.,* **2008**, 16: 3352-3360.

Novel Retinoic Acid Metabolism Blocking Agents Endowed with Multiple Biological Activities Are Efficient Growth Inhibitors of Human Breast and Prostate Cancer Cells in Vitro and a Human Breast Tumor Xenograft in Nude Mice

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Novel retinoic acid metabolism blocking agents (RAMBAs) have been synthesized and characterized. The synthetic features include introduction of nucleophilic ligands at C-4 of *all-trans*-retinoic acid (ATRA) and 13-*cis*-retinoic acid, and modification of terminal carboxylic acid group. Most of our compounds are powerful inhibitors of hamster liver microsomal ATRA metabolism enzyme(s). The most potent compound is methyl (2*E*,4*E*,6*E*,8*E*)-9-(3-imidazolyl-2,6,6-trimethylcyclohex-1-enyl)-3,7-dimethylnona-2,4,6,8-tetraenoate (**5**) with an IC_{50} value of 0.009 nM, which is 666,667 times more potent than the well-known RAMBA, liarozole (Liazal, IC_{50} = 6000 nM). Quite unexpectedly, there was essentially no difference between the enzyme inhibitory activities of the two enantiomers of compound **5**. In MCF-7 cell proliferation assays, the RAMBAs also enhance the ATRA-mediated antiproliferative activity in a concentration dependent manner. The novel atypical RAMBAs, in addition to being highly potent inhibitors of ATRA metabolism in microsomal preparations and in intact human cancer cells (MCF-7, T47D, and LNCaP), also exhibit multiple biological activities, including induction of apoptosis and differentiation, retinoic acid receptor binding, and potent antiproliferative activity on a number of human cancer cells. Following subcutaneous administration to mice bearing human breast MCF-7 tumor xenografts, **6** (VN/14-1, the free carboxylic acid of **5)** was well-tolerated and caused significant tumor growth supression (∼85.2% vs control, $p = 0.022$). Our RAMBAs represent novel anticancer agents with unique multiple mechanisms of action. The most potent compounds are strong candidates for development as therapeutic agents for the treatment of a variety of cancers.

Introduction

Retinoids, natural and synthetic analogues of *all-trans*-retinoic acid (ATRA) play key roles in many biological functions, including induction of cellular proliferation, differentiation, and apoptosis as well as developmental changes.[1] ATRA exerts its activity through binding with transcription-regulatory factors, known as the retinoic acid receptors (RAR), of which there are three subtypes, RARα, -β, and -γ. ATRA and several synthetic retinoids are currently used in cancer differentiation therapy, cancer chemoprevention, and treatment of dermatological diseases. One of the most impressive effects of ATRA is on acute promyelocytic leukemia (APL). Treatment of many APL patients with high doses of ATRA results in complete remission.[2,3] However, the clinical use of ATRA in the treatment of cancers is significantly hampered by the prompt emergence of resistance, which is believed to be caused at least in part by increased ATRA metabolism.[4−6]

One of the strategies for preventing in vivo catabolism of ATRA is to inhibit the P450 enzyme(s) responsible for this process. Inhibitors of ATRA metabolism (also referred to as retinoic acid metabolism blocking agents, RAMBAs) may prove useful for the chemoprevention and/or treatment of various kinds of cancer[5,6] and also for the treatment of dermatological diseases.[7,8] The major pathway of metabolic deactivation of ATRA starts with hydroxylation at C-4 to form 4-hydroxy-ATRA, which is oxidized into 4-oxo-ATRA, which is further transformed into more polar metabolites.[9] The first and rate-limiting step in the process is catalyzed by a cytochrome P450 dependent 4-hydroxylase enzyme. Although several human CYPs have been shown to be capable of converting ATRA to more polar metabolites,

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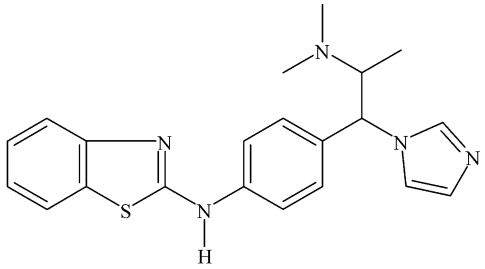
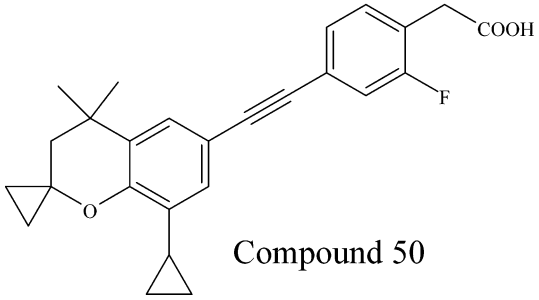
Chart 1. Structures of Two Potent ATRA 4-Hydroxylase Inhibitors: R116010, [*S*-(*R*,*R*)]-*N*-[4-[2-(Dimethylamino)-1-(1*H*-imidazol-1-yl)-propyl]phenyl]-2-benzothiazolamine, and Compound **50**, 2-{4-[2-(8-Cyclopropyl-4,4-dimethylspiro[chromane-2,1′-cyclopropane]-6-yl)ethynyl]-2-fluorophenyl}acetic Acid



R116010



Compound 50

their specificity for ATRA is generally moderate. Recently, while CYP2C8 was reported to be a major contributor to ATRA 4-hydroxylation in the human liver,[10,11] Marill et al.[12] identified CYP3A7 as the most active enzyme responsible for ATRA metabolism. In addition, CYP26 has been identified as the most dedicated ATRA 4-hydroxylase.[13−17] CYP26 recognizes only ATRA as its substrate, and its expression and/or activity can be induced by ATRA both in vitro and in vivo. In adult humans, CYP26 is expressed in several tissues, mainly in liver, adrenals, heart, and hypophysis.[18] Perhaps of more significance to this work is the realization that a variety of human cancer cells and tumors have been shown to possess ATRA 4-hydroxylase activity.[19−26] *Irrespective of the CYP isozyme(s) involved*, increased metabolism of ATRA could generate a condition of retinoid (ATRA) deficiency, which is implicated in cancers and dermatological diseases. Agents that can prolong and intensify the action of endogenous ATRA by inhibiting ATRA metabolizing enzymes would have potential as clinical agents in the treatment of the aforementioned diseases.

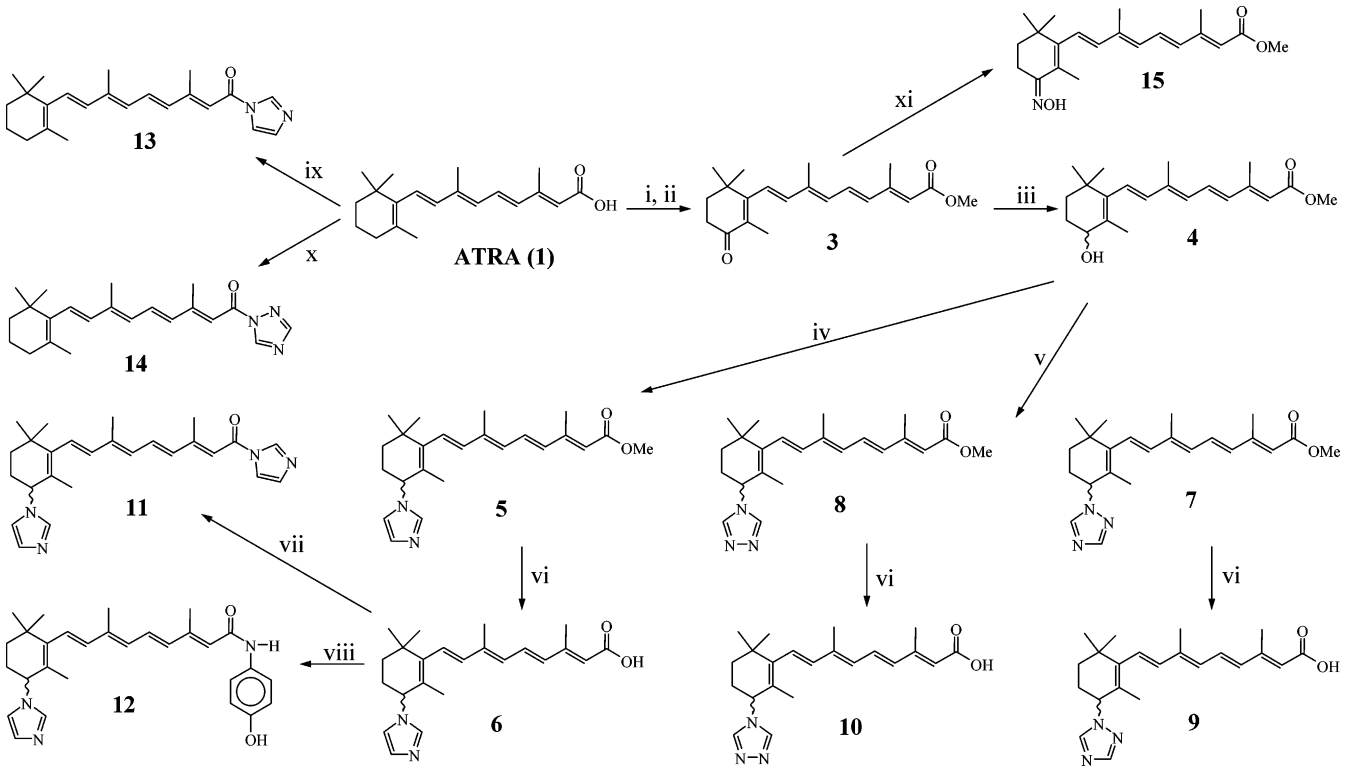
Whereas several categories of nonretinoidal RAMBAs have been reported,[6,26−29] only a few retinoidal RAMBAs, developed by our group, are known.[6,30] Some RAMBAs reported by the Johnson & Johnson Pharmaceutical Research and Development group[7,26,27] (e.g. R116010, Chart 1) and those recently discovered by Allergan Sales Inc.[28] (e.g. compound **50**, a benzeneacetic acid derivative, Chart 1) are highly potent inhibitors, while those reported by the University of Cardiff group[29] are similar to ketoconazole (a weak inhibitor of ATRA-4-hydroxylase; cf. Table 1). As our approach to inhibition of ATRA metabolism enzyme(s), we have designed and synthesized substrate-like molecules with nucleophilic groups (e.g. azoles) at the 4-position of ATRA and of 13-*cis*-retinoic acid (13-CRA) (the major site of enzymatic hydroxylation). These substrate-like compounds not only are expected to interact with the ATRA-binding site of the enzyme, thus introducing high specificity, but also will provide a sixth ligand to the enzyme's heme iron, resulting in tight binding.[30] This paper describes the syntheses and in vitro and preliminary in vivo antitumor activity of novel azolyl retinoids. Several of these compounds, in addition to being potent RAMBAs, are endowed with potent antiproliferative, prodifferentiation, proapoptotic, and antitumor activities. A preliminary account of part of this work has already been published,[30] and two patents (US and World) are pending.

Table 1. In Vitro Inhibition of ATRA Metabolism in Hamster Liver and T47D Microsomes, and in Intact Breast Cancer Cells (MCF-7 and T47D)

	hamster liver microsomal assay		MCF-7 cells	T47D cells	
compd	IC_{50} (nM)[a]	K_i (nM)[b,c]	cellular assay: IC_{50} (nM)[a]	cellular assay: IC_{50} (nM)[a]	microsomal assay: IC_{50} (nM)[a]
5	0.009 ± 0.0007	4.60×10^{-5}	200.0 ± 2.50	215.0 ± 10.00	40.00 ± 3.00
(−)-**5**	−	−	−	6000.0 ± 32.00	680.00 ± 20.00
(+)-**5**	−	−	−	6000.0 ± 30.00	800.00 ± 25.00
6	2.33 ± 0.72	0.22	10.90 ± 0.52	6.3 ± 0.50	2.40 ± 0.12
7	2.00 ± 0.05	−	−	−	−
8	21.67 ± 0.30	−	−	−	−
9	5.84 ± 0.48	−	−	−	−
10	46.67 ± 3.30	−	−	−	−
11	0.050 ± 0.002	6.20×10^{-5}	24.70 ± 0.2	10.0 ± 0.60	5.20 ± 0.32
12	43.73 ± 4.70	0.35	56.00 ± 0.90	24.0 ± 0.2	−
13	61.25 ± 6.50	−	−	−	−
14	51.67 ± 4.40	−	−	−	−
15	23.00 ± 1.63	−	−	−	−
21	119.0 ± 20.2	4.12	−	−	−
22	57.50 ± 8.5	−	−	−	−
23	76.67 ± 13.36	0.78	−	−	−
for comparison:					
liarozole	6000.00 ± 30.00	−	ni	ni	−
ketoconazole	34000.00 ± 170	−	−	−	−
4-HPR	31850.00 ± 150	−	−	−	−

[a] Mean ± SDM of at least two experiments. [b] K_i values were determined as described in the Experimental Section. [c] K_m for substrate, ATRA = 160 nM; ni = no inhibition up to 10 μM; − = not determined.

Scheme 1. Synthesis of RAMBAs with ATRA Scaffold[a]



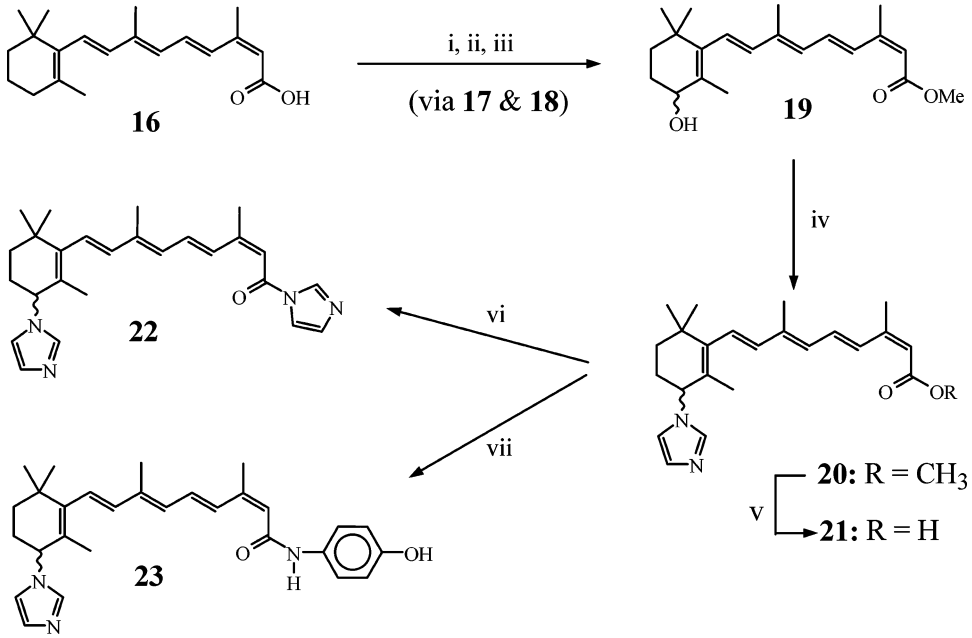
[a] Reagents and conditions: (i) $TMSCHN_2$/benzene, MeOH, Ar, rt; (ii) activated MnO_2/CH_2Cl_2, rt; (iii) $NaBH_4$/MeOH, rt; (iv) CDI/CH_3CN, rt; (v) CDT/CH_3CN, rt; (vi) 10% KOH/MeOH, Ar, reflux; (vii) CDI/CH_3CN, rt; (viii) DCC, HOBT, DMF, 4-APR; (ix) CDI/CH_3CN, reflux; (x) CDT/CH_3CN, reflux; (xi) $NH_2OH{\cdot}HCl$, NaOAc/EtOH, rt.

Chemistry

The azolyl compounds of this class of new RAMBAs were prepared in high yield in a four-step sequence (Scheme 1). Entry into the C-4 ATRA azoles started from ATRA (**1**) itself. Protection[31] of the carboxylic acid group as the methyl ester **2** followed by allylic oxidation[32] with MnO_2 provided the 4-oxo intermediate **3**, which was reduced with $NaBH_4$ to yield the key intermediate (±)-4-hydroxymethylretinoate (**4**). Treatment of **4** with carbonyldiimidazole (CDI) at room temperature gave the corresponding (±)-(1*H*-imidazol-1-yl)methylretinoate (**5**) in near quantitative yield. Alkaline hydrolysis of **5** resulted in (±)-(1*H*-imidazol-1-yl)retinoic acid (**6**) in 80% yield. The triazole derivatives, (±)-4-(1*H*-1,2,4-triazol-1-yl)methylretinoate (**7**) and (±)-4-(4*H*-1,2,4-triazol-4-yl)methylretinoate (**8**) were obtained as previously described by treatment of **4** with carbonylditriazole (CDT). Hydrolysis of **7** and **8** gave their corresponding free acids, (±)-4-(1*H*-1,2,4-triazol-1-yl)retinoic acid (**9**) and (±)-4-(4*H*-1,2,4-triazol-4-yl)-retinoic acid (**10**), respectively. The ease of transfer of imidazole and triazole from CDI and CDT, respectively, in excellent yields to the carbinol carbon of **4** are attributable to the activated nature of the allylic OH. It should be stated that transfer of imidazole from CDI to benzylic, vinylogous, and benhydryl carbinol carbons in modest to excellent yield has been previously reported.[33] Treatment of compound **6** with CDI at room temperature gave the 4-azolyl retinamide **11** in near quantitative yield, while the arylretinamide **12** was synthesized by coupling **6** with *p*-aminophenol (*p*-AP) by the active ester method using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT). Retinamides **13** and **14** were also prepared by treatment of ATRA (**1**) with CDI or CDT, respectively. The 4-keto oxime (**15**) was synthesized in near quantitative yield by treatment of ketone **3** with hydroxylamine hydrochloride. The stereochemistry of the oxime **15** about the C=N bond was assigned the *E* geometry because the signal of the two C-3 hydrogens in the ^1H NMR spectrum shifted downfield to δ 2.74. This demonstrates that the oxime possesses the geometry in which the C-3 hydrogens are proximate to the oxime OH. That the downfield shifts of these two hydrogens are not simply attributable to the anisotropy of the exocyclic π system at C-4 is apparent from analysis of the ^1H NMR spectrum of the 4-oxo derivative **3**, in which the C-3 hydrogens appear relatively upfield at δ 2.52. RAMBAs with the 13-CRA scaffolds, compounds **21**, **22**, and **23** (Scheme 2), were also synthesized using reactions described above for the synthesis of corresponding RAMBAs with ATRA scaffolds, but starting from 13-CRA (**16**). Hitherto unreported ^{13}C NMR data for all compounds are presented in the Experimental Section. The ^{13}C NMR chemical shifts were assigned by comparison with reported values for closely related retinoids.[34, 35]

The 4-azole derivatives described in this paper are racemates. Because their enantiomers may show differences in enzyme inhibitory potencies, it was of interest to separate the enantiomers of our most potent inhibitor (racemate **5**, vide infra) for testing. Compound **5** enantiomers, (4*R*)-(−)- and (4*S*)-(+)-**5** (Figure 1), were readily separated by HPLC on a chiral column Chiralpak AD using UV detection. The chromatograms for racemate **5** and (4*R*)-(−)- and (4*S*)-(+)-**5** are presented

Scheme 2. Synthesis of RAMBAs with 13-CRA Scaffold[a]



[a] Reagents and conditions: (i) $TMSCHN_2$/benzene, MeOH, Ar, rt; (ii) activated MnO_2/CH_2Cl_2, rt; (iii) $NaBH_4$/MeOH, rt; (iv) CDI/CH_3CN, rt; (v) 10% KOH/MeOH, Ar, reflux; (vi) CDI/CH_3CN, reflux; (vii) *p*-AP, HOBT, DMF, DCC.

in Figure 1. Tentative assignment of the chirality (absolute configuration) at C-4 of the two enantiomers was achieved on the basis of their optical rotations in comparison with the knowledge of the absolute configurations of related (4*S*)-(+)-4-hydroxyretinal and (4*R*)-(−)-4-hydroxyretinal.[36]

Biological Results and Discussion

Enzyme Inhibition Studies. A potent inhibitor of ATRA hydroxylases, the enzyme complex responsible for ATRA metabolism would be expected to increase the levels of endogenous ATRA, enhancing the "ATRA-mimetic" effects without the need for ATRA administration. In humans, the target enzymes involved in ATRA metabolism are the nonspecific liver CYPs, among which CYP2C8 and 3A7[10−12] are the major contributors, and the ATRA-inducible CYP26.[13−17] Although some investigators[7,26−28] have targeted inhibition of CYP26, it seems more realistic that both the nonspecific CYPs and specific CYP26 would need to be targeted since without initial ATRA accumulation due to nonspecific CYP action, ATRA levels would be insufficient to induce CYP26. Smith and co-workers[29] have also recently articulated this alternative strategy. With these considerations, we have tested our compounds against the more readily available hamster liver microsome and ATRA-induced CYP26 in MCF-7 and T47D breast cancer cells. It should be emphasized that several groups[22,24−26] have clearly demonstrated that CYP26 is the major ATRA-hydroxylating enzyme in both MCF-7 and T47D cells.

RAMBAs in Hamster Liver Microsomal Assays. The prospective inhibitors were evaluated using microsomal preparations of male hamster liver fortified with NADPH, using [11,12-^{3}H]-ATRA as substrate as we have previously described.[30a] The results are given as IC_{50} values (determined from dose response curves) and are presented in Table 1. All of our compounds exhibited potent inhibitory activity at nanomolar concentration with IC_{50} values of 0.009−119.00 nM. Our best compound **5** showed a 666,667-fold stronger inhibitory activity (IC_{50} = 0.009 nM) than liarozole (IC_{50} = 6000 nM), the only RAMBA to undergo phase III clinical studies in prostate cancer and in psoriasis. After careful evaluation in repeated assays, some of our compounds (i.e., **6**, **9**, and **10**) were found to be more potent inhibitors of hamster liver microsomes than our earlier tests indicated.[30a] As expected, compounds with the ATRA scaffold (**5**−**15**) were more potent than those with the 13-CRA scaffold (**21**−**23**). Furthermore, the results suggest that the nature of the C-4 substituent is important in determining affinity for the enzyme (compare IC_{50} values of **6**, **7**, **8** versus **13** and **14**; see Table 1) and also reveal that the corresponding methyl esters (**5**, **7**, and **8**) and imidazole amide (**11**) are significantly (24- to 48-fold) more potent than the corresponding free acids (**6**, **9**, and **10**). Compounds with 4-imidazole substitutions (**5**, **6**, and **11**) are the most potent three inhibitors. Thus, it would appear that the imidazolyl nitrogen lone pair makes the strongest coordination to the iron atom of the heme in the active site of the enzyme. Compound **12** (IC_{50} = 43.7 nM) was synthesized to determine the effect of increasing the size of the terminal amide group. The modification resulted in a considerable 875-fold decreased potency compared to **11** (IC_{50} = 0.05 nM), suggesting limited bulk tolerance at the active site of the enzyme. Because ketoconazole is used as a standard inhibitor of ATRA metabolism[10−12,26−29] and 4-HPR has recently been suggested as an inhibitor of ATRA metabolism,[37] we also tested these two compounds for comparison. As shown in Table 1, ketoconazole and 4-HPR are very weak inhibitors of this enzyme complex.

Following the determination of IC_{50} values, some seven representative inhibitors (**5**, **6**, **11**−**13**, **15**, and **21**) were evaluated further to determine their apparent

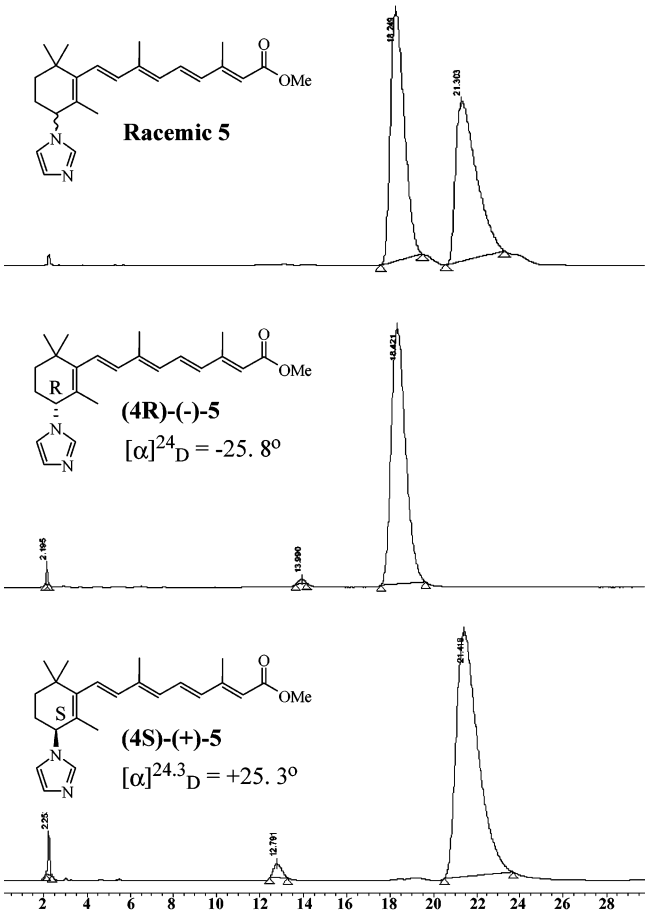


Figure 1. HPLC resolution of the enantiomers of (±)-**5** at 25 °C and structures of racemate **5** and its enatiomers. Conditions: column, Chiralpak AD (4.6 × 250 mm); eluent, hexane/isopropyl alcohol (95:5, v/v); flow rate, 1.5 mL/min. Chromatograms: top to bottom, UV detection, of racemic **5**, (−)-**5** (t_R = 18.42 min), and (+)-**5** (t_R = 21.42 min), respectively. Optical purity was determined by chiral HPLC (see above) and found to be >99.9% ee for both enantiomers.

K_i values (from Lineweaver−Burk plots, e.g. Figure A, Supporting Information) and also the type of enzyme inhibition kinetics. The apparent K_i values are presented in Table 1. Except for **12**, which exhibited uncompetitive-type inhibition (caused decrease in both V_{max} and K_m to the same extent), all other compounds were excellent noncompetitive-type inhibitors of the enzyme complex as shown in Figure A. The nature of inhibition kinetics exhibited by a majority of the compounds was that in which the V_{max} decreased but the apparent K_m was unchanged. This is one of the two characteristics of a noncompetitive enzyme inhibitor and indicates destruction of the catalytic activity of the enzyme.

Enzyme kinetic studies of ATRA metabolism revealed a K_m value of 160 nM, which is lower than the K_m values of 1.1 and 12.5 μM determined by Roberts et al.[38] and Van Wauwe et al.,[39] respectively. Nadin and Murray[10] and McSorley and Daly[12] reported K_m values of 9.0 and 0.9 μM, respectively, for human liver microsomes. It is not clear whether these different K_m values reflect real differences in affinity for ATRA between human and rodent ATRA hydroxylase. The conversion of ATRA to polar metabolites in hepatic microsomal preparations is catalyzed by more than one CYP, but with major contributions from CYPs 2C8, 2C9, 3A4, and 26. Therefore, the kinetic parameters reported in this manuscript do not represent the kinetics of a single enzyme. Our novel compounds are also expected to be potent inhibitors of human liver enzymes because the same CYP isoforms have also been identified as the major contributors in ATRA metabolism.

To the best of our knowledge, the most inhibitory compounds in the present study are far more potent than any inhibitor of ATRA metabolism for which comparable data have been previously described. The IC_{50} value for **5** was 0.009 nM, whereas the most potent inhibitor of yeast microsomes expressing CYP26 reported to date is R115866, with an IC_{50} value of 4.0 nM.[27] The imidazole R116010 inhibits ATRA metabolism in intact T47D cells with an IC_{50} value of 8.7 nM.[26] Compound **50** (Chart 1) has also been developed as a competitive inhibitor of CYP26 expressed in HeLa cells, with an IC_{50} value of 14.0 nM.[28] Furthermore, the exceptionally wide range of IC_{50} values (0.000045−36000 nM, spanning 11 orders of magnitude) obtained in this study will enable us to utilize the Catalyst molecular modeling program to create a general pharmacophore model that can differentiate compounds as active or inactive inhibitors of the enzyme complex. These studies, which are currently underway, could lead to the discovery of other potent RAMBAs with nonretinoidal scaffold.

RAMBAs in Cell-Based Assays. To assess the ability of our novel RAMBA to inhibit ATRA metabolism in intact cells, we chose **5**, **6**, **11**, **12**, and **23** to evaluate their inhibitory potencies in two human breast MCF-7 and T47D carcinoma cells. These two breast cancer cell lines are well-known to have inducible ATRA metabolism,[22−26] which closely correlates with the expression levels of CYP26.

Human T47D breast carcinoma cells cultured under control conditions are unable to metabolize ATRA into more polar metabolites (Figure 2A). However, after pretreatment with 1 μM ATRA for 12−15 h, the cells show extensive ATRA metabolism (Figure 2B), converting ATRA into highly polar metabolites (HPM, retention time, R_t = 2−6 min) and prominent metabolites of medium polarity (MMP, R_t = 11−14 min), including 4-oxo- and 4-hydroxy-ATRA. ATRA metabolism is inhibited dose-dependently by **14** (Figure 2C,D). Identical results were also obtained for experiments with MCF7 breast cancer cells and with the other four compounds tested. The IC_{50} values for compounds were determined from dose−response curves and are presented in Table 1. The compounds inhibited intracellular ATRA metabolism to the same extent in both cell lines, with decreasing activity in the order **6** > **11** > **12** > **23** > **5**. Compound **6** was the most active, with an IC_{50} value of 6.3 and 10.9 nM for the T47D and MCF-7 cell lines, respectively. Surprisingly, compound **5**, which was the most potent inhibitor of hamster liver microsomal enzyme, was the least potent, with IC_{50} values of approximately 200 nM in both cell lines. The inhibitory potency of our best RAMBA, **6** (IC_{50} = 6.3 nM), is comparable to the potency of R116010 (IC_{50} = 8.7 nM)[26] in intact human T47D breast cancer cells.

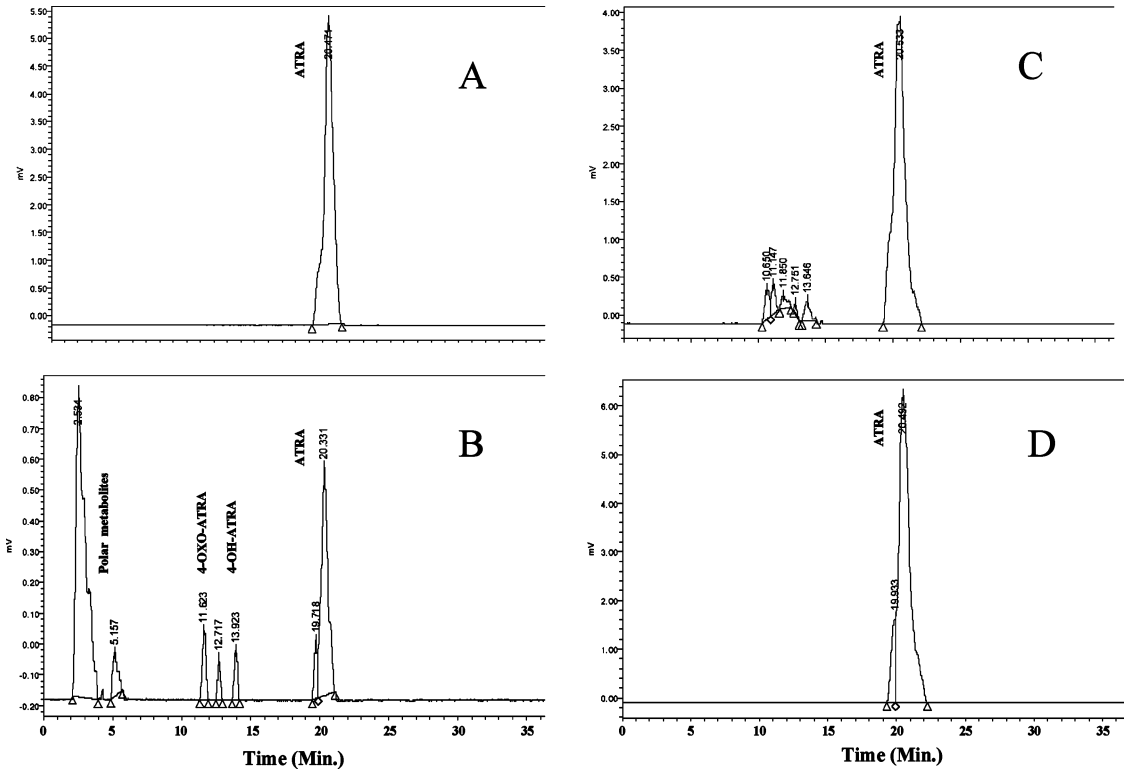


Figure 2. HPLC analysis of [11,12-^{3}H]-ATRA metabolites formed in human T47D breast cancer cells. Human T47D breast cancer cells were cultured under basal conditions (A) or pretreated with 1 μM ATRA (B−D). Thereafter, cell were collected, washed, and incubated with 0.1 μM [11,12-^{3}H]-ATRA, either in the absence (A and B) or in the presence (C and D) of **6** at concentrations of 10 and 100 nM, respectively. The cells and media were extracted and analyzed by reverse phase HPLC as described in the Experimental Section.

Our compounds also inhibited ATRA metabolism of microsomes prepared from T47D cells previously exposed to ATRA in a concentration-dependent manner, with IC_{50} values of 40.0, 2.4, and 5.3 nM for **5**, **6**, and **11**, respectively (Table 1). The inhibition trend of these RAMBAs in microsomes was similar to that in cells, but with 2−5-fold higher potencies. These differences may be due to the different capabilities of the various RAMBAs to penetrate the cell membranes of the T47D breast cancer 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.[40]

Inhibition of ATRA Metabolism by 5 and Its Enantiomers. Because the enantiomers of the 4-azole derivatives may show differences in enzyme inhibitory potencies, we separated the enantiomers of our most potent inhibitor, racemate **5**, to give (4*R*)-(−)-**5** and (4*S*)-(+)-**5** and then tested their abilities to inhibit ATRA metabolism in intact T47D breast cancer cells and in their microsomal preparations. The IC_{50} values are shown in Table 1. Here again, the inhibition trend of the three compounds in microsomes was similar to that in intact cells, but with 5−9-fold higher potency. Surprisingly, the racemate was considerably (up to 28-fold) more potent than the enantiomers, and, quite unexpectedly, there was essentially no difference in activity between the two enantiomers. This lack of stereoselectivity is obviously not due to racemization of the enantiomers in the assay medium, for, if this were the case, then their activities would be the same as that of the racemate. Although the other racemates were not separated into their respective enantiomers and tested for enzyme inhibition, it is likely that they may also behave in a similar fashion. The reason(s) underlying this lack of stereoselectivity is unknown at this time, but might be the consequence of phenomena occurring at the active site of the enzyme. However, it should be stated that there is precedent for this kind of phenomenon with a few examples of enantiopure azole derivatives having the azole moiety directly linked to the stereogenic center.[41]

Overall, these enzyme studies confirm our novel compounds as potent inhibitors of ATRA metabolism not only in liver microsomes but also in ATRA-induced ATRA metabolism in intact cells and their microsomes. Notably, we observed a significant difference between the inhibitory potencies of our compounds toward microsomal enzymes from hamster liver and from T47D and MCF-7 human breast cancer cells. These differences most probably reflect the unique nature of the ATRA-metabolizing CYP(s) present in the two systems. Thus, our potent inhibitors might be superior to other RAMBAs in animal models.

Effects of 6 and 11 on the Antiproliferative Activity of ATRA. The ability of **6** and **11** to enhance the antiproliferative activity of ATRA in MCF-7 breast cancer cells was also studied. MCF-7 cells were continuously incubated with ATRA (0.1 to 10000 nM) alone and in combination with low doses (10 nM each, doses that exhibit low [~10%] antiproliferative effects, see Figure 3B) of **6** and **11**. ATRA inhibited MCF-7 cell proliferation in a dose-dependent manner (Figure 3A) with an IC_{50} value of 584.50 nM. Both **6** and **11** each in combination with ATRA significantly enhanced the antiproliferativ

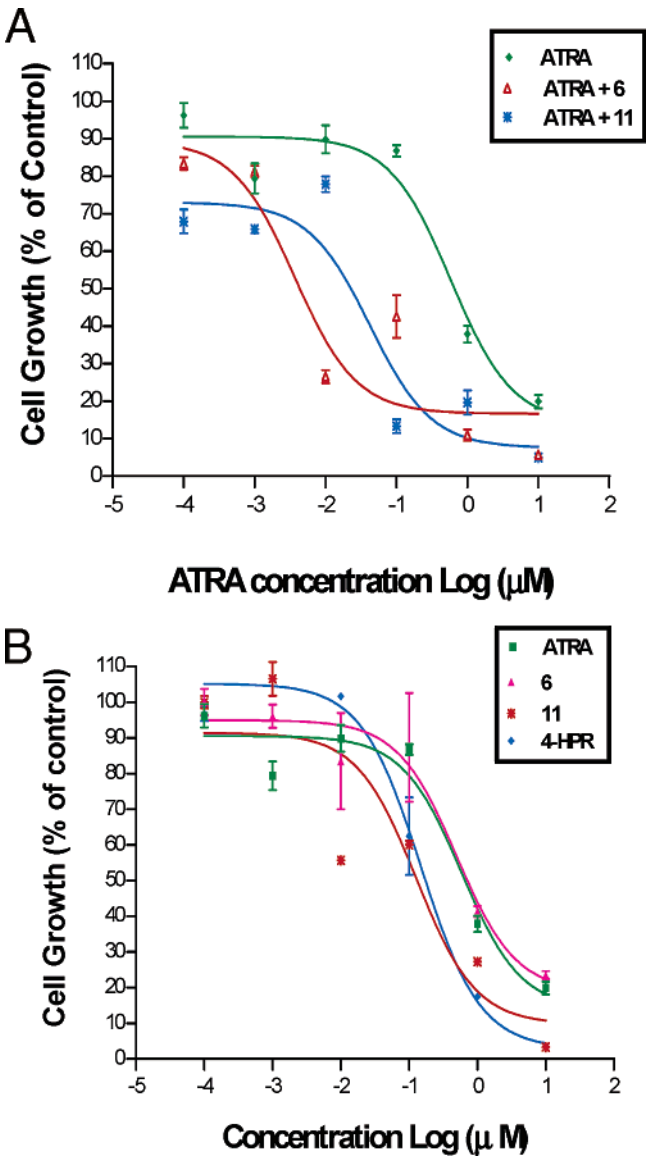


Figure 3. (A) Antiproliferative effects of ATRA alone and ATRA in combination with **6** or **11** at 10 nM each. MCF-7 cell proliferation was measured after 6 days of treatments using a MTT assay as described in the Experimental Section. Results are presented as SEM of three independent experiments. (B) Antiproliferative effects of ATRA, 4-HPR, and RAMBAs **6** and **11**. MCF-7 cell proliferation was measured after 6 days of treatments using a MTT assay as described in the Experimental Section. Results are presented as SEM of three independent experiments.

activity of ATRA, by 159- and 14-fold, respectively (for **6**, IC_{50} from 584.50 to 3.69 nM; and for **11**, IC_{50} from 584.50 to 41.74 nM) (Figure 3A). As expected, concentrations effective in enhancing the biological activity of ATRA are identical to the concentrations required to inhibit ATRA metabolism in intact cells. These results appear to be the most impressive of any RAMBA for which comparable data have been previously described. R116010 (Chart 1), at a concentration 1 μM (*100 times higher than the concentration of* ***6*** *or* ***11***), enhanced the antiproliferative activity of ATRA in human breast T47D cancer cells by only 3-fold.[26] In the same study, liarozole, tested up to a concentration of 10 μM, was unable to enhance the antiproliferative activity of ATRA. Taken together, our results support the hypothesis that our novel RAMBAs are able to enhance the biological activity of ATRA through the inhibition of ATRA metabolism in MCF-7 cells.

Table 3. Inhibitory Concentrations (IC_{50} μM)[a] of RAMBAs and Reference Compounds, ATRA and 4-HPR, in Nuclear Retinoic Acid Receptor (RAR) Binding

	IC_{50} (μM)		
RAMBA[b]	RARα	RARβ	RARγ
6	20	175	45
9	>1000	1000	700
10	410	250	300
13	≫1000	~500	~500
14	20	90	80
21	100	175	65
for comparison:			
ATRA[c]	9	3	10

[a] The IC_{50} values were determined from dose−response curves compiled from at least two independent experiments and represent the compound concentration (nM) required to inhibit cell proliferation by 50%. [b] The other RAMBA (**8**, **11**, **12**, **15**, **22** and **23**) to any of the three RARs at concentrations up to 500 nM. [c] IC_{50} values were taken from Idres et al.[43]

Other Biological Activities. Given the retinoidal nature of our RAMBAs, it seemed logical to investigate their effects on the growth of cancer cells. The antiproliferative effects of ATRA and 4-hydroxyphenyl-retinamide (4-HPR) were also studied for comparison using a MTT assay.[42] Continuous exposure of MCF-7 cells to various doses of the RAMBAs and the two reference compounds for 6 days led to dose-dependent inhibition of cell growth as shown in Figure 3B. The calculated IC_{50} values (defined as the concentration of compounds required to inhibit cell growth by 50%) from these dose−response curves are listed in Table 2. These compounds were also studied in a panel of other human cancer cell lines—breast (T47D and MD-MB-231) and prostate (LNCaP and PC-3)—and their IC_{50} values are also presented in Table 2. The compounds inhibited cell growth to varying degrees, with two breast cancer cells (T47D and MCF-7) exhibiting exquisite sensitivity to most of the RAMBAs. This suggests that the observed

Table 2. Inhibitory Concentrations (IC_{50} μM)[a] of RAMBAs and Reference Compounds, ATRA and 4-HPR, on the Growth of Human Cancer Cell Lines in Vitro

		IC_{50} (μM)						
cell line	tumor type	ATRA	4-HPR	**5**	**6**	**11**	**12**	**13**
MCF-7	breast carcinoma	0.58	0.15	–	0.49	0.13	0.61	–
T47D		0.006	–	–	0.003	0.009	0.57	–
MDA-MB-231		ni	7.7	7.5	ni	ni	6.3	7.0
LNCaP	prostate carcinoma	10.0	7.5	–	10.0	10.0	9.0	–
PC-3		2.0	3.6	–	7.7	5.8	1.5	–

[a] The IC_{50} values were determined from dose−response curves (by a nonlinear regression analysis using GraphPad Prism) compiled from at least two independent experiments and represent the compound concentration (μM) required to inhibit cell proliferation by 50%; ni = no inhibition up to 10 μM; − = not determined.

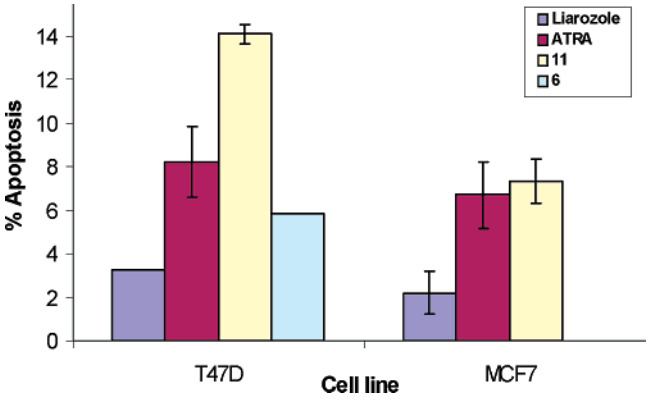


Figure 4. Effects of ATRA, liarozole and RAMBAs **6** and **11** on apoptosis in human breast cancer (T47D and MCF-7) cells. Cells were treated with 1 μM each of ATRA, liarozole, and RAMBAs (**6** and **11**) for 6 days; apoptosis was determined by APO-BRDU (TUNEL assay) and analyzed using flow cytometry.

cancer cell growth inhibition is specific and not due to "general toxicity" of the active RAMBAs. Furthermore, the data suggest that our RAMBAs might also possess retinoidal and/or *4-HPR-like* activities that could strengthen their therapeutic potentials.

The capacity of some RAMBAs to bind to retinoic acid receptors (RARs) was briefly investigated in competitive binding assays using 1 nM [11,12-^{3}H]-ATRA and various concentrations of the RAMBAs ranging from 1 to 1000 nM. The specific binding of ATRA in the absence of RAMBA was set at 100. The IC_{50} values determined from dose−response curves are presented in Table 3, and examples of the competitive binding with **6** and **14** are depicted in Figure B (Supporting Information). The best competitive binders for the three RARα, -β, and -γ were **6** with IC_{50} values of 20, 175, and 45 nM, respectively, followed by **14** (IC_{50} values: 20, 90, and 80 nM) and **21** (IC_{50} values = 100, 175, and 65 nM). These IC_{50} values are ~2-fold (for RARα), 30-fold (for RARβ), and ~4.5-fold (for RARγ) higher than those of ATRA and its natural isomers for binding to RAR receptors.[43] With the exception of **14**, the RAMBAs that do not have the free terminal carboxylic acid moiety did not bind to any of the three RARs in vitro at concentrations up to 500 nM. The low IC_{50} values for **14** were unexpected because of the absence of a terminal free carboxylic acid group in this molecule. However, it is plausible that the terminal triazole group is relatively acidic to interact favorably with RAR active site groups. Collectively, these results suggest that some of our RAMBAs may possess RAR receptor-dependent/independent mechanism of action as well as inhibition of ATRA 4-hydroxylase activity. Studies to assess the transactivation activities of **6**, **14**, and **21** toward RARα, -β, and -γ are planned.

The cellular effects of **6** and **11** were studied briefly in two breast cancer cell lines, MCF-7 and T47D, and one prostate cancer cell line, LNCaP. The effects of **6** and **11** with ATRA as a reference on breast cancer cell apoptosis were monitored using a TUNEL assay to detect intranuclear DNA damage in situ.[44] Figure 4 indicates that both compounds were effective apoptosis inducers, but **11** was more effective. Because of their potent RAMBA activity, these two compounds are expected to be strong enhancers of the proapoptotic action of ATRA. The potential effects of these two compounds on cell differentiation were also briefly studied in LNCaP human prostate cancer cells by assessing the expression of cytokeratin 18 (CK18).[45] Figure 5 shows the results obtained by treating LNCaP cells with ATRA, **6**, and **11** (1 μM each) alone and ATRA in combination with either **6** or **11**. Both compounds induced differentiation and also significantly enhanced ATRA-induced differentiation in this cell line. These results are encouraging, and more detailed cellular experiments are planned. Liarozole, a weak inhibitor of ATRA metabolism, has been shown to effectively enhance the proapoptotic and prodifferentiation activities of ATRA in a variety of in vitro and in vivo

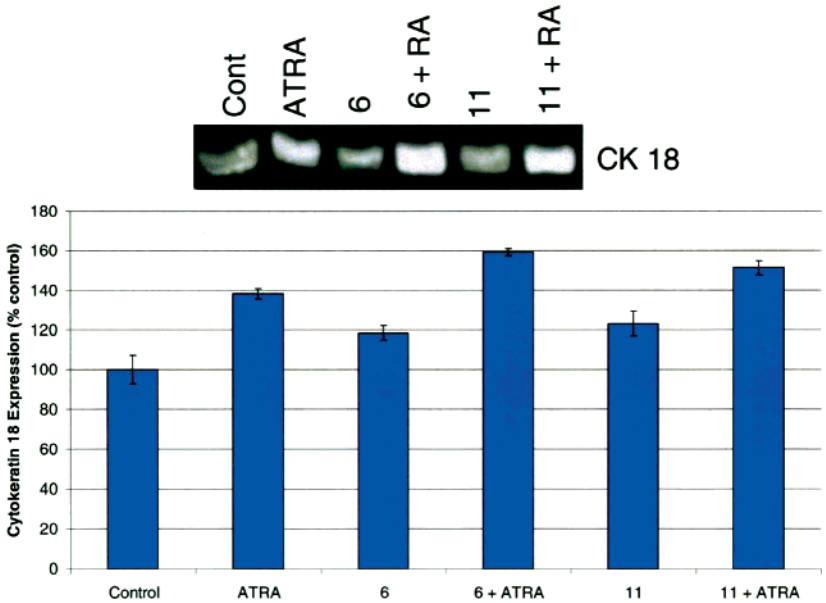


Figure 5. Effects of ATRA and RAMBAs **6** and **11** on levels of cytokeratin 18 (CK18, a differentiation marker) in human prostate LNCaP cells. Cells were incubated with ATRA alone or in combination with **6** or **11** for 6 days. Lysates were subjected to SDS−PAGE and Western blotting. Membranes were probed with CK18 antibody, and the intensities of bands were analyzed by densitometry.

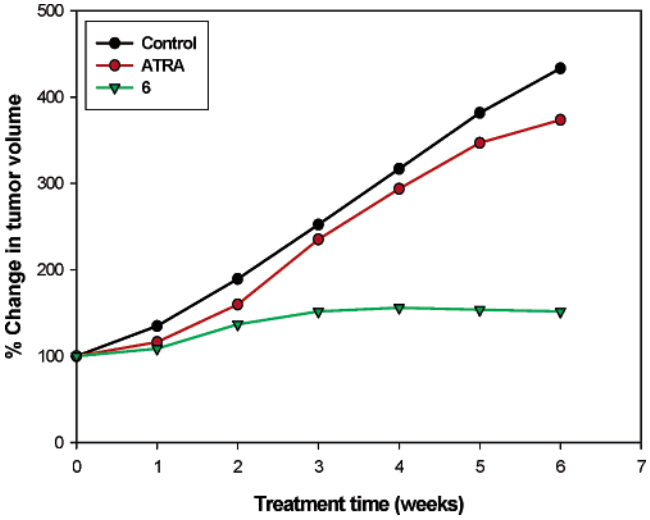


Figure 6. The effects of ATRA and RAMBA **6** (VN/14-1) on the growth of estrogen-dependent human MCF-7 breast tumor in ovariectomized nude mice supplemented with estradiol. Groups of five mice with MCF-7 tumors were treated with the compounds (ATRA and **6**) at 0.033 μmol/kg/day (6 days per week) for 6 weeks. Tumor volumes and body weights were measured weekly, and the percentage change in tumor volume was determined.

models.[5,6,46] Because our RAMBAs are significantly more potent than liarozole, low doses would be required to attain optimal effects and therefore are less likely to produce other unwanted effects.

In Vivo Antitumor Studies. Female ovariectomized nude mice bearing MCF-7 tumor xenografts (approximately 300 mm^3) were treated once daily with 0.033 mmol/kg each of **6** (VN/14-1) or ATRA for six weeks. As shown in Figure 6, VN/14-1 caused a significant reduction of 85.2% in the mean final tumor volume compared with vehicle-treated control animals (p = 0.022). Interestingly, the two tumors that were present in one of the five VN/14-1-treated mice were not palpable (from week 4 to 6), indicating a complete regression of these tumors after drug treatment. VN/14-1 did not alter body weights of mice, and other signs of retinoid clinical toxicity were not observed. In contrast, ATRA did not exhibit significant tumor growth inhibition in this xenograft model (Figure 6). It was recently reported[26] that the growth of murine estrogen-independent TA3-Ha mammary tumors was significantly inhibited by R116010. In addition, ATRA also exhibited strong antitumor efficacy, albeit at a higher dose. This study demonstrates the antitumor efficacy of R116010 in an estrogen-independent model of unestablished tumors. However, it is not known whether they are effective in estrogen-dependent models. Given that VN/14-1 exhibited potent antitumor efficacy in the estrogen-dependent mammary carcinoma model and also on well-established tumors of about 300 mm^3, it seems probable that VN/14-1 may be a more effective anticancer agent.

Conclusions

We have shown that C-4 azolyl RAMBAs, rationally designed analogues of retinoic acid, potently inhibit ATRA metabolism catalyzed by liver microsomes and CYP26 in intact breast cancer cells, but in contrast to other RAMBAs, they are endowed with multiple desirable anticancer activities. Because of their unique characteristics, these novel compounds may have anticancer activity not possible with other RAMBAs. The lead compound **6** (VN/14-1) inhibits the growth of human breast MCF-7 tumors in nude mice. In this model, VN/14-1 showed superior antitumor activity to ATRA. This and related RAMBAs warrant further evaluation as potential therapeutic agents in breast cancer as well as in other varieties of cancers.

Experimental Section

Chemistry. General procedures and techniques were identical with those previously reported.[28] ^{1}H NMR spectra were recorded in $CDCl_3$ at 300, 500, or 600 MHz with Me_4Si as an internal standard. ^{13}C NMR spectra were performed in $CDCl_3$ and obtained using a Varian Inova 500 MHz spectrometer operating at 125 MHz or Bruker Advance DMX600 spectrometer operating at 150 MHz. High-resolution mass spectra (HRMS) were determined on a Karatos-Aspect Systems instrument, EI mode. Low-resolution mass spectra (LRMS) were determined on a Finnegan LCR-MS. Optical rotations were measured at the sodium line using a Perkin-Elmer 141 polarimeter and are the average of seven values. Retinoids (*all-trans*-retinoic acid and *N*-(4-hydroxyphenyl)retinamide (4-HPR) were purchased from Sigma-Aldrich, St. Louis, MO, and from LKT Laboratories, Inc., St. Paul, MN. Liarozole was a gift from Dr. Marcel Janssen of Johnson and Johnson Pharmaceutical Research and Development, Beerse, Belgium.

Although the retinoidal intermediates and final products appeared to be relatively stable to light, precautions were taken to minimize exposure to any light source and to the atmosphere. Thus, all operations were performed in dim light, with reaction vessels wrapped with aluminum foil. All compounds were stored in an atmosphere of argon and in the cold (−20 or −80 °C) and dark without significant decomposition.

4-(±)-(1*H*-Imidazol-1-yl)-(*E*)-methylretinoate (5)[47] and Resolution to (4*R*)-(−)-5 and (4*S*)-(+)-5. To a solution of 4-hydroxy-(*E*)-methylretinoate (**4**, 3.0 g, 9.09 mmol) in dry CH_3CN (50 mL) was added 1,1′-carbonyldiimidazole (CDI) (1.95 g, 12.0 mmol, 1.3 equiv), and the reaction mixture was stirred at room temperature for 30 min, at which time the reaction was complete as determined by TLC. Following addition of cold water (100 mL), the reaction mixture was extracted with 10% MeOH in $CHCl_3$ (50 mL × 3). The combined extract was washed with brine, dried, and concentrated to give a yellow viscous oil. This crude product was dissolved in 10 mL of CH_2Cl_2/EtOAc/Et_3N (7:3:0.3, v/v/v) and filtered through a 3 in. column of silica gel (70−230 mesh). The column was washed with the same solvent, and four fractions of 50, 100, 150, and 150 mL were collected. Pure product was present in the third fraction, which was concentrated to give a viscous yellow oil that crystallized on storage at −20 °C after approximately 12 h. Following trituration in 10 mL of EtOAc/pet ether (9:1, v/v), the yellow crystals were filtered off and dried under vacuum to give the title compound **5** (3.4 gm, 89.5%): mp 118−120 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.09 (s, 3H, 16-CH_3), 1.12 (s, 3H, 17-CH_3), 1.60 (s, 3H, 18-CH_3), 2.02 (s, 3H, 19-CH_3), 2.36 (s, 3H, 20-CH_3), 3.72 (s, 3H, 15-OCH_3), 4.53 (s, 1H, 4-H), 5.80 (s, 1H, 14-H), 6.25 (m, 4H, 7-, 8-, 10- and 12-Hs), 6.91 (s, 1H, 4^1-H), 6.98 (t, 1H, J = 14.7 Hz, 11-H), 7.07 (s, 1H, 5^1-H), 7.50 (s, 1H, 2^1-H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 167.5 (C-15), 152.6 (C-13), 144.9 (C-9), 139.1 (C-8), 138.5 (C-6), 136.8 (C-2^1), 136.1 (C-12), 131.1 (C-11), 130.6 (C-10), 129.1 (C-7), 126.7 (C-5), 118.7 (C-14), 125.0 (C-4^1), 118.2 (C-5^1), 58.1 (C-4), 52.0 (15-OCH_3), 34.7 (C-2), 34.6 (C-1), 29.0 and 28.2 (C-16 and C-17), 27.8 (C-18), 18.9 (C-3), 13.8 (C-20), and 12.9 (C-19). HRMS: calcd 380.2464 ($C_{24}H_{32}O_2N_2$), found 380.2451.

Resolution of (±)-5. A racemic sample of **5** (40 mg) was dissolved in 8 mL of *n*-hexane/isopropyl alcohol, 90:10; v/v. This solution (100 μL/run) was charged to the chiral column (Chiralpak AD, 10 μm, 4.6 × 250 mm) and eluted with

n-hexane−isopropyl alcohol (95:5, v/v) at a flow rate of 0.75 mL/min, using a photodiode array (PDA) detector. The retention times for the first (A) and second (B) peaks were 25.19 and 28.89 min, respectively. Eluents of each peak were carefully collected manually. The fractions were checked for enantiomeric purity, using conditions essentially similar to those described above, but with a flow rate of 1.5 mL/min, with retention times of peaks A and B of 18.42 and 21.42 min, respectively. The pure fractions for each peak were combined and evaporated to dryness under vacuum to give 12 mg of peak A and 16 mg of peak B. The chemical structures were confirmed by ^{1}H NMR spectrum and identical as expected. Optical rotations were measured at the sodium line using a Perkin-Elmer 141 polarimeter and are averages of seven values. For peak A, (4*R*)-(−)-**5**, $[\alpha]^{24}_D = -25.8°$ ($c = 0.46$, $CHCl_3$), and for peak B, (4*S*)-(+)-**5**, $[\alpha]^{24}_D = +25.3°$ ($c = 0.46$, $CHCl_3$), respectively.

4-(±)-(1*H*-Imidazol-1-yl)-(*E*)-retinoic Acid (6). To a solution of **5** (2.5 g, 6.57 mmol) in methanol (25 mL) was added 2 N KOH (40 mL, solution in MeOH/H_2O, 9:1, v/v), and the mixture was stirred under an Ar atmosphere at reflux for 2 h, then concentrated to 1/5 the original volume, cooled, poured into cold water, neutralized (to pH ∼ 7) with 6 N HCl, and extracted with 5% MeOH in EtOAc (50 mL × 3). The combined extract was washed with brine (×3), dried (Na_2SO_4), and concentrated. Trituration in petroleum ether gave pure **6** (2.2 g, 91%). Details of most physical and spectroscopic data have been previously reported.[28] Hitherto unreported ^{13}C NMR data are provided below. ^{13}C NMR (150 MHz, $CDCl_3$ + DMSO-d_6): δ 170.1 (C-15), 155.2 (C-13), 147.5 (C-9), 141.7 (C-8), 141.1 (C-6), 139.4 (C-2^1), 138.7 (C-12), 133.6 (C-11), 133.2 (C-10), 131.7 (C-7), 129.3 (C-5), 121.3 (C-14), 127.6 (C-4^1), 120.8 (C-5^1), 60.7 (C-4), 37.3 (C-2), 37.2 (C-1), 31.6 and 30.8 (C-16 and C-17), 30.4 (C-18), 21.5 (C-3), 16.4 (C-20), and 15.5 (C-19).

4-(±)-(1*H*-1,2,4-Triazol-1-yl)-(*E*)-methylretinoate (7) and 4-(±)-(4*H*-1,2,4-Triazol-4-yl)-(*E*)-methylretinoate (8).[47] To a solution of **4** (1.0 gm, 3.0285 mmol) in dry CH_3CN (15 mL) was added 1,1′-carbonyldi(1,2,4-triazole) (CDT) (693 mg, 4.22 mmol), and the reaction mixture was stirred at room temperature for 30 min, was poured into cold water (50 mL), and then was extracted with 10% MeOH in EtOAc (50 mL × 3). The combined extract was washed with brine (×2), dried, and evaporated to give a yellow viscous oil, which was purified by FCC [silica gel, CH_2Cl_2/EtOH, (35:1, v/v)] to give first **7** (705 mg, 61.1%): mp 105−108 °C. NMR (300 MHz, $CDCl_3$): δ 1.10 (s, 3H, 16-CH_3), 1.13 (s, 3H, 17-CH_3), 1.63 (s, 3H, 18-CH_3), 2.02 (s, 3H, 19-CH_3), 2.36 (s, 3H, 20-CH_3), 3.72 (s, 3H, 15-OCH_3), 4.82 (s, 1H, 4-H), 5.80 (s, 1H, 14-H), 6.30 (m, 4H, 7-, 8-, 10- and 12-Hs), 6.99 (t, 1H, $J = 14.7$ Hz, 11-H), 7.99 (s, 1H, 3^1-H), 8.02 (s, 1H, 5^1-H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 167.4 (C-15), 152.6 (C-13), 151.9 (C-3^1), 142.5 (C-5^1), 146.0 (C-9), 139.2 (C-8), 138.4 (C-6), 136.3 (C-12), 131.1 (C-11), 130.5 (C-10), 126.4 (C-7), 123.6 (C-5), 118.5 (C-14), 61.1 (C-4), 51.0 (15-OCH_3), 34.8 (C-2), 34.0 (C-1), 29.1 and 28.2 (C-16 and C-17), 26.3 (C-18), 19.0 (C-3), 13.8 (C-20), and 12.9 (C-19). HRMS: calcd 381.2416 ($C_{23}H_{31}O_2N_3$), found 381.2442.

Further elution with CH_2Cl_2/EtOH, 20:1, v/v, afforded **8** (390 mg, 33.8%): mp 62−65 °C. NMR (300 MHz, $CDCl_3$): δ 1.10 (s, 3H, 16-CH_3), 1.13 (s, 3H, 17-CH_3), 1.64 (s, 3H, 18-CH_3), 2.02 (s, 3H, 19-CH_3), 2.36 (s, 3H, 20-CH_3), 3.72 (s, 3H, 15-OCH_3), 4.64 (s, 1H, 4-H), 5.81 (s, 1H, 14-H), 6.25 (m, 4H, 7-, 8-, 10- and 12-Hs), 6.98 (t, 1H, $J = 14.7$ Hz, 11-H), 8.15 (s, 1H, 3^1- and 5^1-H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 167.4(C-15), 152.5 (C-13), 146.5 (C-9), 142.3 (C-2^1 and C-3^1), 139.6 (C-8), 137.5 (C-6), 136.5 (C-12), 131.4 (C-11), 130.4 (C-10), 125.9 (C-7), 123.1 (C-5), 118.9 (C-14), 57.0 (C-4), 51.0 (15-OCH_3), 34.7 (C-2), 33.8 (C-1), 29.2 and 28.2 (C-16 and C-17), 27.5 (C-18), 19.1 (C-3), 13.8 (C-20), and 12.9 (C-19). HRMS: calcd 381.2416 ($C_{23}H_{31}O_2N_3$), found 381.2423.

4-(±)-(1*H*-1,2,4-Triazol-1-yl)-(*E*)-retinoic Acid (9). The method followed that described for **6** but used **7** (500 mg, 1.31 mmol). Trituration in petroleum ether gave pure **9** (409 mg, 85%). Details of most physical and spectroscopic data have been previously reported.[28] Hitherto unreported ^{13}C NMR data are provided below. ^{13}C NMR (150 MHz, $CDCl_3$ + DMSO-d_6): δ 167.4 (C-15), 152.6 (C-13), 151.9 (C-3^1), 142.5 (C-5^1), 146.0 (C-9), 139.2 (C-8), 138.4 (C-6), 136.3 (C-12), 131.1 (C-11), 130.5 (C-10), 126.4 (C-7), 123.6 (C-5), 118.5 (C-14), 61.1 (C-4), 34.8 (C-2), 34.0 (C-1), 29.1 and 28.2 (C-16 and C-17), 26.3 (C-18), 19.0 (C-3), 13.8 (C-20), and 12.9 (C-19).

4-(±)-(1*H*-1,2,4-Triazol-4-yl)-(*E*)-retinoic Acid (10). The method followed that described for **6** but used **8** (250 mg, 0.656 mmol). Trituration in petroleum ether gave pure **10** (193 mg, 80%). Details of most physical and spectroscopic data have been previously reported.[28] Hitherto unreported ^{13}C NMR data are provided below. ^{13}C NMR (150 MHz, $CDCl_3$ + DMSO-d_6): δ 168.4 (C-15), 152.5 (C-13), 146.5 (C-9), 142.3 (C-2^1 and C-3^1), 139.6 (C-8), 137.5 (C-6), 136.5 (C-12), 131.4 (C-11), 130.4 (C-10), 125.9 (C-7), 123.1 (C-5), 118.9 (C-14), 57.0 (C-4), 34.7 (C-2), 33.8 (C-1), 29.2 and 28.2 (C-16 and C-17), 27.5 (C-18), 19.1 (C-3), 13.8 (C-20), and 12.9 (C-19).

4-(±)-(1*H*-Imidazol-1-yl)-*N*-(imidazolyl)-(*E*)-retinamide (11). To a suspension of **6** (800 mg, 2.18 mmol) in dry CH_3CN (20 mL) was added CDI (460.6 mg, 2.84 mmol), and the mixture was stirred at room temperature for 1 h, poured into cold water (100 mL), and then extracted with 5% MeOH in ether (50 mL × 3). The combined extract was washed with brine, dried (Na_2SO_4), and concentrated to give an oily dark red product. This crude product was dissolved in a mixture of CH_2Cl_2/MeOH/Et_3N (15:1:0.2, v/v/v) and filtered through a 3 in. silica gel column that was eluted with the same solvent mixture. The fraction that contained pure **11** (as determined by TLC) was concentrated, dried under vacuum, and stored at −20 °C for approximately 12 h to give the desired **11** (850 mg, 95%): mp 82−85 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.15 (s, 3H, 16-CH_3), 1.18 (s, 3H, 17-CH_3), 1.65 (s, 3H, 18-CH_3), 2.11 (s, 3H, 19-CH_3), 2.55 (s, 3H, 20-CH_3), 4.59 (t, 1H, $J = 5$ Hz, 4-H), 6.26 (d, 1H, $J = 26.5$ Hz, 8-H), 6.28 (s, 1H, 14-H), 6.46 (m, 3H, 7-, 10-H and 12-Hs), 6.96 (s, 1H, 4^1-H), 7.12 (s, 1H, 5^1-H), 7.14 (s, 1H, 4^{11}-H), 7.25 (dd, 1H, $J_1 = J_2 = 11$ Hz, 11-H), 7.55 (s, 1H, 2^1-H), 7.58 (s, 1H, 5^{11}-H), 8.24 (s, 1H, 2^{11}-H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 162.0 (C-15), 159.0 (C-13), 145.1 (C-9), 141.0 (C-2^1), 139.1 (C-8), 136.9 (C-6 and C-2^1), 136.3 (C-12 and C-2^{11}), 135.4 (C-10), 133.8 (C-11), 130.7 (C-7), 131.0 (C-5), 129.1 (C-4^1), 118.5 (C-14), 116.5 (C-5^{11}), 115.9 (C-5^1), 58.4 (C-4), 34.8 (C-2), 29.3 (C-1), 28.3 (C-17), 28.0 (C− 16), 22.8 (C-18), 19.1 (C-3), 15.3 (C-20), and 13.3 (C-19). HRMS: calcd 416.2576 ($C_{26}H_{32}ON_4$), found 416.2354.

4-(±)-(1*H*-Imidazol-1-yl)-*N*-(4-hydroxyphenyl)-(*E*)-retinamide (12). Compound **6** (740 mg, 2.02 mmol), *p*-aminophenol (264.6 mg, 2.424 mmol), and 1-hydroxybenzotriazole (HOBT) (327.6 mg, 2.424 mmol) were dissolved in dry DMF (5 mL) and cooled to 0 °C. Dicyclohexylcarbodiimide (DCC), (500.2 mg, 2.424 mmol) was added, and the mixture was allowed to warm to room temperature. After stirring for approximately 20 h, the precipitated dicyclohexyl urea was filtered off and washed with 5% MeOH in EtOAc. The filtrate was washed with water, brine, dried (Na_2SO_4), and concentrated to give crude product (1.2 g), which was purified by FCC (silica gel, CH_2Cl_2/MeOH/Et_3N, 15:1:0.2, v/v/v) to give **12** (510 mg, 55%): mp 125−129 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.09 (s, 3H, 16-CH_3), 1.10 (s, 3H, 17-CH_3), 1.59 (s, 3H, 18-CH_3), 1.99 (s, 3H, 19-CH_3), 2.38 (s, 3H, 20-CH_3), 4.53 (t, 1H, $J = 5$ Hz, 4-H), 5.89 (s, 1H, 14-H), 6.20 (m, 4H, 7-, 8-, 10-H and 12-Hs), 6.81 (d, 2H, $J = 9$ Hz, aromatic Hs), 6.90 (dd, 1H, $J_1 = J_2 = 11$ Hz, 11-H), 6.93 (s, 1H, 4^1-H), 7.09 (s, 1H, 5^1-H), 7.37 (d, 2H, $J = 8.5$ Hz, aromatic Hs), 7.54 (s, 1H, 2^1-H), 7.80 (s, 1H, NH). ^{13}C NMR (150 MHz, $CDCl_3$): δ 165.2 (C-15), 154.0 (C-13), 149.3 (C-9), 145.4 (C-1^{11} and 4^{11}), 139.5 (C-8), 137.7 (C-6), 137.6 (C-2^1), 136.9 (C-12), 134.4 (C-11), 131.4 (C-10), 130.4 (C-7), 126.1 (C-5), 126.1 (C-4^1), 124.5 (C-5^1), 122.0 (2 aromatic Cs), 115.9 (2 aromatic Cs), 118.6 (C-14), 58.5 (C-4), 34.7 (C-2), 34.6 (C-1), 29.2 and 28.2 (C-16 and C-17), 27.8 (C-18), 19.0 (C-3), 12.9 (C-20), and 13.7 (C-19). HRMS: calcd 457.2729 ($C_{29}H_{35}O_2N_3$), found 457.2634.

4-Hydroxyimino-(*E*)-methylretinoate (15). A solution of ketone **3** (400 mg, 1.2192 mmol) in ethanol (8 mL) was treated with a solution of hydroxylamine hydrochloride (184 mg, 2.65

mmol) and anhydrous sodium acetate (138 mg, 1.68 mmol) in 50% aqueous ethanol (12 mL), and the resulting mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure at 35 °C, and the residue was diluted with cold water. The resulting yellow precipitate was filtered and dried under vacuum to afford the oxime **15** (339 mg, 85%): mp 172−174 °C. ^{1}H NMR ($CDCl_3$): δ 1.14 (s, 6H, 16- and 17-CH_3), 1.90 (s, 3H, 18-CH_3), 2.07 (s, 3H, 19-CH_3), 2.41 (s, 3H, 20-CH_3), 2.74 (t, 2H, J = 6.5 Hz, 3-CH_2), 3.76 (s, 3H, 15-OCH_3), 5.85 (s, 1H, 14-H), 6.36 (m, 4H, 7-, 8-, 10, and 12-Hs), 7.04 (dd, 1H, $J_1 = J_2$ = 11.5 Hz, 11-H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.7 (C-15), 158.0 (C-4), 152.0 (C-13), 149.7 (C-9), 139.4 (C-8), 138.8 (C-12), 136.6 (C-11), 131.5 (C-10), 130.9 (C-7), 127.2 (C-6), 125.2 (C-5), 118.9 (C-14), 51.2 (15-OCH_3), 36.2 (C-2), 35.2 (C-1), 27.8 (C-16 and C-17), 19.6 (C-18), 15.2 (C-3), 14.0 (C-20), and 13.1 (C-19). ESI-MS: m/z 343.2 (M^+).

4-(±)-Hydroxy-(13*Z*)-methylretinoate (19). The method followed that described for **4** (Supporting Information) but used the 13-*cis*-**16** (5.0 gm, 16.65 mmol) via the corresponding methyl ester **17** and the 4 keto ester **18**. Chromatography FCC [silica gel, CH_2Cl_2/EtOH, (75:1, v/v)] afforded pure 4-hydroxy-(13*Z*)-methylretinoate **19** (1.5 g, 35% from 16) as a yellow semisolid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.02 (s, 3H, 16-CH_3), 1.05 (s, 3H, 17-CH_3), 1.84 (s, 3H, 18-CH_3), 1.99 (s, 3H, 19-CH_3), 2.08 (s, 3H, 20-CH_3), 3.71 (s, 3H, OCH_3), 4.01 (s, 1H, 4-H), 5.66 (s, 1H, 14-H), 6.27 (m, 3H, 7-, 8- and 10-Hs), 6.97 (t, 1H, J = 12.6 Hz, 11-H), 7.85 (d, 1H, J = 15 Hz, 12-H). ESI-MS: m/z 330.1 (M^+).

4-(±)-(1*H*-Imidazol-1-yl)-(13*Z*)-methylretinoate (20). The method followed that described for **5** but used **19** (1.3 g, 3.94 mmol). The crude product was dissolved in 10 mL of CH_2Cl_2/EtOAc/Et_3N (7:3:0.3, v/v/v) and filtered through a 3 in. column of silica gel (70−230 mesh). The column was washed with the same solvent, and four fractions of 50, 100, 150, and 150 mL were collected. Pure product was present in the third fraction, which was concentrated to give a viscous yellow oil that crystallized on storage at −20 °C after approximately 12 h. Following trituration in 10 mL of EtOAc/petroleum ether (9:1, v/v), the yellow crystals were filtered off and dried under vacuum to give the title compound **20** (1.1 g, 89.5%) as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.08 (s, 3H, 16-CH_3), 1.13 (s, 3H, 17-CH_3), 1.60 (s, 3H, 18-CH_3), 2.01 (s, 3H, 19-CH_3), 2.08 (s, 3H, 20-CH_3), 3.71 (s, 3H, OCH_3), 4.53 (s, 1H, 4-H), 5.67 (s, 1H, 14-H), 6.25 (m, 3H, 7-, 8- and 10-Hs), 6.91 (s, 1H, 4^1-H), 6.96 (t, 1H, J = 12 Hz, 11-H), 7.07 (s, 1H, 5^1-H), 7.50 (s, 1H, 2^1-H), 7.80 (d, 1H, J = 14.7 Hz, 12-H). HRMS: calcd 380.2464 ($C_{24}H_{32}O_2N_2$), found 380.2458.

4-(±)-(1*H*-Imidazol-1-yl)-(13*Z*)-retinoic Acid (21). The method followed that described for **6** but used **20** (1.1 g, 2.89 mmol). Following workup of reaction as described for **6**, the combined extract was concentrated to approximately 20 mL and then diluted with petroleum ether. After standing overnight at −20 °C, the yellow crystals were filtered off and dried under vacuum to afford pure **21** (700 mg, 73%): mp 127−130 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.10 (s, 3H, 16-CH_3), 1.12 (s, 3H, 17-CH_3), 1.58 (s, 3H, 18-CH_3), 2.0 (s, 3H, 19-CH_3), 2.08 (s, 3H, 20-CH_3), 4.58 (t, 1H, J = 5.5 Hz, 4-H), 5.74 (s, 1H, 14-H), 6.24 (m, 3H, 7-, 8-, and 10-Hs), 6.92 (s, 1H, 5^1-H), 6.94 (dd, 1H, $J_1 = J_2$ = 11 Hz, 11-H), 7.17 (s, 1H, 4^1-H), 7.76 (s, 1H, 2^1-H), 7.86 (d, 1H, J = 15 Hz, 12-H). ^{13}C NMR (125 MHz, $CDCl_3$ + DMSO-d_6): δ 170.1 (C-15), 150.6 (C-13), 145.9 (C-9), 139.9 (C-6 and C-8), 138.3 (C-2^1), 132.5 (C-12), 132.5 (C-11), 131.3 (C-7 and C-10), 126. 1(C-4^1 and C-5), 124.5 (C-5^1), 118.8 (C-14), 59.0 (C-4), 34.9 (C-2), 29.3 (C-1), 28.3 (C-16), 28.1 (C-17), 21.2 (C-18), 19.1 (C-3), 14.3 (C-20), and 13.0 (C-19). HRMS: calcd 366.3061 ($C_{24}H_{30}O_2N_2$), found 366.3045.

4-(±)-(1*H*-Imidazol-1-yl)-*N*-(imidazolyl)-(13*Z*)-retinamide (22). The method followed that described for **11** but used **21** (307 mg, 0.84 mmol). Purification of the crude product by FCC [CH_2Cl_2/MeOH/Et_3N (15:1:0.2)] afforded the title compound **22** (279 mg, 80%), a yellow semisolid. NMR (500 MHz, $CDCl_3$): δ 1.12 (s, 3H, 16-CH_3), 1.17 (s, 3H, 17-CH_3), 1.63 (s, 3H, 18-CH_3), 2.09 (s, 3H, 19-CH_3), 2.28 (s, 3H, 20-CH_3), 4.58 (t, 1H, J = 4.5 Hz, 4-H), 6.32 (m, 3H, 7-, 10-H, and 14-Hs), 6.94 (s, 1H, 4^1-H), 7.10 (s, 1H, 5^1-H), 7.13 (s, 1H, 4^{11}-H), 7.22 (dd, 1H, $J_1 = J_2$ = 11 Hz, 11-H), 7.53 (s, 1H, 2^1-H), 7.56 (s, 1H, 5^{11}-H), 7.83 (d, 1H, J = 15.5 Hz, 12-H), 8.23 (s, 1H, 2^{11}-H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 161.1 (C-15), 157.1.0 (C-13), 144.9 (C-9), 141.1 (C-2^1), 139.1 (C-8), 136.8 (C-6), 136.3 (C-2^1), 135.2 (C-12), 131.5 (C-2^{11}), 130.7 (C-4^1), 130.1 (C-11), 129.0 (C-7), 128.0 (C-5), 125.4 (C-4^{11}), 118.3 (C-14), 116.4 (C-5^{11}), 113.8 (C-5^1), 58.2 (C-4), 34.8 (C-2), 34.7 (C-1), 29.1 (C-17), 28.2 (C− 16), 27.9 (C-18), 21.7 (C-3), 18.9 (C-20), and 13.1 (C-19). HRMS: calcd 416.2576 ($C_{26}H_{32}ON_4$), found 416.2526.

4-(±)-(1*H*-Imidazol-1-yl)-*N*-(4-hydroxyphenyl)-(13*Z*)-retinamide (23). The method followed that described for **12** but used **21** (200 mg, 0.55 mmol). Purification of the crude product by FCC [CH_2Cl_2/MeOH/Et_3N (15:1:0.2)] afforded the title compound **23** (150 mg, 60%): mp 116−120 °C. NMR (500 MHz, $CDCl_3$): δ 1.06 (s, 3H, 16-CH_3), 1.07 (s, 3H, 17-CH_3), 1.10 (s, 3H, 18-CH_3), 1.58 (s, 3H, 19-CH_3), 2.00 (s, 3H, 20-CH_3), 4.53 (t, 1H, J = 4 Hz, 4-H), 5.72 (s, 1H, 14-H), 6.15 (m, 3H, 7-, 8-, and 10-Hs), 6.81 (d, 2H, J = 9 Hz, aromatic Hs), 6.85 (dd, 1H, $J_1 = J_2$ = 11 Hz, 11-H), 6.93 (s, 1H, 4^1-H), 7.09 (s, 1H, 5^1-H), 7.38 (d, 2H, J = 8 Hz, aromatic Hs), 7.53 (s, 1H, 2^1-H), 7.79 (s, 1H, NH), 7.91 (d, 1H, J = 15.5 Hz, 12-H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 165.2 (C-15), 154.0 (C-13), 149.3 (C-9), 145.4 (C-1^{11} and 4^{11}), 139.7 (C-8), 137.6 (C-6), 137.6 (C-2^1), 136.5 (C-12), 132.4 (C-11), 131.0 (C-10), 130.5 (C-7), 128.4 (C-5), 125.7 (C-4^1), 124.5 (C-5^1), 122.0 (2 aromatic Cs), 118.6 (C-14), 116.0 (2 aromatic Cs), 58.3 (C-4), 34.5 (C-2), 29.1 (C-1), 28.2 (C-16), 27.8 (C-17), 20.9 (C-18), 19.0 (C-3), 12.8 (C-20), and 11.1 (C-19). HRMS: calcd 457.2729 ($C_{29}H_{35}O_2N_3$), found 457.2634.

Enzyme Preparations and Assay Procedures for ATRA Metabolism. Preparation of Microsomes. Microsomes were prepared from the livers of male Syrian golden hamsters and also from human breast cancer cells (MCF-7 and T47D) pretreated with 1 μM ATRA as described by Van Wauwe et al.[39] and Stoppie et al.,[27] respectively. Protein concentrations were determined with a Bio-Rad protein assay kit, obtained from Bio-Rad Laboratories. They were stored at −80 °C.

ATRA Metabolism Assay. We assessed ATRA hydroxylase activity in incubations containing liver or cancer cell microsomes and the inhibitors by measuring the radiolabed polar metabolites produced from [11,12-^{3}H]-ATRA as we have previously described.[30]

All enzymatic studies were performed in 0.1 M phosphate buffer, pH 7.4, at a final incubation volume of 0.40 mL. The incubation mixture contained 100 μL of microsomes (500 μg/mL dissolved in buffer); 100 μL of NADPH (20 mM dissolved in dH_2O); 40 μL of inhibitor (dissolved in DMSO); and 140 μL of assay buffer [0.01 M $MgCl_2$ and 0.02 (w/v) bovine serum albumin (BSA) in phosphate buffer solution]. After a 3 min preincubation at 37 °C, the reaction was initiated by the addition of 20 μL of [11,12-^{3}H]-ATRA (20 μCi/mL) and the incubation was carried out for 30 min under oxygen with shaking in a water bath at 37 °C. The reaction was terminated by the addition of 100 μL of formic acid. The retinoid products were extracted (2×) with 1 mL of ethyl acetate containing 10% methanol and 0.05% butylated hydroxyanisole (BHA). The organic and aqueous phases are separated by centrifugation at 3000*g* for 10 min at 4 °C. The organic phase, containing the retinoids, was dried with a stream of argon and dissolved in 100 μL of methanol for HPLC analysis.

HPLC Analysis. Chromatographic separations and quantification of the retinoids were achieved by a reverse phase HPLC method on a Waters Novapak C_{18} column (3.9 × 300 nm) protected by a Waters guard cartridge packed with pellicular C_{18} as previously described.[30] The HPLC system used in this study consisted of Waters solvent delivery system, Waters controller (Milford, MA), coupled to a Waters 717^{plus} autosampler and a Waters radiomatic detector. Analysis was performed at ambient temperature, and data acquisition and management was achieved with a Waters millennium chromatography manager. On the basis of our procedure,[30] the retinoids (10 μL) were analyzed on a 10 μm C_{18} Bondapak

column eluted with a multilinear gradient solvent system: MeOH−H_2O (60:40) containing 20 mM ammonium acetate (100% → 0%) at 0.8 mL/min for 20 min and MeOH (0% → 100%) at 1 mL/min for the remaining 10 min, during which time the retinoids were eluted with 100% MeOH. Radioactivity was measured by a radiomatic detector. Percent metabolism was obtained by dividing the areas under the curve (AUC) of the metabolite peaks by the AUC of all peaks. IC_{50} values were determined as the concentration of RAMBA that yielded 50% metabolism.

***K*i Assay Procedure.** This procedure is essentially similar to that employed in the dose−response curve IC_{50}-determination assay, except that the substrate concentration was varied between 2 and 10 nM using 250 μg of protein to ensure a constant initial velocity, even at the lowest substrate concentration. Control samples with no inhibitors were also incubated simultaneously. Each inhibitor was examined at one concentration (10 pM, 0.1 nM, 10 pM, 1 nM, 10 nM, and 1 nM for **5**, **6**, **11**, **12**, **21**, and **23**, respectively). Data from the various assays were used to obtain Lineweaver−Burk plots (e.g. Figure 2). From these plots, K_i values for the RAMBA and the K_m for ATRA (substrate) were determined (Table 1).

Cell Culture. Human mammary-carcinoma cell lines MCF-7 and T47D (both ER +ve) and MDA-MB-231 (ER -ve) as well as LNCaP (AR +ve) and PC-3 (AR -ve) were used. MCF-7 and MDA-MB-231 cells were cultured in improved Dulbecco's modified Eagle's medium (IMEM), with glutamine and supplemented with 5% fetal bovine serum, 1% penicillin−streptomycin. T47D, LNCaP, and PC-3 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin−streptomycin. Cells were grown as a monolayer in T75 or T150 tissue culture flasks in a humidified incubator (5% CO2, 95% air) at 37 °C.

ATRA Metabolism Assay. ATRA Metabolism Induction and Inhibition. MCF-7 and T47D cells (1×10^6 cells per 1 mL per well) were seeded in 6 well plates (Corning Costar plates). Cells were treated with 1 μM ATRA for 12−15 h to induce the cytochrome P450 enzyme. Cells were then washed with PBS and media and then incubated with different concentrations (0.0001−10 μM) of RAMBAs as well as with 0.001 μM [11,12 ^{3}H]-ATRA for a further period of 5 h (all of the drugs were dissolved in 95% ethanol to make the stock solution). The media and the trypsinized cells were then collected in glass tubes; radiolabed ATRA and metabolites were then extracted using ethyl acetate (with 10% methanol and 0.05% BHA). The extracted samples were dried and analyzed by reverse phase HPLC as described above.

Cell Growth Inhibition Assay (MTT Colorimetric Assay). Breast Cancer Cells. MCF-7 and T47D cells (1×10^4 cells per well per 1 mL of medium) were seeded in 24 well plates (Corning Costar plates). Cells were allowed to adhere to the plates for about 18 h and then treated with different concentrations (0.0001−10 μM) of drug (ATRA, and/or the RAMBAs dissolved in 95% ethanol) for 6 days, with renewal of media and drug on day 4. On day 7, medium was renewed and 100 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide from Sigma) solution (5 mg MTT/mL of PBS) was added to the medium such that the ratio of MTT: medium was 1:10. The cells were incubated with MTT for 2 h. The medium was aspirated and 500 μL of DMSO was added to solubilize the blue-violet MTT-formazan product. The absorbance at 540 nm was measured by spectrophotometery (Victor 1420 multilabel counter, Wallac). For each concentration of the drug there were triplicate wells in every individual experiment. The data presented are mean ± SEM for 2−3 experiments. IC_{50} values were calculated by nonlinear regression analysis using Prism GraphPad software.

Prostate Cancer Cells. To measure cell viability, 24-well plates were coated with a 0.001% poly-L-lysine solution for 30 min. The wells were then washed with sterilized dH_2O. 1×10^4 LNCaP cells were seeded in the plates and maintained in RPMI 1640 medium. The cells were allowed to attach for 36 h. 1×10^4 PC-3 cells were seeded without poly-L-lysine treatment in the plates and maintained in RPMI 1640 medium. The cells were allowed to attach for 24 h. After attachment, fresh medium was added, and the cells were treated with a concentration range of either ATRA or one of the RAMBAs for 6 days. The medium was changed every 3 days. Cell were then treated with MTT and analyzed as described above.

Apoptosis APO-BRDU (TUNEL Assay). Apoptosis was determined by APO-BRDU (TUNEL assay) kit from "Pharmingen" and analyzed by flow cytometery. MCF-7 and T47D cells were treated with 1 μM ATRA, RAMBAs, and liarozole for 6 days in T75 flasks. Treated and untreated control cells were trypsinized, and $(1-2) \times 10^6$ cells were suspended in 0.5 mL of PBS. Cells were fixed with 1% w/v of paraformaldehyde and then again washed with PBS, suspended, and then stored overnight at −20 °C in 70% ethanol. Cells were then treated with DNA labeling solution consisting of TdT enzyme and BrdUTP. Next the cells were treated with fluorescein labeled anti-BrdU (antibody) and PI/RNase. Fluorescein labeled apoptotic cells were determined by flow cytometric analysis.

Differentiation Assay. LNCaP cells were incubated in the RPMI 1640 medium preparation containing 1 μM ATRA alone or in combination with a 1 μM concentration of one of the RAMBAs (**6** and **11**) for 6 days. LNCaP cells were scraped, and cell lysates were prepared. Fifty micrograms of cell lysate was run on a 10% SDS−PAGE gel at 90 V at room temperature. The separated lysates on 10% SDS−PAGE gel were transferred onto nitrocellulose membrane (Hybond ECL) overnight at 20 V at 4 °C. Western blot analysis was performed on the nitrocellulose membrane. The membrane was blocked for 1 h in 5% nonfat dried milk and PBS containing 0.5% Tween 20 (PBS-T) at room temperature. Following washing in PBS-T, the membrane was incubated with mouse monoclonal IgG antibody to cytokeratin 8/18 or β-actin (Santa Cruz Biotechnology and Oncogene, respectively) dissolved in 5% nonfat dried milk (1:5000 or 1:1000, respectively) for 1 h at room temperature. Following washing, the membrane was incubated with horseradish peroxidase linked sheep anti-mouse IgG antibody (Amersham and Oncogene, respectively) dissolved in 5% nonfat dried milk (1:1000) for 1 h at room temperature. The membrane was incubated in 4 mL ECL Western blotting analysis system (Amersham) for 1 min. The membrane was resolved on chemiluminescence film (Amersham Hyperfilm high performance chemiluminescence film), and the film was developed using an X-ray developer. The intensity of the bands on film was analyzed using ImageQuant 5.0 software (Amersham). The band intensity corresponds to the level of protein expression of cytokeratin 8/18, which is expressed in differentiated cells of epithelial origin.[45]

Retinoid Binding Assays. The IC_{50} values of each compound for RARα, RARβ, and RARγ were determined essentially as previously described using recombinant S-Tag RAR fusion proteins.[48−52] Binding assays were performed with receptor extracts diluted with binding buffer (40 mM HEPES, pH 7.9, 120 mM KCl, 10% glycerol, 0.1% (w/v) gelatin, 1 mM EDTA, 4 mM dithiothreitol (DTT), and 5 μg/mL each of the protease inhibitors aprotinin and leupeptin) to a final concentration of 10−30 μg/mL of total protein (0.1−0.3 pmol). [^{3}H]-*all-trans*-RA (1 nM) (1.82−1.92 TBq/mmol or 49.2−52.0 Ci/mmol; DuPont NEN) and various concentrations of each RAMBA (0−500 nM) were added to the binding reaction and incubated for 3 h at 27 °C. Nonspecific binding was determined in the presence of a 200-fold molar excess of unlabeled *all-trans*-RA. Bound RA was separated from free by extraction with 3% (w/v) equal particle size charcoal−dextran. All steps in the procedure were performed under yellow light. Specific RA binding was determined by subtracting the nonspecific binding, always less than 12% of the total binding, from the total binding. The IC_{50} value for each RAR subtype represents the RAMBA concentration that resulted in 50% inhibition of the binding of *all-trans*-RA. All binding assays were repeated at least three times.

In Vivo Antitumor Studies (MCF-7 Human Mammary Carcinoma Xenograft 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. Female ovariectomized athymic nude mice 4−6 weeks of age were obtained from the National Cancer Institute−Frederick Cancer Research and Development Center (Frederick, MD). The mice were maintained in a controlled environment with food and water supply.

Estrogen pellets (1.7 mg/pellet, 90 day continuous release obtained from Innovative Research of America) were implanted in the dorsal interscapular region of the mice using a trochar to facilitate tumor growth. Mice were then inoculated with MCF-7 cells (2×10^6 cells in Matrigel per tumor growth site) subcutaneously at one site on the right and left flanks. Tumors were allowed to grow for about 4−5 weeks till they were of measurable size (200−300 mm^3). The mice were then assigned to groups ($n = 5$) so that the total tumor volume was similar in each group. Treatment was then started, with compound **6** and ATRA formulated in 0.3% HPC (dose: 0.033 μmol/kg once a day, 6 days per week, 200 μL sc injection). Once every week the mice were weighed and tumors were measured using a caliper. Tumor volume was calculated according to the formula $(4/3)\pi r_1^2 r_2$ ($r_1 < r_2$). The tumor treatment study was continued for 6 weeks. At the end of 6 weeks, the mice were sacrificed and the tumors were collected, weighed, and stored until required.

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Supporting Information Available: Experimental details for preparation of intermediate compounds **3** and **4** and for preparation of compounds **13** and **14**. Procedure for statistical analysis, Lineweaver−Burk analysis plot (Figure A), and representative competition of [^{3}H]ATRA by RAMBAs (Figure B). This material is available free of charge via the Internet at http://pubs.acs.org.

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Quantification of a novel retinoic acid metabolism inhibitor, 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA) and other retinoids in rat plasma by liquid chromatography with diode-array detection

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Abstract

A simple reversed phase high performance liquid chromatographic (HPLC) method was developed for the separation and quantification of a novel retinoic acid metabolism inhibitor, 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA), and other retinoids in rat plasma. VN/14-1RA, alone or in combination with ATRA, is effective at inhibiting the proliferation of prostate and breast cancer cell lines in vitro. Aliquots of rat plasma were spiked with the retinoids followed by addition of acetonitrile for precipitation of plasma proteins. The decanted supernatant was evaporated under a stream of nitrogen and reconstituted in acetonitrile. Analysis was accomplished by injection of an aliquot of the reconstituted sample into an HPLC system consisting of a Zorbax Rx-C18 column and a diode array detector. A mobile phase composed of ammonium acetate (0.1 M), acetic acid solution (2% (v/v)) and methanol at a flow rate of 1.0 mL/min was used for gradient elution. The recoveries for all compounds ranged from 65 to 85% regardless of the concentrations examined. The HPLC assay was linear over the range 0.10–5.0 μg/mL (CV < 10%) with a limit of quantification of 100 ng/mL for VN/14-1RA. A one-compartment model with apparent first-order elimination was used to describe the plasma concentration-time profile for VN/14-1RA after intravenous administration. The mean terminal elimination half-life ($t_{1/2}$) was 19.0 ± 3.2 min. This HPLC method is useful for the analysis and evaluation of the pharmacokinetics of VN/14-1RA in rats.

Keywords: All-*trans*-retinoic acid; Retinoic acid metabolism blocking agent; Reversed-phase HPLC

1. Introduction

Retinoids comprise a family of polyisoprenoid lipids, which include vitamin A (retinol) and its natural and synthetic analogues. All-*trans*-retinoic acid (ATRA) is a natural oxidative metabolite and biologically active form of retinol. Retinoids are currently the subject of intense biological interest stimulated by the discovery and characterization of retinoid receptors [1]. It is well established that retinoids such as ATRA control normal cell growth, differentiation, and apoptosis within epithelial tissues. The ability of this class of agents to function as differentiating agents provides a viable approach to cancer treatment [2]. However, current systemic therapy and clinical success with ATRA is limited due to toxicity and development of resistance. The development of resistance to retinoid therapy is thought to result from, at least in part, induction of oxidative metabolism mediated by cytochrome P450 enzymes. Consequently, it has been postulated that inhibition of oxidative metabolism of ATRA by retinoic acid metabolism blocking agents (RAMBAs) in vivo may increase circulating levels of ATRA due to a decrease in its clearance. RAMBAs elevate or maintain

Abbreviations: VN/14-1RA, 4-(1H-imidazol-1-yl)retinoic acid; RAMBA, retinoic acid metabolism blocking agent; ATRA, all-*trans*-retinoic acid; 4-OH-RA, 4-hydroxy all-*trans* retinoic acid; 4-oxo-RA, 4-oxo all-*trans* retinoic acid; HPLC, high performance liquid chromatography

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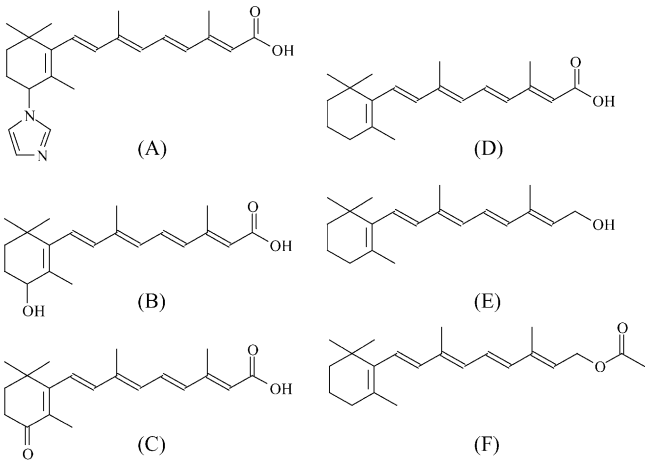

Fig. 1. Chemical structure of the analytes: (A) 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA); (B) 4-hydroxy all-*trans* retinoic acid (OH-RA); (C) 4-oxo all-*trans* retinoic acid (oxo-RA); (D) all-*trans*-retinoic acid (ATRA); (E) retinol; (F) internal standard (all-*trans*-retinol acetate).

endogenous ATRA levels by inhibiting ATRA 4-hydroxylase mediated catabolism and may help overcome the problem of resistance [3,4]. It has been argued that the action of RAMBAs may improve the control of differentiation and growth and possibly enhance the antitumor activity of ATRA. This rationale has been extensively tested with liarozole fumarate (Liazal™) in vitro and in vivo [4–8]. Unfortunately, liarozole is not selective and has been shown to inhibit other cytochrome P450 enzymes. Thus, there is a need to identify more selective inhibitors of retinoic acid metabolism and evaluate them as potential treatments for prostate and breast cancers. Recently, novel (±)-4-azoyl retinoic acid analogues have been synthesized and shown to be potent inhibitors of microsomal ATRA 4-hydroxylase(s). It was demonstrated that 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA) (Fig. 1) is significantly more potent than liarozole in blocking the activity of ATRA 4-hydroxylase(s) [7]. In addition, VN/14-1RA, alone or in combination with ATRA, was effective at inhibiting the proliferation of prostate and breast cancer cell lines in vitro. VN/14-1RA could be more selective and effective than current inhibitors of retinoic acid metabolism [8].

Although several HPLC methods have been reported in the literature for the measurement of retinoids [9–11], there are no reports that described the recovery, separation and quantification of 4-azolyl retinoids such as VN/14-1RA in biological matrices. The present work describes a rapid and facile reversed phase HPLC method with diode-array detection (DAD) for the simultaneous separation and quantification of VN/14-1RA, retinol, ATRA and its metabolites; 4-hydroxy all-*trans* retinoic acid (4-OH-RA) and 4-oxo all-*trans* retinoic acid (4-oxo-RA); in rat plasma and some aspects of the pharmacokinetic properties of VN/14-1RA after intravenous administration in rats. This HPLC method may have general application in the analysis and pharmackinetic evaluation of other 4-azolyl retinoic acids.

2. Experimental

2.1. Materials and reagents

All-*trans*-retinoic acid (ATRA), all-*trans*-retinol, all-*trans*-retinol acetate (internal standard), and hydroxypropyl-β-cyclodextrin (HPβCD) were purchased from Sigma–Aldrich (St. Louis, MO, USA). VN/14-1RA, 4-OH-RA and 4-oxo-RA were provided by Drs Brodie and Njar of the University of Maryland, School of Medicine, Baltimore. Distilled water was obtained from an in-house Barnstead NANOpure® apparatus (GenTech Scientific Inc., Arcade, NY, USA). Methanol, acetonitrile, ammonium acetate and glacial acetic acid were obtained from VWR Scientific Products (Bridgeport, NJ, USA). All chemicals and solvents were of analytical or HPLC grade.

2.2. Preparation of standard solutions and formulations for dosing

Stock solutions of all analytes were prepared with acetonitrile (1.0 mg/mL). Solutions were kept in capped test tubes, wrapped with aluminium foil, and stored at −70 °C. Appropriate working standard solutions of all compounds were obtained by sequential dilutions of the respective stock solutions with acetonitrile.

Formulations of ATRA (5 mg/mL) and VN/14-1RA (10 mg/mL) were prepared in HPβCD (5 mg/mL in saline), sonicated until completely dissolved, and stored at 0 °C. These formulations were diluted with saline to obtain required concentrations for animal treatment. The dosing solutions were placed in a water bath and allowed to reach a temperature of 37 °C before dosing.

2.3. Sample preparation

Whole blood (250 μL) was collected via the jugular vein from male Sprague-Dawley rats into heparinized Eppendorf centrifuge tubes. The blood was immediately centrifuged at 3200 g for 5 min using an Abbott laboratories centrifuge (model 3531) min) and plasma was transferred into clean Eppendorf centrifuge tubes with snap cap for storage at −20 °C. Aliquots (100 μL) of thawed plasma sample were transferred into a 1.5 mL centrifuge tubes using Eppendorf® pipette (Hamburg, Germany). A mixture (200 μL) of acetonitrile containing acetic acid (1% (v/v)) and the internal standard (0.5 mg/mL) was added to the samples to denature plasma proteins and minimize variability, respectively. The samples were vortexed for 10 s and centrifuged at 3200 g for 10 min using an Abbott laboratories centrifuge (model 3531); the supernatant (290 μL) was transferred into clean 5 mL glass test tubes and evaporated to dryness using nitrogen at room temperature. The residue was dissolved in acetonitrile (15 μL), transferred into amber colored HPLC vials and injected into the HPLC for analysis. All handling of retinoids and biological samples was performed in a room

with dim yellow light. Sample preparation was performed in duplicate on three occasions ($n = 3$).

2.4. Stability studies

Retinoids are known to be sensitive to light, heat and oxygen. Therefore, the stability of the analytes was monitored. A set of standard solution in plasma was stored at room temperature (20 °C) and another in a refrigerator (0 °C). Each sample ($n = 3$) was analyzed periodically at day 1, 2, 7 and 14 after preparation of the standard solution and compared to freshly prepared standards. Before HPLC analysis, a mixture (200 μL) of acetonitrile containing acetic acid (1% (v/v)) and the internal standard (0.5 mg/mL) was added to each sample and prepared as described above.

2.5. Chromatographic conditions

The analysis was performed on an HP-1100 HPLC system equipped with a vacuum degasser, quaternary pump, autosampler, column heater, and a diode array detector (DAD). HPLC analysis was conducted on a 5 μm Zorbax-Rx C_{18} column (25 cm × 4.6 mm i.d., Agilent Technologies, Wilmington, DE) with a C_{18} μBondapak guard cartridge (Waters, Milford, MA). Agilent ChemStation® (revision A.06.01) was used for data collection and integration. The mobile phase consisted of three solvent: solvent A: ammonium-acetate solution in water (0.1 M); solvent B: glacial acetic acid solution in water (2% (v/v)); solvent C: methanol (100%). Elution was started at a composition of 25% of solvent A, 15% of solvent B and 60% of solvent C. The mobile phase composition was changed linearly to 15% of solvent A, 0% of solvent B, and 85% of solvent C from the initial time to 12 min. The mobile phase composition was changed linearly to 0% of solvent A, 0% of solvent B, and 100% of methanol from 12 to 22 min and kept at 100% methanol for 4 min. The column was then equilibrated in a mobile phase composed of 25% of solvent A, 15% of solvent B, and 60% of solvent C for 8 min before the next sample was injected. The temperature was maintained at 25 °C. The diode array detector (DAD) was set at wavelengths of 324 nm (retinol, and internal standard), 346 nm (VN/14-1RA, 4-OH-RA, and ATRA) and 358 nm (4-oxo-RA) to monitor all the compounds simultaneously. The optimum wavelengths of all the compounds were determined by Agilent ChemStation® three-dimension spectral analysis module. Quantitation was based on peak areas.

2.6. Calibration curves and assay validation

Aliquots (10 μL) of appropriate dilutions of standard solutions of ATRA and VN/14-1RA were spiked into clean test tubes, evaporated to dryness and reconstituted into blank plasma (100 μL) in order to generate concentrations in the range of 50 ng/mL to 10 μg/mL. The following concentrations were used for the construction of calibration curves for ATRA and VN/14-1RA: 0.0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 3.0, 5.0 μg/mL). Similarly, aliquots (10 μL) of appropriate dilutions of standard solutions of the other retinoids were spiked into clean test tubes, evaporated to dryness and reconstituted into blank plasma (100 μL) in order to generate concentrations in the range of 20 ng/mL to 3 μg/mL. The following concentrations were used for the construction of calibration curves for 4-OH-RA, 4-oxo-RA and retinol: 0.0, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 3.0 μg/mL). Before HPLC analysis, a mixture (200 μL) of acetonitrile containing acetic acid (1% (v/v)) and the internal standard (0.5 mg/mL) was added to each sample and prepared as described above. The reconstituted extract was injected into the HPLC system. Calibration curves for all the compounds were calculated from the least-squares linear regression analysis of peak area ratio (analyte/internal standard) versus sample concentrations using Microsoft® Excel. The ratios of the peak areas for VN-14/1RA, ATRA, 4-OH-RA, 4-oxo-RA, and retinol to the peak area for the internal standards were determined from the chromatograms. The area under the peak of each analyte was normalized for the area under the peak of the internal standard to minimize variability of the assay. The concentrations of the analytes in the samples were computed from the regression parameters. The limit of detection was defined as the smallest concentration of the analyte that produced a peak size that was three times greater than the standard deviation of the HPLC detector noise level (LOD = 3 × S/N). The limit of quantification was defined as the concentration of the analyte that produced a peak size that was ten times greater than the standard deviation of the HPLC detector noise level (LOQ = 10 × S/N). Retinol and its active metabolite (ATRA) are normally present in rat plasma. Although physiologic levels of ATRA in plasma were not detected by this HPLC assay, endogenous retinol was routinely detected in rat plasma. The endogenous concentration of retinol was determined from the mean intercept of the calibration curve for retinol. The recovery of each analyte was determined from the ratio of chromatographic peak area after extraction from plasma to that of an equivalent working standard. Each sample was evaluated in duplicate on three occasions ($n = 3$).

To determine the precision and accuracy of the HPLC assay, known concentrations of the analytes, ranging from 50 to 5000 ng/mL, were spiked in blank plasma samples and analyzed as described above. The validation study was performed in duplicate on three occasions ($n = 3$) over a 2-week period. The coefficient of variation (a measure of precision) and the relative error (a measure of accuracy) were calculated from the spiked concentrations, measured concentrations and the standard deviations of the measured concentrations of the analytes in plasma.

2.7. Preliminary pharmacokinetics of VN/14-1RA

Male Sprague Dawley rats (230–250 g) were obtained from Charles River Laboratories (Wilmington, MA, USA). The animals were maintained in a controlled environment of constant temperature (20 °C), 50% relative humidity and

12 h light/dark cycles for at least 4 days prior to use. The rats were surgically prepared, under ketamine (90 mg/kg) and xylazine (10 mg/kg) anaesthesia, by implanting indwelling cannula into the jugular vein 24 h prior to drug administration and blood sampling. A single dose of VN/14-1RA (5 mg/kg) combined with ATRA (0.5 mg/kg) was administered through the jugular vein to the rats (n = 3). Blood samples (200 μL) were collected in heparinized tubes before treatment and at 5, 10, 20, 30, 45, 60, 90, 120, 150, 180, 240, 300 min, and 24 h after drug administration. Plasma levels of ATRA and VN/14-1RA were determined by HPLC with UV detection as described above. The resulting plasma level data for VN/14-1RA were used to define the pharmacokinetic model and to calculate the basic pharmacokinetic parameters of VN/14-1RA in rats using WinNonlin (Pharsight Corporation Inc., Mountain View, CA).

3. Results

3.1. Chromatography

The maximum UV absorption for the analytes in the mobile phase were as follows; retinol acetate and retinol at 324 nm; VN/14-1RA, 4-OH-RA and ATRA at 346 nm; and 4-oxo-RA at 358 nm. The chromatographic conditions used for the assay provided a well-defined separation (R_s > 1.5) between the compounds and the internal standard. Under the conditions described, retention times for VN/14-1RA, 4-oxo-RA, 4-OH-RA, ATRA, retinol and retinol acetate were approximately 12.51, 13.36, 13.82, 19.58, 22.47 and 25.27 min, respectively. The standard deviations of the retention times were within 0.05 min. The chromatograms for blank plasma and blank plasma spiked with VN/14-1RA, 4-OH-RA, 4-oxo-RA, ATRA, and retinol acetate are shown in Fig. 2. A chromatogram of an extract of plasma obtained from an animal treated with VN/14-1RA (5.0 mg/kg) in combination with of ATRA (0.5 mg/kg) is shown in Fig. 3.

3.2. Stability

When the analytes (1000 ng/mL in plasma), placed in ambler HPLC vials, were stored at room temperature with normal lighting, no detectable degradation occurred within 2 days. However, on day 7 and 14, significant degradation of the analytes occurred based on measurements of the chromatographic peak size (Table 1). The degradation was less than 20% after 14 days for all the compounds. In order to minimize degradation of analytes and to obtain accurate results, HPLC analysis of all samples was completed within 2 days of sample collection.

3.3. Calibration curves and assay validation

The recovery for each analyte was obtained by comparing the peak area from plasma with peak area from standard solutions at three different concentrations on three occasions. The recoveries for all compounds ranged from 65 to 85% regardless of the concentrations examined (Table 2).

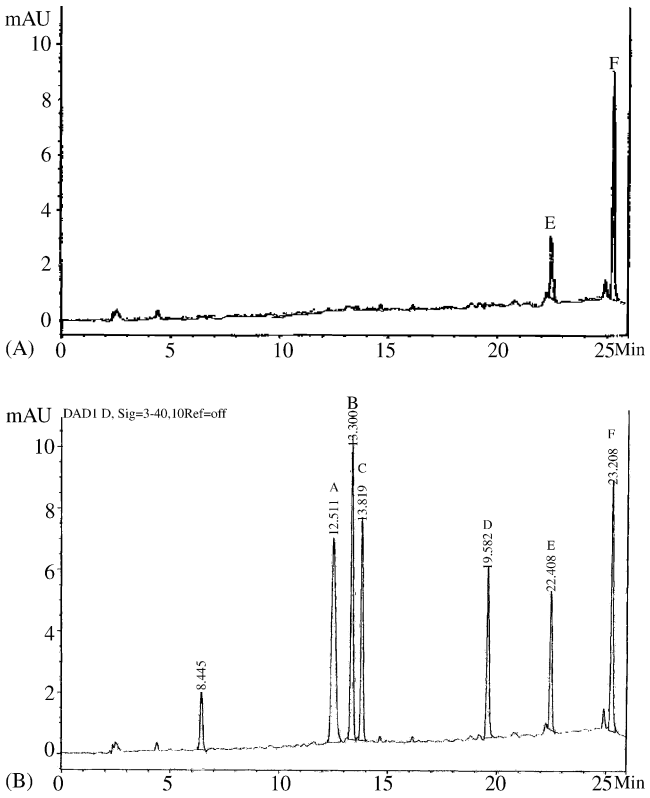

Fig. 2. Typical chromatogram of (a) blank plasma spiked with internal standard; (b) plasma spiked with standards (1.0 μg/mL). (A) VN/14-1RA; (B) oxo-RA; (C) OH-RA; (D) ATRA; (E) retinol; (F) internal standard.

Linear calibration curves were obtained over the range of 0.10–5.0 μg/mL for VN/14-1RA ($y = 0.1081x - 10.176$, $r^2 = 0.9976$). Similarly, linear calibration curves were obtained over the range of 0.05–5.0 μg/mL for ATRA ($y = 0.1877x + 1.2047$, $r^2 = 0.9962$). Linear calibration curves were also

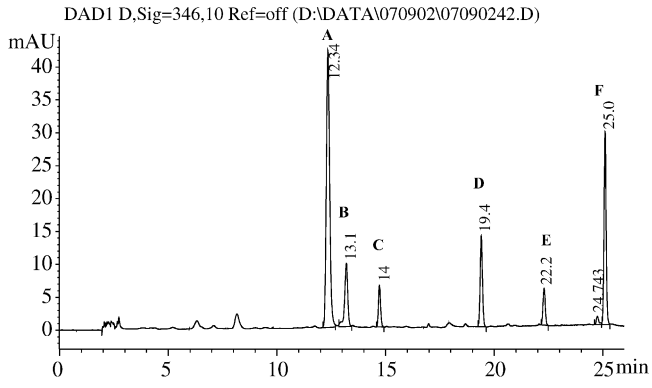

Fig. 3. Typical chromatogram of an extract of a plasma sample obtained 20 min after intravenous administration of VN/14-1RA (5 mg/kg) in combination with ATRA (0.5 mg/kg). The concentrations of VN/14-1, ATRA, OH-RA, oxo-RA and retinol in this sample were 5000, 3200, 775, 1125, and 600 ng/mL, respectively. (A) VN/14-1RA; (B) oxo-RA; (C) OH-RA; (D) ATRA; (E) retinol; (F) internal standard.

Table 1
Stability profile of 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA) and other retinoids in rat plasma

Analytes (1.0 μg/mL)	Refrigerator (0 °C)			Room temperature (20 °C)		
	% Loss			% Loss		
	2nd day	7th day	14th day	2nd day	7th day	14th day
VN/14-1RA	0.4	0.9	2.2	1.1	12.4	17.4
ATRA	−0.3	−0.2	1.2	0.4	7.1	11.9
Retinol	−1.5	0.1	0.9	−0.4	4.1	4.5
OH-RA	1.3	2.2	3.0	0.9	8.3	12.2
Oxo-RA	0.5	2.0	1.9	0.7	13.5	17.9
IS	0.4	−0.3	2.5	0.5	10.0	14.6

The values represent the percentage of loss for each analytes after 2, 7 and 14 days of storage. Loss (%) = ((1000 ng/mL − determined concentration (ng/mL))/1000 ng/mL) × 100. Negative values indicated that the concentrations calculated from calibration curve were higher than 1000 ng/mL.

Table 2
The recovery of the 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA) and other retinoids from rat plasma

Analyte	Recovery (%)
VN/14-1RA	70.1 ± 6.5
ATRA	78.9 ± 7.2
4-OH-ATRA	68.2 ± 7.8
4-oxo-RA	69.2 ± 8.2
Retinol	79.1 ± 5.7

Table 3
The accuracy and precision of the HPLC measurement of 4-(1H-imidazol-1-yl)retinoic acid (VN/14-1RA) and other retinoids in rat plasma

Analyte	Spiked concentration (ng/mL)	Measured concentration (ng/mL)	CV (%)	Relative error (%)
VN/14-1RA	100	106 ± 3.0	2.8	6.0
	200	206.4 ± 8.1	3.9	3.2
	350	361.2 ± 15.2	4.2	3.2
	1000	1050 ± 26.3	2.5	5.0
	2300	2378 ± 78.5	3.3	3.8
	5000	5305 ± 281	5.3	6.1
ATRA	50	51.4 ± 2.0	3.9	2.8
	100	103 ± 2.6	2.5	3.0
	200	202.2 ± 3.8	1.9	1.1
	350	360.5 ± 7.9	2.2	3.0
	1000	1021 ± 11.2	1.1	2.1
	2300	2318 ± 34.8	1.5	0.8
	5000	5160 ± 216.7	4.2	3.2
4-OH-RA	100	103 ± 3.0	2.9	3,0
	200	204.6 ± 10	4.9	2.3
	350	362.3 ± 8.7	2.4	3.5
	1000	1039 ± 33.3	3.2	3.9
	2300	2364 ± 54.4	2.3	2.8
4-oxo-RA	100	101 ± 4.0	3.9	1.0
	200	203 ± 6.9	3.4	1.4
	350	360.2 ± 3.6	1.0	2.9
	1000	1019 ± 22.4	2.2	1.9
	2300	2325 ± 113.9	4.9	1.1
Retinol	200	202.6 ± 2.0	1.0	1.3
	350	354.6 ± 3.9	1.1	1.3
	1000	1007 ± 3.0	0.3	0.7
	2300	2314 ± 11.6	0.5	0.6

The values represent the mean from three separate determinations (n = 3).

obtained over the range of 0.10–3.0 μg/mL for 4-OH-RA (y = 0.0893x + 0.1455, r^2 = 0.9847), 4-oxo-RA (y = 0.1709x + 1.5987, r^2 = 0.9983) and retinol (y = 0.0799x + 0.002, r^2 = 0.9915). The limit of quantification for VN/14-1RA was estimated to be about 100 ng/mL. The limit of quantification of ATRA and its metabolites were estimated to be about 50 and 100 ng/mL, respectively.

The precision and accuracy of the assay were satisfactory; the coefficient of variations (CV (%)) and relative error were less than 10% for all analytes (Table 3).

3.4. Preliminary pharmacokinetics

This HPLC method was used to characterize the pharmacokinetics of VN/14-1RA in rats after co-administration with ATRA. A single dose of VN/14-1RA (5 mg/kg) in combination with ATRA (0.5 mg/kg) was administered intravenously to male Sprague-Dawley rats (n = 3). The plasma concentrations of VN/14-1RA (5.0 mg/kg) showed a monoexponential decline following intravenous administration (Fig. 4) in rats. VN/14-1RA was rapidly eliminated with a mean terminal elimination half-life ($t_{1/2}$) of about 19.0 ± 3.2 min. The lev-

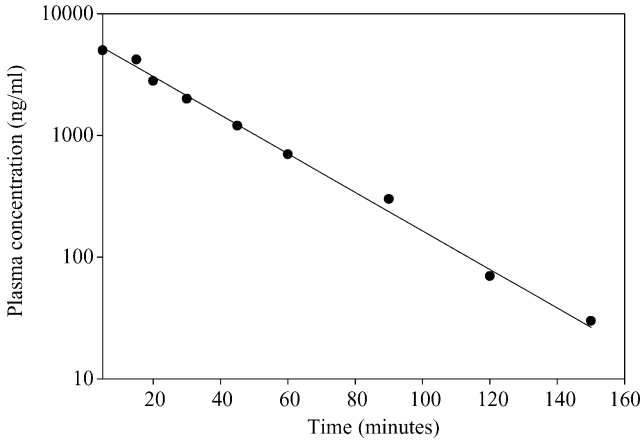

Fig. 4. Pharmacokinetics profile of VN/14-1RA (5 mg/kg) after intravenous administration (in combination with ATRA) in male Sprague-Dawley rats. The values represent the mean plasma concentration from three rats. The terminal elimination half-life = 19.0 ± 0.2 min.

els of 4-OH-RA in rat plasma were very low as a result of rapid conversion of 4-OH-RA to 4-oxo-RA.

4. Discussion

The HPLC method achieved good separation of VN/14-1RA, ATRA, 4-oxo-RA, and 4-OH-RA, with sharp and symmetrical peaks and was useful to monitor the plasma levels of the retinoids at multiple wavelengths simultaneously. Protein precipitation using acetonitrile with acetic acid enhanced the recovery of the analytes from rat plasma. Although ATRA and its metabolites are normally present in rat plasma, physiologic levels of these retinoids in blank plasma samples were not detected by this method. On the other hand, endogenous retinol was routinely detected in blank rat plasma samples. The calibration curves for all analytes were linear in the concentration range examined and the assays were reproducible and allowed accurate determination of the retinoids in rat plasma.

This HPLC method is suitable for the study of the chemical stability, enzymatic metabolism and pharmacokinetics of VN/14-1RA and ATRA in rats. It appears that VN/14-1RA has a relatively short half-life in rats. The findings that have arisen from the preliminary pharmacokinetic studies have been helpful in understanding the disposition of VN/14-1RA and other 4-azolyl retinoids in rats.

Acknowledgements

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Review

Retinoic acid metabolism blocking agents (RAMBAs) for treatment of cancer and dermatological diseases

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Abstract—The naturally occurring retinoids and their synthetic analogs play a key role in differentiation, proliferation, and apoptosis, and their use/potential in oncology, dermatology and a variety of diseases are well documented. This review focuses on the role of all-*trans*-retinoic acid (ATRA), the principal endogenous metabolite of vitamin A (retinol) and its metabolism in oncology and dermatology. ATRA has been used successfully in differentiated therapy of acute promyelocytic leukemia, skin cancer, Kaposi's sarcoma, and cutaneous T-cell lymphoma, and also in the treatment of acne and psoriasis. However, its usefulness is limited by the rapid emergence of acquired ATRA resistance involving multifactoral mechanisms. A key mechanism of resistance involves ATRA-induced catabolism of ATRA. Thus, a novel strategy to overcome the limitation associated with exogenous ATRA therapy has been to modulate and/or increase the levels of endogenous ATRA by inhibiting the cytochrome P450-dependent ATRA-4-hydroxylase enzymes (particularly CYP26s) responsible for ATRA metabolism. These inhibitors are also referred to as retinoic acid metabolism blocking agents (RAMBAs). This review highlights development in the design, synthesis, and evaluation of RAMBAs. Major emphasis is given to liarozole, the most studied and only RAMBA in clinical use and also the new RAMBAs in development and with clinical potential.

Contents

Keywords: All-*trans* retinoic acid (ATRA); Retinoic acid (RA); CYP26; Retinoic acid metabolism blocking agents (RAMBAs); Differentiation; Retinoid resistance; Cancer; Dermatology.
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1. Introduction

Retinoids (vitamin A and its natural metabolites and synthetic analogs) comprise a family of polyisoprenoid compounds. They are currently the subject of intense biological interest stimulated by the discovery and characterization of retinoid receptor and the realization of these compounds as nonsteroidal small-molecule hormones.[1] All-*trans*-retinoic acid (ATRA), the biologically most active metabolite of vitamin A, plays a major role in the regulation of gene expression, in cellular differentiation, and proliferation of epithelial cells.[2] Differentiating agents redirect cells toward their normal phenotype and therefore may reverse or suppress evolving malignant lesions or prevent cancer, and indeed represent an attractive target for medicinal intervention. ATRA is being used in differentiation therapy of cancer, in cancer chemoprevention, and for the treatment of dermatological diseases, including, acne, psoriasis, and ichthyosis.[3] Recently, ATRA has proven useful in cancer chemotherapy.[4] One of the most impressive effects of ATRA is on acute promyelocytic leukemia. Treatment of acute promyelocytic leukemia patients with high dose of ATRA resulted in complete remission.[5,6] Furthermore, several experiments in animals have demonstrated that ATRA inhibited the induction and caused the disappearance of prostate tumors.[7] In spite of these encouraging results, the effects of prolonged ATRA therapy on human cancers in the clinic have been scarce and disappointing.[8] Although the use of retinoids in the treatment of dermatological diseases has met with relatively better outcomes, side effects constitute a limit to the chronic use of these systemic agents. It has been suggested that the therapeutic effects of ATRA are undermined by its rapid in vivo metabolism and catabolism by cytochrome P450 enzymes.[9] An important consideration is that two cellular retinoic acid-binding proteins (referred to as CRABP-I and CRABP-II) are believed to be involved in the presentation of ATRA to metabolizing CYP enzymes and its channeling to the RAR receptors.[10,11] This topic will be briefly discussed in the next section. It should be stated that the two natural isomers of ATRA, 9-*cis*-retinoic acid (9-CRA) or 13-*cis*-retinoic acid (13-CRA), are also being investigated for cancer chemoprevention and/or therapy. In general, few P450 enzymes are known to be involved in retinoid metabolism.

One of the strategies for preventing in vivo catabolism of ATRA is to inhibit the P450 enzymes responsible for this process. Indeed, this seems to be an emerging approach that may yield effective agents for the chemoprevention and/or treatment of cancers and dermatological diseases.[3a,12] In this review, we will highlight development in the design, synthesis, and evaluation of RAMBAs since 1987. Major emphasis is given to liarozole, the most studied and first RAMBA to undergo clinical investigation and also the recently developed novel and potent 4-azolyl retinoids, the benzeneacetic acid derivatives and the 2,6-disubstituted naphthalenes. The potential role of a new family of cytochrome P450 enzymes, CYP26, with specificity toward ATRA is also discussed. For recent presentations of work in this field, the reviews by Miller[3a] and Njar[12] are recommended. To our knowledge, this represents the second comprehensive review of RAMBAs. Since the last comprehensive review on RAMBAs,[12] several manuscripts and patents on novel RAMBAs have appeared and gratifyingly, a pioneering RAMBA called liarozole was recently (2004) approved in Europe and USA as an orphan drug for the treatment of congenital ichthyosis.[13] Studies were identified for this review by searching the MEDLINE® and PubMed databases for appropriate papers published in the last 15 years up to December 2005 and by reviews of bibliographies from articles identified through that search. This review is complementary to the first comprehensive review on RAMBAs that appeared in 2002.[12] In addition, we include some of our unpublished data.

2. Mechanism of action of retinoids and retinoic acids

2.1. The role of retinoid and retinoic acid receptors

With current knowledge, the pleiotropic action of retinoic acids (RAs) and retinoids might be explained mechanistically by the actions of the six known nuclear receptors, the retinoic acid receptors (RARα, β, and γ) and the retinoid X receptor (also called rexinoids (RXRα, β, and γ)).[1,2,3b,10] Each of these receptors is encoded by distinct genes and are members of the steroid/thyroid hormone receptor superfamily. It is also thought that each receptor mediates a set of unique biological functions in certain cell or tissue types. ATRA is the natural ligand of the RARs, while 9-CRA is the ligand for the RXRs and it also has a high affinity for the RARs. The binding of the other ATRA stereoisomers, 11-*cis*-retinoic acid (11-CRA) and 13-CRA, to these receptors is still unclear. However, because of the reported antitumor efficacy of 13-CRA[14–19] it is plausible that 13-CRA is isomerized intracellularly to ATRA, or it may act without obvious interaction with the known retinoid receptors (Fig. 1). Clearly, more research is needed in this area.

Most of the pleiotropic activities of the RAs and other retinoids are elicited by the binding of these agents to the RAR site of RAR/RXR heterodimers. RXRs are the silent partners of the RARs, as the RXR ligands alone are unable to activate the RAR/RXR heterodimers. However, recent studies using RAR- and RXR-selective ligands have revealed that the RXR ligands allosterically increase the potencies of the RAR ligands.[20–22] Furthermore, RXRs form heterodimers with various nuclear receptors, such as estrogen receptors (ERs), vitamin D_3 receptors (VDRs), thyroid hormone receptors (TRs), and peroxisome proliferator-activated receptors (PPARs). Because of these unique properties of the RXRs, the RXR ligands are able to modulate the activities of other hormone receptors, in addition to their retinoidal activities.[23]

These receptors, as heterodimers (RAR/RXR) or homodimers (RXR/RXR), function as RA-inducible transcriptional regulatory proteins by binding to DNA

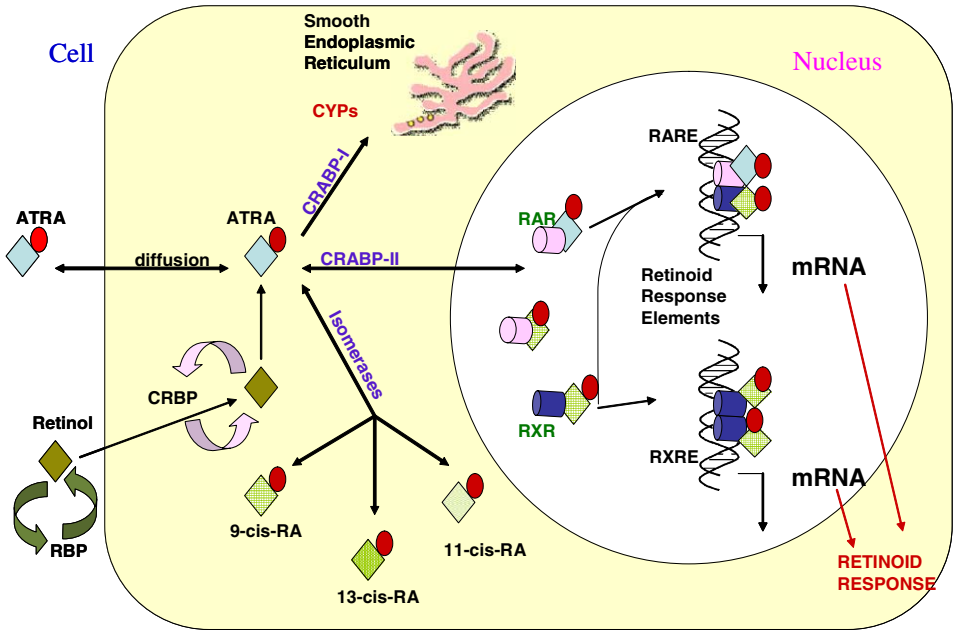


Figure 1. Fate of ATRA in the cell. ATRA enters the cell by simple diffusion or by conversion from retinol that has been absorbed from the gastrointestinal tract, bound in circulating form to retinol-binding proteins (RBPs), and rebound intracellularly to cellular retinol-binding proteins (CRBPs). ATRA can be immediately metabolized upon binding to cellular retinoic acid-binding proteins (CRAPBPs) and oxidized by CYPs located in the endoplasmic reticulum. Alternatively, ATRA and its 9-*cis*-isomer enter the nucleus and bind to RARs or RXRs, respectively. Upon dimerization of these receptors, that is, RAR/RXR heterodimer or RXR/RXR homodimer, the activated receptors bind with high affinity to specific DNA retinoic acid response element (RARE) and effect mRNA transcription. Ultimately, the retinoid response is mediated by primary target genes, by interference with other transcription factors or by control of certain post-transcriptional actions.

regions called retinoic acid response elements (RAREs) or retinoid X response elements (RXREs) located within the promoter of target genes. RAREs consist of direct repeats of the consensus half-site sequence AGGTCA separated most commonly by five nucleotides (DR-5), whereas RXREs are typically direct repeats of AGGTCA with one nucleotide spacing (DR-1). In the absence of ligand (ATRA or 9-CRA), the apo-heterodimer (RAR/RXR) binds to the RARE in the promoter of the target genes and RAR recruits corepressors (CoRs) such as nuclear receptor corepressors (NCoR) or/and silencing mediator for retinoid and thyroid receptors (SMRT). These corepressors by recruiting histone deacetylase complexes (HDACs) cause target gene repression due to compaction of chromatin, causing DNA to be inaccessible to the transcriptional machinery. However, in the presence of ATRA or an agonist, there is a conformational change in the structure of the ligand-binding domain that results in destabilization of the CoR-binding with concomitant recruitment and interaction with coactivators (CoAs). Some coactivators interact directly with the basal transcriptional machinery to enhance transcriptional activation, while others encode histone acetyl transferase (HAT) activity. HAT acetylates histone proteins, causing the opening of the chromatin and activation of transcription of the associated gene. The mechanism of transcriptional repression and activation of RAR/RXR heterodimer is summarized in Figure 2. Other complexes, such as the thyroid receptor associated protein are also involved in this process. It should be stated that whereas the RARα is involved in myeloid leukemias, a growing body of evidence indicate that RARβ is involved in a diverse range of solid tumors.[3b] For more details on the mechanism of action of ATRA, reviews by Chambon[2] and Altucci and Gronemeyer[3b] should be consulted.

2.2. The role of cellular retinoic acid proteins I and II

Two binding proteins, cellular retinoic acid-binding proteins I and II (CRABP-I and CRABP-II), are implicated in the tightly controlled nuclear receptor-mediated mechanism of action of ATRA.[10,11,24] CRABP-I and CRABP-II as well as other retinoid-binding proteins are believed to share a common role, in that they act to solubilize and stabilize their hydrophobic and labile ligands in aqueous milieu. However, in addition to this general role, specific retinoid-binding proteins have distinct functions in regulation of the transport, metabolism, and action of the particular retinoids with which they associate. Furthermore, the distinct patterns of expression of CRABP-I and II suggest that they serve different functions in the biology of ATRA, and/or, perhaps that they allow for accommodating different requirements for ATRA in different tissues.[25] A growing body of information indicates that CRABP-I moderates cellular response to ATRA by facilitating catabolism and/or by sequestering ATRA, rendering it unavailable to nuclear receptors. On the basis of elegant studies by Noy and co-workers,[25–27] it is currently believed that the CRABP-II/RAR complex mediates ligand channeling from the binding protein to the receptor, thereby facilitating the ligation of RAR and potentiating its transcriptional activity (Fig. 3). These investigators have also recently clearly demonstrated that CRABP-II plays a critical role in sensitizing MCF-7 human breast tumors

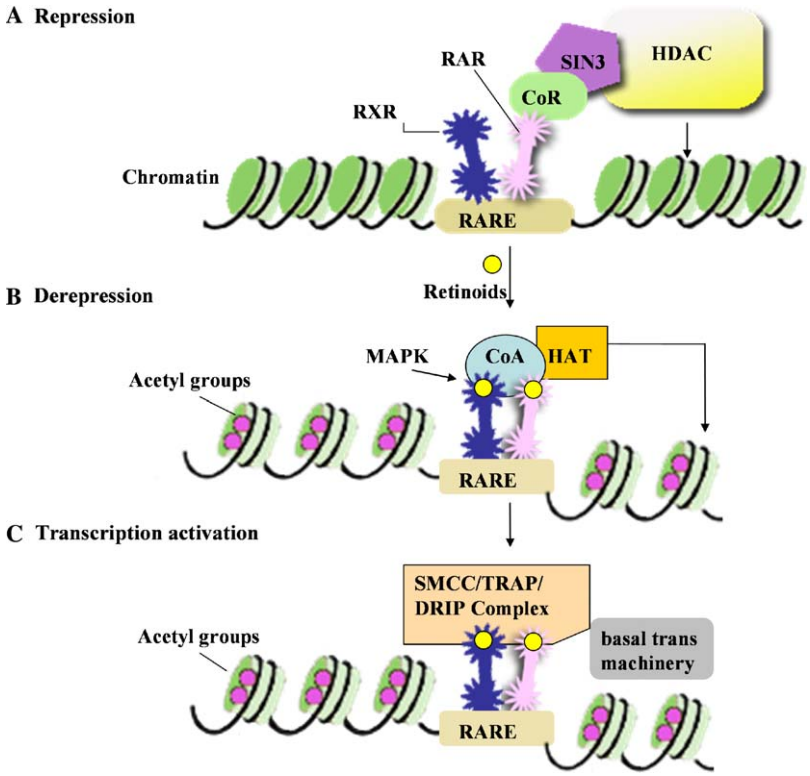


Figure 2. Mechanism of transcriptional repression and activation by RAR–RXR heterodimers. Figure adapted from Ref. 3b.

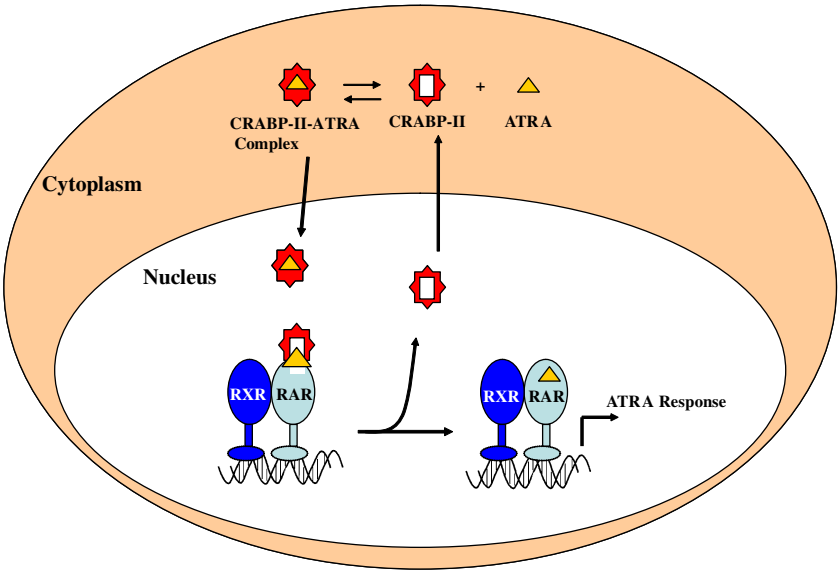


Figure 3. Mechanism of action of cellular RA-binding protein (CRABP-II). Following the binding of ATRA in the cytoplasm, CRABP-II moves into the nucleus where it channels ATRA to the RAR. RAR is then activated, resulting in the up-regulation of multiple target genes. Figure adapted from Ref. 27.

to the growth-suppressive activities of ATRA in vivo.[28] In support of the association between CRABP-II and retinoid receptors, another group has demonstrated that CRABP-II can interact with RARα and RXRα.[29] Taken together, these studies define a novel mechanism of transcriptional control that establish the function of CRABP-II in modulating the RAR-mediated biological activities of ATRA.

3. Cytochrome P450 enzymes (CYPs) involved in retinoic acid metabolism

ATRA is rapidly metabolized by cytochrome P450 (CYP)-dependent enzymes via several routes leading to a variety of polar metabolites (Scheme 1); the immediate products include 4-hydroxy-ATRA (4-OH-ATRA), 18-hydroxy-ATRA (18-OH-ATRA), and 5,6-

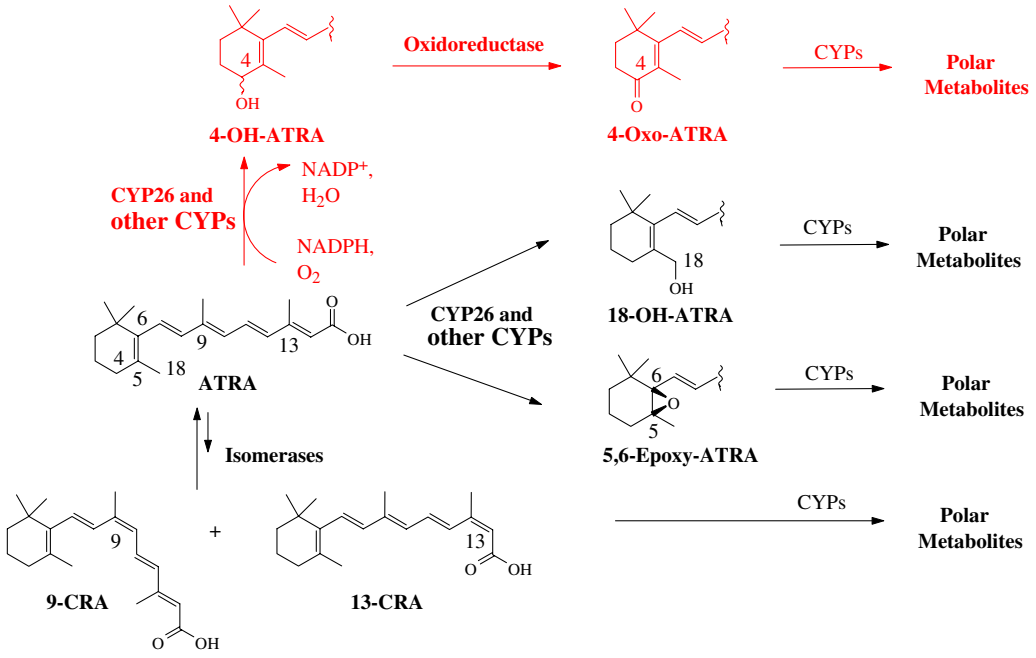
epoxy-ATRA.[30] However, it is believed that the physiologically most prominent pathway starts with the rate-limiting hydroxylation at C-4 position of the cyclohexenyl ring leading to formation of 4-hydroxy-ATRA. It should be stated that the stereochemistry at C-4 of 4-hydroxy-ATRA is yet to be determined. The latter compound is converted by a reductase enzyme into 4-oxo-ATRA that is then further transformed by CYP(s) into more polar metabolites (Scheme 1).[31] Although most of these ATRA metabolism studies have been conducted with rodent liver microsomes, similar results have also been obtained using human liver microsomes.[30,31] Four independent groups have established that of the several human liver CYP isoforms capable of metabolizing ATRA via the 4-hydroxylation route, CYP2C8 is the major contributor, though CYP3A4 and, to a lesser extent CYP2C9, also make contributions.[32–35]

A growing body of experimental data indicates that ATRA is also biotransformed into its isomers, 9-CRA and 13-CRA, but as indicated in Scheme 1, bio-conversions to ATRA are thermodynamically favorable.[36] As expected, these 9-CRA and 13-CRA also undergo CYP metabolism to give their corresponding 4-hydroxy and 4-oxo metabolites. Recent studies by Chabot and co-workers[37] have identified adult human CYPs 2C9, 2C8, and 3A4 to be involved in the 9-CRA metabolism, whereas CYPs 3A4 and 2C8 are active in 13-CRA metabolism. For recent presentations of work in this field, the review by Marill et al.[38] is recommended.

Although several CYPs have been shown to be involved in the catalysis of ATRA 4-hydroxylation, their specificity for ATRA is generally low.[32–34] However, a new family of cytochrome P450 enzymes CYP26A1 has recently been cloned and characterized in zebrafish, human, and mouse tissues.[39–44] In addition to this new enzyme, a second human CYP26 (referred to as CYP2B1) which is 44% identical to CYP26A1 has also been identified.[45] CYP26A1 and CYP26B2 are ATRA-inducible and appear to be the most dedicated ATRA 4-hydroxylase enzymes known. Interestingly, these CYPs do not hydroxylate the closely related isomeric 9-CRA or 13-CRA.[44,46] Of significance also is the recent report of 4-hydroxyphenyl retinamide (4-HPR) induction of CYP26A1 in A2780 human ovarian carcinoma cells, that is responsible for the metabolism of 4-HPR to the biologically active 4-oxo-HPR.[47] A third member of the CYP26 family named CYP26C1 has recently been identified, cloned, and characterized.[48] Although CYP26C1 shares extensive sequence similarity with CYPA1 and CYPB1, its catalytic activity appears distinct from those of other CYP26 family members. Specifically, CYP26C1 can also recognize and metabolize 9-CRA and is much less sensitive than the other CYP26 family members to the inhibitory effects of ketoconazole. These enzymes display high specificity toward ATRA and may function as important regulators of differentiation and as possible modulator of disease states by controlling retinoid concentrations and homeostasis. Recent reviews on the cloning and characterization of CYP26 enzymes[49,50] and also the regulation of CYP genes by nuclear receptors have appeared.[51] It should be noted that the pioneering paper by Petkovich and co-workers[39] opened the way for the recent exciting studies on CYP26 enzymes.

4. Distribution and role of CYP26 in cancer and dermatology

CYP26 is expressed in the liver, heart, pituitary gland, adrenal gland, testis, duodenum, colon, and in specific



Scheme 1. Metabolic pathways of all-*trans*-retinoic acid (ATRA). The major metabolic pathway is shown in red, and partial structures for some metabolites are presented for clarity.

regions of the brain and the placenta [reviewed in Refs. 45, 50, and 52]. Based on recent studies,[50] it is suggested that the major role of CYP26A1 is a protective one, that is, the regulation of intracellular ATRA steady-state levels, exhibiting a similar negative feedback as has been demonstrated for CYP24, which is involved in cholecalciferol catabolism.[53] Although the major retinoid products (4-hydroxy- and 4-oxo-ATRA) of CYP26 were originally considered to be inactive retinoids, there is compelling evidence which suggest that they are highly active modulators of positional specification in amphibian embryonic development and they bind and activate retinoic acid receptors (RAR) subtypes as efficiently as ATRA.[54,55] Thus, in development CYP26A1 may fulfil functions distinct from metabolic inactivation of ATRA.

CYP26 is readily induced by ATRA in a variety of normal and some cancer cells (MCF7, T47D, NB4, HepG2, HPK1A, and LNCaP) and the enzyme efficiently converts ATRA into its oxygenated derivatives. Although the therapeutic potential of ATRA has been demonstrated (reviewed in Refs. 7 and 56) a major drawback to its clinical application is the prompt emergence of resistance, attributed to the induction of oxidative catabolism through CYPs,[9,57–60] and CYP26 could be a major contributor. Because ATRA deficiency is associated with the progression of some cancers,[61–63] it is possible that ATRA-induced CYP26 is involved in rapid metabolism of ATRA in cancer patients. In addition, it is firmly established that inappropriate metabolism of ATRA by CYPs can generate a condition of retinoid deficiency, which is characterized by hyperkeratinization and desquamation as seen in dermatological diseases such as acne, psoriasis, and ichthyosis.[64]

The cloning and characterization of CYP26 represents an important development in ATRA (retinoid) biochemistry and molecular biology. The enzyme's inducibility by ATRA and its ATRA metabolic/catabolic activity define a feedback loop, which may be critical in regulating both normal and therapeutic levels of ATRA. This emphasizes the importance of maintaining stable physiological levels of ATRA. Thus, compounds designed to inhibit CYP26 activity may be useful in elevating normal tissue ATRA levels or maintaining high therapeutic levels of ATRA. As stated earlier, since ATRA has proven useful in the treatment and/or chemoprevention of some cancers and skin disorders, it is now possible to investigate the contributions of the expression/activity of CYP26 (or lack thereof) in various diseases. CYP26A1 has recently been mapped to human chromosome 10q23–q24,[65] a region where several suppressor gene loci have been described[66] as well as the split-hand-split foot syndrome (SHSF-3).[67] Thus, it is possible that mutations in CYP26A1 may play a role in these diseases. On the other hand, CYP26B1 is localized on chromosome 2P12 with 6 exons and codes 512 amino acid proteins.[45,68] CYP26C1 is not widely expressed in the adult but is inducible by ATRA in HPK1a, transformed keratinocyte cell lines, and it is suggested that it may play a specific role in catabolizing both ATRA and 9-CRA.[48]

5. ATRA 4-hydroxylase inhibition—development of retinoic acid metabolism blocking agents (RAMBAs)

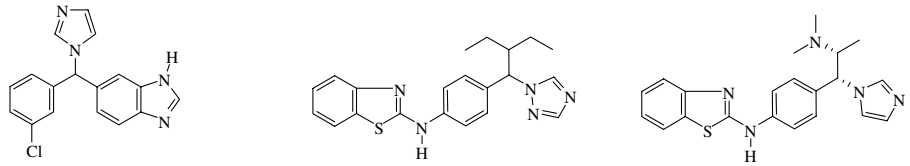
The realization that the metabolism of ATRA may be responsible for its limited efficacy in the clinics (CYP26-mediated resistance?) provided the impetus behind the search for inhibitors of the CYP-mediated metabolism of ATRA. As the enzymes responsible for ATRA metabolism are P450 proteins, most of the intial compounds tested for their inhibition were the known P450 inhibitors, such as clotrimazole and ketoconazole. Because the early studies on the inhibitors of these enzymes have recently been discussed,[12] we will focus on the important developments in the design, synthesis, and testing of the newer inhibitors of ATRA 4-hydroxylases (RAMBAs).

A potent RAMBA would be expected to modulate the levels of endogenous or co-administered ATRA, enhancing the 'ATRA-mimetic' effects. In humans, the target enzymes involved in ATRA metabolism are the nonspecific liver CYPs, among which CYP2C8 and 3A4[33–35] are the major contributors, and the ATRA-inducible CYP26.[39–45] Although some investigators[69–72] have targeted inhibition of CYP26, it seems more realistic that both the nonspecific CYPs and specific CYP26 would need to be targeted since without initial ATRA accumulation due to nonspecific CYP action, ATRA levels would be insufficient to induce CYP26. Smith and co-workers[73] have also recently articulated this alternative strategy.

Since the last review on this topic, a large number of new RAMBAs have been reported in the literature and in patent disclosures by researchers from academia, including the University of Cardiff group, and our University of Maryland group and by pharmaceutical companies such as Johnson and Johnson Pharmaceutical Research and Development, Allergan Sales Inc., and OSI Pharmaceuticals, Inc. It should be stated that ATRA hydroxylase inhibition assays are performed differently in various laboratories and inhibitory data reported here are based on assays with hamster or rat liver microsomes, and also cell-based/microsomal assays using ATRA-induced CYP26 in MCF-7 of T47D human breast cancer cells and CYP26 stably transfected HeLa cells. The following is an overview of the different types of RAMBAs and a perspective on the significance of the various classes as potential therapeutic agents in oncology and dermatology.

6. Liarozole (Liazal™) and related compounds—R115866 and R116010

Following extensive studies by researchers of Janssen Research Foundation (now called Johnson and Johnson Pharmaceutical Research and Development) liarozole (**1**, Liazal™, Fig. 4) was identified as a modest inhibitor (IC_{50} 2.2–6.0 μM) of ATRA-4-hydroxylase (CYP26).[69,74–80] On the other hand, the compound was shown to be a good inhibitor of rat CYP17 (IC_{50} = 260 nM) and a potent inhibitor of CYP19.[77]



1: Liarozole (Liazal TM) **2: R115866** **3: R116010**

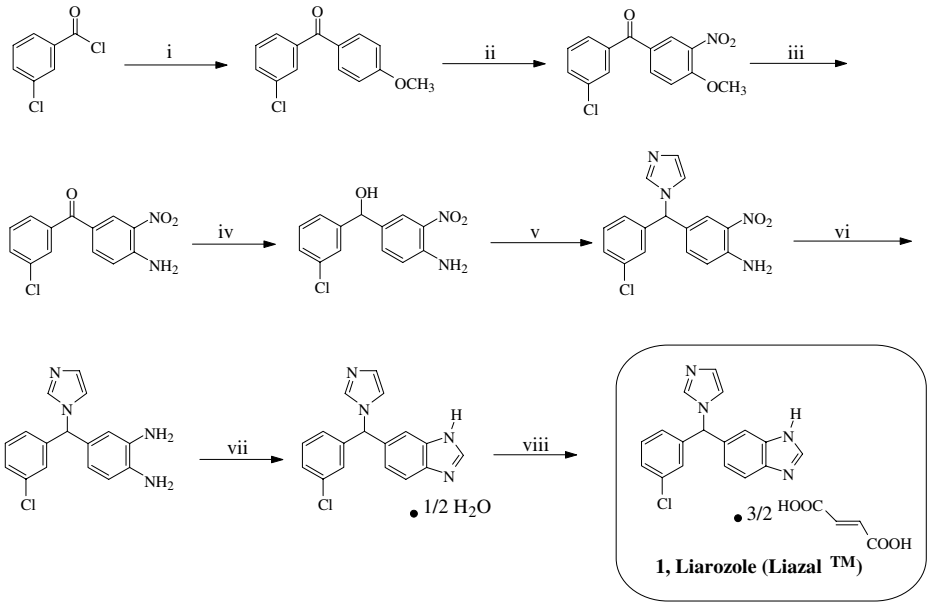
Figure 4. Structures of liarozole (**1**), R115866 (**2**), and R116010 (**3**).

It should be noted that liarozole is the most studied RAMBA and remains to date the only RAMBA to have been evaluated clinically in patients with cancer and also dermatological diseases and, as such, is a standard against which future RAMBAs may be judged. A practical eight-step synthesis of liarozole has been reported (Scheme 2).[69]

Anti-tumoral action was detected in androgen-dependent and androgen-independent rat prostate carcinoma models.[14,78,81] Remarkable anti-tumor activity was observed against prostate cancer xenografts in immunodepressed mice[82,83] and further studies revealed that the anti-tumor properties of liarozole correlate with an increase in tumor differentiation, following accumulation of ATRA.[84] These studies established that the anti-tumoral properties of the compound are related to its inhibition of ATRA metabolism and that the previously demonstrated inhibition of CYP17 (inhibition of androgen synthesis) is marginal in vivo.

A large phase III international study was completed comparing liarozole 300 mg twice daily with cyproterone acetate (CPA) 100 mg twice daily in a total of 321 patients with metastatic prostate cancer in relapse after first-line endocrine therapy.[85] The adjusted hazard ratio for survival was 0.74 in favor of liarozole ($P = 0.039$), indicating a 26% lower risk of death than in patients treated with CPA. Liarozole was superior to CPA in terms of prostate-specific antigen (PSA) response, PSA progression, and survival, and was capable of maintaining patients' quality of life. The observed adverse events were relatively mild to moderate in nature. The results indicate that liarozole might be a possible treatment option for prostate cancer (PCA) following failure of first-line endocrine therapy.

Although most experiences of liarozole as an anti-cancer agent have been limited to prostate cancer, a few experiments with breast cancer have also been conducted. In cultured human breast cancer MCF-7 cells, liarozole potentiated the antiproliferative and differentiative effects of ATRA.[86–89] This enhancement of ATRA effects could be explained by the inhibition of enzymatic degradation of ATRA in these cells. Liarozole has proven antitumor activity in steroid-insensitive TA3-mouse mammary carcinoma and in NUM-induced mammary carcinoma in rats.[90] A recent study of three phase II clinical studies of liarozole in the treatment of ER negative, tamoxifen refractory or chemotherapy-resistant postmenopausal metastatic breast cancer patients only reported a modest response.[91]



Scheme 2. Synthesis of liarozole. Reagents and conditions: (i) $PhOCH_3$, $AlCl_3$, CH_2Cl_2, 5–10 °C; (ii) HNO_3/H_2SO_4, CH_2Cl_2, 10–15 °C, 1 h; (iii) NH_3(g), *i*-PrOH, 100 °C; (iv) $NaBH_4$, *i*-PrOH, reflux, 1 h; (v) CDI, CH_2Cl_2, reflux, 1 h; (vi) H_2, Pt/C, 5% thiophene soln, MeOH, rt; (vii) HCOOH, 4 N HCl, reflux; (viii) EtOH, 50 °C, fumaric acid.

Despite these encouraging preclinical and clinical results, the usefulness of liarozole cancer therapy is considered limited due to adverse side effects that are attributed to its lack of CYP isozyme specificity and its moderate potency of ATRA 4-hydroxylases. Consequently, Janssen have since discontinued clinical development of liarozole as an anti-cancer agent.[91,92]

Inappropriate metabolism of ATRA could generate a condition of retinoid deficiency, which is characterized by hyperkeratinization and desquamation as seen in acne, psoriasis, and ichthyosis.[64] Because of these reasons, liarozole has also been extensively investigated as a potential agent for the treatment of dermatological diseases.[3d,3e,76] Studies in mice revealed that liarozole is able to mimic the antikeratinizing effects of ATRA.[76] In open clinical studies, liarozole was found to be therapeutically effective in patients with psoriasis[93,94] and with ichthyosis.[95] A double-blind, randomized clinical study involving 20 patients with severe plaque-type psoriasis was conducted where half of the patients were treated with oral liarozole (75 mg, twice daily) and the other half were treated with oral acitretin (25 mg/day).[3d] After 12 weeks of treatment, both groups responded with a similar decrease in the PASI (psoriasis area severity index) score from ~20 to ~10. It is gratifying to state that liarozole was recently (2004) approved in Europe and USA as an orphan drug for the treatment of congenital ichthyosis.[13,96] Finally, in a most recent (2005) paper, Lucker and co-workers reported that topical liarozole was effective in the treatment of ichthyosis.[96]

6.1. R115866 and R116010

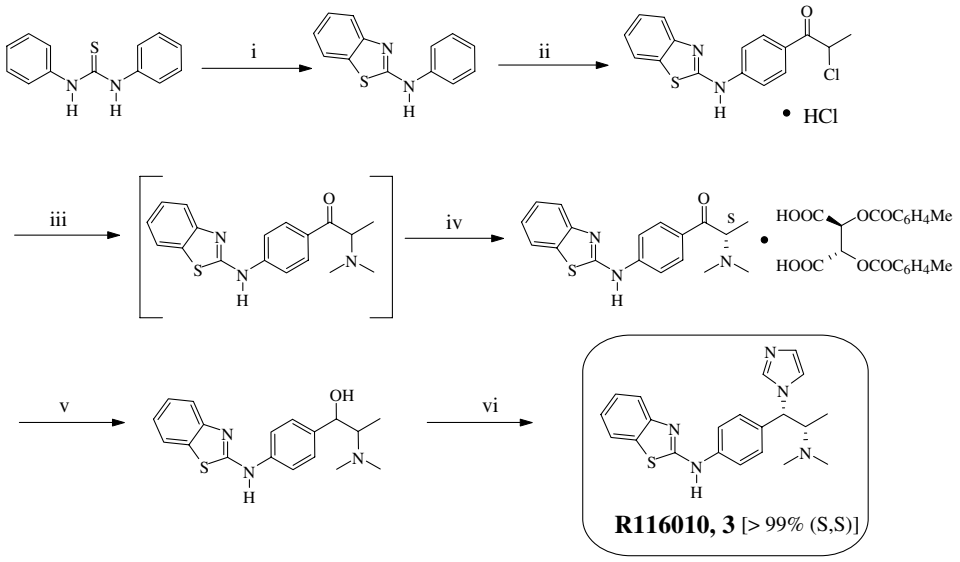
Recently, researchers of this same company have identified two novel benzothiazolamines, R115866, **2**[70] and R116010, **3**[71] (Fig. 4) as highly potent and selective second-generation inhibitors of ATRA metabolism. R115866 is a potent inhibitor of human CYP26A1 (IC_{50}, 4 nM), being 750 times as potent as liarozole (IC_{50}, 3 μM).[70] R115866 is highly selective for CYP26 as it exhibited mediocre inhibitory effects on aromatase, CYP17, CYP211, CYP3A, and CYP2A1, respectively. In vivo administration of R115866 (2.5 mg/kg po) to rats induced significant and transient increase of endogenous ATRA levels in plasma, skin, fat, kidney, and testis. Consequently, the compound exerted retinoidal effects, for example, inhibition of vaginal keratinization in rats. Although these studies with R115866 seem to be focused on dermatological therapy,[3e,70] their potential as agents for the treatment of cancers is warranted.

R116010 (**3**) is a dimethylamino derivative of R115866 and it has only been investigated as an anti-cancer agent.[71a,b] In vitro R116010 inhibits ATRA metabolism in intact T47D human breast cancer cells with an IC_{50} value of 8.7 nM and was selective against several CYPs. In combination with ATRA, R116011 enhanced ATRA-mediated antiproliferative activity in a concentration-dependent manner. In vivo, the growth of murine estrogen-independent TA3-Ha mammary tumors was significantly inhibited by R116010 at a dose as low as 0.16 mg/kg. A facile and large-scale preparation (commercial process) of R116010 (3) has recently been developed (Scheme 3).[71b]

Although these two agents are clearly superior to liarozole, it is unclear whether they are being developed further given that the last publication on R115866 and R116011 appeared in 2000 and 2002, respectively. Because of these compounds, high inhibitory potencies and selectivity for CYP26A1, they should be considered less likely to produce unwanted side effects as those experienced with liarozole therapy.

7. Azolyl retinoids and related compounds

The emerging role of RAMBAs as potential agents in the treatment of both hormone-dependent and

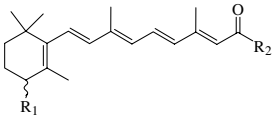


Scheme 3. Synthesis of R116010. Reagents and conditions: (i) aq HBr, Br_2; (ii) $ClCOCHClCH_3$, $AlCl_3$, CH_2Cl_2; (iii) aq Me_2NH, *i*-PrOH; (iv) (+)(D)-ditoluoyl tartaric acid, MeOH, refux, 6 h; (v) $NaBH_4$, *i*-PrOH, NaOH; (vi) CDI, imidazole, EtOAc.

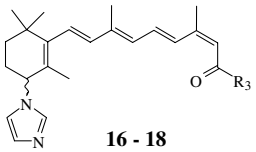
hormone-independent cancers[3a,12,97] has led to our interest in this area. Given the significance of azole group of many drugs that are P450 enzyme inhibitors,[98–100] we reasoned that introducing azole group at C-4 (the site of initial enzymatic hydroxylation) of ATRA should yield specific and potent inhibitors of ATRA 4-hydroxylase. Indeed, we very recently described the synthesis of a number of novel 4-azolyl ATRA derivatives, some of which are amongst the most potent inhibitors of this enzyme (Table 1, **4**–**14**, **16**–**18**).[12,101–103] This series of compounds were evaluated against microsomal preparations of male hamster liver (ATRA 4-hydroxylase CYPs) and microsomal preparations from T47D cells induced to express CYP26[103]. As shown in Table 1, all of our compounds exhibited potent inhibitory activity versus hamster liver microsomal CYPs at nanomolar concentration with IC_{50} values ranging from 0.009 to 119.00 nM. Our best compound **4** showed a 666,667-fold stronger inhibitory activity (IC_{50} = 0.009 nM) than liarozole (IC_{50} = 6000 nM) in the same assay. As expected, compounds with ATRA scaffold (**4**–**14**) were more potent than those with 13-CRA scaffold (**16**–**18**). Furthermore, the results suggest that the nature of C-4 substituent is important in determining affinity for the enzyme (compare IC_{50} values of **5**, **6**, **7** vs **12** and **13**; see Table 1) and also reveal that the corresponding methyl esters (**4**, **6**, and **7**) and imidazole amide (**10**) are significantly (24- to 48-fold) more potent than the corresponding free acids (**6**, **9**, and **10**). Compounds with 4-imidazole substitutions (**4**, **5**, and **10**) are most potent three inhibitors. Thus, it would appear that the imidazolyl nitrogen lone pair makes the strongest coordination to the iron atom of the heme in the active site of the enzyme. Compound **11** (IC_{50} = 43.7 nM) was synthesized to determine the effect of increasing the size of the terminal amide group. The modification resulted in a considerable 875-fold decreased potency compared to **10** (IC_{50} = 0.05 nM), suggesting limited steric tolerance at the active site of the enzyme. Because ketoconazole is used as a standard inhibitor of ATRA metabolism and 4-hydroxyphenyl retinamide (4-HPR, **15**) has recently been suggested as an inhibitor of ATRA metabolism,[104] we also tested these two compounds for comparison. As shown in Table 1, ketoconazole and 4-HPR are very weak inhibitors of this enzyme. Some compounds were further tested against microsomal preparations from T47D cells induced to express CYP26. The trend in activity against T47D CYP26 is different from those observed in the hamster liver microsomal CYPs. For example, **5** is the most active of the series against T47D CYP26, but it was less active than **4** against the hamster liver CYPs. Another interesting and surprising observation was that the racemate **4** was considerably more potent than the enantiomers (−)-**4** and (+)-**4** and quite unexpectedly, there was essentially no difference in activity between the two enantiomers.

The exceptionally potent inhibitory activities versus hamster liver microsomal CYPs of our RAMBAs have

Table 1. Structures and activities of novel azolyl RAMBAs (**4**–**18**)



4 - 15



16 - 18

Compound	R_1	R_2	R_3	IC_{50} value[a] (nM)	
				Hamster liver	T47D cells
4	–1*H*-Imidazole	–OMe	—	0.009 ± 0.0007	40.00 ± 3.00
(−)-**4**	–1*H*-Imidazole	–OMe	—	—	680.00 ± 20.00
(+)-**4**	–1*H*-Imidazole	–OMe	—	—	800.00 ± 25.00
5	–1*H*-Imidazole	–OH	—	2.33 ± 0.72	2.40 ± 0.12
6	–1*H*-1,2,4-Triazole	–OMe	—	2.00 ± 0.05	—
7	–4*H*-1,2,4-Triazole	–OMe	—	21.67 ± 0.30	—
8	–1*H*-1,2,4-Triazole	–OH	—	5.84 ± 0.48	—
9	–4*H*-1,2,4-Triazole	–OH	—	46.67 ± 3.30	—
10	–1*H*-Imidazole	–1*H*-imidazole	—	0.050 ± 0.002	5.20 ± 0.32
11	–1*H*-Imidazole	–4-Aminophenol	—	43.73 ± 4.70	—
12	–H	–1*H*-Imidazole	—	61.25 ± 6.50	—
13	–H	–1*H*-1,2,4-Triazole	—	51.67 ± 4.40	—
14	–Keto-oxime	–OMe	—	23.00 ± 1.63	—
16	—	—	–OH	119.0 ± 20.2	—
17	—	—	–1*H*-Imidazole	57.50 ± 8.5	—
18	—	—	–4-Aminophenol	176.67 ± 13.36	—
For comparison					
Liarozole (**1**)				6000.00 ± 30.00	—
Ketoconazole				34000.00 ± 170	—
4-HPR (**15**)	–H	–4-Aminophenol	—	31850.00 ± 150	—

'—,' not determined.

[a] Means ± SDM of at least two experiments.

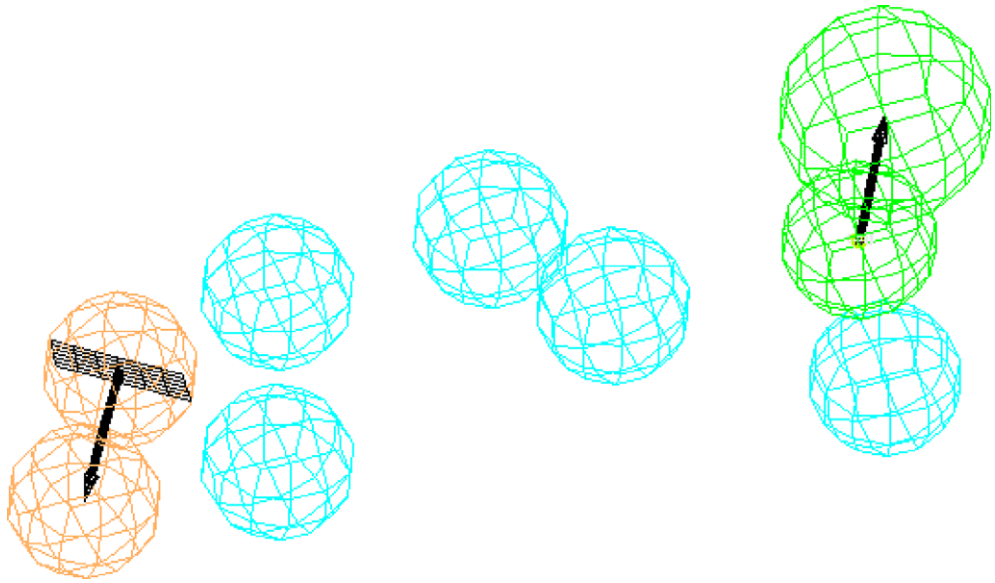


Figure 5. Common feature-based (catalyst/HipHop) pharmacophore model of novel RAMBAs. The model contains seven features: five hydrophobes (cyan), one hydrogen bond acceptor (green), and one aromatic ring (brown).

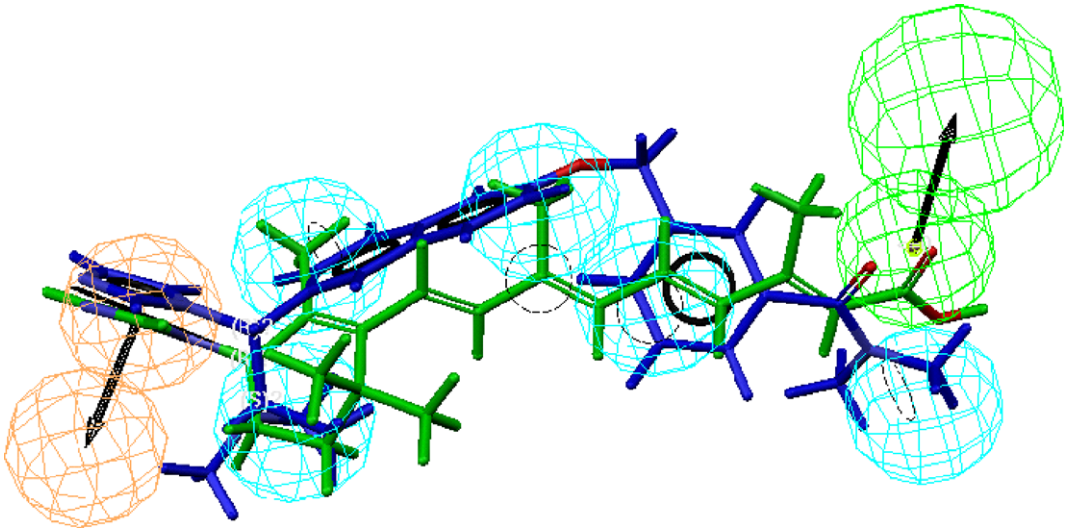
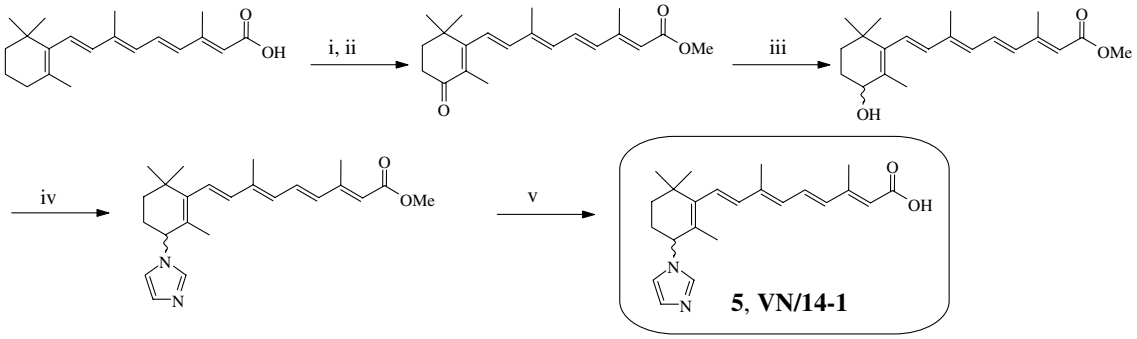


Figure 6. Alignment of common-feature pharmacophore model with compounds **4** (green) and **39** (blue).

enabled us to utilize the Catalyst molecular modeling program to create a common feature-based pharmacophore model (Fig. 5; consisting of five hydrophobic groups, one hydrogen bond acceptor, and one ring aromatic group) that can differentiate compounds as active or inactive inhibitors of this enzyme.[105] It is interesting to note that a recently identified RAMBA (**39**, IC_{50} = 14.4 nM, vide supra) by OSI Pharmaceuticals Inc. researchers[106] overlays perfectly with our most potent RAMBA, **4** (an isosteric relationship between the two compounds) and maps all the seven pharmacophores of our model (Fig. 6). Their current lead compound **35** (IC_{50} = 20.0 nM) maps to six of our seven pharmacophores.

The novel atypical RAMBAs, in addition to being highly potent inhibitors of ATRA metabolism in microsomal preparations and in intact human cancer cells (MCF-7, T47D, and LNCaP), also exhibit multiple biological activities, including induction of apoptosis and differentiation, retinoic acid receptor binding, and potent antiproliferative activity on a number of human cancer cells.[103,107–110] The combination of **11** with SAHA [a histone deacetylase inhibitor (HDACI)] inhibited LNCaP cell proliferation in an additive manner compared with single agents.[111] In a recent study, **5** was shown to be a potent microsomal human placental aromatase inhibitor (IC_{50} = 8.0 pM) and it significantly inhibited the growth of MCF-7Ca and LTLC (letrozole-resistant MCF-7Ca) with IC_{50} values of 8.5 and 0.83 nM, respectively.[108] Amongst these RAMBAs, compound **5** has been shown to significantly suppress MCF-7 tumor growth (~90.4% vs control, $P < 0.0005$ at 66.0 μM/kg/day dose) in female ovariectomized nude mice. In addition, the molecular mechanisms underlying the activities of these novel RAMBAs in



Scheme 4. Synthesis of compound **5** (**VN/14–1**). Reagents and conditions: (i) $TMSCHN_2$/benzene, MeOH, Ar, rt; (ii) activated MnO_2/CH_2Cl_2, rt; (iii) $NaBH_4$, MeOH, rt; (iv) CDI, CH_3CN, rt; (v) 10% KOH/MeOH, Ar, reflux.

human breast and prostate cancer models have been reported.[107–110] These atypical RAMBAs are yet to be tested as potential agents for the treatment of dermatological diseases. In this series of novel RAMBAs, compound **5** (code name **VN/14–1**) has been identified as the lead anticancer agent, and its synthesis has been reported (Scheme 4).[101a,102,103] An Australian patent[112] has recently been insured to protect these compounds and several other patents are pending.

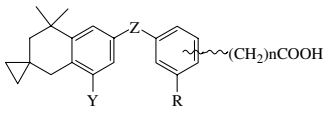
8. Benzeneacetic acid derivatives

Researchers at Allergan Sales Inc. have described in about 12 US patents on the synthesis and evaluation of several new benzeneacetic acid derivatives as inhibitors of human CYP26 stably transfected in HeLa cells.[72] Although they did not disclose the inhibitor design strategy, their compounds appear to overlay in a complementary fashion with the structure of ATRA. The structures and CYP26 inhibitory activities of their most potent inhibitors (**19**–**27**) are presented in Table 2. These compounds have a linker or tethering group designated Z covalently connecting an aromatic moiety and substituted chromain. The most potent CYP26 inhibitor was **23** (IC_{50} = 14 nM). The synthesis of this compound has also been reported (Scheme 5).[72] With regard to structure–activity relationship the preferred linker is the acetylene moiety and aromatic *para* substitution of terminal carboxylic acid with a one-carbon spacer. Introduction of fluorine in the aromatic ring and substitution of cyclopropyl ring at position Y of chromain moiety result in increase in potency. These researchers also demonstrated that their RAMBAs are not agonists or antagonists for both the RAR and RXR receptors. In addition, topical application of some of these agents caused an increase in the endogenous levels of ATRA that resulted in ATRA-induced irritation in skin of hairless mice. To the best of our knowledge, these agents have not been investigated in preclinical in vitro or in vivo cancer models. Because of the potent CYP26 inhibitory activities of these agents, they may be considered as potential therapeutics that may be useful in dermatology.

9. 2,6-Disubstituted naphthalenes

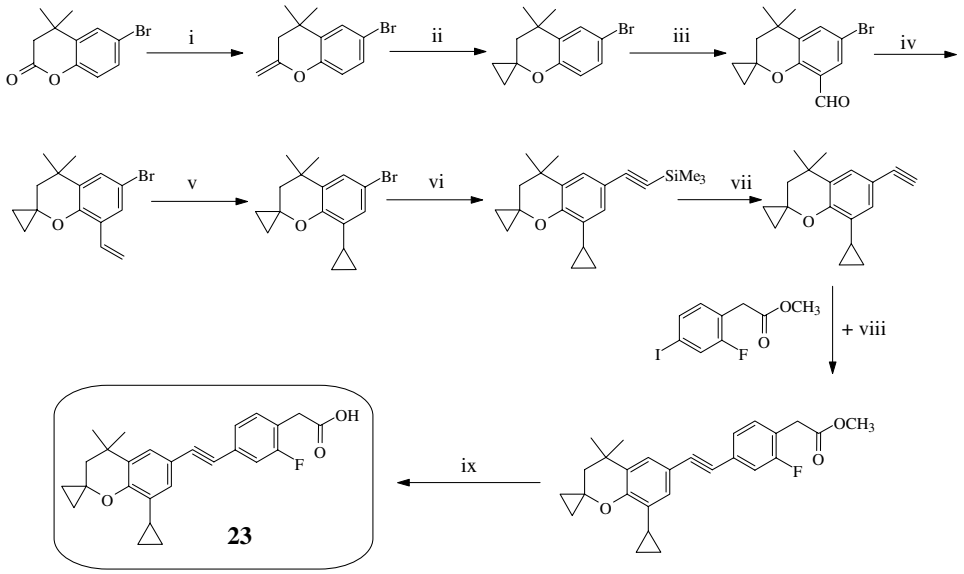
In the most recent publication on novel RAMBAs, researchers of OSI Pharmaceuticals Inc. reported the synthesis of a series of 2,6-disubstituted naphthalenes (**28**–**42**, Table 3) as inhibitors of microsomal preparations

Table 2. Structures and activities of benzeneacetic acid derivatives (**19**–**27**)



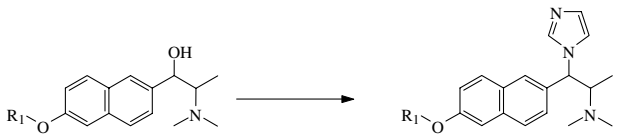
19 - 27

Compound	Y	Z	R	*n*	Position of $(CH_2)_nCOOH$	CYP26, IC_{50} (nM)
19	H	—≡—	H	0	4	1700
20	H	—≡—	H	1	4	190
21	H	—≡—	F	0	4	270
22	Cyclopropyl	—≡—	H	1	4	33
23	Cyclopropyl	—≡—	F	1	4	14
24	Cyclopropyl	—≡—	H	0	4	50
25	Cyclopropyl	—≡—	F	0	4	22
26	Cyclopropyl		H	1	3	1600
27	Cyclopropyl		H	1	4	180



Scheme 5. Synthesis of compound **23**. Reagents and conditions: (i) Tebbe reagent; (ii) CH_2I_2, Et_2Zn, CH_2Cl_2; (iii) $TiCl_4$, $Cl_2CH(OCH_3)$, CH_2Cl_2, $PhCH_3$; (iv) $CH_2{=}PPh_3$, THF; (v) CH_2N_2, $Pd(OAc)_2$, ether; (vi) $Pd(PPh_3)_2Cl_2$, HCC–TMS, CuI, NEt_3, THF, 70 °C; (vii) K_2CO_3, MeOH; (viii) $Pd(PPh_3)_2Cl_2$, THF, NEt_3, CuI; (ix) IM NaOH, EtOH, 80 °C.

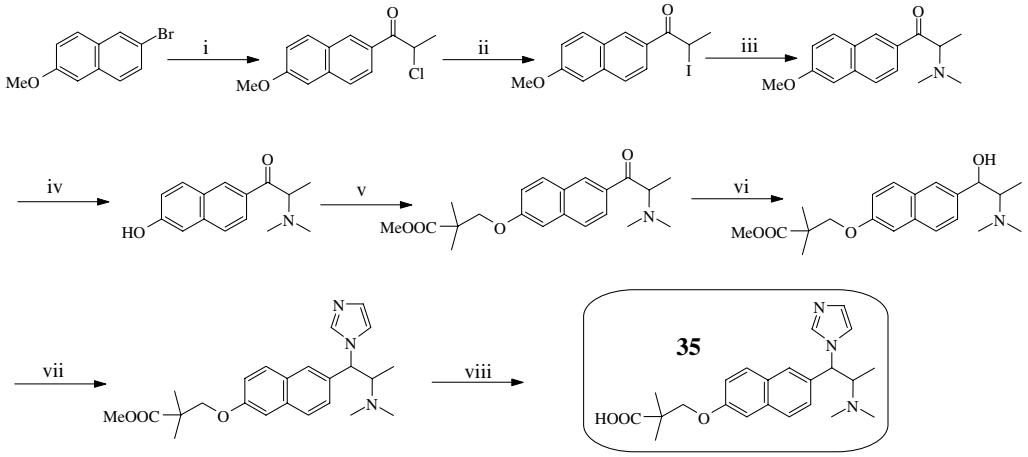
Table 3. Structures and activities of 2,6-disubstituted naphthalenes (**28**–**42**)



Aminoalcohol **28 - 42**

Compound	OR^1/O–Z–CO_2H/–O–Z–$CONR^2R^3$	Stereochemistry	CYP26 in T47D (IC_{50}, nM)
28	(O)–CH_2CO_2H	Mix	335
29	(O)–CH_2–Ph–4-(CH_2CO_2H)	Mix	25.0
30	(O)–CH_2–Ph–4-(OCH_2CO_2H)	*syn*	8.00
31	(O)–CH_2–Ph–3-(CO_2H)	*syn*	3.50
32	(O)–CH_2–Ph–3-(CO_2H)	*anti*	6.30
33	(O)–CH_2–Ph–4-(CO_2H)	*syn*	3.30
34	(O)–CH_2–Ph–4-(CO_2H)	*anti*	12.0
35	(O)–$CH_2C(CH_3)_2CO_2H$	*syn*	20.0
36	(O)–$CH_2C(CH_3)_2CO_2H$	*anti*	46.0
37	(O)–CH_2–Ph–3-($CONH_2$)	*syn*	48.0
38	(O)–CH_2–Ph–3-($CONHCH_3$)	*syn*	37.0
39	(O)–CH_2–Ph–3-($CON(CH_3)_2$)	*syn*	14.4
40	(O)–$CH_2C(CH_3)_2CONH_2$	*syn*	47.0
41	(O)–$CH_2C(CH_3)_2$–($CONHCH_3$)	*syn*	54.0
42	(O)–$CH_2C(CH_3)_2$–($CON(CH_3)_2$)	*syn*	35.0

from T47D cells induced to express CYP26.[106] These compounds are based on fusion of the imidazolyl propylamino moiety of R116010 (**3**)[71] with naphthalene core with a substitution (called CYP26 selectivity handle) at the 6-position. This series of compounds (Table 3) demonstrated that the *syn*-isomers were generally more potent than the *anti*-isomers. The imidazolyl moiety proved to be a critical element for CYP26 activity as seen by the lack of activity in the aminoalcohol precursors (Table 3), but a variety of tethers and substituents were tolerated. While in some cases only a moderate degree of selectivity for CYP3A4 was observed, the terminal carboxylate moiety in their lead compounds, **33** and **35**, afforded a large degree of selectivity (CYP3A4 IC_{50} values for **33** and **35** were 640 and 6300 nM, respectively). Conversion of the carboxylate moieties of **33** and **35** to their corresponding amides, **37**–**42**, caused a reduction in both CYP26 inhibitory potency and selectivity for CYP3A4. These two compounds also inhibited (nanomolar range) the growth of T47D breast cancer cells as well as the AT6.1 rat prostate cancer cells. The excellent pharmacokinetic properties of **35** (IC_{50} = 20 nM) and



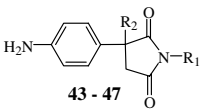
Scheme 6. Synthesis of compound **35**. Reagents and conditions: (i) Mg/THF, $CH_3CHClCOCl$, −78 °C; (ii) NaI, acetone; (iii) Me_2NH, MeOH; (iv) HBr/AcOH, 120 °C; (v) DIAD, Ph_3P, $MeOOC(CH_3)_2CH_2Br$; (vi) $NaBH_4$, MeOH; (vii) CDI, CH_3CN, 65 °C; (viii) NaOH, THF/H_2O.

its favorable selectivity for CYP26 over CYP3A4, 1A2, 2D6, and 2C9 make it a suitable candidate for further development as a therapeutic agent in oncology and dermatology. The synthesis of compound **35** is outlined in Scheme 6.

10. Miscellaneous structures

Researchers at the University of Cardiff, UK, have had a long interest in the development of inhibitors of ATRA metabolism. These investigators first reported on a series of (±)-3-(4-aminophenyl)pyrrolidine-2,5-diones substituted in the 1-, 3-, or 1,3-position with an aryl or long chain alkyl function and found them to be relatively weak inhibitors of ATRA metabolism by rat liver microsomes (68-80% inhibition at 100 μM) compared to ketoconazole (85% inhibition) (Table 4: **43-47**).[113] Although there appears to be no obvious structure activity relationship (SAR) in the limited series of compounds, the unsubstituted compounds were found to be inactive. A related phenylamine compound [(±) teralone A, **48**, Fig. 7] based on the teralin structure was found to be 2-fold more potent than ketoconazole (i.e., $IC_{50} = 12.75$ vs 22.15 μM).[114] Interestingly, the (+)- and (−)- forms of **48** had similar activity, and it was ~8-fold more potent than the previously reported compound **43**. In a recent study, Angotti et al. showed that intraperitoneal administration of **48** (100 mg/kg) to rats induced a marked and transient increase (from 0.11 to 0.15 ng/ml) of endogenous ATRA levels in plasma.[115] Another recent related study by the University of Cardiff group reported on a series of tetralone derivatives. The most potent compounds versus cellular MCF-7 CYP26 were 2-(hydroxybenzyl)-6-methoxytetralone (**49**) and the corresponding benzylidene (**50**) with IC_{50} values of 7 and 5 μM, respectively, which were comparable with liarozole ($IC_{50} = 7$ μM).[116] These compounds were further investigated with a CYP26A1 homology model.

Table 4. Structures and activities of aminophenyl pyrrolidines (**43**–**47**)



43 - 47

Compound	R_1	R_2	% inhibition[a]
43	Ph–	H	75
44	C_6H_{13}	H	79
45	H	C_5H_{11}	71
46	C_7H_{15}	H	80
47	C_6H_{13}	Ph–NH_2	68
Ketoconazole			85

[a] % inhibition versus rat liver microsomal CYPs.

From another series of 70 azoles, compound **51** (Fig. 7) was the most potent and was equipotent with ketoconazole.[117] The same group also examined some 1,2-ethanediones, 2-hydroxyethanones, and 1-ethylenedioxyethanones based on aryl-substituted 1,2-diphenylethane.[118] This study identified the 2-hydroxy ethanone (**52**, Fig. 7) with a 1-(4-dimethylaminophenyl) substituent as the most potent compound for rat liver microsomal enzyme ($IC_{50} = 52.1$ μM; ketoconazole, 2.8 μM). However, some compounds in this series were found to be moderate inhibitors of the ATRA-induced enzymes in cultured human genital fibroblasts. Finally, another recent report identified seven compounds (**53**–**59**, Fig. 7) that were equipotent with ketoconazole.[73]

It should be stated that this group has investigated several compounds so far, with at least five different scaffolds as inhibitors of ATRA metabolism. As their most potent inhibitors are at best equipotent with ketoconazole (a weak inhibitor of ATRA-hydroxylase, cf. Table 1), it would appear that these series of compounds and possibly their derivatives are unlikely to yield potent inhibitors of this enzyme complex. Therefore, future inhibitor design strategies with these scaffolds would not be wise.

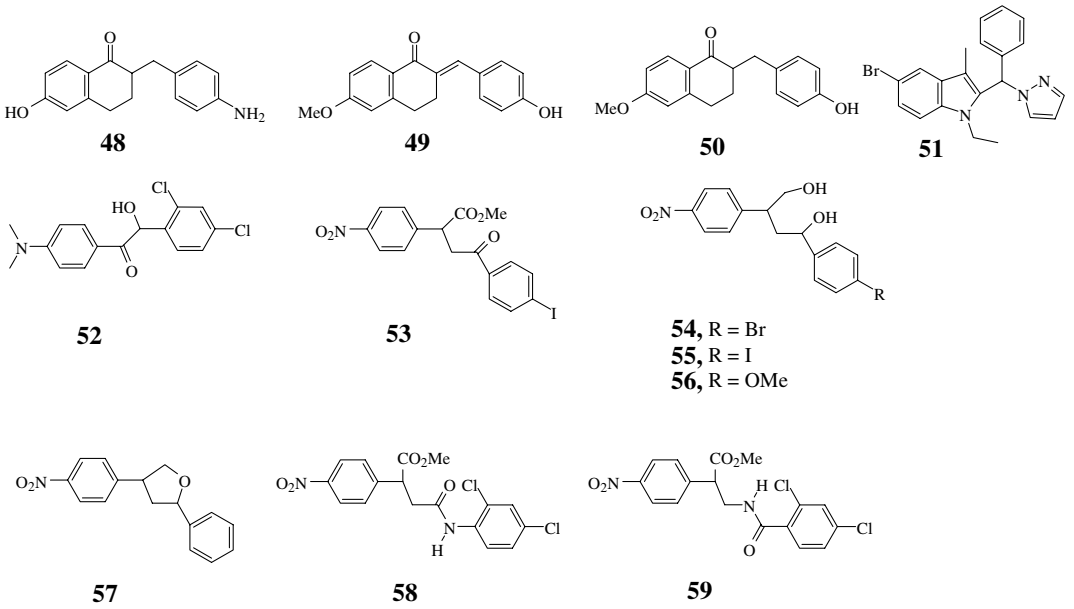


Figure 7. Structures and activities of **48**–**59**.

11. Concluding remarks

Retinoid therapy is based on differentiation of premalignant and malignant cells with the potential of redirecting the cells toward their normal phenotype. However, exogenous retinoid therapy is yet to fulfill the expectations raised by in vitro and in vivo studies in cancer models in the clinics. It is evident from this review that there is currently a high level of interest in the rational design of new RAMBAs, several of which have IC_{50} values in the nanomolar range. The recent approval of a first-generation RAMBA, liarozole, for the treatment of congenital ichthyosis will undoubtedly boost research efforts to develop other potentially useful RAMBAs. Modulation of endogenous ATRA and possibly its natural sterioisomers with the use of new RAMBAs may present an additional cancer therapy strategy and treatment of dermatological diseases. Just as with ATRA, it will be of interest to study RAMBA-HDACI synergy and the possibility of restoring retinoid signaling in ATRA-resistant cells. In addition to HDAC (particularly HDACs 3 and 4) inhibition, reversal of DNA hypermethylation by demethylating agents has been shown to restore ATRA-mediated differentiation/growth inhibition in some leukemia and solid tumor cells in vitro. Thus, studies on the molecular basis and selectivity of the complexes that modulate epigenetic events during tumorigenesis/dermatological insults and their effects on differentiation and apoptogenic pathways might provide new tools to fight cancer and dermatological diseases.

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Retinoids in Clinical Use

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Abstract: Retinoids have been investigated for their therapeutic potential for the past 3 decades. They have a reputation for being both beneficial in the treatment of several diseases and detrimental due to toxic and/or teratogenic side effects. The purpose of this review is to highlight retinoids that are currently used in the clinic. We also discuss their mechanisms of action and research strategies to develop new and safer retinoid-based therapies.

Key Words: Retinoids, rexinoids, retinoic acid metabolism blocking agents (RAMBAs), RAR/RXR, clinical utility.

INTRODUCTION

Retinoids are a class of polyisoprenoids that are derived by oxidative cleavage of β-carotenes of plant origin to yield vitamin A (retinol). Dietary sources of vitamin A include eggs, milk, butter and fish-liver oils [1,2]. Retinoids are essential for embryonic development and play important physiological functions, particularly in the brain and reproductive system, by regulating organogenesis, organ homeostasis, and cell growth, differentiation and apoptosis [1,2]. The naturally occurring and synthetic retinoids are currently the subject of intense biological interest stimulated by the discovery of retinoid nuclear receptors and the realization of these compounds as non-steroidal small-molecule hormones [3]. However, it should be stated that before the discovery of the nuclear retinoid receptors, a number of therapeutically useful retinoids were identified [4,5]. Indeed, most retinoids that are currently used in dermatology and in oncology, such as all-trans-retinoic acid (ATRA, tretinoin), 13-cis retinoic acid (13-CRA, isotretinoin), etretinate and acitretin, were discovered by chemical modifications on the basis of vitamin A structure and by biological evaluations in suitable pharmacological models. This review pre-supposes familiarity with the retinoid field in general. For those seeking more background information, many recent and comprehensive reviews are available [2, 6-8]. Our purpose here is to provide the reader with an astute review of retinoids that are in clinical use and also those retinoids that are currently in clinical trials. This review mainly is based on perspectives, reviews and abstracts published in the last 12 years up to December 2005. Most of the original articles cited were also consulted.

MECHANISM OF ACTION OF RETINOIDS AND RETINOIC ACIDS

With current knowledge, the pleiotropic action of retinoic acids (RAs) and retinoids might be explained mechanistically by the actions of the six known nuclear receptors, the retinoic acid receptors (RARα, β, γ) and the retinoid X receptor (also called rexinoids, (RXRα, β, γ).[2,3,9,10] Each of these receptors are encoded by distinct genes and are members of the steroid/thyroid hormone receptor superfamily. It is also thought that each receptor mediates a set of unique biological functions in certain cell or tissue types. ATRA (Fig. **1**) is the natural ligand of the RARs, while 9-CRA is the ligand for the RXRs and it also has a high affinity for the RARs. The binding of the other ATRA stereoisomers, 11-cis-retinoc acid (11-CRA) and 13-CRA to these receptors is still unclear. However, because of the reported antitumor efficacy of 13-CRA [11-15], it is plausible that 13-CRA is isomerized intracellularly to ATRA, or it may act without obvious interaction with the known retinoid receptors. Clearly, more research is needed in this area.

Most of the pleiotropc activities of the RAs and other retinoids are elicited by the binding of these agents to the RAR site of RAR-RXR heterodimers. RXRs are the silent partners of the RARs, as the RXR ligands alone are unable to activate the RAR-RXR heterodimers. However, recent studies using RAR- and RXR-selective ligands have revealed that the RXR ligands allosterically increase the potencies of the RAR ligands [16-18]. Furthermore, RXRs form heterodimers with various nuclear receptors, such as estrogen receptors (ERs), vitamin D_3 receptors (VDRs), thyroid hormone receptors (TRs), peroxisome proliferators-activated receptors (PPARs), liver X receptors (LXRs), and farnesoid X receptors (FXRs). Because of these unique properties of the RXRs, the RXR ligands are able to modulate the activities of other hormone receptors, in addition to their retinoidal activities [19].

These receptors, as heterodimers (RAR/RXR) or homodimers (RXR-RXR), function as RA-inducible transcriptional regulatory proteins by binding to DNA regions called retinoic acid response elements (RAREs) or retinoid X response elements (RXREs) located within the promoter of target genes. RAREs consist of direct repeats of the consensus half-site sequence AGGTCA separated most commonly by five nucleotides (DR-5), whereas RXREs are typically direct repeats of AGGTCA with one nucleotide spacing (DR-1). In

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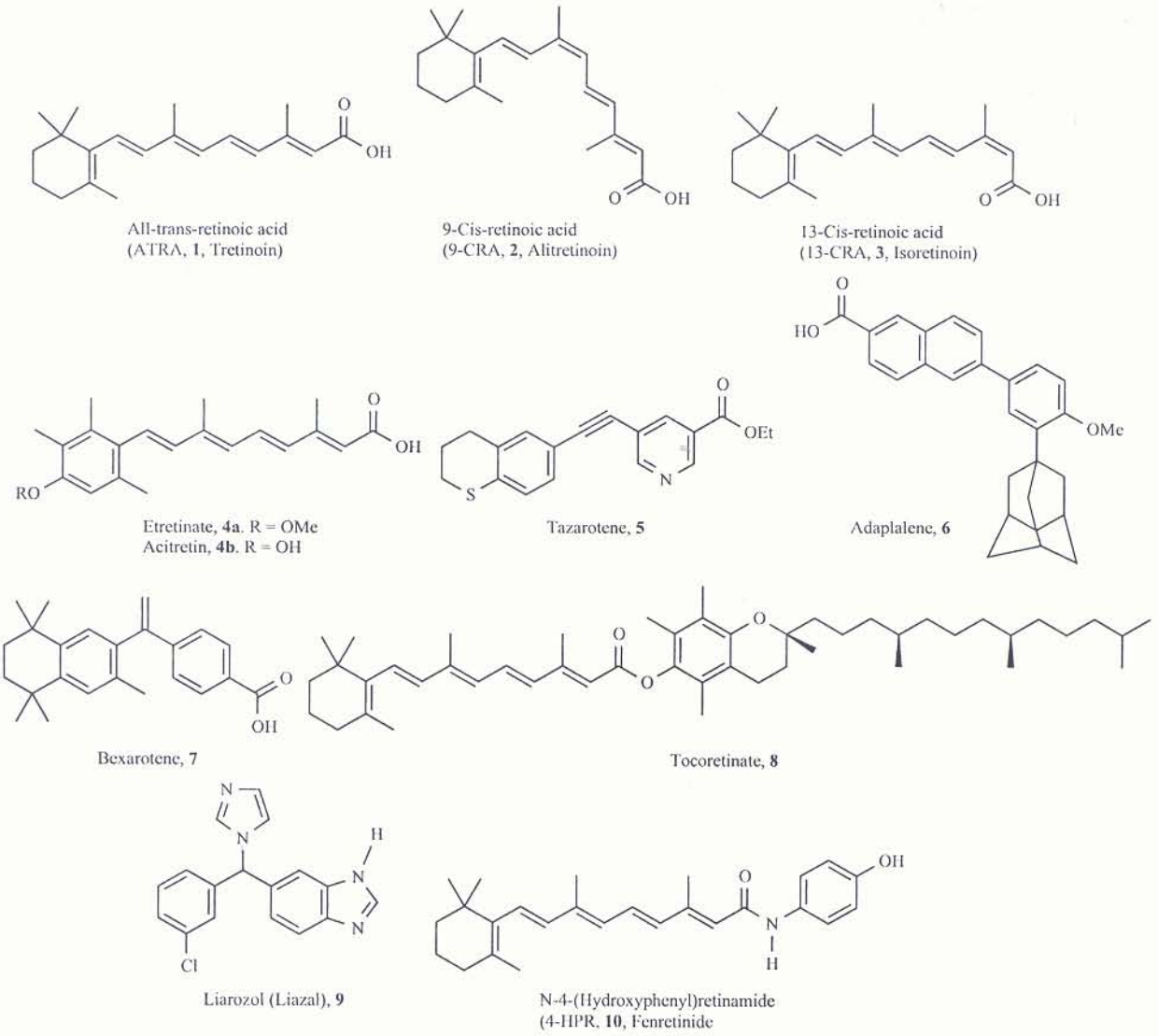

Fig. (1). Chemical structures of retinoids and related compounds.

the absence of ligand (ATRA or 9-CRA) the apo-heterodimer (RAR-RXR) binds to the RARE in the promoter of the target genes and RAR recruits co-repressors (CoRs) such as nuclear receptor co-repressors (NCoR) or and silencing mediator for retinoid and thyroid receptors (SMRT). These co-repressors function by recruiting histone deacetylase complexes (HDACs), causing target gene repression due to compaction of chromatin, making DNA inaccessible to the transcriptional machinery. However, in the presence of ATRA or an agonist, there is a confor-mational change in the structure of the ligand binding domain that results in destabilization of the CoR-binding with concomitant recruitment and interaction with co-activators (CoAs). Some co-activators interact directly with the basal transcriptional machinery to enhance trancscrip-tional activation, while others encode histone acetyl transferase (HAT) activity. HAT acetylates histone proteins, causing the opening of the chromatin and activation of transcription of the associated gene. Other complexes, such as the thyroid receptor associated protein are also involved in this process. It should be stated that, whereas the RARα is involved in myeloid leukemias, a growing body of evidence indicates that RARβ is involved in a diverse range of solid tumors [2]. For more details on the mechanism of action of ATRA, reviews by Chambon [9] and Altucci and Gronemeyer [2] should be consulted.

RETINOIDS IN THE CLINIC AND IN CLINICAL INVESTIGATIONS

Although retinoids have shown immense translational potential because of their activities *in vitro* and *in vivo*, their use in the clinic has resulted in limited responses. Part of the problem is that most of the retinoids studied in the clinic (Table **1**) were stand-alone therapies and not geared towards an optimal therapeutic regimen where they are used in combination with other therapeutic or disease-modifying agents. In addition, most of the promising newer generation of receptor and/or function selective retinoids has not yet been investigated in the clinic [7,8].

Table 1. Retinoids in the Clinic or in Clinical Trials

Compound (Drug Name)	Indication	Pharmacology	Clinical Status	Company Name
All-trans-retinoic acid (1) (Tretinoin)	Acne, Photodamage, Acute promyelocytic Leukemia (AML)	RAR agonist, Protein synthesis antagonist, microbial collagenase inhibitor	Launched	AP Pharma, Johnson & Johnson, Hoffmann-La Roche, Myland Labs.
9-cis-retinoic acid (2) (Alitretinoin)	Psoriasis, Kaposi's sarcoma, AML	RAR and RXR agonist, apoptosis agonist	Lunched	Ligand
13-cis-retinoic acid (3) (Isotretinoin)	Acne	RAR agonist, Protein synthesis antagonist, microbial	Lunched	Hoffmann-La Roche
Etretinate (4a) Acitretin (4b)	Breast cancer, non-small cell lung cancer, Kaposi's sarcoma, T-cell lymphoma	RXR agonist	Lunched	Hoffmann-La Roche
Tazarotene (5)	Acne, psoriasis, cancer	RAR_ agonist	Lunched	Allergan
Adapalene (6)	Acne, psoriasis	RAR agonist, Protein synthesis antagonist, microbial collagenase inhibitor	Lunched	Galderma
Bexarotene (7)	Psoriasis, keratosis, eczema, head & neck, renal, prostate, ovarian and colorectal cancers	RXR agonist	Lunched	Ligand
Tocoretinate (8)	Ulcer	RAR antagonist, Protein synthesis antagonist, microbial collagenase inhibitor	Lunched	Nisshin Pharma
Liarozol (9), (Liazal)	Laminar ithciosis	Inhibitor of ATRA catabolism	Lunched	Johnson & Johnson
Fenretinide (10)	Prostate & CNS cancer, breast cancer chemoprevention	RAR agonist, Apoptosis agonist	Phase III	Johnson & Johnson

It is now generally believed that retinoids have promising potential for a number of indications, including various dermatological diseases, cancers, ulcer, type II diabetes and HIV infection. However, the reality is that these agents are only currently effective in man for the treatment of various dermatological diseases such as acne, psoriasis and other keratinizing dermatoses and also in the treatment of a few types of cancers. We will focus our attention on retinoic acids and derivative(s), synthetic RAR agonist and antagonist and then those molecules able to increase the endogenous retinoic acid by inhibiting the cytochrome P450-mediated catabolism of retinoic acid, also known as the retinoic acid metabolism blocking agents (RAMBAs). Specifically, we will discuss the clinical agents (see Fig. **1**), including all-*trans* retinoic acid (ATRA, **1**), 9-*cis*-retinoic acid (9-CRA, **2**), 13-*cis*-retinoic acid (13-CRA, **3**), etretinate/ acitrtine (**4a/4b**), tazarotene (**5**), adapelene (**6**), bexarotene (**7**), tocoretinate (**8**), liarozole (**9**) and those in clinical trials, including N-4-(hydroxyphenyl)retinamide (4-HPR, fenretinide, **10**). The recent review by Berrie and Goldhill [3] provides a comprehensive list of retinoids and related compounds in the clinic and in clinical trials.

All-*trans*-retinoic Acid (ATRA, Tretinoin, 1)

Tretinoin (**1**) is an RARα,β,γ agonist and was the first retinoid approved for the treatment of acne and has been in clinical use for almost 3 decades. It is used as a monotherapy in patients with non-inflammatory comedones, and in combination with other topical or systemic drugs in mild, moderate and severe inflammatory acne [21,21]. Tretinoin acts by increasing the turnover of follicular epithelial cells and by accelerating the shedding of corneocytes. These processes help normalize keratinization, which leads to drainage of comedones and inhibition of new comedone formation. A major concern with the use of early formu-lations of tretinoin was excessive skin irritation associated with its hydro-alcoholic vehicle and the high concentration of the drug. This side-effect has now been corrected by use of various creams/gels vehicles, and with low drug concentrations (e.g., 0.0025, 0.05 and 0.01%) [22,23]. Topical formulations of ATRA are currently used for treatment of acne, psoriasis and ichthyosis [24].

Best defined among the clinical oncological application of retinoids is the use of ATRA for treatment of acute promyelocytic leukemia (APL). Oral administration (45 mg/m^2/day p.o.) of this drug to APL patients is currently approved in several countries worldwide. More than 90% of APL patients achieve complete remission with ATRA therapy [25,26]. The basis for the dramatic efficacy of ATRA against APL is the ability of pharmacological doses of ATRA to overcome the repression of signaling caused by the PML-RARα fusion protein at physiological ATRA concentrations. Restoration of signaling leads to differentiation of APL cells and then to postmaturation apoptosis [17]. Several randomized clinical trials have now defined the utility of ATRA as maintenance therapy [28,29] and also the benefits

of combining ATRA with chemotherapy [30]. The National Cancer Institute (USA) is currently evaluating ATRA as an anticancer agent in phase II trials for brain, head and neck, and prostate caners [6].

9-*Cis*-retinoic Acid (9-CRA, Alitretinoin, 2)

9-Cis-retinoic acid has been detected in humans [31] and was the first RAR/RXR pan-agonist discovered [32-34]. It is the only retinoic acid isomer not approved for the common dermatological diseases. However, it has recently been launched in the USA as adjuvant topical treatment of AIDS-associated Kaposi's sarcoma [35-39]. This agent is the first RXR ligand to be approved for the treatment of a dermatological disease. In a randomized study with 268 AIDS-associated Kaposi's sarcoma patients, 35% treated with alitretinoin (0.1% gel) had a positive response, compared with 18% treated with vehicle gel irrespective of the number of concurrent anti-retroviral therapies [35]. 9-CRA is in clinical trials for the treatment of various cancers, including breast cancer [40], renal-cell carcinoma [41,42] and squamous-cell carcinoma [43-45].

13-*Cis*-retinoic Acid (Isotretinoin, 13-CRA, 3)

13-CRA is a metabolite of ATRA [46] that binds poorly to the RARs. Recent studies suggest that 13-CRA is a prodrug, activated in human sebocytes *via* a selective intracellular isomerization to high levels of ATRA and subsequent binding to RARs [47]. This agent has been available in topical formulations in Europe since the early 1970s for the treatment of acne. In the USA, oral isotretinoin greatly advanced the treatment of severe acne after an important discovery by Peck and Yoder [48]. Isotretinoin gained approval from the US Food and Drug Administration (FDA) for the treatment of resistant nodular acne in 1982 [49]. Numerous clinical studies do not show a fundamental difference between 13-CRA and ATRA [50-52], although the former is apparently better tolerated and it is the only retinoic acid isomer used in systemic form [53]. On the basis of several clinical trials (reviewed in reference [54]), systemic isotretinoin may be considered as an alternative drug in some dermatological diseases unresponsive to conventional therapy. Nevertheless, more randomized clinical trails to determine the role of systemic isotretinoin therapy in dermatological diseases, including skin cancers other than acne are required. Isotretinoin also represents a potentially useful drug in many dermatological diseases other than acne and also skin cancers, due to its immunomodulatory, anti-inflammatory and anti-tumor activities [54].

Etretinate (4a, Ethyl all-*trans*-3,7-dimethyl-9-(4-methoxy-2,3,6-trimethylphenyl)nona-2,4,6,8-tetraenoate) and Acitretin (4b, all-*trans*-3,7-dimethyl-9-(4-methoxy-2,3,6-trimethylphenyl)nona-2,4,6,8-tetraenoate)

Etretinate (**4a**) is considered as a second-generation retinoid with a characteristic substituted aromatic ring in place of the substituted cyclohexenyl ring in retinoic acids. It was first lunched by Hoffmann-La Roche in the USA in 1982 as the first systemic retinoid for psoriasis. Etretinate was replaced by its hydrolyzed metabolite free acid–acitretin (**4b**) in 1997. Acitretin was found to be clinically as effective as etretinate, but with a much shorter elimination half-life, advantageous for clinical use. Acitretin does not bind to, but activates, the RARs [55], and it has a high affinity for both cellular retinoic acid binding proteins I and II (CRABP I and II) [56]. Systemic treatment with acitretin is effective in several disorders of keratinization, due to its action in promoting keratinocytes differentiation in several skin disorders [57]. A review of acitretin as a systemic retinoid for the treatment of psoriasis has recently appeared [58]. Oral acitretin is currently being investigated in several clinical trials for the prevention of skin cancers in solid organ transplant patients [59-61]. Paradoxically, in spite of the similar therapeutic efficacies of acitretin and etretinate, the latter has been reported to succeed in cases where acitretin has failed [62]. In addition, a recent study reported the successful use of etretinate for long-term management of a patient with cutaneous-type adult T-cell leukaemia/lymphoma [63]. Reports of this nature may warrant the resurgence of etretinate.

Tazarotene (Ethyl 6-[2-(4,4-dimethylthiochroman-6-yl)-ethynyl] Nicotinate, 5, Tazarac®, Allergan, Inc.)

Tazarotene (**5**) was first approved in 1997 by the FDA for the treatment of acne [64], but it is also currently used for the treatment of plaque psoriasis [65] and photodamage [66]. It is a synthetic acetylinic retionoid that is readily hydrolyzed to its active form, tazarotenic acid in keratinocytes. Unlike its parent compound, tazarotenic acid has the ability to bind and activate RARβ and RARγ (RARβ> RARγ) with less effect on RARβ and, no effect on the RXRs [67]. However, because RARβ is not expressed in human keratinocytes, the effect of this drug on the major cell type of the epidermis is clearly attributed to its interaction with RARγ. Through regulation of gene expression in a specific manner, tazarotenic acid modulates abnormal differentiation of keratinocytes, increased keratinocyte proliferation and inflammation [64]. Clinical responses are seen after 2 weeks, with significant clearing after 6-12 weeks of treatment with topical gel or cream formulations of tazarotene [68]. Combination of tazarotene with topical corticosteroids of low potency appears to increase overall therapeutic potential with reduced side effects, such as local skin irritation, erythema, and burning sensation [69]. A review of the use of topical tazarotene in the treatment of plaque psoriasis has recently been published [70].

Following a clinical study which suggested that tazarotene may be effective treatment of cutaneous basal cell carcinoma (BCC) [71], a recent study of 30 patients with small superficial and nodular BCC was conducted to assess the efficacy and mechanism of action of tazarotene (0.1% gel). Overall, 76.7% of treated tumors showed > 50% regression, while complete healing was observed in 46.7% of all treated BCC. Induction of tazarotene-induced BCC regression was attributed to synergistic RARβ-dependent anti-proliferative and pro-apoptotic activities [72].

Adapelene (6-[3-(1-adamantyl)-4-methoxyphenyl]-2-naphthoic acid, 6, Differin®, Galderma Labratories)

Adapelene (**6**) is a naphthoic acid derivative with a methoxyphenyl adamant side chain and is a commonly used

anti-acne drug [73]. Similar to the action of tazarotene in its hydrolyzed form - tazarotenic acid, adapelene interacts selectively with RARβ and RARγ, and its activity on proliferation and differentiation can be blocked by a RARγ antagonist [74]. In addition, adapelene has anti-inflammatory potential due to its anti-AP1 activity [74]. Although its efficacy is similar to that of other retinoids, it has an improved therapeutic ratio due to its better tolerance [reviewed in ref 75].

Bexarotene (7, 4-[1-(5,6,7,8-tetrahydro-3,5,58,8-pentamethyl-2-naphthalenyl)ethenyl]benzoic Acid)

Bexarotene (**7**) is a selective RXR agonist (classified as a rexinoid) whose exact mechanism of action in cancer therapy and chemoprevention is poorly understood [8]. In a multinational phase II-III clinical trials, oral bexarotene (300 mg/m2/day) showed 55% response rate in patient with refractor advanced stage cutaneous T-cell lymphoma (CTCL) [76]. Bexarotene (1% Targretin gel) is approved for the topical treatment of cutaneous lesions in patients with state 1A and 1B CTCL who have not tolerated other therapies or who have refractory or persistent disease [77,78]. The ability of bexarotene to activate RXRs and their heterodimer partners results in modulation of gene-expression pathways, which ultimately modulate converging signaling pathways responsible for cell differentiation and apoptosis [79]. This multi-targeted approach of mediating cell differentiation, apoptosis, and proliferation suggest that bexarotene may be particularly active in the treatment of malignancies, especially in combination with chemothera-peutic agents. Thus, following acceptable phase II response rates (25%) in combination with cisplatin and vinorelbine in non-small-cell lung cancer (NSCLC) [80,81], oral bexa-rotene in combination with paclitaxel and carboplatin or vinorelbine is currently being evaluated in multi-center phase III studies in previously untreated patients with NSCLC patients [82]. A recent preclinical study by Yen and Lamph suggests a role of bexarotene in combination with paclitaxel in prevention and overcoming acquired drug resistance in advanced prostate cancer [83].

Tocoretinate (Tretinoin tocoferil, (±)-3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyl-tridecyl)-2*H*-1-benzopyran-6-yl (2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexene-1-yl)-2,4,6,8-nontetraenoate), 8)

Tocoretinate (**8**) is a unique α-tocopherol ester of ATRA and has been safely used in Japan for the treatment of decubitus and skin ulcer, acting *via* stimulation of the proliferation of human skin fibroblast [84,85]. The agent is characterized as an RAR antagonist, protein synthesis antagonist, and microbial collagenase antagonist. Unex-pectedly, although tocoretinate is an α-tocopherol ester of ATRA, it was reported to be stable *in vitro* and *in vivo* [86]. This characteristic of tocoretinate has support from observations that tocoretinate enhances the growth of human skin fibroblasts and stimulates the formation of granulation tissue in ulcers, effects that are different from those of either ATRA or α-tocopherol [86]. Furthermore, toxicity tests in animal models have shown that tocoretinate is at least 150 times less toxic than ATRA [87-89]. Tocoretinate is actively being investigated for the possible treatment and chemoprevention of leukemia [90-92].

Liarozole (9, Liazal™)

Following extensive studies by researchers at Janssen Research Foundation (now called Johnson and Johnson Pharmaceutical Research and Development) liarozole (**9**) was identified as a modest inhibitor (IC_{50} = 2.2 – 6.0 μM) of ATRA-4-hydroxylase (CYP26) [93, 94-100]. On the other hand, the compound was shown to be a good inhibitor of rat CYP17 (IC_{50} = 260 nM) and a potent inhibitor of CYP19 [97]. Although liarozole has undergone phase III clinical trials for the treatments of patients with metastatic prostate cancer [101] and also phase II trials for the treatment of ER negative metastatic breast cancer patients [102], its development for these indications have been discontinued [102,103].

Inappropriate metabolism of ATRA could generate a condition of retinoid deficiency, which is characterized by hyperkeratinization and desquamation as seen in acne, psoriasis, and ichthyosis [104]. Because of these reasons, liarozole has also been extensively investigated as a potential agent for the treatment of dermatological diseases [96,105, 106]. Studies in mice revealed that liarozole is able to mimic the antikeratinizing effects of ATRA [96]. In open clinical studies, liarozole was found to be therapeutically effective in patients with psoriasis [107,108] and with ichthyosis [108]. A double-blind, randomized clinical study involving 20 patients with severe plaque-type psoriasis was conducted; half of the patients were treated with oral liarozole (75 mg, twice daily) and the other half were treated with oral acitretin (25 mg/day) [105]. After 12 weeks of treatment, both groups responded with a similar decrease in the PASI (psoriasis area severity index) score from ~20 to ~10. It is gratifying to state that liarozole was recently (2004) approved in Europe and USA as an orphan drug for the treatment of congenital ichthyosis [109,110]. Finally, in a most recent (2005) paper, Lucker and co-workers reported that topical liarozole was effective in the treatment of ichthyosis [110].

***N*-(4-hydroxyphenyl)retinamide (4-HPR, Fenretinide, 10)**

The synthetic *N*-(4-hydroxyphenyl)retinamide (4-HPR, **10**) was first synthesized in the USA by Sporn and colleagues 27 years ago [111]. Although 4-HPR is derived from the natural ATRA, it is less toxic and substantially less tetratogenic [111,112]. 4-HPR is considered to be a non-classical or atypical retinoid, because its biological effects have been shown to act through both retinoid receptor-dependent and –independent mechanisms [reviewed in 113, 114]. Unlike classical retinoids that often induce differentiation, 4-HPR elicits distinct biological effects, such as generation of reactive oxygen species (ROS) and the promotion of apoptosis. This property has led to suggestions that 4-HPR may exert greater therapeutic activity than a classical retinoid [113,114]. As a result of several promising *in vitro* and *in vivo* studies in a wide variety of tumor cells [3,114], clinical testing of the effectiveness of 4-HPR against neuroblastoma, breast, prostate, ovarian and bladder cancers has been conducted, with modest outcomes. In a large phase II study of Italian women (2,972) aged 30-70 years with surgically removed Stage I breast cancer or ductal carcinoma

in situ, oral 4-HPR (200 mg/day) caused no difference in the incidence of breast cancer 7 years after treatment compared to untreated patients [115]. However, there was a beneficial trend (35% reduction of contralateral breast cancer) in premenopausal women and no effect in postmenopausal patients. Phase III trials of 4-HPR as a chemopreventive agent for breast cancer are currently in progress in the USA, and alone in Phase I trials for prostate cancer [6]. Recent reviews on the potential of 4-HPR as a cancer preventive agent should be consulted [116,117].

DEVELOPMENT OF NEW RETINOIDS AND THE FUTURE FOR RETINOID-BASED THERAPIES

Most of the retinoids that are currently in clinical use are RAR or RXR agonists/antagonist. However, in the desire to generate new retinoids/rexinoids that may exhibit fewer side-effects, the goal of chemists and biologist is to develop RAR- and RXR-specific ligands. The generation of these agents has been made possible by recent progress in crystallographic studies on nuclear receptor ligand binding domains that has enabled useful information of ligand-receptor interactions at the molecular level [118]. Thus, various ligands have been developed by computer-assisted procedures using virtual libraries and/or molecular databases. Studies in this area have recently been reviewed by Kagechika and Shudo [8] and will not be discussed further in this review.

Recent developments in the understanding of gene regulation by nuclear receptors and chromatin organization have increased interest among researchers in the cancer field in the identification of agents that modulate gene expression through chromatin re-organization. Retinoids fit the profile of these agents since they can induce, for example, the expression of a number of tumor/growth suppressor genes, which otherwise are transcriptionally silent in cancer cells. A number of tumor/growth suppressor genes such as RARβ, TIG1, etc, are epigenetically silenced because of DNA hypermethylation in their promoter regions [119,120]. As loss of RARβ has been linked to retinoid resistance, and RARβ is a tumor suppressor as well as an intracellular effector of retinoid action, a therapy involving a combination of retinoids and histone deacetylase and/or histone methy-transferase inhibitors may show synergistic efficacy in cancers. As a proof of concept, reversal of transcriptional silencing of RARβ gene and increased growth inhibition has been observed, in the treatment of t(15;17) ATRA-resistant patient with a combination of the HDACi sodium butyrate and ATRA [121]. In addition to HDAC inhibition, reversal of DNA hypermethylation by demethylating agents, 5-aza-2'-deoxycytidine has been shown to restore ATRA-mediated differentiation/growth inhibition in many head and neck squamous cell carcinomas [120]. In addition, there are several studies that document synergistic efficacy in some leukemia and solid tumor cells *in vitro* and *in vivo* [2, 122, 123].

As stated earlier, RXR is a promiscuous dimerization partner for several nuclear receptors, including those related to lipid physiology, such as PPARs, LXRs, and FXRs [124]. Since RXR selective ligands (agonists, also called rexinoids) can elicit similar activities to ligands of the heterodimer partner receptors, it is believed that these agents may be useful as anti-diabetic and anti-obesity agents [125].

CONCLUSION

There is now compelling evidence from the number of retinoids in the clinic and in clinical studies (Table **1**) that these molecules exhibit efficacy in human diseases. The use of ATRA for the treatment of acute promyelocytic leukemia is considered a successful therapy in our view. It is the hope that the application of retinoids, most probably more receptor specific retinoids/rexinoids, in combination with other chemotherapeutic agents, will lead to broad clinical utility in many diseases. We anticipate that the retinoid field will continue to expand as researchers gain more information about new levels of retinoid/rexinoid biology and their relevance to human diseases.

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Effects of Novel Retinoic Acid Metabolism Blocking Agent (VN/14-1) on Letrozole-Insensitive Breast Cancer Cells

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Abstract

Aromatase inhibitors are proving to be more effective than tamoxifen for postmenopausal estrogen receptor (ER)-positive breast cancer. However, the inevitable development of resistance to treatment is a concern. We investigated the effects of novel retinoic acid metabolism blocking agent, VN/14-1, in overcoming letrozole resistance in long-term letrozole cultured (LTLC) cells. Compared with MCF-7 cells stably transfected with aromatase (MCF-7Ca), LTLC cells were no longer sensitive to growth inhibition by aromatase inhibitors. The HER-2/phosphorylated mitogen-activated protein kinase (pMAPK) growth factor signaling pathways were activated, and ERα and coactivator amplified in breast cancer 1 (AIB1) were up-regulated ~3-fold in LTLC cells. VN/14-1 inhibited aromatase activity and growth values of in MCF-7Ca cells with IC_{50} of 8.5 and 10.5 nmol/L, respectively. In human placental microsomes, aromatase activity was inhibited with IC_{50} of 8.0 pmol/L. The IC_{50} in LTLC cells was 0.83 nmol/L, similar to letrozole (IC_{50}, 0.3 nmol/L) in MCF-7Ca cells. LTLC cells were 10-fold more sensitive to growth inhibition by VN/14-1 than MCF-7Ca cells. VN/14-1 treatment effectively down-regulated ERα, AIB1, pMAPK, HER-2, cyclin D1, cyclin-dependent kinase 4 (CDK4), and Bcl2 and up-regulated cytokeratins 8/18, Bad, and Bax. Tumor growth of LTLC cells in ovariectomized nude mice was independent of estrogens but was inhibited by VN/14-1 (20 mg/kg/d; $P < 0.002$). Decreases in ERα, cyclin D1, CDK4, and pMAPK and up-regulation of cytokeratins, Bad, and Bax with VN/14-1 in tumor samples may be responsible for the efficacy of this compound in inhibiting LTLC cell growth *in vitro* and *in vivo*. (Cancer Res 2006; 66(23): 11485-93)

Introduction

Breast cancer is the second leading cause of cancer deaths in women today (after lung cancer) and is the most common cancer among women. The role of estrogens in the progression of breast cancer in both premenopausal and postmenopausal women is well established (1). The effects of estrogens on tumor growth are mediated by the estrogen receptor (ER), mainly ERα. The binding of estrogen to ERα induces a cascade of events leading to transcription of estrogen-responsive genes, such as *cyclin D1*, which are known to stimulate mammary tumor cell proliferation (2). Although estrogens affect both premenopausal and postmenopausal breast cancer, following menopause, breast tumors become more sensitive to estrogens as the concentration of ER increases with age (3). Thus, antiestrogens, such as tamoxifen, which block ER are effective in these patients. Whereas the ovary is no longer the main source of estrogen in postmenopausal women, estrogen production is increased in peripheral sites, such as adipose tissue and breast tissue, and contributes to stimulation of breast cancer (4). Although plasma estrogen concentrations are very low in postmenopausal women, levels in breast cancers from postmenopausal patients are reported to be 10-fold higher than in plasma and normal tissue (5). Synthesis of estrogens from androgens, which is a rate-limiting step in estrogen biosynthesis pathway, is catalyzed by the enzyme aromatase. Inhibition of this conversion by selective aromatase inhibitors is now proving to be a valuable approach for reducing the growth-stimulatory effects of estrogens in estrogen-dependent breast cancer (6). Aromatase inhibitors, such as exemestane and letrozole, have advantages over tamoxifen as the latter is a weak estrogen agonist as well as antagonist (7, 8).

Greater benefits of aromatase inhibitor treatment have recently been shown in patient survival and tolerability in studies comparing aromatase inhibitors with tamoxifen as first-line and adjuvant treatments for postmenopausal patients with hormone-dependent advanced breast cancer (9–11). However, the inevitable development of drug resistance presents a significant hurdle in all cancer therapies. Although tamoxifen has proved to be a successful breast cancer therapy, patients eventually relapse, showing a hormone-independent and more invasive cancer phenotype. Several mechanisms have been proposed that may contribute to the development of resistance. These comprise activation of growth factor receptor survival pathways leading to ligand-independent activation of the ER and ER-mediated transcription (12, 13). Thus, it is possible that abnormally increased growth factor signaling pathways and/or cross-talk between these signaling pathways and steroidal receptors may play an important role in endocrine resistance and may account for loss of some estrogen dependence, resulting in resistant tumors (14–17). Indeed, acquired resistance of MCF-7 cells *in vitro*, after long-term treatment with tamoxifen, is shown to be associated with increased levels of epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) activity (18). In addition, studies carried out in our laboratory indicate that MCF-7Ca cells (MCF-7 cells stably transfected with the human aromatase gene) deprived of estrogen loose their ability to respond to the mitogenic effects of estrogen. However, these cells still retain some sensitivity to the inhibitory effects of the ER down-regulator fulvestrant (at a dose 10-fold higher than needed to inhibit the growth of MCF-7Ca cells), indicating that ER is still functional in growth regulation. Nevertheless, these cells develop resistance to tamoxifen and several aromatase inhibitors accompanied by increased expression and activity of erbB-2 tyrosine kinase receptor and proteins in the phosphatidylinositol 3-kinase/AKT signaling pathway (19). Previous

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studies have been carried out in our laboratory to investigate the effects of resistance to aromatase inhibitor letrozole *in vivo*. The results suggested that tumor cells adapt to estrogen deprivation during letrozole treatment by activation of alternate signaling pathways to increase transcription. Adapter proteins [phosphorylated Shc and growth factor receptor binding protein 2 (Grb2)] as well as all of the signaling proteins in the MAPK cascade [phosphorylated Raf, phosphorylated MAPK/extracellular signal-regulated kinase kinase 1/2 (pMEK1/2), and phosphorylated MAPK (pMAPK)], but not AKT, were increased in tumors no longer responsive to letrozole (20, 21). The current study was undertaken to determine loss of sensitivity to aromatase inhibitor letrozole *in vitro* and to identify agents to which aromatase inhibitor refractory tumors would be responsive.

Besides endocrine therapies, another class of well-tolerated chemotherapeutic agents used in the treatment of breast cancer are retinoids. All-*trans*-retinoic acid (ATRA) and its isomers as well as other retinoids, such as fenretinide [*N*-(4-hydroxyphenyl)-retinamide (4-HPR)], are differentiation agents known to play an important role in the control of tumor cell proliferation and differentiation (22). However, the rapid metabolism of ATRA in the body is believed to be one of the major reasons for limited efficacy of ATRA. Thus, retinoic acid metabolism blocking agents (RAMBA) represent a promising approach for various diseases responsive to ATRA, including breast cancer, especially in patients heavily pretreated with hormone therapies (23).

Several novel potent RAMBAs that are structural analogues of ATRA and 13-*cis*-retinoic acid have been designed and synthesized in our laboratory (24). They have been shown to compete with ATRA, thus preventing ATRA metabolism and leading to increased levels of endogenous ATRA. VN/14-1 (Fig. 1) was found to be the most potent and effective compound among several RAMBAs studied in human breast cancer MCF-7 and T47D models both *in vitro* and *in vivo* (25). This novel compound was found to possess various biological properties, including induction of differentiation, apoptosis, as well as cell cycle arrest (25). Therefore, we investigated the possible effects of VN/14-1 on these processes in breast cancer cells that are no longer responsive to aromatase inhibitor letrozole. Our studies suggest that VN/14-1 might be a promising treatment following development of resistance to aromatase inhibitors in breast cancer patients.

Materials and Methods

Materials

DMEM, penicillin/streptomycin (10,000 IU each), 0.25% trypsin-1 mmol/L EDTA solution, Dulbecco's PBS, and geneticin (G418) were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) and dextran-coated charcoal-treated serum were obtained from Hyclone (Logan, UT). Androstenedione, DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Tween 20 (polyoxyethylene sorbitan monolaurate) were obtained from Sigma Chemical Co. (St. Louis, MO). Tritiated androst-4-ene-3,17-dione ([1β-^{3}H]4A; specific activity 25.3 Ci/mmol) for aromatase enzyme studies was purchased from PerkinElmer Life Sciences (Boston, MA). Antibodies against cytokeratins 8 and 18 were from Santa Cruz Biotechnology (Santa Cruz, CA). MCF-7 human breast cancer cells stably transfected with the human placental aromatase gene (MCF-7Ca) were kindly provided by Dr. S. Chen (Duarte, CA; ref. 26). ECL chemiluminescence kit and Hybond-ECL nitrocellulose membranes were purchased from Amersham Biosciences (Piscataway, NJ). Letrozole (CGS20267, Femara) was kindly provided by Dr. D. Evans (Novartis Pharma, Basel, Switzerland; Fig. 1). We have previously reported the synthesis of VN/14-1 (Fig. 1; ref. 25).

Cell Lines

The MCF-7 human breast cancer cell line stably transfected with the human placental aromatase gene (designated as MCF-7Ca; ref. 27) was routinely maintained in T75 or T150 tissue culture flasks in a humidified incubator (5% CO_2, 95% air) at 37°C in DMEM with 5% FBS, 1% penicillin/streptomycin solution, and 700 μg/mL G418.

The long-term letrozole cultured (LTLC) cells were obtained by culturing early passage of MCF-7Ca cells (passage 7) in steroid-depleted medium (phenol red–free improved MEM) supplemented with 5% dextran-coated charcoal-treated serum, 1% penicillin/streptomycin, 700 μg G418, 25 nmol/L of aromatase substrate androstenedione, and 1 μmol/L of aromatase inhibitor letrozole.

Cell Proliferation (Growth) Inhibition—MTT Assay

The MTT assay is a colorimetric assay used to measure cell proliferation. Growth studies were done on synchronized cells. This was achieved by transferring the parental MCF-7Ca cells into steroid-free medium and the LTLC to low-serum Opti-MEM medium 72 hours before plating. Cells (1×10^4 per well) were plated into 24-well plates (Corning Costar) and allowed to attach for 24 hours. The cells were then washed with Dulbecco's PBS and treated with steroid-free medium containing vehicle or indicated concentrations of estrogens, androstenedione, aromatase inhibitors, or RAMBAs. The medium was changed every 3 days, and the cells were counted on the 10th day using the MTT powder (Sigma Chemical). MTT (500 μg) was added to each well in serum-free medium, and cells were incubated for 2 hours. Medium was then removed and the formazan dye trapped in the living cells was dissolved in DMSO and absorbance was measured in a spectrophotometer at 560 nm. The results are expressed as a percentage of the cell number in the vehicle-treated control wells (26–28). IC_{50} values were determined using SigmaPlot 2000.

Preparation of Human Placental Microsomes

The human placental microsomal assay was used for measuring the inhibition of aromatase activity (29). Microsomes were isolated from human term placenta and stored in 0.1 mol/L phosphate buffer (pH 7.4) in −70°C until required as described elsewhere (30).

Radiometric ^3H_2O Release Assay for Measuring the Aromatase Activity

Microsomal and cellular assay. This assay was done as reported earlier (31, 32). Tritiated water (^3H_2O) formed during the aromatization of [1β-^{3}H]4A to estrogen was measured after incubation with microsomes or cells and extraction of steroids from the medium with organic solvent (33). The protein concentration of the homogenate was measured using the

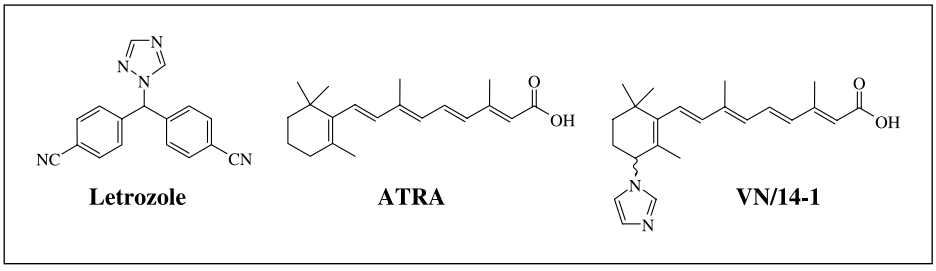

Figure 1. Structures of letrozole, ATRA, and VN/14-1.

Bradford method (Bio-Rad, Hercules, CA), and 25 μg protein was used in the assay (33, 34).

Western Imunoblotting

Cells were harvested on the 10th day of treatment. The cells were washed with ice-cold Dulbecco's PBS, scraped, and processed, and the supernatant was separated and stored at −80°C. The protein concentration in the cell lysates was measured using Bio-Rad method. Western immunoblotting was done on the whole-cell lysates as described previously (19).

Tumor Growth in Ovariectomized Female Athymic Nude Mice

All animal studies were done according to the guidelines approved by the Institution of Animal Care and Use Committee of the University of Maryland School of Medicine (Baltimore, MD). Female ovariectomized BALB/c athymic nude mice 4 to 6 weeks of age were obtained from the National Cancer Institute (NCI; Frederick, MD). The animals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water *ad libitum*.

LTLC cells were routinely maintained in improved MEM with 5% charcoal-stripped FBS, 1% penicillin/streptomycin solution, 700 μg/mL G418, 1 μmol/L letrozole, and 25 nmol/L androstenedione. Subconfluent cells were scraped into Dulbecco's PBS, collected by centrifugation, and resuspended in Matrigel (10 mg/mL) at 5.0×10^7 cells/mL. Each animal received s.c. inoculations in one site per flank with 100 μL of cell suspension. Animals were randomly grouped into three. One group ($n = 7$) was injected daily s.c. with 4-androstenedione (100 μg/d) for the duration of treatment. The second group ($n = 7$) was injected daily s.c. with 4-androstenedione (100 μg/d) along with letrozole (10 μg/d) for the duration of treatment. The third group ($n = 14$) was injected with vehicle (0.3% hydroxypropylcellulose). Tumors were measured twice weekly with calipers, and tumor volume was calculated by the following formula: $4/3\pi r_1^2 \times r_2$, where r_1 is the smaller radius and r_2 is the larger radius. Treatments began when the tumors reached a measurable size (~100 mm^3), which was ~6 weeks after cell inoculation. Mice from the vehicle group were then regrouped in two. One group ($n = 7$) was injected s.c. 20 mg/kg/d with VN/14-1. The second group ($n = 7$) continued to receive the vehicle and served as control group. Letrozole, 4-androstenedione, and VN/14-1 were prepared in sterile conditions as suspensions in 0.3% hydroxypropylcellulose.

Statistical Analysis

All experiments were carried out thrice in replicates of six, and the results are expressed as mean ± SE where applicable. The effects of treatment were compared with MCF-7Ca control cells using either GraphPad Prism 4.0 software or Student's t test on SigmaPlot 2000, and Ps < 0.05 were considered statistically significant.

Results

Progression of hormone-dependent, letrozole-sensitive MCF-7Ca cells to hormone-independent, letrozole-insensitive LTLC cells. Early passage of MCF-7Ca cells (passage 7) was cultured in steroid-depleted medium (phenol red–free improved MEM) supplemented with 5% dextran-coated charcoal-treated serum, 1% penicillin/streptomycin, 700 μg G418, 25 nmol/L of aromatase substrate androstenedione, and 1 μmol/L letrozole. The cells became quiescent for 6 to 8 weeks before they began to proliferate slowly in presence of 1 μmol/L letrozole. These cells were designated the LTLC cells. Growth studies were done at various time points to evaluate the effects of a range of concentrations of letrozole (10^{-12} to 10^{-4} mol/L) on proliferation of LTLC cells versus the parental MCF-7Ca cells. We observed a gradual loss of sensitivity of LTLC cells to letrozole compared with the parental cells that show a dose-dependent inhibition of growth following treatment with letrozole (Fig. 2*A*). By 50 to 52 weeks, 1 μmol/L letrozole (the concentration in which the cells were growing) no longer inhibited the growth of these cells (Fig. 2*A*). However, higher concentrations (10 and 100 μmol/L) of letrozole were inhibitory, although to a significantly less extent than in the parental MCF-7Ca cells (Fig. 2*A*). This clearly indicates that the cells have become less responsive to letrozole compared with the parental MCF-7Ca cells.

As shown previously, the rate of proliferation of MCF-7Ca cells slows down when cultured in estrogen-deprived medium but is increased in response to estradiol (E_2; ref. 33). MCF-7Ca cells show maximum growth stimulation in response to 1 nmol/L E_2 and 25 nmol/L androstenedione (33, 35). To examine the response of LTLC cells to these hormones, growth studies were carried out on these cells and results were compared with those of the parental MCF-7Ca cells. Cells were synchronized by transferring them to steroid-depleted medium for 3 days. After prolonged estrogen deprivation caused by long-term letrozole treatment, LTLC cells had acquired the ability to grow in an estrogen-deprived environment and did not respond to treatment with E_2 or androstenedione, indicating that their growth was no longer dependent on estrogen (Fig. 2*B*). The LTLC cells were not only insensitive to letrozole but were also found to be no longer sensitive to growth inhibition by other clinically used aromatase inhibitors, such as exemestane and anastrazole (data not shown), indicating cross-resistance to other aromatase inhibitors.

Mechanism of resistance. Studies have shown the involvement of growth factor pathways in proliferation of breast cancer cells after prolonged estrogen deprivation (36). It is known that estrogens can stimulate growth factor production, which in turn can regulate the process of ER-mediated transcription. Therefore, we examined the expression of the growth factor receptor erbB-2, an EGFR that is activated in a ligand-independent manner. HER-2 protein is found to be overexpressed in 20% to 30% of metastatic breast cancer patients and is a negative prognostic factor (37, 38). HER-2 protein was found to be increased 4.5-fold in the LTLC cells compared with the MCF-7Ca cells (Fig. 2*C*). It has been shown that overexpression of HER-2 in MCF-7 breast cancer cells results in MAPK hyperactivity. MAPK hyperactivity promotes increased association of ER with coactivators and reduces association with corepressors, thus favoring estrogen-inducible gene transcription (39, 40). Therefore, we also examined the expression of ERα and its coactivator protein amplified in breast cancer 1 (AIB1). The levels of ERα and AIB1 proteins were both increased ~3-fold compared with the MCF-7Ca cells (Fig. 2*C*). In addition, evidence of HER-2/MAPK growth factor signaling pathways driving the growth of LTLT-Ca cells (20) prompted us to investigate whether this signaling pathway was also responsible for insensitivity of LTLC cells to letrozole *in vitro*. As reported for the LTLT-Ca cells, LTLC cells also showed up-regulation of HER-2 as well as overexpression of Grb2, pMEK1/2, and pMAPK1/2 proteins (Fig. 2*C*) compared with the parental MCF-7Ca cells (21).

Effect of VN/14-1 on growth of MCF-7Ca and LTLC cells. The effect of VN/14-1 on growth of cells that had become less responsive to letrozole and other aromatase inhibitors was examined and compared with the parental MCF-7Ca cells. VN/14-1 inhibited the growth of MCF-7Ca with IC_{50} of 10.5 nmol/L, whereas letrozole inhibited growth with an IC_{50} of 0.4 nmol/L (Fig. 3*A*). Interestingly, LTLC cells were exquisitely sensitive to VN/14-1 and growth was inhibited with an IC_{50} of 0.83 nmol/L (Fig. 3*B*), whereas letrozole was essentially ineffective with an IC_{20} of ~100 μmol/L. Thus, LTLC cells were significantly more

sensitive to VN/14-1 than the parental cells. More importantly, the potency of VN/14-1 was ~10,000-fold greater than that of letrozole in LTLC cells.

Effect of VN/14-1 on aromatase inhibition in MCF-7Ca cells and human placental microsomes. The effect of the chemopreventive synthetic retinoid 4-HPR on aromatase activity inhibition in microsomes of JEG3 cells and in MCF-7 cells has been shown previously (41). Because VN/14-1 also possesses retinoidal properties and, in addition, also inhibits retinoic acid metabolism by blocking CYP-mediated catabolism of ATRA, we investigated its effect on CYP-19 (aromatase) activity. We found that VN/14-1 was a potent inhibitor of intracellular aromatase activity in MCF-7Ca cells (Fig. 4*A*), with an IC_{50} value of 8.5 nmol/L. Thus, VN/14-1 is comparable with other potent aromatase inhibitors, such as letrozole, anastrazole, and exemestane, whose IC_{50} values range from 1 to 50 nmol/L (42).

To confirm whether VN/14-1 has a direct effect on aromatase, the enzyme assay was repeated using human placental microsomes. In microsomes, VN/14-1 inhibited aromatase with an IC_{50} of 8.0 pmol/L. Although letrozole was ~10 times less potent in microsomes than in cells (IC_{50}, 0.6 pmol/L; Fig. 4*B*), it is a potent aromatase inhibitor in addition to being a potent RAMBA (25).

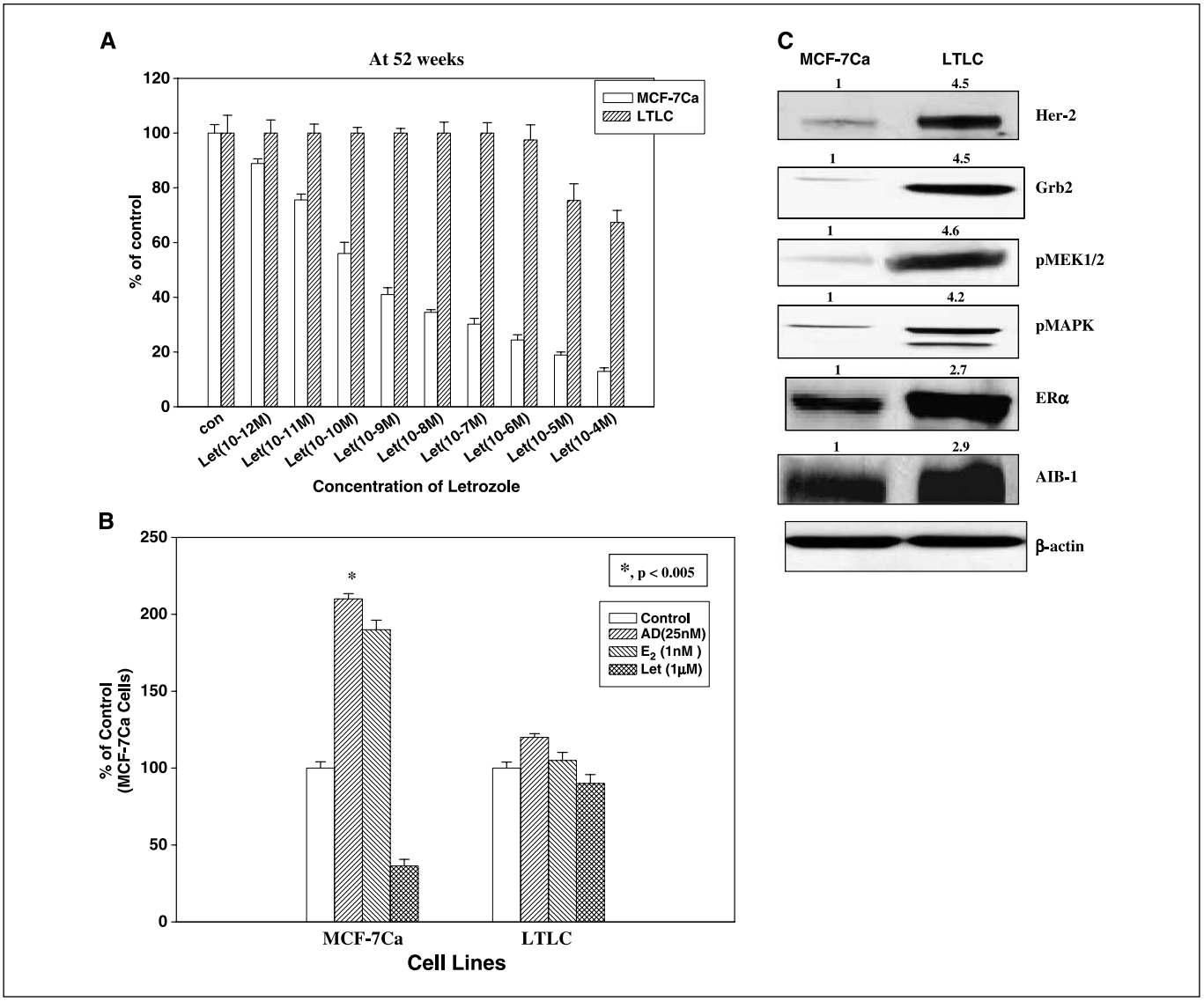

Figure 2. *A,* effect of letrozole (*Let*) on the growth of MCF-7Ca and LTLC human breast cancer cells *in vitro*. MCF-7Ca cells were cultured in steroid-free medium for 3 days before plating, and LTLC cells were cultured in reduced serum medium Opti-MEM 3 days before plating. Triplicate wells were then treated with the indicated concentrations of letrozole for 9 days, and the media were refreshed every 3 days. Cell proliferation was measured on day 10 using the MTT assay as described in Materials and Methods. Cell viability is expressed as the percentage of the cells compared with the control wells. *Columns,* mean of three experiments; *bars,* SE. *B,* effect of E_2 and androstenedione on the growth of MCF-7Ca and LTLC human breast cancer cells *in vitro*. MCF-7Ca cells were cultured in steroid-free medium for 3 days before plating, and LTLC cells were cultured in reduced serum medium Opti-MEM 3 days before plating. Triplicate wells were then treated with the indicated concentrations of E_2 and androstenedione for 9 days, and the media were refreshed every 3 days. Cell proliferation was measured on day 10 using the MTT assay as described in Materials and Methods. Cell viability is expressed as the percentage of the cells compared with the control wells. *Columns,* mean of triplicate experiments; *bars,* SE. For MCF-7Ca cells, E_2 or androstenedione treatment significantly increased cell viability ($P < 0.005$) *C,* growth factor receptor pathway adopted by the LTLC human breast cancer cells *in vitro*. Western immunoblotting analysis of whole-cell lysates from MCF-7Ca and LTLC cells cultured *in vitro* for HER-2, Grb2, MEK1/2, pMAPK, ERα, and AIB1 proteins. Experimental protocol was as described in Materials and Methods. Blots were stripped and probed for β-actin to verify equal amount of protein loaded in each lane. Representative of three independent experiments.

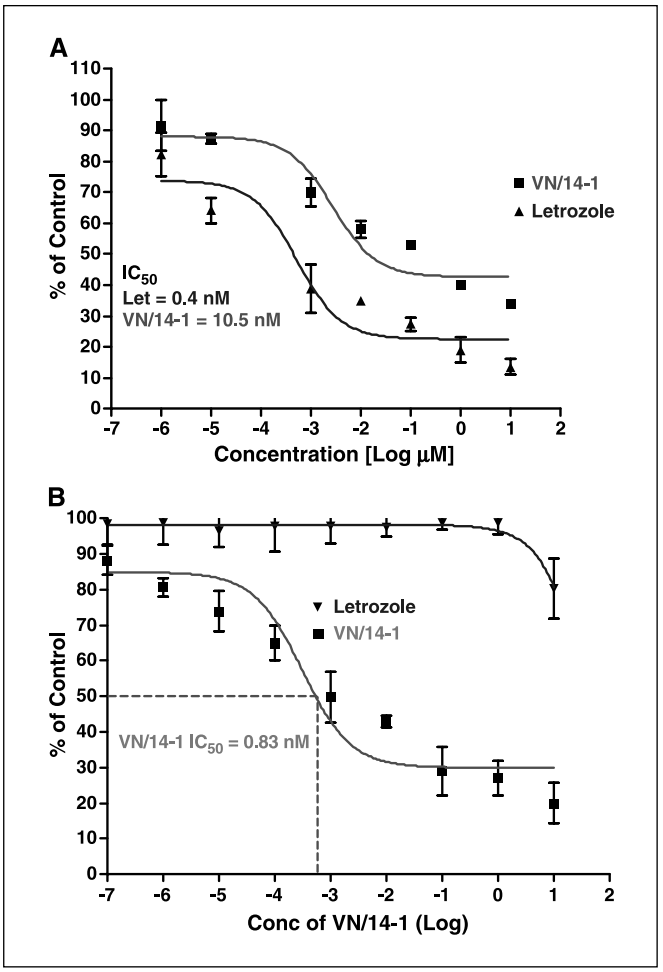

Figure 3. *A,* effect of letrozole and VN/14-1 on the growth of MCF-7Ca human breast cancer cells *in vitro*. MCF-7Ca cells were cultured in steroid-free medium for 3 days before plating. Triplicate wells were then treated with the indicated concentrations of letrozole or VN/14-1 for 9 days, and the media were refreshed every 3 days. Cell proliferation was measured on day 10 using the MTT assay as described in Materials and Methods. Cell viability is expressed as the percentage of the cells compared with the control wells. *Points,* mean of triplicate experiments; *bars,* SE. *B,* effect of VN/14-1 on the growth of LTLC human breast cancer cells *in vitro*. LTLC cells were cultured in reduced serum medium for 3 days before plating. Triplicate wells were then treated with the indicated concentrations of VN/14-1 for 9 days, and the media were refreshed every 3 days. Cell proliferation was measured on day 10 using the MTT assay as described in Materials and Methods. Cell viability is expressed as the percentage of the cells compared with the control wells. *Points,* mean of triplicate experiments; *bars,* SE. Treatment with VN/14-1 caused a dose-dependent inhibition in LTLC cells with IC_{50} of 0.83 nmol/L.

Effect of VN/14-1 on growth factor signaling pathways. As seen from the growth study results, LTLC cells were more sensitive to growth-inhibitory effects of VN/14-1 compared with the parental MCF-7Ca cells. Because the growth of the LTLC cells seems to be driven by the MAPK survival pathway, we investigated the effects of VN/14-1 on HER-2 and pMAPK proteins in LTLC cells. VN/14-1 caused a significant down-regulation of pMAPK at 1 μmol/L (1.2-fold) and HER-2 at 10 μmol/L (3.35-fold; Fig. 5).

Down-regulation of ERα and AIB1 following treatment with VN/14-1. To explore the mechanism of VN/14-1 in MCF-7Ca as well as LTLC cells, we investigated the effect of VN/14-1 on the ER expression by examining ERα protein and coactivator AIB1 by Western blotting. As indicated above, ERα protein was found to be increased 2.72-fold in the LTLC cells compared with the parental MCF-7Ca cells. VN/14-1 caused 1.4-fold decrease in ERα expression (Fig. 5). AIB1 was also up-regulated ~3-fold in the LTLC cells but was almost completely inhibited by VN/14-1 treatment (Fig. 5).

Down-regulation of cell cycle proteins (cyclin D1 and cyclin-dependent kinase 4) after treatment with VN/14-1. Treatment with retinoids inhibits cell cycle progression usually by causing arrest in the G_1 phase by affecting different cell cycle proteins, such as cyclins and cyclin-dependent kinases (CDK; refs. 43–47). Cyclin D1 is overexpressed in about one third of breast cancer cell lines (46). CDK2 and CDK4 are also known to be up-regulated, resulting in increase kinase activities (47). Because *cyclin D1* is an estrogen-responsive gene and because the ER was down-regulated

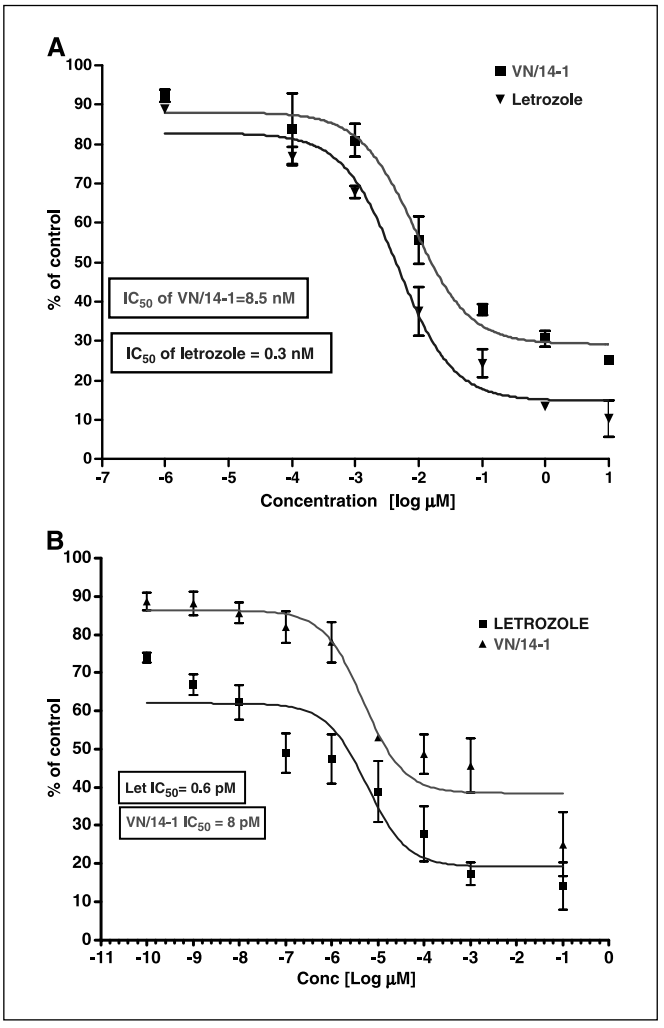

Figure 4. *A,* effect of letrozole and VN/14-1 on aromatase activity in MCF-7Ca human breast cancer cells *in vitro*. MCF-7Ca cells were cultured in steroid-free medium for 3 days before plating. Triplicate wells were then treated with the indicated concentrations of letrozole or VN/14-1 for 24 hours. Tritiated water formed was measured as described in Materials and Methods. Aromatase enzyme activity is expressed as the percentage of the cells compared with the control wells. *Points,* mean of triplicate experiments; *bars,* SE. *B,* effect of letrozole and VN/14-1 on aromatase activity in human placental microsomes *in vitro*. Microsomes were extracted from human placenta as described in Materials and Methods. Triplicate wells were then treated with the indicated concentrations of letrozole or VN/14-1 for 30 minutes in the presence of oxygen. Tritiated water formed was measured as described in Materials and Methods. Aromatase enzyme activity is expressed as the percentage of the cells compared with the control wells. *Points,* mean of triplicate experiments; *bars,* SE.

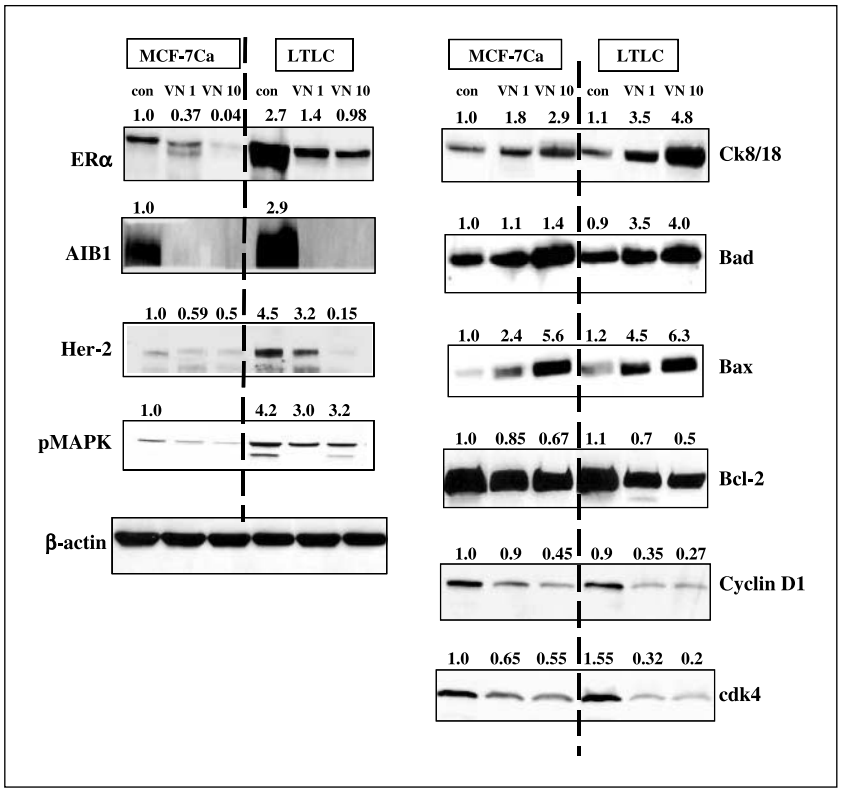

Figure 5. Effect of VN/14-1 on protein expression of ERα, AIB1, HER-2, pMAPK, cytokeratins 8 and 18, Bad, Bax, Bcl2, cyclin D1, and CDK4 in MCF-7Ca and LTLC cells. Western immunoblotting analysis of whole-cell lysates from MCF-7Ca and LTLC cells cultured *in vitro. Left,* ERα at 66 kDa, AIB1 at 160 kDa, HER-2 at 180 kDa, and pMAPK at 4,442 kDa; *right,* cytokeratins 8 and 18 at 47 kDa, Bad at 23 kDa, Bax at 20 kDa, Bcl2 at 28 kDa, cyclin D1 at 36 kDa, and CDK4 at 30 kDa. *Left* and *right, lane 1,* MCF-7Ca cells (control); *lanes 2* and *3,* 1 and 10 μmol/L VN/14-1 in MCF-7Ca cells; *lane 4,* LTLC cells (control); *lanes 5* and *6,* VN/14-1 treatments at 1 and 10 μmol/L in LTLC cells. Experimental protocol was as described in Materials and Methods. Blots were stripped and probed for β-actin to verify equal amount of protein loaded in each lane. Blots are representative of three independent experiments.

after treatment with VN/14-1, we examined the level of this protein after treatment with VN/14-1. The level of cyclin D1 was decreased by 0.8- and 0.45-fold in MCF-7Ca and 0.35- and 0.27-fold in LTLC cells after treatment with 1 and 10 μmol/L VN/14-1, respectively (Fig. 5). The level of CDK4 was increased by ~1.7-fold in LTLC cells compared with the parental MCF-7Ca cells, indicating increased kinase activity. Its levels were also down-regulated in both the cell lines by 0.65- and 0.55-fold in MCF-7Ca and 0.32- and 0.20-fold in LTLC cells compared with the untreated cells, respectively (Fig. 5). These results suggest that LTLC cells are more sensitive to the effect of VN/14-1 compared with the parental MCF-7Ca cells. Down-regulation of cyclin D1 and CDK4 after VN/14-1 treatment indicates that VN/14-1 causes arrest of the cells in G_1 phase of the cell cycle, thus preventing the proliferation of the tumor cells.

Effect of VN/14-1 on differentiation proteins, cytokeratin 8, and cytokeratin 18. Cytokeratins have been identified as one of the differentiation marker proteins also known as structural marker proteins for epithelial cells (48). Elevated levels of cytoskeletal proteins indicate a favorable prognosis and are useful predictors for overall survival of breast cancer patients (49, 50). Recent studies have shown that retinoids enhance the expression of cytokeratin in breast cancer cells. Therefore, we examined the expression of cytokeratins 8 and 18 after treatment with VN/14-1. Cell lysates of MCF-7Ca and LTLC cells were obtained on the 10th day after treatment with 1 and 10 μmol/L VN/14-1 and then probed by Western blotting for cytokeratins 8 and 18 using antibody at 1:2,500 dilution in 10% milk-PBS-Tween 20 for 1 hour at room temperature. Treatment with 1 and 10 μmol/L VN/14-1 showed dose-dependent increases of 2.0- and 6.2-fold in MCF-7Ca cells and 4.1- and 9.9-fold in LTLC cells of cytokeratins 8 and 18, respectively (Fig. 5).

Effect of VN/14-1 on apoptotic proteins (Bad, Bax, and Bcl2). The mechanisms underlying the anticarcinogenic activity of retinoids seem to be associated with the ability of retinoids to modulate growth, differentiation, and apoptosis in different malignancies, including breast cancer (43, 44, 46). Therefore, we investigated the apoptotic proteins Bad, Bax, and Bcl2 after treatment with VN/14-1. The expression of proapoptotic protein Bad showed dose-dependent increases of about 1- to 2-fold after treatment with 1 and 10 μmol/L VN/14-1 in both cell lines. Another proapoptotic protein Bax showed much greater increases of 2.4- and 5.56-fold in MCF-7Ca cells and 4.5- and 6.3-fold increase in LTLC cells after treatment with 1 and 10 μmol/L VN/14-1, respectively. The antiapoptotic protein Bcl2 was down-regulated by 0.85- and 0.67-fold in MCF-7Ca cells and 0.68- and 0.5-fold in LTLC cells after treatment. Thus, these results indicate that VN/14-1 is causing apoptosis in both the cell lines but to a greater extent in LTLC cells (Fig. 5).

Effect of VN/14-1 in female athymic ovariectomized nude mice. To confirm our *in vitro* findings, we inoculated LTLC cells in athymic ovariectomized nude mice. The hormone-independent nature of these cells was evident when one group was injected with 100 μg/d androstenedione from day 1 of inoculation. Unlike MCF-7Ca tumors that require estrogens to grow, LTLC tumors grew without hormones (Fig. 6*A*). After a period of 6 weeks, when the tumors had reached a size of 100 mm^3, the vehicle-treated group was divided into two. One group continued to receive vehicle (control group), whereas the other group received 20 mg/kg/d VN/14-1 s.c. five times weekly. The dose of VN/14-1 was selected based on previous studies with this novel compound done in our laboratory (25). Tumors of the mice treated with letrozole and androstenedione grew like the control tumors, indicating that the LTLC tumors were insensitive to the effects of letrozole as shown

in vitro. Thus, VN/14-1 inhibited the LTLC tumor growth significantly. Tumor growth resumed, but even after 60 days, tumors had not doubled in volume and were significantly smaller than the control group, androstenedione alone, as well as letrozole plus androstenedione group.

Effect of VN/14-1 on LTLC tumor protein expressions. Expression of the same proteins was examined in tumor samples as studied earlier *in vitro*. ERα, AIB1, cyclin D1, and CDK4, including pMAPK, MEK1/2, and phosphorylated ribosomal protein S6 kinase (90-kDa protein; p90RSK), were down-regulated in the tumors of VN/14-1 treatment group. Similarly Bad, Bax, and cytokeratins 8/18 were up-regulated (Fig. 6*B*). However, HER-2 protein was not affected unlike our *in vitro* finding where treatment with VN/14-1 caused down-regulation of HER-2. This may be due to differences in doses of VN/14-1 *in vitro* and *in vivo*.

Discussion

Aromatase inhibitors are now showing greater efficacy than antiestrogen tamoxifen. However, as resistance eventually develops to all forms of treatment, it is relevant to investigate other strategies to control tumor proliferation. In this study, we investigated the growth effects of a novel RAMBA on breast cancer cells using cells that were made refractory to aromatase inhibitor letrozole. Aromatase-expressing MCF-7Ca cells were cultured in the presence of 1 μmol/L letrozole (concentration ~ 1,000 times higher

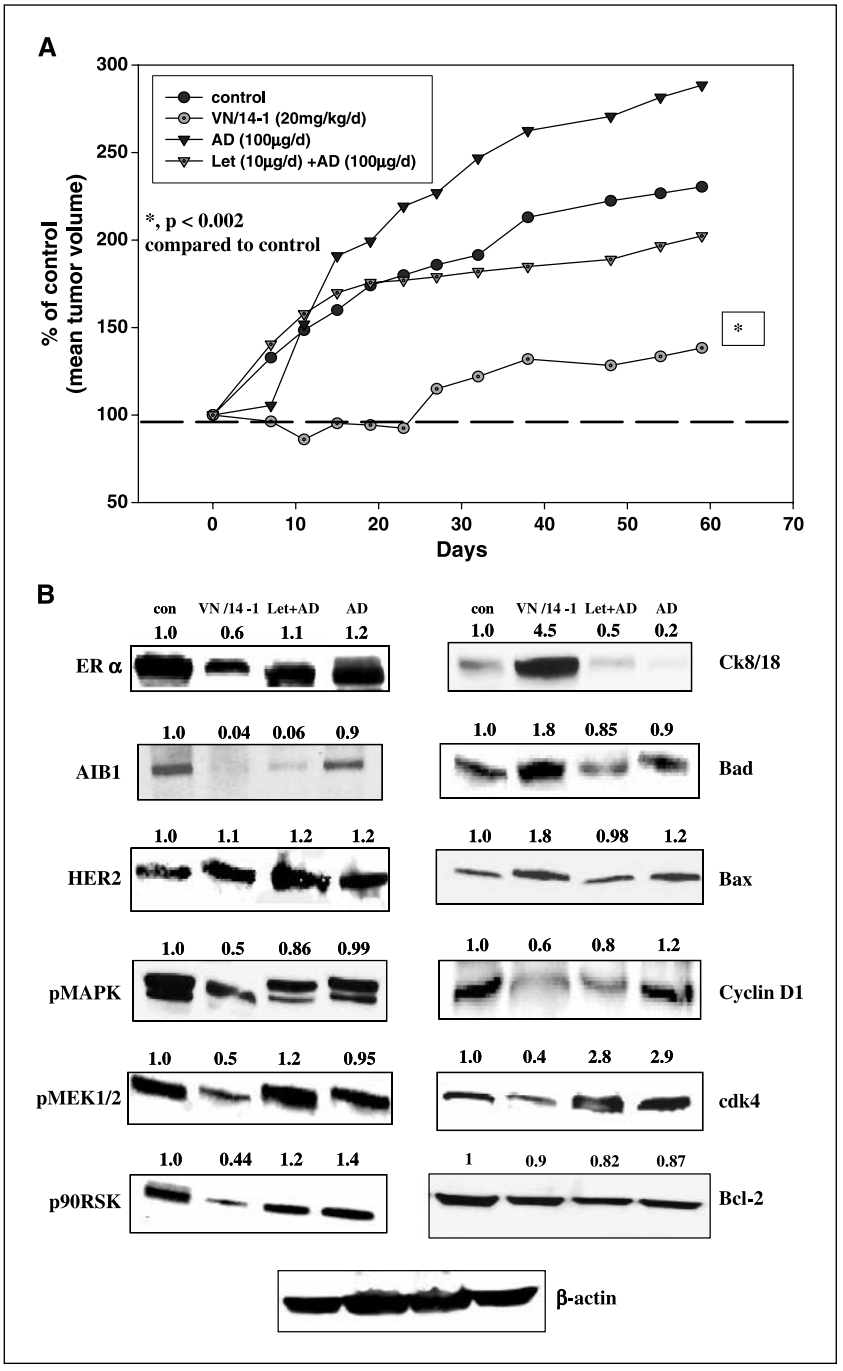

Figure 6. *A,* effect of letrozole plus androstenedione (*AD*), androstenedione alone, and VN/14-1 in LTLC xenograft model. Female ovariectomized nude mice were inoculated with LTLC cells as described in Materials and Methods. Mice were randomly grouped (n = 10 control; n = 5 in other two groups) and treated with vehicle only for control, androstenedione (100 μg/mouse/d), and letrozole (10 μg/mouse/d) plus androstenedione (100 μg/mouse/d). After the tumors were 100 mm^3, control mice were regrouped (n = 5). One group continued on vehicle and other group switched to VN/14-1 (20 mg/kg/mouse/d). Treatment was continued for 70 days. The mice were sacrificed at the end of the study, and tumor samples were collected for further studies. *B,* effect of letrozole plus androstenedione, androstenedione alone, and VN/14-1 on protein expression of ERα, AIB1, HER-2, pMAPK, pMEK1/2, p90RSK, cytokeratins 8 and 18, Bad, Bax, Bcl2, cyclin D1, and CDK4 in LTLC tumor samples. Western immunoblotting analysis of LTLC tumor lysates. *Left,* ERα at 66 kDa, AIB1 at 160 kDa, HER-2 at 180 kDa, pMAPK at 4,442 kDa, pMEK1/2 at 45 kDa, and p90RSK at 90 kDa; *right,* cytokeratins 8 and 18 at 47 kDa, Bad at 23 kDa, Bax at 20 kDa, Bcl2 at 28 kDa, cyclin D1 at 36 kDa, and CDK4 at 30 kDa. Experimental protocol was as described in Materials and Methods. Blots were stripped and probed for β-actin to verify equal amount of protein loaded in each lane. Blots are representative of three independent experiments.

than its IC_{50} value) and the aromatase substrate androstenedione. The latter is converted by aromatase in the cells into E_2. As previously reported, letrozole inhibited the growth of the cells initially for a period of 6 to 8 weeks. The growth-inhibitory effects of letrozole (10^{-12} to 10^{-4} mol/L) were evaluated at several time points. Eventually, after 50 to 52 weeks, the cells then began to proliferate slowly and were no longer inhibited by 1 μmol/L letrozole, although they were marginally inhibited by 10 and 100 μmol/L letrozole. Thus, these cells (designated as LTLC) were less sensitive than the parental MCF-7Ca cells to the growth-inhibitory effects of letrozole. The LTLC cells were also insensitive to antiestrogens tamoxifen and Faslodex (data not shown). Compared with the LTLT-Ca cells previously reported (33), LTLC cells retain some sensitivity to letrozole. LTLT-Ca cells were isolated from MCF-7Ca tumors of mice with letrozole for 56 weeks. The LTLT-Ca cells, like the LTLC cells, also showed up-regulation of the proteins in MAPK pathway. However, the level of ER, which is up-regulated in the LTLC cells, was up-regulated initially but subsequently decreased in tumors that were unresponsive to letrozole. This suggests that the LTLT-Ca cells were subjected to more severe estrogen deprivation than the LTLC cells. We have also reported studies of MCF-7Ca cells that were deprived of estrogen in culture (UMB-1Ca; refs. 19, 33). These cells were only sensitive to higher concentrations of letrozole like the LTLC cells (Fig. 2*A*; refs. 19, 33).

The results in the present study indicate that after prolonged estrogen deprivation caused by letrozole treatment *in vitro*, MCF-7Ca cells have increased expression of proteins in the estrogen signaling pathway (increase ER, AIB1, cyclin D1, and CDK4). The cells had up-regulated proteins in the MAPK growth factor pathway for survival. LTLC cells were less responsive to the inhibitory effects of letrozole as well as other aromatase inhibitors: anastrazole (nonsteroidal aromatase inhibitor) and exemestane (steroidal aromatase inhibitor). Thus, MCF-7Ca cells that have developed resistance to letrozole also tend to be unresponsive to other aromatase inhibitors (data not shown).

Using the LTLC breast cancer cells that were no longer responsive to the growth-inhibitory effect of letrozole, the goal was to identify agent(s) that would inhibit the growth of these cells. As reported previously, we have discovered several RAMBAs with multiple biological and inhibitory activities against several human breast and prostate cancer cells (25). Our results presented here show that the lead compound in this series of novel RAMBAs, VN/14-1, was a remarkably potent inhibitor of the growth of LTLC and MCF-7Ca cells. However, the other RAMBAs (VN/12-1, VN/50-1, and VN/66-1) were ineffective in the LTLC cells (data not shown). Our results indicate that VN/14-1 is also a potent aromatase inhibitor and down-regulates ERα and steroid coactivator AIB1. In LTLC cells, estrogen signaling was significantly increased. Figure 5 shows increased ERα, its coactivator AIB1, *cyclin D1* (estrogen-responsive gene), and CDK4. Treatment with VN/14-1 caused marked down-regulation of ERα, the related coactivator AIB1, as well as cyclin D1 and CDK4 in the MCF-7Ca cells as well as LTLC cells. Our findings suggest that, similar to other retinoids, VN/14-1 mediates its effects in part through interference with coactivator AIB1 and ER signal transduction, thus affecting estrogen-responsive genes, such as *cyclin D1* (51).

In addition, VN/14-1 also showed several other significant effects on cell differentiation, cell cycle, and apoptosis. Hormone independence and letrozole insensitivity of LTLC cells were further confirmed in the xenograft model. Parental MCF-7Ca xenografts are hormone dependent and need androstenedione supplementation to grow (35, 52). However, LTLC cells grew without any supplementation (androstenedione or estrogens) as well as in the presence of letrozole. It should be noted that letrozole (10 μg/d) has shown to be the most effective inhibitor of MCF-7Ca tumor growth (53). Treatment with 20 mg/kg/d VN/14-1 caused significant tumor growth suppression ($P < 0.002$). In addition, similar *in vitro* and *in vivo* findings (alteration in the levels of apoptotic proteins, cell cycle proteins, differentiation proteins, ERα, and AIB1) further strengthen the effectiveness of this compound. Down-regulation of HER-2 at 10 μmol/L and pMAPK at 1 μmol/L proteins *in vitro* as well as pMEK1/2, pMAPK, and p90RSK *in vivo* following VN/14-1 treatment suggests interference of VN/14-1 in this signaling pathway. This may partly explain why VN/14-1 is more effective in LTLC cells, although the exact reason for VN/14-1 being more effective in LTLC cells than in MCF-7Ca cells is unclear at this time.

In conclusion, we induced letrozole insensitivity by prolonged treatment of MCF-7Ca cells with letrozole *in vitro* (LTLC). These cells grew without hormone supplementation and showed up-regulation of proteins in the estrogen and MAPK signaling pathways. VN/14-1 has potent antiproliferative effects against estrogen-dependent MCF-7Ca cells. VN/14-1 was found to be a potent inhibitor of the aromatase activity as well as of growth in the parental MCF-7Ca cells. We observed that the anticancer effects of VN/14-1 seem to be due to its multiple biological properties. These include significant down-regulation of proteins in the MAPK pathway in the LTLC cells as well as marked ER and AIB1 down-regulation in both the cell lines. In addition, VN/14-1 caused induction of apoptosis, differentiation, and cell cycle arrest *in vitro* and *in vivo*. Furthermore, treatment with VN/14-1 induced significant arrest of growth of LTLC tumors in the xenograft model. These multiple anticancer properties of this novel RAMBA, VN/14-1, can be exploited clinically. The compound has potential as a new therapeutic agent for hormone-dependent breast cancer as well as following resistance to aromatase inhibitors.

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ORIGINAL ARTICLE

Murine toxicology and pharmacokinetics of novel retinoic acid metabolism blocking agents

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Abstract
Purpose Novel potent C-4 azolyl retinoic acid metabolism blocking agents (RAMBAs)—VN/14-1, VN/50-1, VN/66-1, VN/67-1, and VN/69-1, have been synthesized and investigated for their in vitro and in vivo effects against breast and prostate cancers. These RAMBAs, in addition to being potent inhibitors of all-*trans*-retinoic acid (ATRA) metabolism have potent anti-cancer properties and in vivo anti-tumor efficacies as characterized in breast and prostate cancer models. Here we determined the toxicity and pharmacokinetics (PK) of these various RAMBAs.
Methods Preliminary acute toxicity studies of these RAMBAs were carried out using Swiss NIH mice. The toxicity profile of the RAMBAs was evaluated relative to ATRA. Three different doses (8.3, 33, and 100 μmol/kg/day) of ATRA and RAMBAs were administered on a daily basis subcutaneously for 14 days to the mice. Clinical signs of toxicity alopecia, scaly skin, and loss of body weight in the mice were observed during the study and the maximum tolerated dose was determined. PK of selected agents (VN/14-1, VN/50-1, and VN/66-1) was studied in Balb/C mice after a single dose subcutaneous administration. Plasma concentrations of the agents were quantitatively determined using a high-performance liquid chromatographic method with ultraviolet detection. Plasma concentration versus time profiles were fit to various PK structural models and relevant PK parameters were estimated.
Results VN/66-1 and VN/69-1 were found to be the least toxic even at the highest doses when compared to the other RAMBAs and ATRA. VN/66-1 had the longest half-life, the slowest clearance, and the greatest exposure.
Conclusions Based on PK characteristics and toxicity studies, VN/66-1 appeared to be the most favorable agent. However, both VN/14-1 and VN/66-1 are our leads based on the fact that VN/14-1 has been found to be highly effective in endocrine-sensitive and -resistant breast cancer cells and tumors with little toxicity. Our findings provide valuable information that will be used to select RAMBAs and establish therapeutic regimens that provide optimal efficacy with minimal toxicity.

Jyoti B. Patel and Aakanksha Khandelwal contributed equally to this work.

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Keywords Retinoic acid metabolism blocking agents · Retinoids · Anti-cancer agents · Pharmacokinetics · Toxicology

Introduction

The ability of all-*trans*-retinoic acid (ATRA) and other retinoids to modulate a variety of important functions like cell growth and differentiation, induction of apoptosis and prevention of angiogenesis is well documented [2, 5, 7, 8]. They are effective in the prevention and therapy of a number of proliferative diseases including breast and prostate cancers. However, the clinical use of ATRA in the treatment of malignancies is significantly hindered by the prompt emergence of resistance, which is believed to be caused at least in part by increased ATRA metabolism [9, 15, 17, 18]. The use of high dose ATRA is limited due to toxic and tetratogenic effects [1, 6]. Others [15] and ourselves [16–19] have suggested the use of retinoic acid metabolism blocking agents (RAMBAs) as a viable strategy to increase the endogenous levels and thus potentiate the effect of ATRA without the need for high exogenous doses of ATRA. Indeed, RAMBAs may be used alone or in combination with low doses of ATRA. RAMBAs may prove useful for the chemoprevention and/or treatment of different cancers and also for the treatment of dermatological diseases [15, 18].

Few groups have synthesized and studied non-retinoidal RAMBAs [24–28] and research on retinoidal RAMBAs is even less. Our RAMBAs are structural analogues of ATRA (VN/14-1, VN/50-1), 13-*cis* RA (VN/67-1, VN/69-1) and 4-HPR [*N*-(4-hydroxyphenyl) retinamide; fenretinide] (VN/66-1; Fig. 1). An analog of 4-HPR was made based on previous studies which have found that 4-HPR possesses a longer half-life than either ATRA or 13-*cis* RA [14]. Furthermore, 4-HPR has been previously determined to have lower toxicity than ATRA, thus making it a favorable compound to optimize. These RAMBAs, which have been synthesized in our laboratory, differ from each other by the structural modification at the end of the side chain [16–19]. We have reported previously that these RAMBAs, like ATRA appear to have pleiotropic properties apart from their main mode of action of inhibiting ATRA metabolism in intact breast and prostate cancer cells and microsomes. RAMBAs are able to inhibit breast and prostate cancer cell growth, induce differentiation and apoptosis and have anti-tumor efficacies in vivo in mice bearing human breast and prostate cancer xenografts [3, 11, 19, 20].

For clinical therapeutic and chemopreventive use of any compound, it must not only be effective, but also safe. Therefore, it is essential that we determine the toxicity profiles of compounds early in the drug discovery stage to enable the development of those which are non-toxic. The pharmacological disposition and metabolism of an agent are important in determining its therapeutic activity and helps to decide the frequency of dosing. In this paper we discuss the murine preliminary acute toxicological profiles of several RAMBAs and pharmacokinetic (PK) parameters of three RAMBAs. The data obtained from this study will allow us to optimize the structures, disposition profiles and in vivo activities of promising RAMBAs.

Materials and methods

Chemicals and reagents

All-*trans*-RA and 13-*cis* RA (internal standard) were purchased from LKT Laboratories Inc., St Paul, MN, USA. The RAMBAs, VN/14-1, VN/50-1, VN/66-1, VN/67-1, and VN/69-1 and VN/2-1 (internal standard) were synthesized in our laboratory as previously described [19]. Methanol, acetonitrile, ethyl acetate,

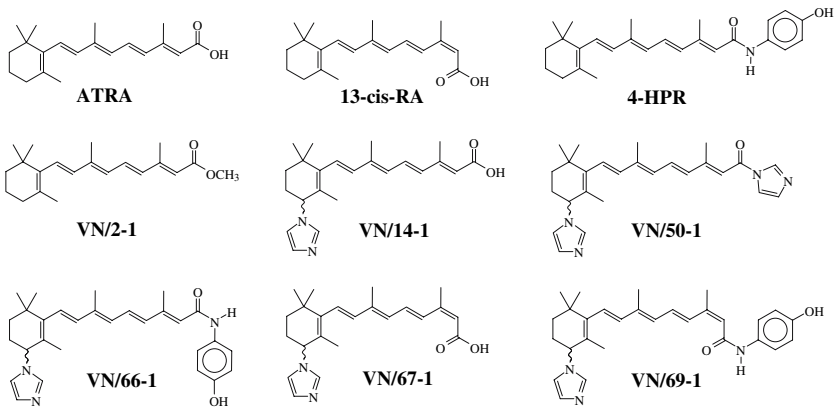

Fig. 1 Chemical structures of retinoids and RAMBAs, ATRA, 13-cis-RA, 4-HPR, VN/2-1, VN/14-1, VN/50-1, VN/66-1, VN/67-1, and VN/69-1

ammonium acetate, and water were purchased from Fisher Scientific, Fair Lawn, NJ, USA. Formic acid, butylated hydroxyl anisole, and hydroxypropyl-β-cyclodextrin (HPβCD) were purchased from Sigma-Aldrich, St Louis, MO, USA. All chemical and solvents were of analytical or high-performance liquid chromatographic (HPLC) grades.

Although the retinoidal compounds and RAMBAs appeared to be relatively stable to light, precautions were taken to minimize exposure to any light source and to the atmosphere. Thus, all operations were performed in dim light, with reaction vessels wrapped with aluminum foil. All compounds were stored in an atmosphere of argon and in the cold (−20 or −80°C) and dark without significant decomposition.

Animal handling

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 followed the NIH guidelines as well. Female NIH Swiss mice (4–6 weeks old) and female Balb/c mice (4–6 weeks old, weighing 20–25 gm) were obtained from NCI (Frederick, MD, USA) and were maintained in a controlled environment of light, humidity, and temperature and were given food and water ad libitum.

Toxicology study

Female NIH Swiss mice were used for a 14 day toxicity study in which the mice were given different doses of ATRA or RAMBAs (VN/14-1, VN/50-1, VN/66-1, VN/67-1, and VN/69-1) (Fig. 1). Three different doses, i.e., 8.3, 33, and 100 μmol/kg/day (formulated in 0.3% hydroxypropyl cellulose in saline) of each compound were administered on a daily basis subcutaneously for 14 days. Each group consisted of five mice. Clinical signs of toxicity (alopecia, scaly skin, and loss of body weight), were observed during the 14 days of dosing and the maximum tolerated dose (MTD) was determined following established procedures [12, 13, 23]. Dosing with ATRA served as a reference. The MTD was essentially the highest dose at which no mortality was observed. Toxicities observed during the course of the study were scored according to their severities. The degrees of ATRA/RAMBA toxicities in each animal at weighing were scored using the rating scale described by Bollag [4]. Three physical parameters were scored on a scale of 0–4 as follows: (1) Weight loss: (10 g = 4, 7–9 g = 3, 4–6 g = 2, 1–3 g = 1, <1 g = 0; (2) hair loss (alopecia): very severe = 4, severe = 3, moderate = 2, slight = 1, none = 0; (3) skin scaling: very severe = 4, severe = 3, moderate = 2, slight = 1, none = 0. The total score for each animal was obtained by adding the three individual scores. Scores are ranked as none (score = 0), mild (score = 1–3), moderate (4–6), and severe (7+).

Pharmacokinetic study

Dosing and sampling

Female Balb/c mice were used for PK studies ($n = 2$ per time point). Mice were administered a single 10 mg/kg dose subcutaneously of RAMBA formulated in 45% HPβCD in water, and blood was collected at different time points ranging from 5 min to 12 h after drug administration. Blood was collected in heparinized tubes after cardiac puncture (VN/14-1) or retro-orbital puncture (VN/50-1 and VN/66-1) using light halothane for anesthesia. The plasma was separated and stored at −20°C until HPLC analysis.

Sample preparation

Sample preparation for various agents involved a liquid–liquid extraction method using VN/1-2 (for VN/14-1) or 13-*cis* RA (for VN/50-1 and VN/66-1) as an internal standard. Two hundred microliters of plasma sample was spiked with 1 μg/ml (VN/1-2) or 0.5 μg/ml (13-*cis* RA) of internal standard and extracted with 2 ml of ethyl acetate + 10% methanol + 0.05% butylated hydroxyl anisole. Samples were vortexed and supernatant was transferred to another tube and dried under nitrogen gas. The samples were reconstituted in 100 μl of methanol, passed through 0.22 μm syringe filters and 50 μl was injected onto the HPLC system. Calibration samples were prepared by spiking control mice plasma with various concentrations of agents (1–10 μg/ml) and processed and analyzed in the same manner as described above.

HPLC bioanalytical conditions

The HPLC system consisted of a—1535 pump, 717 autosampler, and 996 detector (Waters, Miliford, MA, USA). Chromatographic separation was achieved on a reverse phase C_{18} column (3.9 mm × 150 mm × 5 μm) (Novapak) using a gradient mobile phase of various combinations of 20 μM ammonium acetate buffer and methanol at a flow rate of 0.8 ml/min for detection of VN/14-1. A mobile phase of 75% acetonitrile, 25% water, 0.5% formic acid, and 1 mM ammonium acetate at a flow rate of 1.2 ml/min was use for detection of VN/50-1 and VN/66-1. The eluate was monitored with

a UV detector set at an absorption maximum of 350 nm. Calibration curves were prepared by plotting peak area versus spiked concentration. Concentration of analytes in PK samples was obtained by simple linear regression analysis of calibration samples.

Data analysis

The lower limit of quantification (LLOQ) of the assay was determined from calibration samples. Concentrations below the LLOQ were not considered for PK modeling. Compartmental modeling was performed using non-linear regression software, WinNonlin (ver. 4.1, Pharsight Corporation, Mountain View, CA, USA). Various weighting schemes were applied to determine the best model. The goodness of fit of competing models was assessed by the Akaike information criterion (AIC), diagnostic plots, variance, and random distribution of residuals. Weighting schemes were assessed in a similar manner except that weighted sum of squares residual was examined in place of the AIC. The values for area under the curve (AUC) and other PK parameters such as clearance (CL), volume of distribution (V_d), and half-life ($t_{1/2}$) were obtained from the WinNonlin output.

Results

Toxicological evaluations

Six compounds were examined for toxic effects in female Swiss mice. Three different doses (8.3, 33, and 100 μmol/kg/day) were examined for each compound with each dosage group consisting of five mice. It is important to note that the number of moles administered was taken into account when determining the dose for each compound. Each compound was subcutaneously administered daily for 14 days, during which clinical signs of toxicity were recorded. Alopecia, scaly skin, mean change in body weight, and mortality were assessed. At the completion of the study the MTD was determined. ATRA, which has known toxic effects, was used as a reference for which to compare the five RAMBAs (VN/14-1, VN/50-1, VN/66-1, VN/67-1, and VN/69-1). Toxic events were scored on the basis of severity with 1–3 being mildly severe, 4–6 being moderately severe and above 6 as being very severe. A total score was then computed based on the occurrence of individual toxic events at each dose, with a higher score corresponding to higher toxicity. As expected, a greater score was seen with increasing doses for each of the compounds examined. Two compounds which did not exhibit any toxic or deleterious effects at the 8.3 and 30 μmol/kg/day, did exhibit skin scaling at the highest dose, 100 μmol/kg/day (VN/66-1 and VN/69-1) as shown in Table 1. Furthermore, it is important to note that there was a lack of weight loss even after administration of the highest dose with VN/66-1 and VN/69-1, where all the other compounds induced some loss in weight. Alopecia was seen in the two higher doses of ATRA and VN/50-1 and in the 100 μmol/kg/day dose of VN/14-1 and VN/67-1. Scaly skin was found to be the predominant form of toxicity in all of the groups tested. In the various dosage groups, if one mouse exhibited scaly skin or alopecia the others were observed to do the same. To clarify, there was no partial response among the groups, for example for these two toxic events either 0/5 or 5/5, but never 1, 2, 3, or 4, out of 5 mice were observed to have the condition, however, the severity did differ among the groups. The greatest severity of skin scaling and weight loss was seen in the VN/14-1 and VN/50-1 treated mice. Mortality at the highest dose was also seen in these two groups resulting in the death of all five mice at 100 μmol/kg/day. It was found that VN/14-1 and VN/50-1 were the most toxic of the five RAMBAs tested and VN/66-1 and VN/69-1 were the least toxic.

Pharmacokinetics

The pharmacological disposition and metabolism of an agent are important determinants of its pharmacodynamic activity and play a critical role in the development of an optimal dosing regimen. Different PK parameters such as $t_{1/2}$, CL, V_d, and AUC for the plasma concentration versus time profile of different RAMBAs were determined. Three RAMBAs were used for the PK studies, VN/14-1, VN/50-1, and VN/66-1. These three were chosen out of the six compounds examined for toxicity based on previous research done in our lab showing that these three compounds were most potent in vitro in various breast and prostate cancer cell lines.

Balb/c mice were administered 10 mg/kg dose of RAMBA subcutaneously. The dose was determined from the toxicity studies, at this dose there were minimal or no toxic effects of the compounds. Blood plasma was collected and reverse phase HPLC analysis was performed to obtain the PK profile of the various RAMBAs All compounds fit a one compartment model with first order elimination and flip-flop kinetics, where the rate of absorption is equal to the rate of elimination. The mean plasma concentration–time profile of VN/14-1 is shown in Fig. 2a. Following subcutaneous administration of VN/14-1, there was an initial increase in plasma

Table 1 Effects of ATRA and RAMBAs on body weight and clinical observations at termination of study

Compound	Dose level		Clinical observations[a]			Mean body weight change (g)	Total score
	Mg/kg/day	μmol/kg/day	Mortality	Alopecia	Scaly skin		
Vehicle	0	0	–	–	–	+0.11	0
ATRA	2.5	8.3	–	–	5/5 (1)[b]	−0.12	1
	10.0	33.0	–	5/5 (2)	5/5 (2)	+0.004	4
	30.0	100.0	–	5/5 (3)	5/5 (3)	−5.28 (2)	8
VN/14-1	3.1	8.3	–	–	–	+1.04	0
	12.2	33.0	–	–	5/5 (2)	−1.26 (1)	3
	36.6	100.0	5/5	5/5 (4)	5/5 (4)	−6.39 (3)	11
VN/50-1	3.5	8.3	–	–	–	+1.04	0
	13.9	33.0	–	5/5 (2)	5/5 (2)	−1.26 (1)	5
	41.6	100.0	5/5	5/5 (4)	5/5 (4)	−5.68 (3)	11
VN/66-1	3.8	8.3	–	–	–	+1.13	0
	15.2	33.0	–	–	–	+0.37	0
	45.7	100.0	–	–	5/5 (1)	+2.13	1
VN/67-1	3.1	8.3	–	–	–	+1.08	0
	12.2	33.0	–	–	5/5 (1)	−0.22	1
	36.6	100.0	–	5/5 (2)	5/5 (2)	−3.09 (1)	2
VN/69-1	3.8	8.3	–	–	–	+0.95	0
	15.2	33.0	–	–	–	+0.06	0
	45.7	100.0	–	–	5/5 (1)	+1.09	1

– indicates that no death or signs of toxicity were found in any animal within the group of five

[a] Data are presented as the number of mice exhibiting clinical observations/number of mice in the dose group

[b] The numbers in parentheses indicate the range of severity of the indicated lesion, according to the scale indicated above

concentration with a maximum plasma concentration (C_{max}) of 4.3 μg/ml at a time taken to achieve maximum plasma concentration (t_{max}) of 30 min (Table 2). After 30 min the plasma concentration declined exponentially with a mean $t_{1/2}$ of 0.34 h and CL of 1,720 ml/h/kg. VN/50-1 on the other hand was not absorbed as fast and to the same extent as VN/14-1 (Fig. 2b). VN/50-1 also had a faster rate of CL (1,924 ml/hr/kg) and a longer $t_{1/2}$ (0.57 h) thus is not as favorable as VN/14-1. Furthermore, between 0.5 and 2 h (Fig. 2b), the plasma concentration of VN/50-1 appears to have reached a pseudo steady-state condition because the concentrations appear to be saturated and not undergoing elimination. At these time points the concentrations are not near the LLOQ, thus we can assume that the model is accurately predicting the kinetics of VN/50-1. VN/66-1 was found to possess the most favorable PK out of the three compounds examined (Fig. 2c). VN/66-1 had the greatest exposure, having a much higher C_{max} (12.32 μg/ml) and greatest AUC (41.3 h·μg/ml) compared with VN/14-1 and VN/50-1, which both had AUC values less than 6 h·μg/ml. VN/66-1 also showed the slowest elimination as measured by CL and $t_{1/2}$, 242 ml/hr/kg and 0.85 h, respectively. The relative highness of the last two points (6 and 12 h) was very close to the LLOQ and was most likely measured with a fair amount of background noise. Thus, the prediction is not highly influenced by these two points for VN/66-1.

Discussion

Toxicity as determined by alopecia, skin scaling, loss of body weight and ultimate mortality in mice was lowest for VN/66-1 and VN/69-1. VN/14-1 and VN/50-1 had the highest toxicity scores and displayed equal toxicity

Table 2 Pharmacokinetic parameters of various RAMBAs in mice after a single s.c dose of 10 mg/kg

Compound	C_{max} (μg/ml)	t_{max} (h)	$t_{1/2}$ (h)	AUC (h·μg/ml)	V_d (ml/kg)	CL (ml/h/kg)
VN/14-1	4.32	0.50	0.34	5.81	852	1,720
VN/50-1	2.31	0.83	0.57	5.20	1,594	1,924
VN/66-1	12.32	1.23	0.85	41.30	299	242

C_{max} maximum plasma concentration, t_{max} time taken to achieve C_{max}, $t_{1/2}$ elimination half-life, AUC area under the curve, V_d volume of distribution, CL clearance

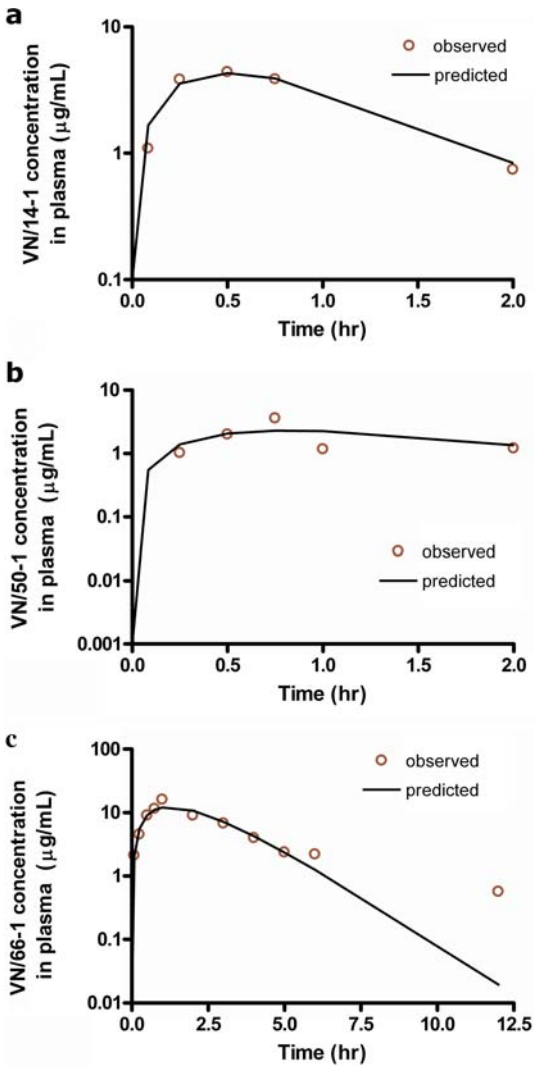

Fig. 2 Plasma concentration–time profiles of VN/14-1 (**a**), VN/50-1 (**b**), and VN/66-1 (**c**) after s.c. administration of a 10 mg/kg dose in Balb/c mice. Observed values are presented with the predicted values. Values represent the mean plasma concentrations from two mice per time point

for each clinical observation, including weight loss. Interestingly, both of these compounds displayed higher toxicity than their parent compound ATRA, which was used as a reference. VN/67-1 displayed relatively low toxicity and was very similar but not as good as VN/66-1 and VN/69-1. These results suggest that analogs of 4-HPR exhibit the lowest toxicity. Furthermore, the two most toxic compounds, VN/14-1 and VN/50-1 and the least toxic compound, VN/66-1 are the three which showed the greatest ability to inhibit breast and prostate cancer cell proliferation as determined by previous experiments in our lab. It is for this reason that VN/14-1, VN/50-1, and VN/66-1 were chosen for the PK study. At the conclusion of the toxicity study, it was found that ATRA, VN/66-1, VN/67-1, and VN/69-1 all had a MTD of 100 μmol/kg/day. VN/14-1 and VN/50-1, the most toxic RAMBAs, were found to have a MTD of 33 μmol/kg/day. However, it should be noted that we have shown in recent anti-tumor xenograft studies in nude mice that VN/14-1 up to 66 μmol/kg/day was not toxic [3, 19, 20]. In contrast, R116010, a non-retinoidal RAMBA in clinical develop was found to have a MTD of 5 mg/kg or 13.25 μmol/kg in syngeneic A/J mice [25].

Pharmacokinetic studies were carried out with VN/14-1, VN/50-1, and VN/66-1 after a single subcutaneous dose of 10 mg/kg. Though VN/14-1 and VN/50-1 showed high toxicity, we wanted to examine the difference in the PK of these two compounds and VN/66-1, a non-toxic compound. The PK parameters for VN/14-1 in mice observed in this study were similar to those reported previously by us in Sprague–Dawley rats [29]. Elimination as measured by CL and $t_{1/2}$ in Balb/c mice was found to be the most favorable for VN/66-1, an analog of 4-HPR. This compound not only had the longest half-life and slowest clearance it also had the greatest exposure when compared to VN/14-1 and VN/50-1. VN/66-1 also showed one of the lowest levels of toxicity out of all the compounds. This result is expected, because 4-HPR itself shows lower clinical toxicity than ATRA [22], thus its analog is also expected to have lower toxic effects than analogs of ATRA and 13-*cis* RA which have very similar structures.

Determining both the toxicity and PK parameters of VN/14-1, VN/50-1, and VN/66-1 will allow us to optimize these compounds in terms of their chemistries, in vivo action and dosing schedules. This comparison would not have been as strong if the PK of only the non-toxic compounds was determined. The toxicological profiles of these RAMBAs appear to be related to the chemical nature of the terminal polar groups of the retinoid side chain. The particularly toxic nature of VN/50-1 may be attributed to the propensity for imidazole amides to undergo facile nucleophilic substitution reactions [21]. Based on these results it would be beneficial to examine the effect of VN/66-1 in various cancer xenograft models and carry out further experiments to determine its mechanism of action. As stated above, VN/14-1 has been shown to be non-toxic up to 66 μmol/kg/day. We also envision that an isosteric replacement for the carboxylic acid moiety of VN/14-1 with 5-substituted-1H-tetrazole would yield its metabolism-resistant analog that is expected to be significantly less toxic than VN/14-1 [10].

In conclusion, a combined evaluation of toxicity and PK of selected RAMBAs has allowed us to select lead candidates for further development as anti-cancer

agents. VN/14-1 and VN/66-1 have been chosen for further studies for the reasons stated above. That is, their significantly superior efficacies in breast or prostate in vitro and in vivo cancer model systems.

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Novel retinoic acid metabolism blocking agents have potent inhibitory activities on human breast cancer cells and tumour growth

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Antitumour effects of retinoids are attributed to their influence on cell proliferation, differentiation, apoptosis and angiogenesis. In our effort to develop useful agents for breast cancer therapy, we evaluated the effects of four representative retinoic acid metabolism blocking agents (RAMBAs, VN/14-1, VN/50-1, VN/66-1 and VN/69-1) on growth inhibition of oestrogen receptor positive (ER +ve, MCF-7 and T-47D) and oestrogen receptor negative (ER −ve, MDA-MB-231) human breast cancer cells. Additionally, we investigated the biological effects/molecular mechanism(s) underlying their growth inhibitory properties as well as their antitumour efficacies against MCF-7 and MCF-7Ca tumour xenografts in nude mice. We also assessed the effect of combining VN/14-1 and all-*trans*-retinoic acid (ATRA) on MCF-7 tumuor xenografts. The ER +ve cell lines were more sensitive (IC_{50} values between 3.0 and 609 nM) to the RAMBAs than the ER −ve MDA-MB-231 cell line ($IC_{50} = 5.6–24.0\ \mu M$). Retinoic acid metabolism blocking agents induced cell differentiation as determined by increased expression of cytokeratin 8/18 and oestrogen receptor-α (ER-α). Similar to ATRA, they also induced apoptosis via activation of caspase 9. Cell cycle analysis indicated that RAMBAs arrested cells in the G1 and G2/M phases and caused significant downregulation (>80%) of cyclin D1 protein. *In vivo*, the growth of MCF-7 mammary tumours was dose-dependently and significantly inhibited (92.6%, $P<0.0005$) by VN/14-1. The combination of VN/14-1 and ATRA also inhibited MCF-7 breast tumour growth *in vivo* (up to 120%) as compared with single agents ($P<0.025$). VN/14-1 was also very effective in preventing the formation of MCF-7Ca tumours and it significantly inhibited the growth of established MCF-7Ca tumours, being as effective as the clinically used aromatase inhibitors, anastrozole and letrozole. Decrease in cyclin D1 and upregulation of cytokeratins, Bad and Bax with VN/14-1 may be responsible for the efficacy of this compound in inhibiting breast cancer cell growth *in vitro* and *in vivo*. Our results suggest that our RAMBAs, especially VN/14-1 may be useful novel therapy for breast cancer.
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The potential of all-*trans*-retinoic acid (ATRA) and other retinoids in prevention and therapy of a number of proliferative diseases including breast cancer was noted almost 20 years ago (Moon *et al*, 1985). The ability of ATRA as well as other natural and synthetic retinoids to modulate a variety of important functions such as cell growth and differentiation, induction of apoptosis and prevention of angiogenesis is well documented (Dragnev *et al*, 2000; Altucci and Gronemeyer, 2001; Boyle, 2001; Fontana and Rishi, 2002). 4-Hydroxyphenyl retinamide (4-HPR) – a synthetic retinoid – is a possible therapeutic option for early breast cancer in premenopausal women (Torrisi and Decensi, 2000).

All-*trans*-retinoic acid is currently being used in the treatment of acne and psoriasis. It is effective in chemotherapy of acute promyelocytic leukaemia and also inhibiting the *in vivo* development of carcinogen-induced carcinoma of the breast, bladder, liver, lung, pancreas, prostate, ovaries and skin (Miller, 1998; Altucci and Gronemeyer, 2001; Njar *et al*, 2006a, b). All-*trans*-retinoic acid and its isomers exert their action by binding to their nuclear receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Both the receptors have three subtypes α, β and γ that regulate the expression of a variety of genes. These receptors also heterodimerise with other nuclear steroid receptors that lead to transcription of various genes.

Although numerous studies have shown the anticancer effect of ATRA against different neoplasms, the clinical use of ATRA has been thwarted by the emergence of resistance (Wouters *et al*, 1992; Van Huesden *et al*, 2002). Several molecular mechanisms may underlie ATRA resistance, including (1) downregulated expression or mutation of RARα or -β receptors (Lin *et al*, 2000; Tanaka *et al*, 2004); (2) different levels of expressions of cellular retinoic acid binding proteins (CRABP I and II) (Arapshian *et al*, 2004) leading

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to rapid metabolism of ATRA (Budhu and Noy, 2002); (3) overexpression of HER2/Grb2/Akt pathway (Mendoza-Gamboa *et al*, 2004); and (4) metabolism of ATRA by cytochrome *P*450 (CYP)-dependent ATRA 4-hydoxylase enzymes namely CYP26 family, CYP2C8 and CYP3A4 (McSorley and Daly, 2000; Marill *et al*, 2002; Njar, 2002; Njar *et al*, 2006a, b). All-*trans*-retinoic acid is responsible for inducing the expression of cytochrome *P*450 enzymes leading to its catabolism. The physiologically most prominent pathway for ATRA metabolism starts with the hydroxylation at the C-4 position of the cyclohexenyl ring leading to the formation of 4-hydroxy-ATRA and other metabolic by-products. This tightly controlled negative feedback mechanism limits the availability of ATRA and consequently its biological activity. Therefore, the strategy to increase intracellular levels of ATRA by inhibition of its metabolism is considered an innovative approach for cancer therapy. Inhibitors of ATRA metabolism are also called retinoic acid metabolism blocking agents (RAMBAs).

Whereas several categories of nonretinoidal RAMBAs have been reported (reviewed in Njar, 2002; Njar *et al*, 2006a), only a few retinoidal RAMBAs developed by our group are known. Our RAMBAs are also considered to be *atypical*, because in addition to being potent inhibitors of ATRA metabolism (able to enhance the antiproliferative action of ATRA), they also possess intrinsic potent cancer antiproliferative activities (Njar *et al*, 2000, 2006a; Njar, 2002; Patel *et al*, 2004; Huynh *et al*, 2006; Belosay *et al*, 2006). We have also found that our RAMBAs appear to have different potencies in different types of human cancer cell lines. For example, VN/14-1 is a weak inhibitor of several prostate cancer cells but the compound has been identified as our most potent RAMBA in several breast cancer cell lines (Patel *et al*, 2004; Belosay *et al*, 2006; Huynh *et al*, 2006). Here, we present data on growth inhibition, molecular mechanisms/biological effects and *in vivo* antitumour effects of these new RAMBAs against breast cancer cell lines and tumours. We report that our RAMBAs are capable of inducing differentiation, apoptosis, affecting the cell cycle proteins, as well as altering the expression of oestrogen receptor-α (ER-α) in breast cancer cells. In addition, we have identified VN/14-1 as a highly potent suppressor of growth of human MCF-7 and MCF-7Ca tumour xenograft (derived from MCF7Ca cells, that is human MCF-7 human breast cancer cells stably transfected with human aromatase gene) in female nude mouse model that is more effective than either ATRA or 4-HPR. VN/14-1 is also as effective as clinically used aromatase inhibitors (AIs) and caused prevention of MCF-7Ca tumour formation.

MATERIALS AND METHODS

Material for cell culture

Improved Dulbecco's modified Eagle's medium (IMEM), RPMI 1640 medium, OPTI-MEM I (low-serum medium), Dulbecco's phosphate-buffered saline (PBS), trypsin/EDTA solution and penicillin/streptomycin were purchased from GIBCO (Invitrogen Corporation, Grand Island, NY, USA). Regular fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). Tissue culture flasks (T-25, T-75 and T-150), six-well plates and 24-well plates were obtained from Corning Incorporated (Corning, NY, USA).

Cell lines

Hormone-dependent/oestrogen receptor positive (ER +ve) MCF-7 cells were a generous gift from Dr Richard Santen (University of Virginia health system, Charlottesville, VA, USA), whereas MDA-MB-231 (oestrogen receptor negative (ER −ve)) cells were purchased from American type cell culture (ATCC). MCF-7 and MDA-MB-231 cells were cultured in IMEM with glutamine and phenol red, supplemented with 5% FBS and 1% penicillin–streptomycin. MCF-7Ca cells were cultured in Dulbeco's modified Eagle's medium supplemented with 5% FBS, 1% penicillin–streptomycin, 700 μg ml^{-1} G_{418}. Cells were grown as a monolayer in T75 or T150 tissue culture flasks in a humidified incubator (5% CO_2, 95% air) at 37°C. Hormone-dependent/ER +ve T47D cells were purchased from ATCC, and cultured in RPMI 1640 medium with glutamine, supplemented with 10% FBS and 1% penicillin–streptomycin. Cells were grown as a monolayer in T75 or T150 tissue culture flasks in a humidified incubator (5% CO_2, 95% air) at 37°C.

Chemicals/test compounds

All-*trans*-retinoic acid was purchased from Sigma Chemical Company (St Louis, MO, USA) and LKT laboratories Inc. (St Paul, MN, USA), whereas [11,12-^{3}H]ATRA was purchased from Perkin Elmer Life Sciences Inc. (Boston, MA, USA). Retinoic acid metabolism blocking agents (VN/14-1, VN/50-1, VN/66-1 and VN/69-1) (Figure 1) were designed and synthesised by us (Njar *et al*, 2000; Patel *et al*, 2004). 4-Hydroxyphenyl retinamide (fenretinide) is available commercially, but was also synthesised in our laboratory. 4-Hydroxyphenyl retinamide was prepared by a literature method (Moon *et al*, 1979), provided spectral and analytical data as described (Moon *et al*, 1979).

Antibodies for Western immunoblotting

Antibodies against cytokeratin 8/18 (CK 8/18), E-cadherin and ER-α were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) Antibodies against poly ADP ribose polymerase (PARP), Cyclin- D1, Caspase-9 and Bad were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibody for β-actin was obtained from Calbiochem (San Diego, CA, USA). Secondary anti-mouse IgG horseradish peroxidase (HRP) conjugate antibody was purchased from Calbiochem and Bio-Rad. Secondary anti-rabbit IgG HRP conjugate antibody was purchased from Bio-Rad as well as from Kirkegaard and Perry laboratories.

Animals and material for *in vivo* studies

Ovariectomised female athymic nude mice (4–6-week old) used for tumour xenograft studies were obtained from NCI/FDRC. The mice were maintained in controlled environment with food and water *ad libitum*. Oestrogen pellets (1.7 mg per pellet, 90 day release) were purchased from Innovative Research of America (Sarasota, FL, USA). Matrigel was purchased from BD Biosciences (Bedford, MA, USA). All the animal studies were performed according to the guidelines and approval of the animal care committee of the University of Maryland School of Medicine, Baltimore, and were consistent with United Kingdom Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia.

Cell growth inhibition assays (MTT colorimetric assay)

These assays were performed as described previously (Patel *et al*, 2004).

Retinoid receptor binding assays

To determine the IC_{50} values for each RAMBA, competition binding experiments were performed as described previously (Soprano *et al*, 2000; Zhang *et al*, 2003). The full-length cDNA clones for RARα, RARβ, RARγ and RXRα were cloned into the prokaryotic expression vector pET29 and recombinant S-Tag protein for each RAR subtype, and RXR were prepared as described previously (Tairis *et al*, 1995; Scafonas *et al*, 1997).

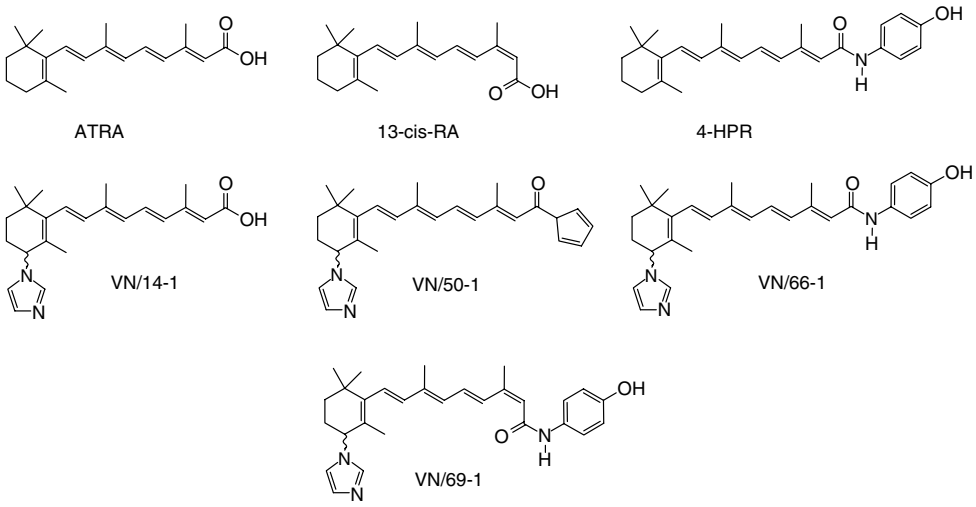

Figure 1 Structures of retinoic acids, RAMBAs and retinoid 4-HPR.

Competition binding experiments were performed using a single concentration of 1.0 nM [^{3}H]-all-*trans*-RA (1.82–1.92 TBq mmol^{-1} or 49.2–52.0 Ci mmol^{-1}; Dupont (Boston, MA, USA) NEN) for RARα, RARβ and RARγ or 1.0 nM [^{3}H]-9-*cis*-RA (1.74 TBq mmol^{-1} or 47.2 Ci mmol^{-1}; Amersham, Piscataway, NJ, USA) for RXRα and various concentrations of each RAMBA ranging from 1 nM to 1 μM. IC_{50} values are the concentration of each VN compound that reduced binding of either [^{3}H]-all-*trans*-RA (RARs) or [^{3}H]-9-*cis*-RA (RXRα) by 50%.

Transactivation assays

The ability of each RAMBA to function as a tanscriptional agonist of RARα, RARβ and RARγ was determined as described previously (Tairis *et al*, 1995; Scafonas *et al*, 1997; Soprano *et al*, 2000; Zhang *et al*, 2003). Briefly, CV-1 cells were cotransfected with 4 μg RAR subtype (RARα, RARβ or RARγ) expression vector construct in pSG5, 4 μg RARE-CAT reporter DNA and 1 μg pCMV-β-gal DNA. Twenty-four hours following transfection, the cells were treated with various concentrations of each RAMBA ranging from 10^{-11}–10^{-5} M along with an ethanol carrier control. Additional cells were treated with 10^{-6} M all-*trans*-RA for normalisation. Twenty-four hours later, the cells were harvested and CAT and β-gal activities were assayed. The EC_{50} value for each RAMBA represents the concentration of the compound that results in 50% of the maximal activity obtained with $10^{-}6$ M all-*trans*-RA.

Anti-AP1 assay

The ability of each RAMBA to inhibit AP1 activity was determined using anti-AP1 assays as we have described previously (Soprano *et al*, 2000). Briefly, CV-1 cells were cotransfected with 1.5 μg RAR subtype (RARα, RARβ and RARγ) expression vector construct in pSG5, 1.5 μg AP-1 CAT reporter DNA and 0.5 μg pCMV-β-gal DNA. Four hours following transfection, the cells were treated with one of the following treatments: ethanol alone, $10^{-}6$ M ATRA, $10^{-}6$ M VN/14-1 or 10^{-6} M ATRA + 10^{-6} M VN/14-1. Twenty-four hours later, the cells were harvested and CAT and β-gal activities were assayed. The percentage of AP-1 activity was calculated using the ethanol carrier control sample as 100%.

CRABP binding assays

Wild-type full-length cDNAs for mouse CRABPI and CRABPII were subcloned into the prokaryotic expression vector pRSETB and transformed into *Escherichia coli* strain BL21(DE3)pLys (Chen *et al*, 1995). Recombinant fusion proteins were prepared essentially as we have described previously using Ni-NTA resin (Chen *et al*, 1995). The binding of each RAMBA to CRABPI and CRABPII was determined by competition binding assays as described previously (Chen *et al*, 1995). Briefly, 50 nM CRABPI or CRABPII protein was incubated with 5 or 25 nM [^{3}H]-all-*trans*-RA (1.82–1.92 TBq mmol^{-1} or 49.2–52.0 Ci mmol^{-1}; Dupont NEN) (near the K_d value for CRABPI or CRABPII, respectively) and various concentrations of each RAMBA ranging from 1 to 500 nM. IC_{50} values were calculated as described above in the RAR binding studies.

Determination of apoptosis in breast cancer cells

TUNEL assay using in situ *cell death detection kit, alkaline phosphatase* Breast cancer cells (5000 cells per chamber) were plated on an eight-chamber slide (Nunc lab-Tek chamber slide system (Fisher Scientific, Pittsburgh, PA, USA). Cells were allowed to adhere for about 24 h and then treated with different concentrations (1 and 5 μM) of ATRA or RAMBAs. The medium and drug were renewed on day 3. After 6 days of treatment, the medium was aspirated; cells were washed with PBS and then fixed with 4% para formaldehyde for about 45 min on the slides. After fixing, the cells were washed with PBS and stored at 4°C until further staining. The cells were then permeabilised using permeabilisation solution containing 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice and then treated with the TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labelling) reaction mixture containing the enzyme terminal transferase and the label solution for 60 min at 37°C in a humidified atmosphere in the dark according to the protocol of *in situ* cell death detection kit, AP (Roche Diagnostics, Basel, Switzerland, Germany). The cells were rinsed with PBS and then the stained-labelled apoptotic cells were mounted using DAPI (diamidino-2-phenylindole 2HCl)-glycerol and visualised with a fluorescent microscope using two filters (1) for TUNEL stained cells, and (2) for DAPI stained cells, which represents all the cells (nuclei). Fluorescent microscope model was Nikon, Eclipse E400 and the software was SPOT advanced version 3.5.5 by Diagnostic Instruments Inc. (Sterling Heights, MI, USA).

Preparation of cell lysates

Breast cancer cells (MCF-7 and T47D) were treated with different concentration (1, 5 or 10 μM) of ATRA or RAMBAs or untreated in

T-75 or T-150 flasks for 6 days. At the end of the treatment, cells were scrapped and collected by washing with ice-cold PBS in a centrifuge tube. Cells were centrifuged at 2500 r.p.m. for 10 min, the pellet was again washed with ice-cold PBS and centrifuged. The cell pellet was then suspended in chilled cell lysis buffer (0.1 M Tris–HCl, 0.5% Triton X-100, protease inhibitor cocktail) and sonicated on ice for 10–15 s. The homogenate was kept on ice for 30 min and then centrifuged at 13 000 r.p.m. for 30 min. The supernatant was used as cell lysate. The cell lysates were stored at −80°C. Protein concentration of the cell lysates was determined using Bio-Rad protein assay reagents.

Gel electrophoresis and Western immunoblotting

Protein lysates from MCF-7 and T47D cells were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted for different proteins. Equal amounts of protein (25–50 μg) were subjected to SDS–PAGE at 60 V for 3 h using the mini PROTEAN3 electrophoresis module assembly (Bio-Rad, Hercules, CA, USA), and transferred to nitrocellulose membranes (Hybond ECL, Amersham). Immunodetections were carried out using antibodies against CK 8/18 (1° antibody – 1 : 8000 in 10% milk PBST), E-cadherin (1° antibody – 1 : 1000 in 5% milk in TBST) and ER-α (1° antibody – 1 : 200 in 5% milk PBST) purchased from Santa Cruz Biotechnology Inc. Antibodies against PARP (1° antibody – 1 : 1000 in 5% milk TBST), cyclin- D1 (1° antibody – 1 : 2000 in 10% milk TBST), caspase-9 (1° antibody – 1 : 1000 in 10% milk TBST) and BAD (1° antibody – 1 : 1000 in 5% BSA TBST) were obtained from Cell Signaling Technology. Antibody for β-actin was obtained from Calbiochem (CA, USA). Secondary anti-mouse IgG HRP conjugate antibody was purchased from Calbiochem and Bio-Rad. Secondary anti-rabbit IgG HRP conjugate antibody was bought from Bio-Rad as well as from Kirkegaard and Perry laboratories (Gaithersburg, MD, USA). Immunoreactive bands were visualised using the enhanced chemiluminescence detection reagents and analysis system (Amersham Bioscences) according to the manufacturer's instructions and quantified by densitometry using ImageQuant software version 5.

Cell cycle analysis

MCF-7 and T47D cells were plated in T-25 flasks and allowed to adhere for about 18 h. The cells were then synchronised by growing them in low-serum OPTI-MEM I medium for 2 days. Then the cells were treated in regular medium for 6 days with different concentrations (1 and 5 μM) of ATRA or RAMBAs with renewal of drug and media on day 3. After 6-day treatment, the cells were trypsinised, washed twice with PBS, centrifuged at 2500 r.p.m. to obtain a cell pellet. The cells (about $1-2\times10^6$) were fixed with 3 ml of 70% ethanol (by adding drop-wise and taking care that the cells are not clumped together). Cells were kept at −20°C until further analysis (at least overnight). Next, cells ($\sim1-2\times10^6$) were stained with 1 ml of propidium iodide (PI) (50 μg ml^{-1})/RNase (100 μg ml^{-1}) solution and after about half an hour the fluorescence of stained nuclei (15 000 events) was analysed by flow cytometry (Becton Dickinson FACScan Instrument and quantification by Modfit LT version 3.1 software) with excitation wavelength of 488 nm. The percentage of G1, S, G2/M and sub-G1 (apoptotic cells) in the population of treated and untreated cells were determined.

In vivo antitumour studies

All the animal studies were performed according to the guidelines and approval of the Animal Care Committee of the University of Maryland, School of Medicine. Ovariectomised female athymic nude 4–6-week-old mice were used. Oestrogen pellets (Wen *et al*, 2000; Patel *et al*, 2004) were implanted in the dorsal interscapular region of the mice using a trochar to facilitate tumour growth. Ovariectomised mice were then inoculated with MCF-7 cells (2×10^6 cells in Matrigel per tumour growth site) subcutaneously (s.c.) on the right and left flank. Tumour volumes were measured weekly with calipers. When the tumour volumes reached 200–300 mm^3, the mice were grouped into control and treatment groups ($n=5$) (Yue *et al*, 1994; Wen *et al*, 2000; Patel *et al*, 2004). Tumour volumes were similar in each group at the start of the treatment. Retinoic acid metabolism blocking agents were formulated in 0.3% HPC (hydroxy propyl cellulose). We used doses equivalent to 5, 10 and 20 mg kg^{-1} of ATRA, that is 16.5 (VN/14-1), 33.0 (ATRA, VN/14-1, VN/50-1 and 4-HPR) and 66.0 μmol kg^{-1} (VN/14-1 and VN/66-1), respectively, in 200 μl of vehicle s.c. injection, once per day. Compounds were injected continuously for three days with a drug holiday of one day after every 3 days of treatment. Twice per week, the mice were weighed and tumours were measured using a caliper. Tumour volume was calculated according to the formula $4/3\pi r_1^2 r_2$ ($r_1<r_2$). The tumour treatment study was continued for 6 weeks. At the end of 6 weeks, mice were killed and blood plasma, as well as tumours, were collected, weighed and stored at −80°C until analysis.

The second experiment was similar to that described above, but consisted of only four groups. When the tumour volume has reached about 100 mm^3, the mice were randomly divided in to four groups of five mice each. The control group received vehicle, whereas the other three groups received ATRA (33.0 μmol kg^{-1} day^{-1}), VN/14-1 (16.5 μmol kg^{-1} day^{-1}) or ATRA (33.0 μmol kg^{-1} day^{-1}) + VN/14-1 (16.5 μmol kg^{-1} day^{-1}). These treatments continued for 36 days and the tumours were measured and processed as described above.

The third *in vivo* antitumour experiment was conducted with MCF-7Ca tumours. MCF-7Ca were provided by Dr S Chen (City of Hope, Duarte, CA, USA). The tumour xenografts were grown in mice as described previously (Yue *et al*, 1994). Subconfluent cells were scraped into Delbecco's phosphate-buffered saline, collected by centrifugation and resuspended in Matrigel (10 mg ml^{-1}) at 2.5×10^7 cells ml^{-1}. Each mouse received s.c. inoculations in two sites per flank with 100 μl of cell suspension. Mice in the tumour formation prevention group ($n=5$) were than injected daily with androstenedione (100 μg day^{-1}) plus VN/14-1 (20 mg kg^{-1} day^{-1}) in the vehicle for the duration of the experiment. The rest of the mice were injected daily with 100 μg day^{-1} of androstenedione. Measurements and treatments began when the tumours reached approximately 300 mm^3, about 6 weeks after cell inoculation. Mice were assigned to groups for treatment so that there was no statistically significant difference in tumour volumes among the groups at the beginning of the treatment. Tumours were measured and volumes calculated as described above. Mice were than injected s.c. daily with the indicated agents: 100 μg per mouse per day (5 × weekly) plus 10 μg per mouse per day (5 × weekly) of letrozole or 200 μg per mouse per day (5 × weekly) of anastrozole and 20 mg kg^{-1} day^{-1} (5 × weekly) of VN/14-1. The doses of letrozole, anastrozole, androstendione and VN/14-1 used are as determined previously (Long *et al*, 2002, 2004; Patel *et al*, 2004). The mice were treated for the indicated times, after which they were killed by decapitation and blood collected. Tumours and uteri were excised, cleaned, weighed and stored at −80°C for additional analyses.

Statistical analysis

The statistical differences among the groups were analysed using Student's *t*-test on Sigma Plot 2000 software or Mann–Whitney *U*-test using GraphPad Prism 4 for tumour statistics. Differences were considered to be statistically significant when $P<0.05$.

RESULTS

Effects of RAMBAs on MCF-7, T-47D and MDA-MB-231 cell growth

We have recently reported the synthesis, CYP26 inhibitions and effects of our novel RAMBAs on the growth of human breast and prostate cancer cell lines (Patel *et al*, 2004). These inhibitory effects of our lead RAMBAs (VN/14-1, VN/50-1, VN/66-1 and VN/69-1), ATRA and 4-HPR (Figure 1) on three breast cancer cells were further investigated in the present study with similar results. The data are summarised in Table 1, and reveal that these RAMBAs potently inhibited the growth of the two ER +ve human breast cancer cell lines (MCF-7 and T-47D) with IC_{50} values in the nanomolar range, with the T-47D cell being more sensitive. VN/14-1 is also a potent inhibitor of proliferation of MCF-7 cells stably transfected with human aromatase gene (MCF-7Ca) and also of the ER +ve letrozole resistant long-term letrozole cultured breast cancer cells (Belosay *et al*, 2006). The ER −ve MDA-MB-231 breast cancer cells were less sensitive to these agents (Table 1). The differential growth inhibitory effects of VN/14-1 are represented in Figure 2. These promising results with the ER +ve breast cancer cells prompted the present study, which examined the molecular mechanisms/biological effects underlying their growth inhibitory potencies. It should be stated that some of the procedure used in this study were identical to those utilised previously (Patel *et al*, 2004; Huynh *et al*, 2006). Except where otherwise stated, all cells were assessed following 6 days of ATRA/RAMBAs treatments. We point out that several researchers (Bollag *et al*, 1997; Toma *et al*, 1997) including us (Patel *et al*, 2004) have found that induction of several biochemical pathways in most cancer cell line become evident only after 6 days of ATRA/retinoid treatment.

Table 1 Inhibitory concentrations (IC_{50} values) of RAMBAs and reference compounds, ATRA and 4-HPR, on the growth of human breast cancer cells

	Cellular IC_{50} (nM)[a,b]		
Compound	**MCF-7**	**T47D**	**MDA-MB-231**
VN/14-1	493.5	3.0	24 000.0
VN/50-1	125.9	9.0	—
VN/66-1	590.5	569.9	5620.0
VN/69-1	609.0	—	—
For comparison			
ATRA	585.2	8.0	22 000.0
4-HPR	147.8	—	1402.0

ATRA = all-*trans*-retinoic acid; RAMBAs = retinoic acid metabolism blocking agents; 4-HPR = 4-hydroxyphenyl retinamide. [a]Data from Patel *et al*. (2004). [b]The IC_{50} values were determined from dose–response curves (by a nonlinear regression analysis using GraphPad Prism) compiled from at least two independent experiments and represents the compound concentration (nM) required to inhibit cell proliferation by 50%; — = not determined.

Effects of ATRA and RAMBAs on binding to CRABPs and retinoid receptors (RAR and RXR) and on transcriptional activation of RAR receptors

The ability of each of the four RAMBAs to bind CRABPs I and II and nuclear retinoid receptors was examined by competition bindings assays (Table 2). Only VN/14-1 displayed significant

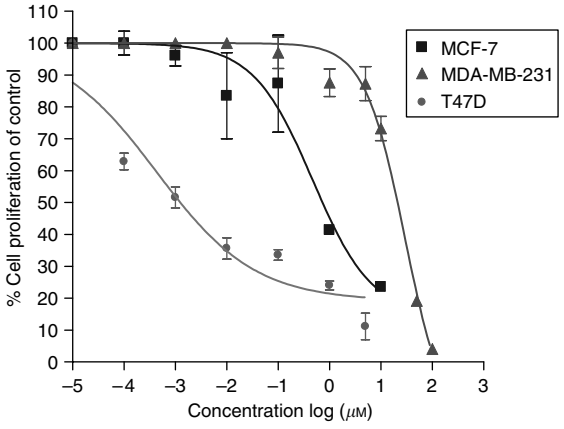

Figure 2 The effects of RAMBA VN/14-1 on proliferation of three human breast cancer cell line. For MCF-7 and T47D cells, 24-well plates were used and for MDA-MB-231 cells, 96-well plates were used. Cells (10 000 cells per well of a 24-well plate and 1000 cells per well of a 96-well plate) were plated and allowed to attach for 24 h. Cells were treated with VN/14-1 dissolved in 95% ethanol on day 1 and day 4 and analysed on day 7 by MTT assay using the spectrophotometer (Victor 1420 multi-label counter, Wallac (Perkin Elmer, Waltham, MA, USA)). For each concentration of the drug there were triplicate wells (24-well plate) and six wells (96-well plate) in every individual experiment. The data presented are mean ± s.e.m. for 2–3 experiments. IC_{50} values were calculated by nonlinear regression analysis using GraphPad Prism software.

Table 2 Binding activities (IC_{50} values) and transactivation activities (EC_{50} values) of RAMBAs to CRABPs, RARs and RXRα

RAMBAs	**CRAPBI**	**CRABPII**	**RARα**		**RARβ**		**RARγ**		**RXRα**
	IC_{50}[a]	IC_{50}[a]	IC_{50}[a]	EC_{50}[a]	IC_{50}[a]	EC_{50}	IC_{50}[a]	EC_{50}	IC_{50}[a]
VN/14-1	NB[b]	NB[b]	16	300	200	1500	16	1000	NB[b]
VN/50-1	NB[b]	NB[b]	NB[c]	>10 000	NB[c]	10 000	NB[c]	>10 000	NB[b]
VN/66-1	NB[b]	NB[b]	NB[c]	>10 000	NB[c]	10 000	NB[c]	>10 000	NB[b]
VN/69-1	NB[b]	NB[b]	NB[c]	>10 000	NB[c]	>10 000	NB[c]	>10 000	NB[b]
For comparison									
	K_d[a]	K_d[a]	K_d[a]	EC_{50}[a]	K_d[a]	EC_{50}[a]	K_d[a]	EC_{50}[a]	K_d[a]
ATRA	4	60	2	20	2	20	2	20	ND
9-CRA	ND	ND	ND	ND	ND	ND	ND	ND	7

CRABPs = cellular retinoic acid binding proteins; RAMBAs = retinoic acid metabolism blocking agents; RARs = retinoic acid receptors; RXRα = retinoid X receptors-α; 4-HPR = 4-hydroxyphenyl retinamide. [a]Values are indicated as nM. [b]NB = No binding up to 500 nM. [c]NB = No binding up to 1000 nM. ND = not determined. IC_{50} values are the concentration of each VN compound that reduced binding of either [^{3}H]-all-*trans*-RA (CRABPs and RARs) or [^{3}H]-9-*cis*-RA (RXRα) by 50%. These values were determined from dose–response curves compiled from at least three independent experiments.The EC_{50} value for each RAMBA represents the concentration of the compound that results in 50% of the maximal activity obtained with 10^{-6} M all-*trans*-RA. These were also determined from dose–response curves compiled from at least two independent experiments. Details of these experiments are described under Materials and Methods.

specific binding to each of the RAR subtypes with IC_{50} values ranging from 16 nM for both RARα and RARγ to 200 nM for RARβ. None of the four RAMBAs displayed binding to CRABPI, CRABPII and RXRα.

We then examined the functional activity of each of the four RAMBAs in transactivation assays with RARα, RARβ and RARγ (Table 2). Again only VN/14-1 induced RAR-dependent transcriptional activity with EC_{50} values of 300 nM for RARα, 1500 nM for RARβ and 1000 nM for RARγ. Since VN/14-1 displayed agonistic activity with each RAR subtypes, we examined the ability of VN/14-1 to inhibit AP1 activity. VN/14-1 at a concentration of 10^{-6} M displayed weak anti-AP1 activity reducing AP1-dependent transcription activity when each of the three RAR subtypes were cotransfected by 20–40%.

Effect of ATRA, 4-HPR and RAMBAs on the expression levels of CK 8/18 and ER-α in MCF-7 and T47D cells

The effects of these agents on the expression levels of markers associated with differentiation (CK 8/18 and ER-α) were investigated following established procedures (Jing *et al*, 1996; Korsching *et al*, 2002; Kim and Freeman, 2003; Woelfle *et al*, 2004). Breast cancer cells were treated with the indicated concentrations of ATRA or RAMBAs (VN/14-1, VN/50-1, VN/66-1 and VN/69-1) for 6 days or 4HPR for 4 days with renewal of media and compounds on day 3. At the end of the treatment, cell lysates were prepared and the expression of cytoskeletal proteins CK 8/18 were evaluated by Western immunoblotting. As shown in Figures 3A and B, expressions of CK 8/18 were increased in both MCF-7 and T47D cells after treatments with ATRA and RAMBAs. Treatments with 1 and 5 μM concentrations of the compounds (ATRA, VN/14-1 and VN/50-1) increased ~2–3-fold the expression of these proteins as compared with the control. However, no further up-modulation of theses markers was observed at higher concentrations of the RAMBAs. Unexpectedly, VN/66-1 and VN/69-1 caused down-regulation in the expression of CK 8/18.

An analogous set of experiments was performed to assess the effects of ATRA (1–10 μM) and VN/14-1 (1–10 μM) on the level of ER-α expression in MCF-7 cells. As demonstrated in Figure 4, there was a significant dose-dependent increase in the expression of ER-α elicited by both ATRA (from two- to eight-fold) and VN/14-1 (from four- to six-fold). Increase in ER is consistent with more differentiation. Together, these data suggest that the growth inhibitory effects of some of our RAMBAs may in part be due to their ability to induce differentiation in these two breast cancer cell lines.

Effects of RAMBAs on cell cycle phase distribution and expression of cyclin D1

To further investigate the causes of the antiproliferative effects of RAMBAs, with ATRA and 4-HPR as positive controls, cell cycle analysis was performed on both MCF-7 and T47D cells treated with

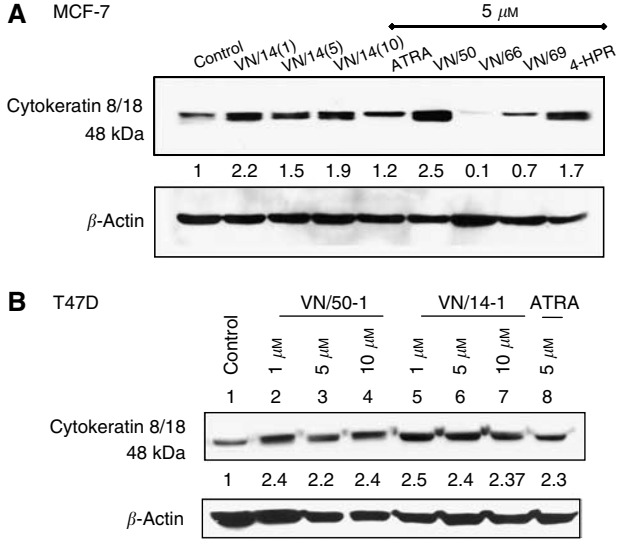

Figure 3 Western immunoblotting analysis of whole-cell lysates of treated MCF-7 and T47D cells for the expression of CK 8/18: (**A**) MCF-7 cells were treated with ATRA or RAMBAs for 6 days or 4HPR for 4 days and then cell lysates were electrophoresed using 10% SDS–PAGE and subjected to Western blotting. Lane 1: control; lanes 2–4: VN/14-1 (1, 5 and 10 μM); lane 5: ATRA (5 μM); lane 6: VN/50-1 (5 μM); lane 7: VN/66-1 (5 μM); lane 8: VN/69-1(5 μM); and lane 9: 4-HPR (5 μM). (**B**) T47D cells were treated with ATRA or RAMBAs (VN/50-1 and VN/14-1) for 6 days and then cell lysates were electrophoresed using 10% SDS–PAGE and subjected to Western blotting. Lane 1: control; lane 2: VN/50-1 (1 μM); lane 3: VN/50-1 (5 μM); lane 4: VN/50-1 (10 μM); lanes 5–7: VN/14-1 (1, 5 and 10 μM); and lane 8: ATRA (5 μM). Numbers below the blot show fold increase in expression of the protein as analysed by ImageQuant densitometry analysis. Membranes were stripped and probed for β-actin to verify equal protein loading. Cytokeratin 8/18 is a 48 kDa protein. Primary antibody CK 8/18 (Santa Cruz Biotechnology) 1 : 8000 in 10% milk in PBST for 1 h, and secondary antibody (anti-mouse) 1 : 2000 in 10% milk in PBST for 1 h at room temperature. The experiments were repeated thrice with similar results.

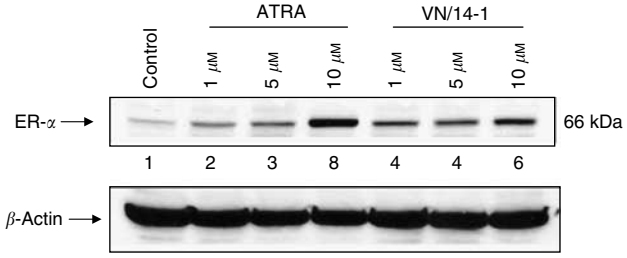

Figure 4 Western immunoblotting analysis of whole-cell lysates of treated MCF-7 cells for the expression of ER-α. MCF-7 cells were treated with ATRA and VN/14-1 for 6 days and then cell lysates were electrophoresed using 10% SDS–PAGE and subjected to Western blotting. Lane 1: control; lanes 2–4: ATRA (1, 5 and 10 μM); and lanes 5–7: VN/14-1 (1, 5 and 10 μM). Numbers below the blot show fold increase in expression of the protein as analysed by ImageQuant densitometry analysis. Membrane was stripped and probed for β-actin to verify equal protein loading. Primary antibody (Santa Cruz Biotechnology), 1 : 200 in 5% milk PBST for 2 h at RT, secondary antibody (anti-rabbit) 1 : 3000 in 5% milk PBST for 1 h at RT. The experiment was repeated twice with similar results.

Table 3 Effects of RAMBAs, ATRA and 4-HPR on T47D cell cycle distribution

Treatment	Sub-G1 (%)	Go/G1 (%)	S (%)	G2/M (%)
Control	8.89±1.59	74.28±3.15	20.42±1.02	5.31±2.13
VN/14-1	22.38±2.95	77.98±2.56	1.18±1.0	20.85±3.75
VN/50-1	21.67±1.56	77.72±4.54	0.51	21.78±5.05
VN/66-1	26.41±1.18	80.44±0.20	0	19.57±0.21
VN/69-1	24.34±1.24	78.99±0.99	0	21.01±0.99
ATRA	17.12±4.06	83.89±0.96	3.1±1.3	13.03±4.06
4-HPR	30.25±6.66	76.94±3.47	6.32±0.93	16.75±4.39

ATRA = all-*trans*-retinoic acid; RAMBAs = retinoic acid metabolism blocking agents; 4-HPR = 4-hydroxyphenyl retinamide. T47D cells were treated with 5 μM of ATRA or RAMBAs for 6 days, or 4-HPR for 4 days. Percentage distribution of cells in each of the cell cycle phases are expressed as mean±s.e. of at least two independent experiments.

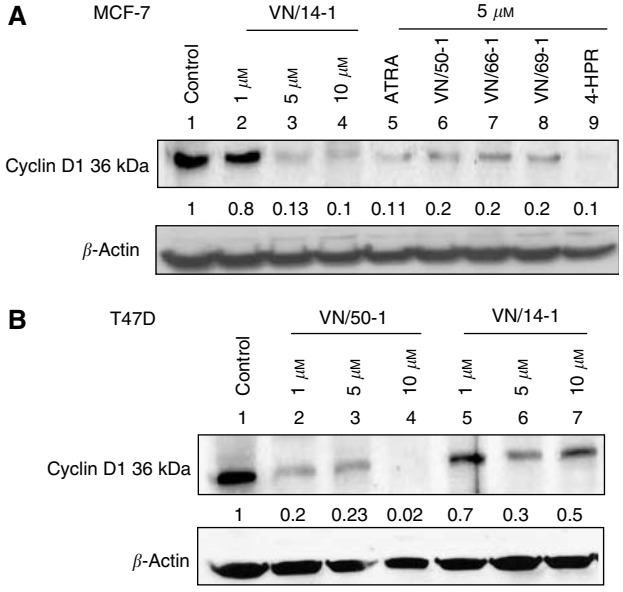

Figure 5 Western blotting of whole-cell lysates of treated MCF-7 and T47D cells for the expression of Cyclin D1. (**A**) MCF-7 cells were treated with ATRA or RAMBAs for 6 days or 4HPR for 4 days and then cell lysates were electrophoresed using 15% SDS–PAGE and subjected to Western blotting. Lane 1: control; lanes 2–4: VN/14-1 (1, 5 and 10 μM); lane 5: ATRA (5 μM); lane 6: VN/50-1 (5 μM); lane 7: VN/66-1 (5 μM); lane 8: VN/69-1(5 μM); and lane 9: 4-HPR (5 μM). (**B**) T47D cells were treated with VN/50-1 and VN/14-1 for 6 days and then cell lysates were electrophoresed using 15% SDS–PAGE and subjected to Western blotting. Lane 1: control; lanes 2–4: VN/50-1 (1, 5 and 10 μM); and lanes 5–7: VN/14-1 (1, 5 and 10 μM). Numbers below the blot show fold decrease in expression of the protein as analysed by ImageQuant densitometry analysis. Membranes were stripped and probed for β-actin to verify equal protein loading. Primary antibody (Cell Signaling Technology) 1 : 2000 in 10% milk TBST ovemight at 4°C and secondary antibody (anti-rabbit) 1 : 2000 in 10% milk TBST for 1 h at RT. The experiments were repeated twice with similar results.

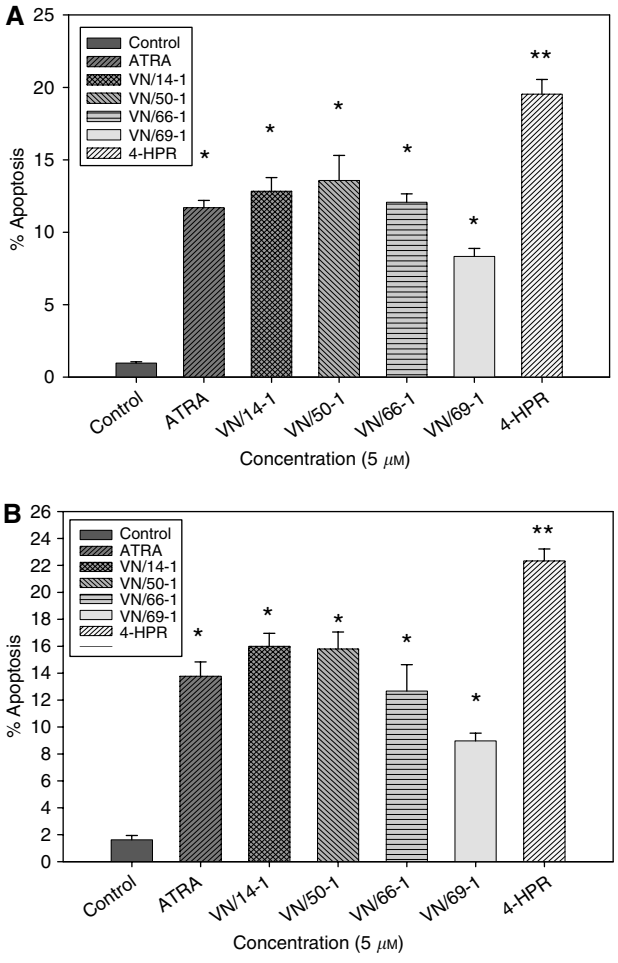

Figure 6 Graphs showing apoptosis induced in (**A**) MCF-7 and (**B**) T47D cells as determined by TUNEL. (**A**) MCF-7 cells and (**B**) T47D cells (4×10^4)/ well were plated in eight-well slide and treated with 5 μM of ATRA, RAMBAs and 4-HPR for 6 days (see Materials and Methods for details). TUNEL-stained apoptotic cells were counted against the DAPI stained cells to obtain percentage apoptosis. The experiments were repeated thrice with similar results. Error bars show s.e.m of three different fields of treated cells, statistically significant $^*P < 0.01$ and $^{**}P < 0.001$ *vs* control.

agents for 6 days. As described in Materials and Methods, cells were stained with PI and cell cycle analysis was performed by flow cytometry (Toma *et al*, 1997; Thiantanawat *et al*, 2003). Histograms were obtained from the analysis of both cell lines (data not shown) and the percentages of T47D cells in each cell cycle phase are presented in Table 3. The cell cycle profiles of the treated cells were more prominent in the T47D cells, as each treatment caused a significant increase in the percentage of cells in the G1 phase and decrease in the percentage of cells in the S phase, compared with the control. Among all treatments, VN/66-1 and VN/69-1 caused the greatest suppression (0%) of cells in the S phase, compared with 20.4% in the untreated control. These treatments also caused increases in the percentages of cells in the G2/M phase, but were more prominent in T47D than in the MCF-7 cells (data not shown). Furthermore, we also observed significant accumulation of cells in the sub-G1 phase of the treated cells, which may represent cells undergoing apoptosis (Table 3).

Given the well-established involvement of cyclins in the regulation of cell cycle progression and the previous findings that cyclin D1 is overexpressed in many cancers and cancer cell lines (Teixeira and Pratt, 1997; Zhou *et al*, 1997; Niu *et al*, 2001), we investigated the effects of our RAMBAs on the level of cyclin D1 expression. As shown in Figure 5A, ATRA, RAMBAs and 4-HPR, each significantly decreased cyclin D1 protein expression by >80% in MCF-7 cells as compared with untreated control. This decrease in the expression of cyclin D1 was also shown to be dose-dependent following treatment with VN/14-1. Similarly, the expression of cyclin D1 was significantly downregulated in T47D cells by treatments with VN/14-1 or VN/50-1 (Figure 5B).

Apoptosis induced by RAMBAs

Because ATRA and most retinoids control cell proliferation via apoptosis among several other mechanisms in a variety of cancers (Bollag *et al*, 1997; Toma *et al*, 1997; Kotake-Nara *et al*, 2002; Afonja *et al*, 2004; Simeone and Tari, 2004) and also because of the cell cycle analysis data that suggests accumulation of cell in the sub-G phase, the apoptotic potential of our RAMBAs was evaluated. Retinoic acid metabolism blocking agents-induced apoptosis in MCF-7 and T47D cells was examined in three independent experiments with ATRA and 4-HPR as positive controls. First, TUNEL assays, involving a fluorescent DNA-binding dye, DAPI (Gavrieli *et al*, 1992; Thiantanawat *et al*, 2003) were used to study the morphology of dying cells following treatment with 5 μM RAMBAs, ATRA or 4-HPR for 6 days. Cells undergoing apoptosis displayed the typical morphologic feature of apoptotic cells with condensed and fragmented nuclei, and in some

cases, membrane blebbing (data not shown). Quantifications of apoptosis caused by the various agents are presented in Figure 6A and B. Following treatment with ATRA and RAMBAs, induction of apoptosis was 8–13% in MCF-7 cells ($P<0.01$) and 9–17% in T47D cells ($P<0.01$) as compared with control which had about 1.5% apoptosis. However, 4HPR caused the most induction of apoptosis in both the cell lines (20–25%; $P<0.001$ *vs* control). These results strongly suggest that our RAMBAs possess intrinsic apoptotic activity.

Proteins involved in RAMBAs-induced apoptosis

To further characterise the apoptosis observed in the RAMBAs-treated breast cancer cells, we used Western blot analysis to assess the activation and cleavage of apoptosis-related proteins, including Bad, caspase 9 and PARP. As VN/14-1 was the most effective of the RAMBAs, we tested VN/14-1 in comparison to ATRA.

We first investigated the effects of VN/14-1 on the expression of the pro-apoptotic Bad protein. Both VN/14-1 and ATRA at concentrations of 1, 5 and 10 μM each induced upregulation of Bad protein by about two-fold, but there was no significant dose-dependent effect (Figure 7A). The possible involvement of Bad implies that induction of apoptosis by these treatments may be via the mitochondrial pathway. We therefore examined whether caspase-9 was activated. This molecule that plays a major role as an initiator caspase in this pathway was activated (Zou *et al*, 1999). As shown in Figure 7B, increased concentration of ATRA and VN/14-1 treatment caused an increase in the active form (37 kDa) and a decrease in the procaspase form (47 kDa). Control cells had a higher expression of the procaspase-9 (47 kDa) than the treated cells, that is 1 (control) *vs* 0.4- and 0.3-fold for 5 and 10 μM ATRA, respectively, and 0.4- and 0.7-fold for 5 and 10 μM of VN/14-1, respectively. The active form of caspase-9 was increased by 1.7- and 1.8-fold with 5 and 10 μM of ATRA, respectively, and by 1.7- and 1.7-fold with 5 and 10 μM of VN/14-1, respectively, compared with untreated control.

Another approach to detect apoptosis was by determining the proteolysis of PARP, the DNA repair enzyme that can be cleaved by effector caspases. As shown in Figure 7C, 5 μM of ATRA, or VN/14-1 caused almost two-fold increases in the level of cleaved PARP (89 kDa) as compared with control in MCF-7 cells. Treatment of T47D cells (Figure 7C) with 1, and 5 μM of VN/14-1 caused 6.3- and 7.5-fold increases, respectively, in the levels of cleaved PARP as compared with the control.

In vivo antitumour study of ATRA, 4-HPR and RAMBAs

To assess the ability of RAMBAs to inhibit tumour growth, we examined their effects in human MCF-7 breast cancer xenograft model with ATRA and 4-HPR as positive controls. Female ovariectomised nude mice bearing established MCF-7 tumour xenografts (200–300 mm^3) were grouped so that the mean tumour volume was similar for each group. They were then treated once daily, 6 days per week for a total of 6 weeks, with vehicle (control), ATRA (33.0 μmol kg^{-1} day^{-1}), 4-HPR (33.0 μmol kg^{-1} day^{-1}), VN/50-1 (33.0 μmol kg^{-1} day^{-1}), VN/66-1 (66.0 μmol kg^{-1} day^{-1}) and

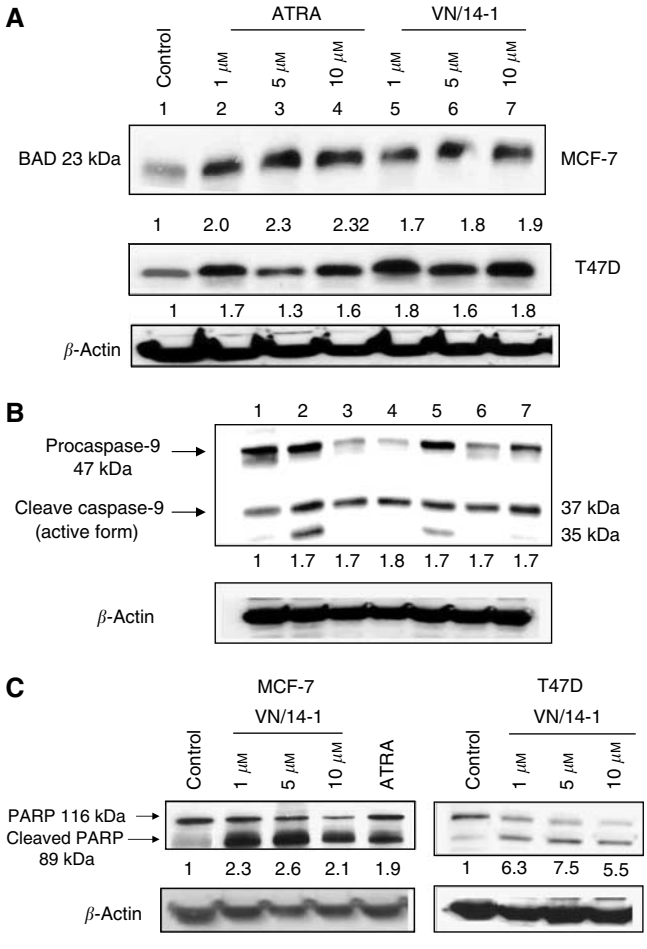

Figure 7 (**A**) Western immunoblotting of whole-cell lysates of treated MCF-7 and T47D cells for the expression of Bad. MCF-7 and T47D cells were treated with ATRA and VN/14-1 for 6 days and then cell lysates were electrophoresed using 15% SDS–PAGE and subjected to Western blotting. Lane 1: control; lanes 2–4: ATRA (1, 5 and 10 μM); and lanes 5–7: VN/14-1 (1 5 and 10 μM). Numbers below the blot show fold increase in expression of the protein as analysed by ImageQuant densitometry analysis. Membranes were stripped and probed for β-actin to verify equal protein loading. Primary antibody (Cell Signaling Technology) 1 : 1000 in 5% BSA TBST overnight at 4°C and secondary antibody (anti-rabbit) 1 : 2000 for 1 h at RT. This experiment was repeated twice with similar results. (**B**) Western blot showing activation of caspase-9 in T47D cells after treatment with ATRA or VN/14-1. T47D cells were treated with ATRA or VN/14-1 for 6 days and whole-cell lysates were electrophoresed using 10% SDS–PAGE and subjected to Western immunoblotting for caspase-9. Treatment with ATRA and VN/14-1 cleaved procaspase-9 to its active form. Lane 1: control; lanes 2–4: ATRA (1, 5 and 10 μM); and lanes 5–7: VN/14-1 (1, 5 and 10 μM). Numbers below the blot show fold increase in the active form of caspase-9 as compared with control. Membrane was stripped and probed for β-actin to verify equal amount of protein loading. The primary and secondary antibodies and their dilutions are as follows: primary antibody (Cell Signaling Technology), 1 : 1000 in 10% milk TBST overnight at 4°C and secondary antibody (anti-rabbit) 1 : 2000 in 10% milk TBST for 1 h at RT. Densitometry analysis was performed by ImageQuant software. This experiment was repeated twice with similar results. (**C**) Western immunoblotting of whole-cell lysates of treated MCF-7 and T47D cells for the expression of full-length and cleaved PARP. MCF-7 cells were treated with ATRA and RAMBAs for 6 days and 4-HPR for 4 days and then cell lysates were electrophoresed using 10% SDS–PAGE and subjected to Western blotting. Lane 1: control; lanes 2–4: VN/14-1 (1, 5 and 10 μM); lane 5: ATRA (5 μM); lane 6: VN/50-1 (5 μM); lane 7: VN/66-1 (5 μM); lane 8: VN/69-1(5 μM); and lane 9: 4-HPR (5 μM). T47D cells were treated with RAMBAs (VN/50-1 and VN/14-1) for 6 days and then cell lysates were electrophoresed using 10% SDS–PAGE and subjected to Western blotting. Lane 1: control; lanes 2–4: VN/50-1 (1, 5 and 10 μM); lanes 5–7: VN/14-1 (1, 5 and 10 μM). Numbers below the blot show fold increase in expression of the protein as analysed by ImageQuant densitometry analysis. Membranes were stripped and probed for β-actin to verify equal protein loading. Primary antibody (Cell Signaling Technology) 1 : 1000 in 5% milk TBST overnight at 4°C and secondary antibody (anti-rabbit) 1 : 2000 for 1 h at RT. The experiments were repeated twice with similar results.

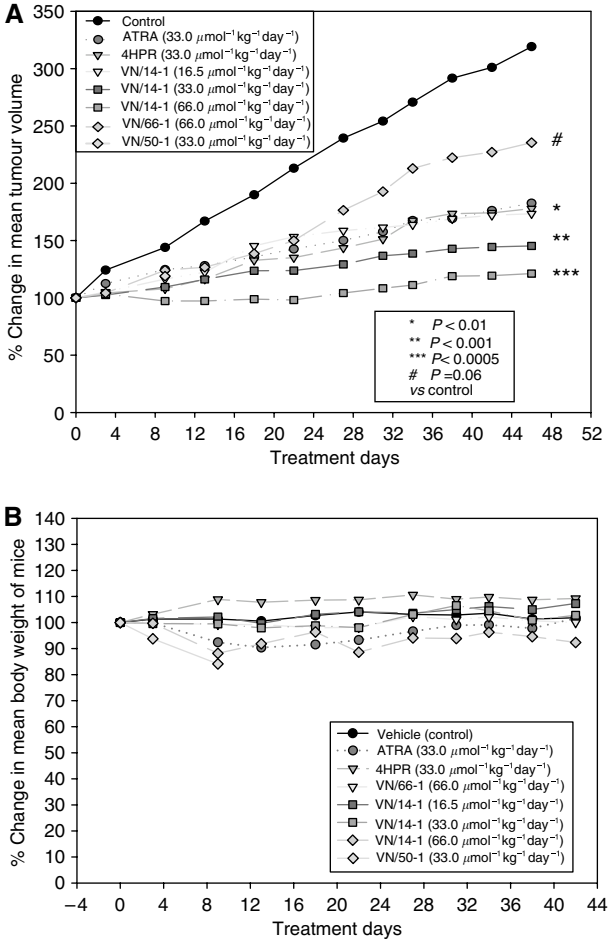

Figure 8 (**A**) Effect of ATRA, 4HPR or RAMBAs on the growth of MCF-7 tumour xenograft in ovariectomised female athymic nude mice. Ovariectomised female athymic nude 4–6-week-old mice were used. Oestrogen pellets (1.7 mg per pellet, 90 day release obtained from Innovative Research of America) were implanted in the mice using a trochar to facilitate tumour growth. Mice were then inoculated with MCF-7 cells (2×10^6 cells in Matrigel per tumour growth site) s.c. on the right and left flank. Tumours were allowed to grow for about 4–5 weeks till they were of measurable size (200–300 mm^3). The mice were then grouped as control and treatment groups. Twice every week the mice were weighed and tumours were measured using a caliper. Tumour volume was calculated according to the formula $4/3\pi r_1^2 r_2$ ($r_1 < r_2$). The tumour treatment study was continued for 6 weeks. (**B**) Effect of vehicle, ATRA 4-HPR and RAMBAs on body weight of ovariectomised female nude mice during the 6-week antitumour study. Mice were weighed twice every week during the 6-week antitumour study.

three doses of VN/14-1 (16.5, 33.0 and 66.0 μmol kg^{-1} day^{-1}). Tumour sizes were measured twice a week after the start of treatment. Because treatment with VN/50-1 was very toxic to the mice, the experiment with this cohort was terminated. As shown in Figure 8A, treatment with VN/14-1 produced a dose-dependent inhibition of tumour growth. Indeed, all three doses of VN/14-1, that is 16.5, 33.0 and 66.0 μmol kg^{-1} day^{-1}, caused significant reductions of 66.5% ($P<0.01$), 79.4% ($P<0.001$) and 92.6% ($P<0.0005$), respectively, in the mean tumour volume compared with the vehicle control. The effect of VN/14-1 (16.5 μmol kg^{-1} day^{-1} equivalent to 5 mg kg^{-1} day^{-1} ATRA) was comparable with the antitumour effect of ATRA or 4-HPR (33.0 μmol kg^{-1} day^{-1}). VN/14-1 (33.0 and 66.0 μmol kg^{-1} day^{-1} equivalent to 10 and 20 mg kg^{-1} day^{-1} of ATRA, respectively)

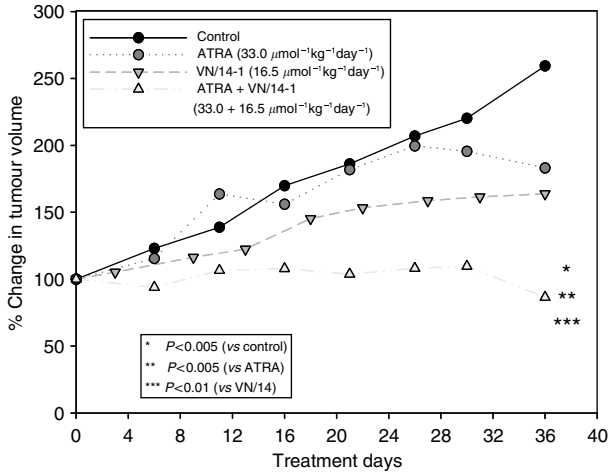

Figure 9 Effects of ATRA or VN/14-1 alone or in combination on the growth of MCF-7 tumour xenograft in ovariectomised female athymic nude mice. Procedure was similar to that described in Figure 8A.

caused a significant reduction of 45% ($P<0.05$) and 75.61% ($P<0.005$), respectively, in the mean tumour volume compared with the ATRA group (10 mg kg^{-1} day^{-1}). The antitumour effect of VN/66-1 (66.0 μmol kg^{-1} day^{-1}) was not better than ATRA. Importantly, except for the VN/50-1-treated group, no significant changes in animal body weight were observed even at the highest concentration of VN/14-1 (Figure 8B), suggesting that the growth-inhibitory effects were tumour specific and that the treatments did not produce general cytotoxicity in the mice.

It was of interest to determine the effects of combination of ATRA and VN/14-1 on the growth of MCF-7 tumour xenografts. Single agents ATRA (33.0 μmol kg^{-1} day^{-1}) or VN/14-1 (16.5 μmol kg^{-1} day^{-1}) resulted in a modest tumour growth inhibition compared with controls (Figure 9). However, the combination of ATRA and VN/14-1 resulted in a significant super additive inhibitory effect (up to 120% growth inhibition, as compared with controls; $P<0.005$ *vs* control). This result supports the hypothesis that RAMBA VN/14-1 is able to enhance the biological activity of ATRA through the inhibition of ATRA *in vivo*. We have demonstrated previously this effect in *in vitro* breast and prostate cancer models (Patel *et al*, 2004; Huynh *et al*, 2006).

Effects of VN/14-1 compared with clinical AIs on growth of MCF-7Ca xenografts

Based on our recent findings that VN/14-1 is also a potent AI (Belosay *et al*, 2006) and also because of the prominence of AIs in breast cancer therapy (Brodie *et al*, 2003), we wished to test the effects of VN/14-1 head to head with clinically used AIs, anastrozole or letrozole. As shown in Figure 10, VN/14-1 was as effective as either anastrozole or letrozole at inhibiting the growth of established MCF-7Ca tumours. In addition, VN14-1 was efficacious in preventing the formation of MCF-7Ca tumours (Figure 10). We also observed that VN/14-1 treatment caused significant reduction in uterine wet weight (data not shown), which indicates that VN/14-1 can effectively block the uterotropic activity of oestrogens produced by peripheral aromatisation of androstenedione (Goss *et al*, 2000; Jelovac *et al*, 2005).

DISCUSSION

We have reported previously that our novel RAMBAs are potent inhibitors of ATRA metabolism and also potent inhibitors of

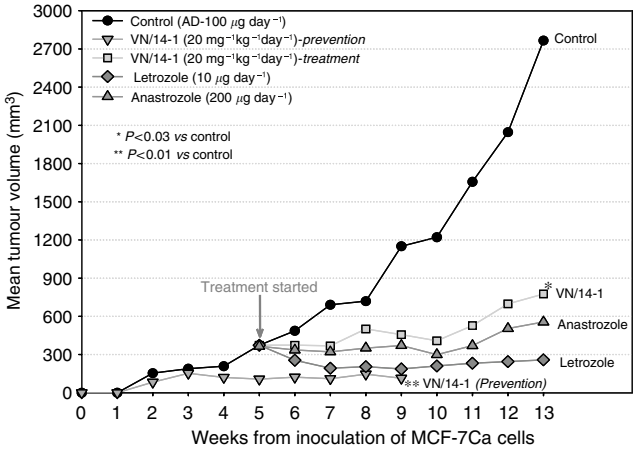

Figure 10 Effects of VN/14-1 on the formation of MCF-7Ca tumours and effects of VN/14-1, anastrozole and letrozole on the growth of MCF-7Ca xenografts in ovariectomised female athymic nude mice. Mice were inoculated with MCF-7Ca cells as described in Materials and Methods. Beginning on the following day, androstendione (100 μg per mouse per day) was supplemented by s.c. injection for the duration of the experiment. For the tumour formation prevention group ($n=5$), VN/14-1 (20 mg kg^{-1} day^{-1}) treatment began from the day after inoculation. For the other groups (VN/14-1, anastrazole and letrozole), treatment began after the tumour had reached approximately 300 mm^3 following procedures described in Materials and Methods.

proliferation of some human breast cancer cells (Patel *et al*, 2004). These results led us to propose that our RAMBAs also possess intrinsic retinoidal antitumour properties. Here, we have examined their molecular mechanisms of action, and shown that administration of some RAMBAs to nude mice bearing human oestrogen-dependent MCF-7 or MCF-7Ca breast tumours can effectively suppress tumour growth at doses that cause no apparent toxicity.

Like ATRA, each of the RAMBAs tested significantly caused up-modulation (two- to three-fold) of CK 8/18 in both MCF-7 and T47D breast cancer cell line, which suggest that our RAMBAs are capable of inducing differentiation in these breast cancer cell lines. Further support for the putative role of our RAMBAs as differentiating agents came from the findings that VN/14-1 caused a dose-dependent up-modulation (from four- to six-fold) of ER-α protein, another differentiation marker. The ability of these RAMBAs to induce differentiation in breast cancer cells may be of significance in light of recent findings which suggest that downregulated expressions of CK 18 (Korsching *et al*, 2002; Woelfle *et al*, 2004; Schaller and Buhler, 2005) and/or E-cadherin/ER-α (Nass *et al*, 2000; Kowalsski *et al*, 2003) promote progression of human breast cancer. It should be noted that differentiated tumour cells exhibit low proliferative and metastatic potential. An elevation in the expression of cytokeratins indicates a favourable prognosis and is a useful predictor for overall survival in breast cancer patients (Korsching *et al*, 2002). We have also shown that VN/14-1 binds and activates the RARs (RARα, -β and -γ), but the other RAMBAs do not activate these receptors. Although it has been suggested that only ligands that activate RARα can induce cell differentiation, our current results suggest that some of the RAMBAs are able to induce differentiation via RAR-independent pathway(s).

VN/14-1 binds (and transactivates) with higher affinity to RARα and RARγ compared with RARβ. Concentrations of VN/14-1 that can activate these two receptors will probably increase the level of CYP26 (along with other RA-inducible transcripts) in the cell. The question is how much of the antiproliferative effect of VN/14-1 is mediated by inhibition of CYP26 activity (increase in ATRA- and RAR-mediated transcription) *vs* some other mechanism leading to apoptosis that does not involve RARs.

Several studies have shown that retinoids inhibit proliferation of various cancers by inducing arrest of cells in a phase of the cell cycle. We have demonstrated here that our RAMBAs induce growth inhibition of both MCF-7 and T47D cells by G1 phase arrest with concomitant decrease of cells in S phase and also significant accumulation of cells in the sub-G1 phase. In addition, we also found increased percentage of cells in the G2/M, but this was consistently higher in the treated T47D cells than in the MCF-7 cells. We also demonstrated that expression of cyclin D1, the positive regulator of cell cycle progression from G1 to S phase, was reduced in MCF-7 and T47D cells after 6-day treatment with RAMBAs (1, 5 and 10 μM). This result is in agreement with the cell cycle analysis, which revealed G1 phase cell arrest.

The ability of ATRA/retinoids to inhibit the growth of cells has previously been shown to result from their ability to induce apoptosis in various cancer cells. In the present investigation, a significant increase in cells in the sub-G1 phase, accounting for 10–20% of the total cell population was detected after exposure to RAMBAs. Cells in this phase are thought to represent cells that have undergone apoptosis. TUNEL analysis confirmed that the RAMBAs did induce a portion of cells (MCF-7 and T47D) to undergo apoptosis in a dose-dependent fashion.

Apoptosis induced by ATRA and RAMBAs was further characterised by assessing the activation and cleavage of apoptosis-related proteins as determined by Western blot analysis. Treatment of breast cancer cells (MCF-7 and T47D) with RAMBAs resulted in proteolysis of full-length PARP – the DNA repair enzyme, which was comparable to ATRA. In T47D cells, VN/14-1 was able to cleave pro-caspase-9 to active caspase-9, an effect similar to ATRA. However, the involvements of other caspases were not investigated. VN/14-1 and VN/50-1 also increased the expression of the pro-apoptotic Bcl-2 family member Bad. Thus, induction of apoptosis in breast cancer cells by the RAMBAs was confirmed by several methods. When compared with 4-HPR (a retinoid known for inducing apoptosis in breast cancer cells) (Pellegrini *et al*, 1995; Wu *et al*, 2001) apoptosis induced by RAMBAs was less than 4-HPR, but comparable to ATRA. Although VN/66-1 exhibited less antiproliferative activity, it induced apoptosis that was comparable to that induced by ATRA. VN/69-1 was the least effective of the RAMBAs in inducing apoptosis in both MCF-7 and T47D cells.

The marked effects that our RAMBAs had on cell proliferation led to an *in vivo* study. We studied the effects of VN/14-1, VN/50-1, VN/66-1 and ATRA. VN/50-1 was found to be toxic to the animals and its antitumour efficacy could not be assessed. Our results show that on a molar basis, ATRA and 4-HPR were each more effective in suppressing the growth of established MCF-7 tumours than VN/66-1, but both agents were each less effective than VN/14-1. Indeed, we demonstrated that VN/14-1 inhibited MCF-7 tumour growth in a dose-dependent fashion. A dose of 66.0 μmol kg^{-1} day^{-1} caused a reduction of 92.6% in the mean final tumour weight compared with that of tumour-bearing mice receiving vehicle alone, with no apparent toxicity as there was no change in body weight of the mice. It was recently reported that the growth of murine oestrogen-independent TA3-Ha mammary tumours were significantly inhibited by a non-retinoidal RAMBA, R116010 (Van Huesden *et al*, 2002). The study demonstrated the antitumour efficacy of R116010 in an oestrogen-independent model of un-established tumours and it is not clear if the agent is effective against oestrogen-dependent breast tumours. Our findings that VN/14-1 is efficacious against well-established breast tumours suggest that the molecule may be a more effective anticancer RAMBA.

In the present study, we also demonstrated for the first time a synergistic inhibitory effect of VN/14-1 and ATRA on the growth of established MCF-7 and MCF-7Ca breast cancer tumour xenografts *in vivo*, and also the prevention of MCF-7Ca tumour formation. Importantly, VN/14-1 was as effective as the clinically

used AIs, anastrozole and letrozole. Both treatments with VN/14-1 caused significant reduction in uteri weights. This suggests that VN/14-1, unlike tamoxifen, may not cause endometrial hyperplasia in patients.

The growth inhibitory effects of cell lines and tumour xenografts especially by VN/14-1 appear to be due to its ability to induce differentiation, apoptosis and cycle arrest. However, in the antitumour efficacy studies VN/14-1 did not cause any tumour regressions. The mechanism underlying the lack of tumour regression is unknown at this time. One possibility is that the treatment with VN/14-1 results mainly in cytostatic but not cytotoxic antitumour effect.

In conclusion, our results clearly suggest that our RAMBAs induce other biochemical pathways in addition to differentiation. It appears that the molecular mechanisms for the antiproliferative activities of our RAMBAs in these breast cancer cells include their ability to induce cell differentiation, apoptosis and effects on cell cycle. Among the RAMBAs tested, VN/14-1 was shown to be the most efficacious in both *in vitro* and *in vivo* studies. Not only are RAMBAs effective against established tumours, but may also be important as differentiating agents in preventing breast cancer. As most cancers become resistant to agents that act on specific targets, the development of molecules that act on multiple cellular targets offer considerable hope for the development of new cancer therapies. These novel RAMBAs endowed with multiple biological activities have potential as therapeutics for breast cancer and possibly other diseases responding to retinoids. Indeed, VN/14-1 has been selected for further preclinical studies. We were recently awarded an Australian patent (Njar *et al*, 2005) to protect these novel and promising anticancer agents and several other patents are pending.

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Review

Targeting cytochrome P450 enzymes: A new approach in anti-cancer drug development

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Abstract—Cytochrome P450s (CYPs) represent a large class of heme-containing enzymes that catalyze the metabolism of multitudes of substrates both endogenous and exogenous. Until recently, however, CYPs have been largely overlooked in cancer drug development, acknowledged only for their role in phase I metabolism of chemotherapeutics. The first successful strategy targeting CYP enzymes in cancer therapy was the development of potent inhibitors of CYP19 (aromatase) for the treatment of breast cancer. Aromatase inhibitors ushered in a new era in hormone ablation therapy for estrogen dependent cancers, and have paved the way for similar strategies (i.e., inhibition of CYP17) that combat androgen dependent prostate cancer. Identification of CYPs involved in the inactivation of anti-cancer metabolites of vitamin D_3 and vitamin A has triggered development of agents that target these enzymes as well. The discovery of the over-expression of exogenous metabolizing CYPs, such as CYP1B1, in cancer cells has roused interest in the development of inhibitors for chemoprevention and of prodrugs designed to be activated by CYPs only in cancer cells. Finally, the expression of CYPs within tumors has been utilized in the development of bioreductive molecules that are activated by CYPs only under hypoxic conditions. This review offers the first comprehensive analysis of strategies in drug development that either inhibit or exploit CYP enzymes for the treatment of cancer.
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Contents

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1. Introduction

Cyotochrome P450s (CYPs) are a large ubiquitous family of proteins containing a single iron protoporphyrin IX prosthetic heme group. The majority of CYPs (designated Class I and II) act as versatile monooxygenases. These enzymes catalyze a multitude of reactions, including the hydroxylation of alkanes to alcohols, conversion of alkenes to epoxides, arenes to phenols, sulfides to sulfoxides and sulfones, and the oxidative split of C–N, C–O, C–C or C–S bonds.

Functionally, CYPs can be classified into two groups: those with specific roles in the metabolism of endogenous molecules such as hormones, and those that non-specifically process exogenous molecules (drugs, chemicals, natural products, etc.). Both classes of CYPs offer potential targets in chemotherapeutic and chemopreventative strategies.

Most CYPs were once considered liver specific enzymes, but now their extrahepatic expression has been well established. It has long been known that CYP17 and CYP19—the enzymes responsible for the production of androgens and estrogens, respectively—are expressed in the testes, ovaries, and adrenals. However, CYP19 has subsequently been found expressed locally in the adipose tissue of the breast, and indirect evidence suggests CYP17 may be expressed in adipose tissue as well.[1,2] It is also now well established that expression of CYPs responsible for the metabolism of anti-cancer metabolites of vitamin A (all-*trans*-retinoic acid; ATRA) and vitamin D (1α,25-dihydroxyvitamin D_3; 1,25-D_3) are induced by their substrates in target cells (including cancer).[3–11] Members of CYP families 1, 2, and 3 have also been identified in both healthy and cancerous extrahepatic tissues.[12–19] The enzymes in these families are involved in the metabolism of xenobiotic substances such as carcinogens, pro-carcinogens, and chemotherapeutics.[20–23] Of note, CYP1B1 and, more recently, CYP2W1 have been identified as having tumor-specific expression.[15,17,18,24,25]

These observations have led to a greater appreciation for the role of CYPs in tumor formation and development. Targeting of these enzymes with natural or synthetic small molecules offers potential benefits in cancer prevention and therapy. Because crystal structures for nearly all CYPs are yet to be determined, drug design strategies rely on the knowledge of substrate structure and the enzyme's mechanism of action. Strategies to target these enzymes include: (i) designing molecules that inhibit the enzymes; (ii) designing prodrugs that are activated by the enzymes; (iii) immuno-based therapies that target immune responses toward the enzymes; (iv) genetic therapy strategies to express specific CYPs in cancer cells.[26] This review will focus on the small molecule based approaches (i and ii) being employed to target CYPs involved in hormone, vitamin, and xenobiotic metabolism for the treatment and prevention of cancer (Fig. 1).

2. CYPs and hormone dependent cancer

2.1. Aromatase (CYP19)

The development of aromatase inhibitors for the treatment of breast cancer (BCa) represents the paradigm of success of CYP inhibition in cancer therapy. For years, the standard pharmacological treatment for hormone dependent BCa in post-menopausal women was blocking estrogen (E) binding to the estrogen receptor (ER) with the anti-estrogen tamoxifen. ER is a nuclear hormone receptor that is normally activated by E to recruit co-activators and induce transcription of target genes. By blocking E binding to the ER, tamoxifen prevents E-induced proliferation. Unfortunately, tamoxifen, although an important advance in BCa therapy, has many drawbacks. First, it is a partial ER agonist in many tissue types,[27] which has been correlated with a threefold increase in the incidences of endometrial cancer in patients receiving the drug.[28] Furthermore, resistance to tamoxifen therapy inevitably results.[27] An alternative approach to tamoxifen treatment is inhibition of estrogen synthesis. The target for such therapy is the enzyme aromatase, which catalyzes the rate limiting step in the conversion (aromatization) of androgens to estrogens (Fig. 2). Aromatase is expressed in many tissues throughout the body, including adipose and muscle, which are the main sites of estrogen synthesis in post-menopausal woman.[1] Therefore, surgery to remove endocrine glands is ineffective, and inhibition of estrogen production requires a systemic pharmacological approach.

The first successful aromatase inhibitor, *4-hydroxyandrostenedione* (4-OHA, *Formestane*), was demonstrated by Harry and Angela Brodie and colleagues to have efficacy against breast cancer tumors in 1977.[29] Since then, several selective inhibitors of aromatase have been developed (Fig. 3). These include fellow steroidal inhibitor *6-methylenandrosta-1,4-diene-3,17-dione* (*exemestane*) as well as two non-steroidal triazoles, *letrozole* (*femara*) and *anastrozole* (*arimidex*). All four of these inhibitors are approved for the treatment of BCa, and have been shown in clinical trials to be more effective as a first line therapy than tamoxifen for post-menopausal women with hormone-sensitive BCa.[30] A great deal of work is still being done in this field to optimize the efficacy of aromatase inhibitors both alone and in combination with other treatments, but these studies are beyond the scope of discussion here. Despite the obstacles that lay ahead, it seems clear that aromatase inhibitors represent the first successful class of cancer therapeutics specifically designed to target a CYP enzyme.

2.2. 17α-Hydroxylase,C17,20-lyase (CYP17)

The clinical success of aromatase inhibitors raises the question of whether a similar strategy could be employed for the treatment of androgen dependent cancers such as prostate cancer (PCa). In 1941, Charles Huggins and colleagues first demonstrated the benefits of hormone deprivation therapy in prostate cancer.[31,32] To this

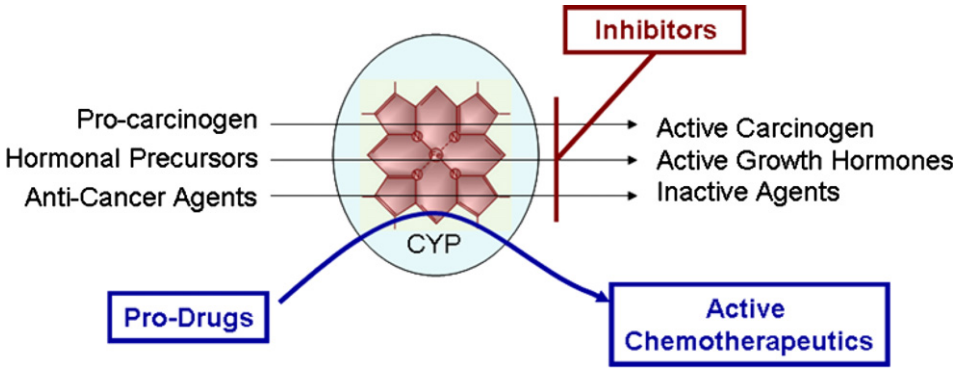

Figure 1. Potential strategies targeting CYPs for cancer therapy and prevention.

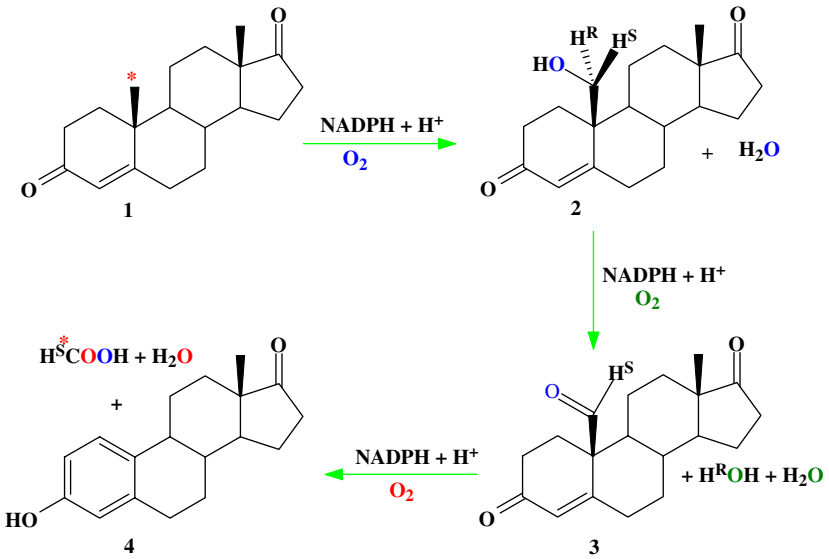

Figure 2. Biosynthesis of estrogens from androgens catalyzed by CYP19 (aromatase).

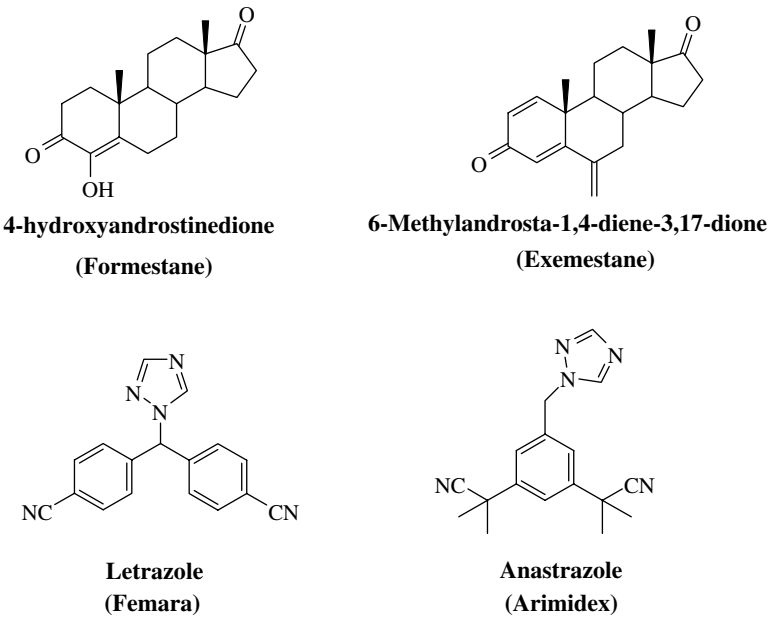

Figure 3. Chemical structures of aromatase inhibitors.

day, androgen ablation remains the standard treatment for advanced PCa.

At the molecular level, androgens, mainly testosterone (T) and dihydrotestosterone (DHT), bind to the androgen receptor (AR) in target cells and initiate transcription of genes involved in cell proliferation and survival.[33,34] Generally, androgen withdrawal therapy is carried out via treatment with LHRH or GnRH agonists and anti-androgens (AR antagonists, e.g., bicalutamide, flutamide). Unfortunately, LHRH and GnRH agonists fail to prevent synthesis of testosterone by the adrenal glands (site of about 10% of total androgen production) and anti-androgens can act as weak agonists in prostate cancer cells expressing mutated and/or over-expressed AR.[35] In addition, combination therapy with anti-androgens seems to fail to extend survival rates in patients with advanced PCa, with response times ranging for only 12–33 months.[36] It has been shown that patients developing resistance to anti-androgen therapy maintain levels of T and DHT in their cancerous tissue at levels sufficient to activate the AR, and that an increase in AR mRNA was the only change consistently associated with anti-androgen resistance.[35,37] Taken together, these results suggest that androgens play a role even in so called hormone-refractory PCa. Furthermore, recent experiments suggest the possibility of androgen production in adipose tissue.[2] Thus, compounds that can systemically inhibit the production of androgens, similar to the systemic inhibition of estrogen production in BCa, may prove to be more effective in the treatment of PCa.

The last step in the production of T requires two sequential reactions both catalyzed by the same enzyme, 17α-hydroxlase/17,20-lyase (CYP17; Fig. 4).[38] Therefore, CYP17 has become the target of interest for systemic inhibition of androgen production. *Ketoconazole* (Fig. 5), an anti-fungal agent that non-specifically inhibits a broad range of CYP enzymes, has been used clinically as a second line therapy for advanced hormone-refractory PCa[39] and showed efficacy in patients no longer responding to treatment with the anti-androgen flutamide.[40] Owing to its lack of specificity for CYP17, *ketoconazole* treatment is, unfortunately, limited by toxicity. Clinical trials using low dose *ketoconazole* (LDK) co-treated with glucocorticoids and chemotherapeutics or androgen withdrawal

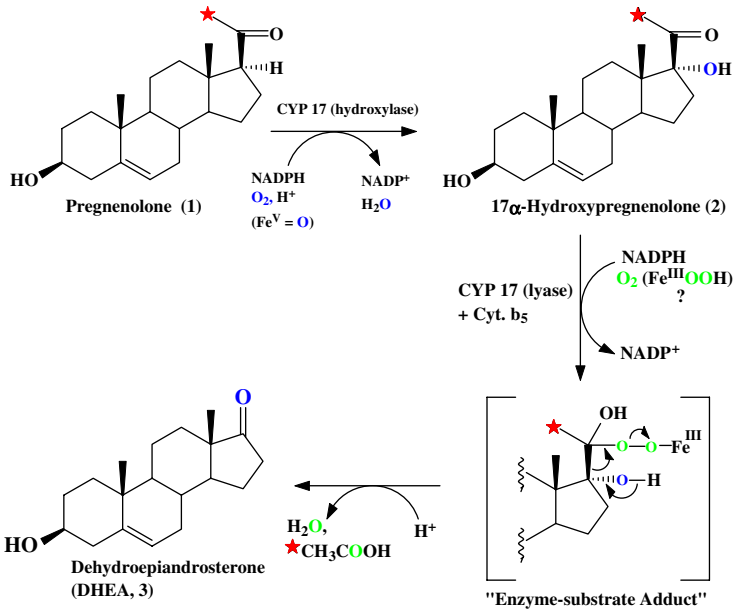

Figure 4. Biosynthesis of androgens.

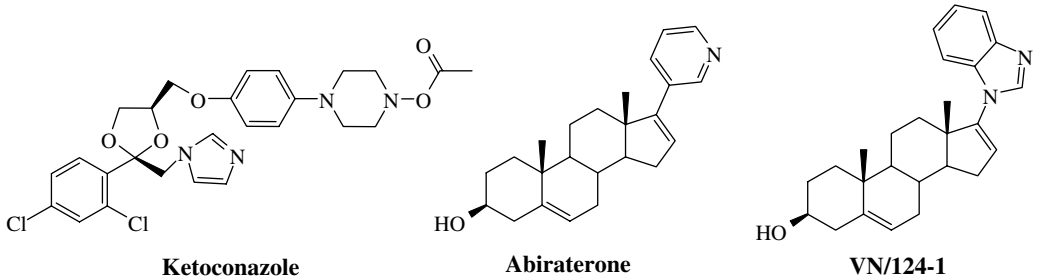

Figure 5. Inhibitors of CYP17. Ketoconazole is a broad range CYP inhibitor while abiraterone and VN/124-1 are designed as specific CYP17 inhibitors.

therapies have shown similar efficacy to high dose *ketoconazole* (HDK) with reduced toxicity.[41–43]

Despite the potential therapeutic benefits of LDK, development of more potent and selective inhibitors of CYP17 would clearly offer a therapeutic advantage over *ketoconazole*. One such molecule, *abiraterone* (Fig. 5), has already entered phase II clinical trials. At the 2007 American Association for Cancer Research Annual Meeting, Attard et al. reported exciting phase II results for *abiraterone acetate* (prodrug of *abiraterone*) showing PSA responses ($\geqslant 50\%$) in 11/18 hormone-refractory patients and a drop in circulating T levels from castrate levels (<50 ng/dl) to undetectable levels (<1 ng/dl).[44] Our laboratory has done extensive research in this field as well, and has developed several molecules that inhibit both CYP17 and the AR directly. One of these compounds, a 17-benzoimidazole called *VN/124-1* (Fig. 5), has shown superb anti-cancer properties both in vitro and in vivo.[45] In fact, *VN/124-1* is the only CYP17 inhibitor/anti-androgen to date that has been shown to inhibit prostate cancer tumor growth in vivo more effectively than castration.[45] *VN/124-1* has recently been licensed to Tokai Pharmaceuticals Inc., Boston, MA, USA, with hopes of following *abiraterone* into the clinic within a few years. A more comprehensive review of this subject can be found in the several reviews written by our group and others regarding the development of CYP17 inhibitors.[46–49]

3. Inhibiting vitamin metabolism

3.1. 25-Hydroxyvitamin D_3-24-hydroxylase (CYP24)

Vitamin D is synthesized in the skin upon exposure to UVB radiation. One of vitamin D's active metabolites, 1,25-D_3 (clinical formulation is known as calcitriol), acts as hormone, and like E and T, it binds to a nuclear receptor, the vitamin D receptor (VDR), and initiates transcription. Unlike T and E, however, 1,25-D_3 has gained attention as an anti-cancer agent because of its ability to inhibit proliferation, promote differentiation, and induce apoptosis in many cancer cell types including colon and prostate.[50–53] The role of 1,25-D_3 in cancer prevention is also supported by epidemiological studies that reveal a negative correlation between occurrence of certain cancers and sunlight exposure.[54–56] This connection has been particularly well documented in colon cancer where it has recently been suggested by Grant and Garland that as much as 20–30% of colorectal cancer incidences are due to insufficient exposure to sunlight.[57] Deactivation of 1,25-D_3 occurs via hydroxylation at C-24 catalyzed by CYP24 (Fig. 6).[58] CYP24 is mainly expressed in the kidney, however, its expression has been demonstrated in other tissue types both healthy and cancerous[3,59–63] and has been identified as a possible oncogene.[64] CYP24's role in tumor development and initiation is further supported by its apparent overexpression in lung and colon cancer compared to the corresponding healthy tissue.[62,63] Importantly, CYP24's expression is inducible by treatment with 1,25-D_3.[3] This negative feedback mechanism limits the amount of 1,25-D_3 present in tumor cells, and consequently the effectiveness of 1,25-D_3 therapy. Therefore, regulation of CYP24 enzymatic activity may potentiate the anti-cancer benefits of 1,25-D_3. In addition, clinical benefits of 1,25-D_3 have been limited due to hypercalciuric and/or hypercalcemic side effects at therapeutically necessary concentrations.[51] Targeting CYP24 provides the opportunity to increase endogenous levels of 1,25-D_3, or reduce the effective dose of exogenous 1,25-D_3, a therapeutic strategy that may help overcome the deleterious side effects associated with 1,25-D_3 treatment.

Strategies to limit CYP24 action include down-regulation of the enzyme's expression, as well as inhibition of the enzyme itself. *Genestein* (Fig. 7a), a naturally occurring isoflavonoid with anti-cancer properties,[65] has been shown to inhibit the transcription of CYP24 as well as CYP27B1 (25-hydroxyvitamin D-1α-hydroxylase),[61,66] which catalyzes the hydroxylation at C-1 of 25-hydroxyvitamin D_3 to 1,25-D_3.[67] Interestingly, co-treatment with the histone deacetylase inhibitor trichostatin A increased inhibition of CYP24 expression while restoring expression of CYP27B1.[66] Because CYP27B1 has been shown to be expressed in cancer cells,[68,69] this co-treatment offers a unique strategy to maximize the amount of 1,25-D_3 present in tumors by suppressing its metabolism while leaving its synthesis unhindered. *Genestein*, and various synthetic derivatives, have been evaluated extensively in pre-clinical studies and have shown promising results as chemopreventative and adjuvant chemotherapies[65,70] prompting the need for evaluation of these compounds in clinical trials.

Recently, Sundaram et al. described the ability of the synthetic 1,25-D_3 analogue, *QW-1624F*$_2$-2 (Fig. 7a), to inhibit the expression of CYP24.[71] *QW-1624F*$_2$-2 was originally described by Posner et al. to mimic the actions of 1,25-D_3 through binding of the VDR and activation of transcription.[72] Importantly, however, it lacks the calcemic side effects of 1,25-D_3. QW-162F$_2$-2 seems to be as effective as 1,25-D_3 in inhibiting cell growth, inducing its effect through modulation of cell cycle and apoptotic proteins.[73] The molecule has also been shown to inhibit neuroblastoma xenografts in nude mice more effectively than the 1,25-D_3 analogue, EB1089 (1α,25-dihydroxy-22,24-diene-24,26,27-trishomovitamin D_3).[74] Furthermore, *QW-1624F*$_2$-2 can inhibit the expression of CYP24 even in the presence of 1,25-D_3 and has been shown to act synergistically with 1,25-D_3 to inhibit cell proliferation.[71] These pre-clinical results are compelling and demonstrate a need to develop *QW-1624F*$_2$-2 as a chemotherapeutic agent.

Obviously, small molecules that can bind to CYP24 and inhibit the enzyme's activity directly may also prove to be effective treatments for some forms of cancer. Currently, the non-selective CYP inhibitor *ketoconazole* (Fig. 5) is being used as an adjuvant therapy for hormone-refractory prostate cancer, mainly for its inhibition of CYP17 (discussed earlier). However, the compound has also been shown to inhibit CYP24 and act synergistically with vitamin D_3 analogues in cell culture[75,76] and is being tried in combination with calcitriol

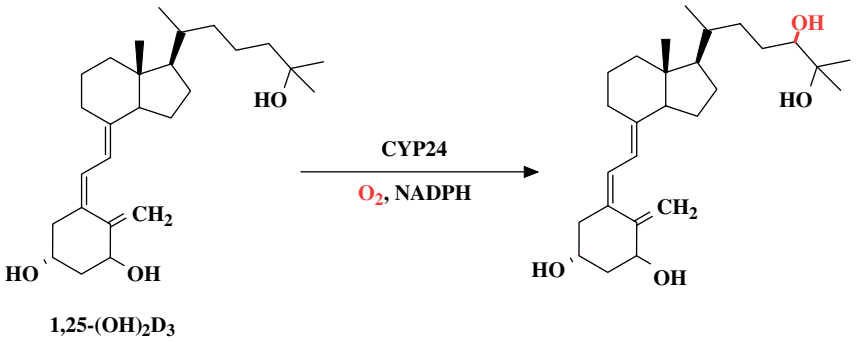

Figure 6. Metabolism of 1,25-D_3 via C-24 hydroxylation catalyzed by CYP24.

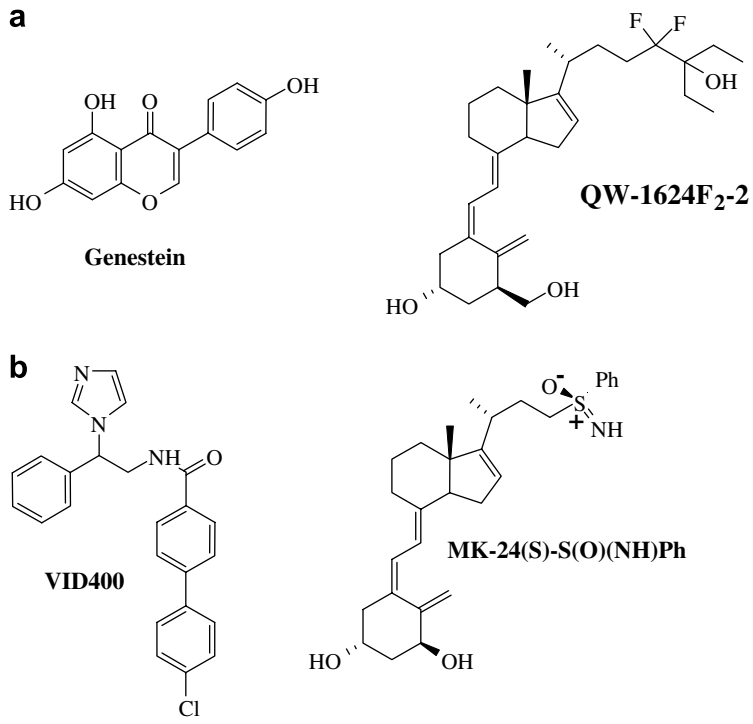

Figure 7. CYP24 Inhibitors. (a) Genestein and QW1624F_2-2 inhibit CYP24 expression; (b) VID400 and MK-24(S)-S(O)(NH)Ph inhibit CYP24 enzyme activity.

in a phase I clinical trial.[77] *Liarozole* (Fig. 9), a CYP inhibitor initially designed to inhibit CYP26, has also been shown to inhibit 1,25-D_3 hydroxylation and act synergistically with 1,25-D_3 in androgen independent DU-145 prostate cancer cells.[78] Unfortunately, both *liarozole* and *ketoconazole* are more potent inhibitors of CYP27B1 than they are of CYP24, greatly limiting their potential efficacy.

Due to these limitations, selective CYP24 inhibitors offer a therapeutic advantage, and several groups have developed compounds to this end.[76,79–81] Schuster and colleagues have developed potent azol containing inhibitors of CYP24, and their lead compound, *VID400* (Fig. 7b), is highly selective for CYP24 over CYP27B1 (IC_{50}'s of 15 and 616 nM, respectively).[82] *VID400* is currently undergoing pre-clinical development as an anti-proliferation agent.[79,82,83] A different class of CYP24 inhibitors, sulfone analogues of 1,25-D_3, have recently been developed by Posner and colleagues.[81,84] Their lead compound, a NH phenyl sulfoximine called *MK-24(S)-S(O)(NH)Ph* (*MK*; Fig. 7b), has shown great specificity for CYP24 with an IC_{50} of 7.4 nM, compared to CYP27B1 (IC_{50} = 554 nM) and CYP27A1 (IC_{50} > 1000 nM). *MK* was recently shown to be effective in pre-clinical models of lung cancer, working synergistically with 1,25-D_3 to inhibit growth of the non-small cell lung cancer cell line 128-88T.[63]

3.2. ATRA hydroxylase (CYP26)

All-*trans*-retinoic-acid (ATRA) is the most active biological metabolite of vitamin A. Through its interaction with nuclear retinoic acid receptors (RAR), ATRA induces cellular differentiation of epithelial cells,[85] and is being used for the treatment and prevention of several types of cancer.[86–89] However, despite ATRA's pre-clinical efficacy and its clinical success in the treatment of

acute promyelocytic leukemia,[90] the overall clinical efficacy of ATRA against human cancer has been disappointing.[91,92] ATRA's success seems to be limited by the development of resistance in patients.[93] Like 1,25-D_3, this resistance seems to be due, in part, to the rapid metabolism of ATRA in vivo via C-4 hydroxylation (Fig. 8).[94,95] This realization has inspired researches to develop new classes of drugs designed to inhibit the metabolism of ATRA. Such drugs are often termed retinoic acid metabolism blocking agents (RAMBAs).

Many CYPs have been identified that show the ability to metabolize ATRA via 4-hydroxylation including CYP2C8, CYP3A4, and CYP2C9.[96–99] However, the specificity of these enzymes for ATRA is quite low.[96–98] CYP26A1 and CYP26B1 have recently been identified as members of a new family of P450 enzymes that seem dedicated to ATRA metabolism.[10,11] Furthermore, CYP26A1 expression has been shown to be induced upon treatment with ATRA in cancer cells,[4–9] and expression of the enzyme limits induction of apoptosis by ATRA.[5] This phenomenon appears to be implicated in clinically acquired resistance to ATRA. In addition, certain cancers including acute promyelocytic leukemia, prostate, breast, and non-small lung carcinomas express CYP26A at constitutively high levels.[5–7,100–102] This has led to a great deal of interest in the specific targeting of RAMBAs toward CYP26. However, non-specific inhibition of all enzymes involved in ATRA metabolism is an alternative strategy that hinges on the idea that non-specific metabolism of ATRA would prevent accumulation of the hormone to levels sufficient enough to induce the expression of CYP26.

Liarazole (Liazal™; Johnson and Johnson Pharmaceutical Research and Development; Fig. 9) is the first and only RAMBA to be evaluated clinically in patients with cancer. Interestingly, *liarazole* is a relatively weak inhibitor of CYP26 ($IC_{50} \sim 2.2–6.0$ μM).[103–107] *Liarazole* showed promise in pre-clinical models of prostate cancer[106,108] and clinically as a second line therapy following failure of androgen deprivation.[109] However, *liarazole's* usefulness as a cancer therapy is unfortunately limited by its lack of specificity for CYP isozymes responsible for ATRA metabolism, as well as its moderate potency against CYP26 and is no longer being developed as an anti-cancer agent.[10] Follow-up compounds *R*115866 and *R*116010 (Fig. 9) are far more potent and selective inhibitors of CYP26 and have shown efficacy in pre-clinical cancer models.[10] Work by our group, researchers at Allergan Sales Inc., and OSI Pharmaceuticals Inc. have yielded novel compounds classified as azolyl retinoids, benzeneacetic acid derivatives, and 2,6-disubstituted naphthalenes, respectively (Fig. 9). All of these compounds have shown strong inhibition of CYP26. Additionally, the azolyl retinoids and the 2,6 disubstituted naphthalenes have shown anti-cancer properties in pre-clinical models.[10,110,111] In fact, one of our RAMBAs, *VN/14-1*, inhibits the growth of letrozole resistant breast cancer cells more potently

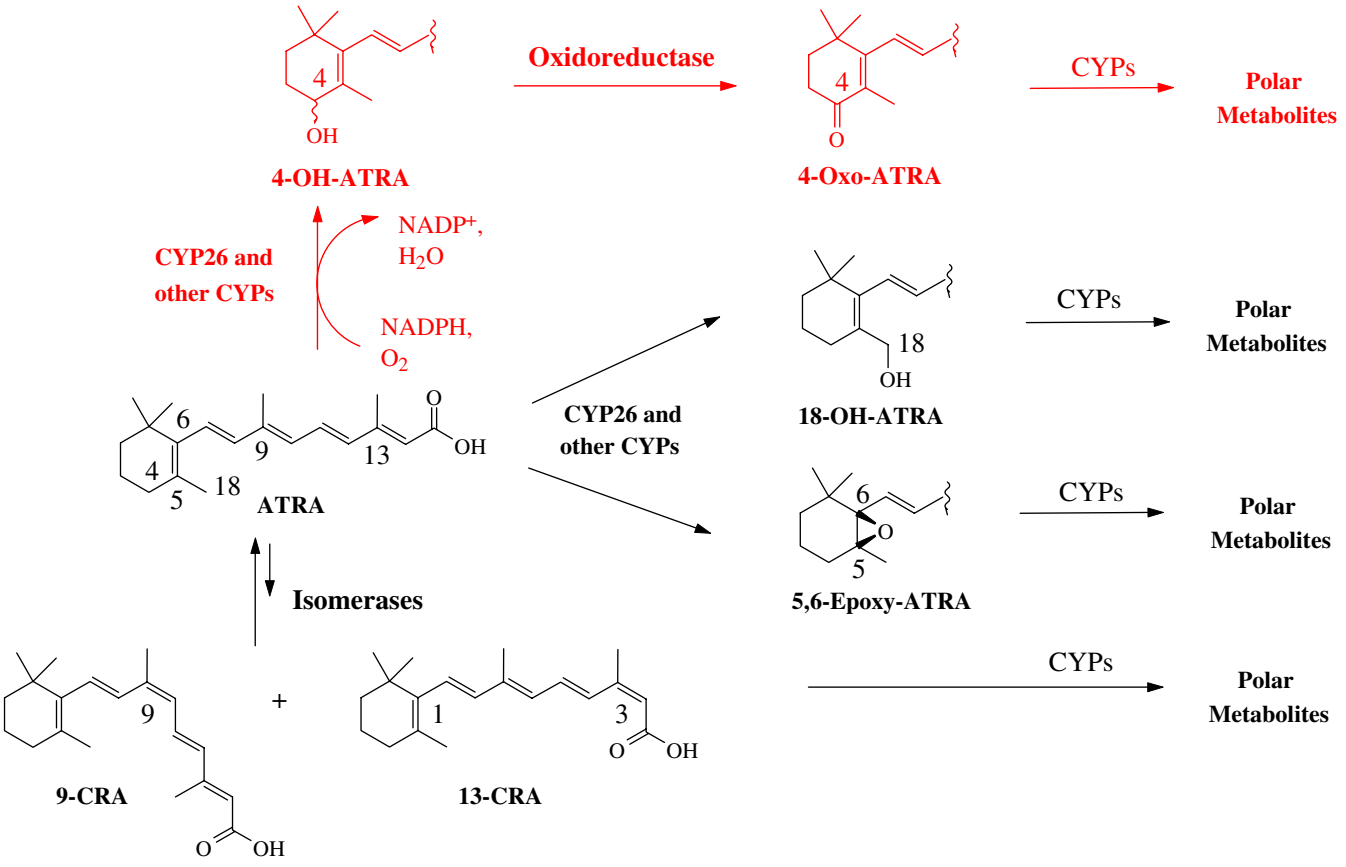

Figure 8. Metabolism of ATRA by CYP26.

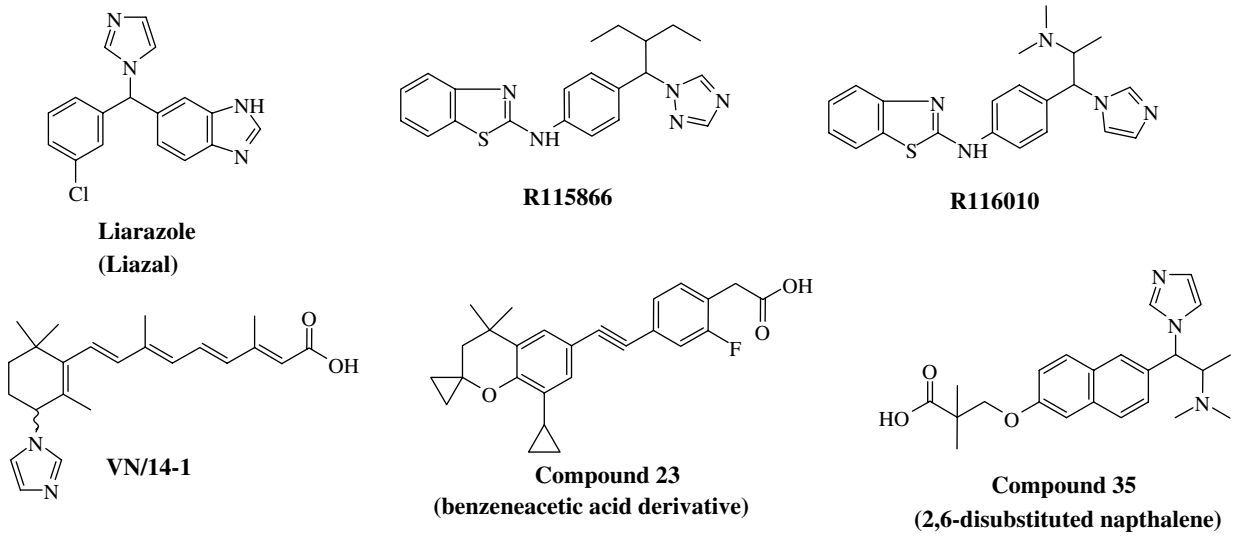

Figure 9. Retinoic acid metabolism blocking agents.

than parental letrozole sensitive cells.[112] These results indicate the potential usefulness of RAMBAs for hormone-refractory cancers. Despite promising published pre-clinical results, no clinical trials have been undertaken to date with any RAMBA other than liarazole. However, plans are underway to advance our novel RAMBAs alone and in combination with histone deacetylase inhibitors for clinical trials in breast and prostate cancer patients. For more information on the development and utility of RAMBAs, please consult our group's recent review on the subject (Njar et al., Ref. 10).

4. Exogenous metabolizing CYPs and cancer

4.1. CYP1

Humans express three types of CYP1 enzymes: CYP1A1, CYP1A2, and CYP1B1. Members of this family are under the transcriptional regulation of the aryl hydrocarbon receptor (AhR) and are known to activate pro-carcinogens such as polycyclic aromatic hydrocarbons (PAHs). All members of the CYP1 family are expressed in extrahepatic tissues,[113] but CYP1B1 is unique in that it is over-expressed in many tumor types relative to normal tissues.[15,17,18] This insight has peaked a great deal of interest into CYP1B1.

Evidence for the role of CYP1B1 in tumorigenesis is supported not only by its increased expression, but also by its ability to activate several carcinogens in the chemical classes of PAHs, heterocyclic amines, aromatic amines, and nitropolycyclic hydrocarbons.[114,115] Furthermore, recent epidemiological studies have linked CYP1B1 polymorphisms to increased or decreased risk of certain cancers.[116–119] It is also likely that CYP1B1, along with CYP1A1, plays a role in advanced carcinoma, as well, as the ability of these enzymes to metabolize chemotherapeutic agents may help tumors avoid chemotherapeutic induced cytotoxicity.[120–122]

Of most importance, however, is CYP1B1's role in estradiol metabolism. It catalyzes the hydroxylation of estradiol primarily at the C-4 position. C-2 hydroxylation can also occur primarily through CYP1A2 and CYP3A4. However, C-4 hydroxylation seems to be the preferred pathway outside of the liver and may play an important role in estrogen-related tumorigenesis.[123] The reasons for this are twofold. First, 4-hydroxyestradiol is a strong ER agonist, with a binding affinity for the estrogen receptor 1.5-fold greater than estradiol.[124] Second, and more importantly, 4-hydroxyestradiol is subsequently converted to estradiol 3,4-quinone, which has been shown to bind DNA and form unstable adducts leading to mutations.[123,125]

An obvious strategy for chemoprevention would therefore be the inhibition of CYP1B1. Studies with CYP1A1, CYP1A2, and CYP1B1 knockout mice demonstrated that animals lacking any one of these genes developed normally and showed no noticeable deficiencies. Furthermore, CYP1B1 knockout mice showed strong resistance to 7,12-dimethylbenz[*a*]anthracene (DMBA) induced tumor formation.[126] These studies provide evidence for the potential efficacy and safety of a chemopreventative agent that blocks CYP1B1 expression or activity.

As previously mentioned, members of the CYP1 family are under the transcriptional control of AhR. AhR binds a diverse set of exogenous molecules, including carcinogens such as PAHs and polyhalogenated hydrocarbons which are present in air pollution and cigarette smoke.[127,128] Upon binding, AhR translocates to the nucleus where it binds aryl hydrocarbon nuclear translocator (ARNT). The AhR/ARNT dimer regulates transcription of target genes.[127] The ability of PAHs and other carcinogens to induce CYP1 family expression is illustrated by experiments demonstrating elevated CYP1A1 and CYP1B1 expression in lung and urothelial tissue of smokers compared to non-smokers.[129,130] Because CYP1A1 and CYP1B1 are necessary for the

activation of many carcinogens that induce CYP1 expression via AhR, treatment with antagonists of AhR seems to be a practical chemopreventative strategy.

Many natural products have shown promise for this indication. Recently, the flavonoid *kaempferol* (Fig. 10a) was shown to inhibit agonist binding to AhR, as well as agonist induced AhR/ARNT/DNA complex formation and induction of CYP1A1 expression.[131] *Kaempferol* also inhibited cigarette smoke condensate induced growth of immortalized lung epithelia cells (BEAS-2B) in a soft agar colony assay. In these studies, *kaempferol* inhibited the AhR with an IC_{50} of 28 nM and cellular responses were seen at 10 μM ($\sim IC_{90}$). Other natural inhibitors of CYP1 family expression include *5,7-dimethoxyflavone*, which inhibits both expression and activity of CYP1A1,[132] and the stilbene phytoestrogen *resveratrol* (Fig. 10a).[133]

In addition to AhR inhibitors, a vast array of compounds, both natural and synthetic, have been evaluated for their ability to directly inhibit CYP1 family enzymatic activity.[115,123,134–137] Molecules that have been identified as potent inhibitors come from diverse chemical families including synthetic aromatics, coumarins, flavonoids, and stilbenes. Table 1 shows the IC_{50} values of various compounds shown to inhibit members of the CYP1 family, and Figure 10b shows the structures of representative compounds. Unfortunately, it remains unclear whether the inhibition of CYP1 family members by these compounds translates into chemoprevention in vivo.

An alternative strategy for CYP1 based chemotherapies involves the activation of an inactive prodrug to a cytotoxic compound. It has been reported that resveratrol can be activated to an active anti-cancer agent, *piceatannol*, by CYP1B1 within cancer cells.[138] Unfortunately, resveratrol has recently been shown to have limited anti-cancer activity in vivo in an athymic mouse cancer model.[139] New synthetic prodrugs specifically designed to be activated by CYP1A1 and CYP1B1 have been developed and have shown promise as novel approaches to cancer therapy.

One such compound, *phortress*, is a benzothiazole prodrug that has entered phase I clinical trials (Fig. 11a).[140] In the absence of cells, *phortress*, a hydrophilic lysil-amide, does not undergo hydrolysis, but is rapidly hydrolyzed in the presence of cells to its lipophilic amide parent compound *5F203*. *5F203* is then taken up by sensitive cells where it acts as a potent AhR agonist, leading to the induction of AhR target genes (i.e., CYP1A1 and CYP1B1). It is believed that CYP1A1 then metabolizes *5F203* to generate reactive electrophillic species, which ultimately leads to DNA damage and cell death. The drug has shown tremendous pre-clinical results both in vitro and in vivo against sensitive tumors.[140] Obviously, only AhR expressing cancer cells are susceptible to *phortress*, so patients will require screening to select those who might benefit from the drug.

Aminoflavone (5-amino-2,3-fluorophenyl-6,8-difluoro-7-methyl-4*H*-1-benzopyran-4-one; Fig. 11b) has recently followed *phortress* into phase I clinical trials. The

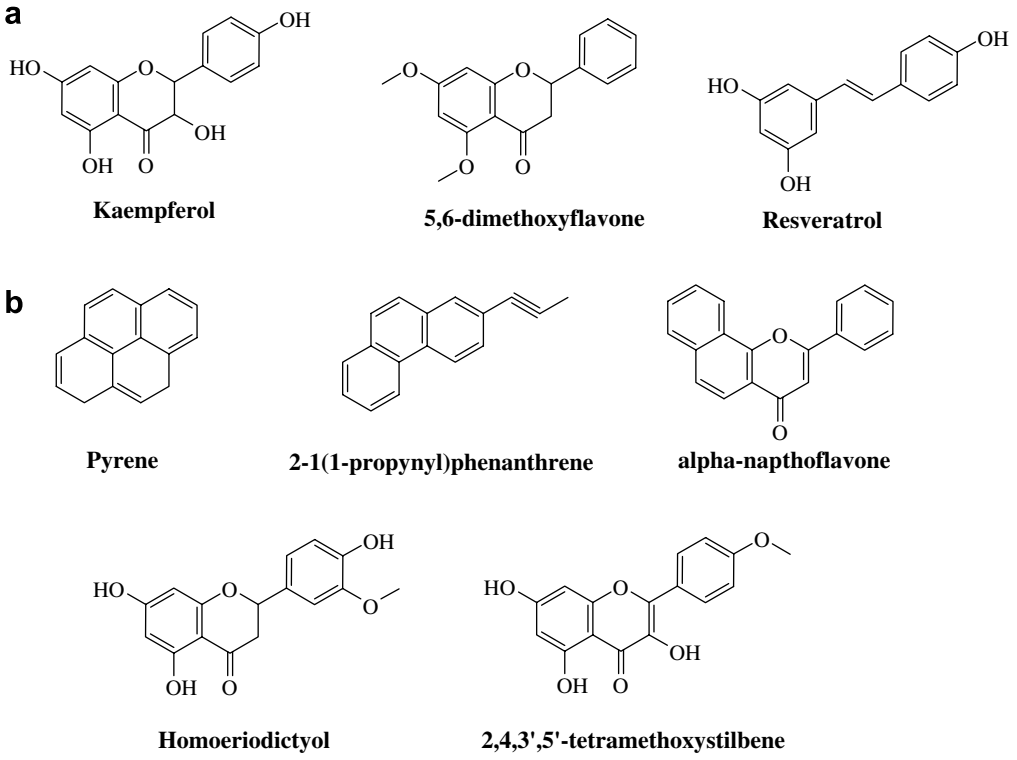

Figure 10. CYP 1 inhibitors. (a) Inhibitors of CYP1 family transcription (Ahr antagonists). (b) Inhibitors of CYP1 family enzyme activity.

Table 1. Inhibitors of the CYP1 family

Compound	CYP1B1 IC_{50} (nM)	CYP1A1 IC_{50} (nM)	CYP1A2 IC_{50} (nM)	Reference
Pyrene	2	41	7	134
2-(1-Propynyl) phenanthrene	30	150	60	134
3,3′,4,4′,5′-Pentachlorobiphenyl	11	ND	ND	135
α-Naphthoflavone	5	60	6	134
Acacetin	7	80	80	136
Homoeriodictyol	240	>4000	>4000	136
2,4,3′,5′-Tetramethoxystilbene	6	300	3000	137
2-[2-(3,5-Dimethoxy-phenyl)-vinyl]-thiopene	2	61	11	137

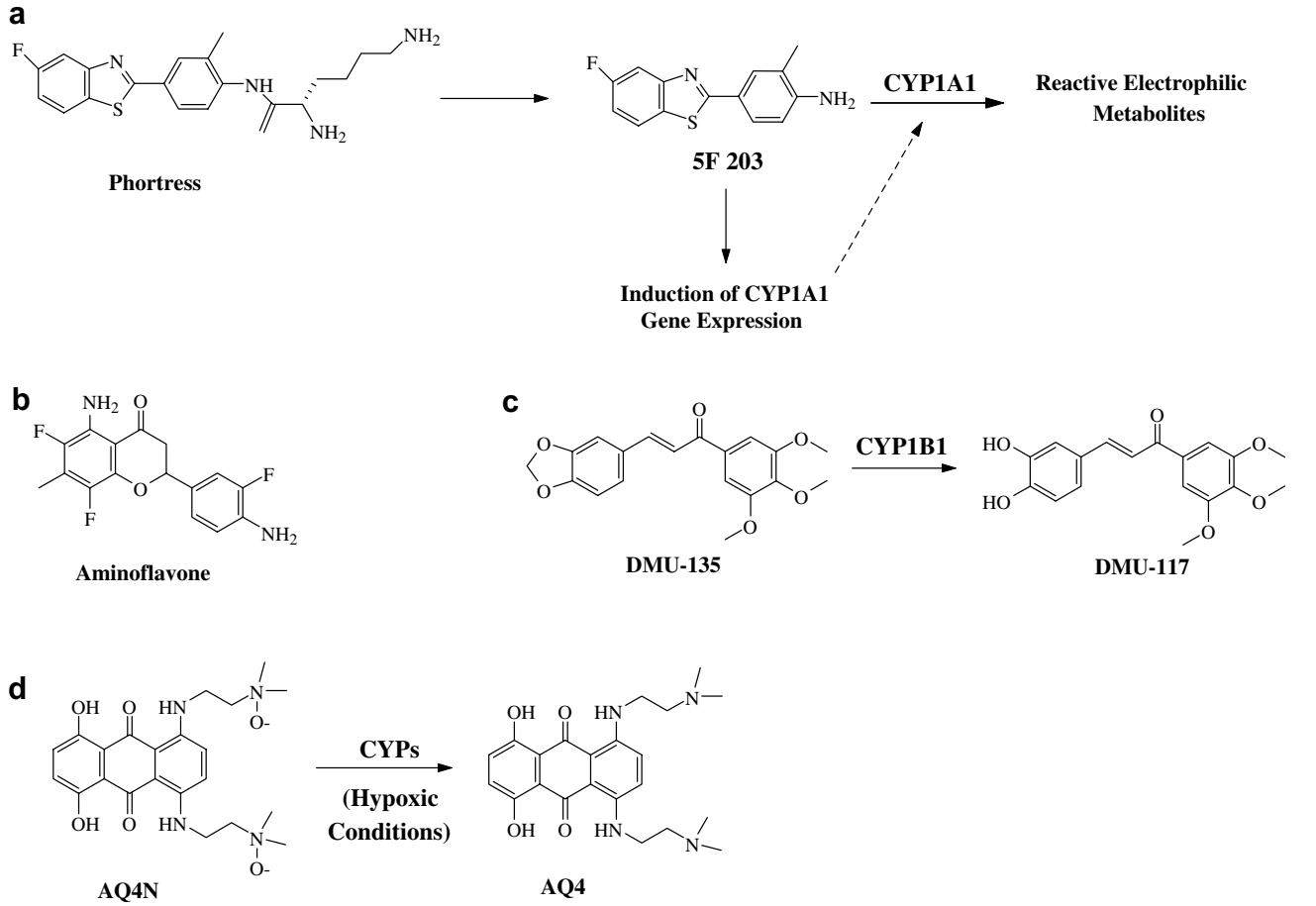

Figure 11. Prodrugs activated by CYPs. (a) Phortress and its metabolite *5F203*. *5F203* activates Ahr inducing transcription of CYP1A1, which activates the drug to a reactive electrophilic species. (b) Aminoflavone is activated by CYP1A1 in a similar fashion as 5F203. (c) DUM-135 and its active metabolite DMU-117. DUM-135 is activated by CYP1B1 to form its active metabolite, DMU-117 (a tyrosine kinase inhibitor). (d) AQ4N and AQ4. AQ4N is activated by CYPs only under hypoxic conditions to form the active topoisomerase inhibitor AQ4.

compound is believed to function through a similar mechanism as *5F203*.[141] Interestingly, however, it has recently been shown that *aminoflavone* requires the expression of sulfotransferase A1 (SULTA1) to elicit a cellular response,[142] suggesting *aminoflavone* activation may be more complex than previously thought.

It remains unclear what the long term effects of treatment with *phortress* or *aminoflavone* will have on patients. As discussed earlier, activation of AhR and induction of CYP1 family proteins is believed to be involved in the development of some cancers. Inducing CYP1 expression may induce more harm than benefit in the long run. Still, the developers of *phortress* have pointed out that the drug's cell specific activation of AhR differs greatly from that of carcinogens, such as PAHs, and pre-clinical safety results demonstrated relatively low toxicity.[140] Only time will tell if the exciting pre-clinical results of *phortress* and *aminoflavone* can translate to clinical efficacy.

Alternatively, prodrugs designed to be explicitly activated by the tumor specific CYP1B1 would offer the added safety advantage of not having to induce CYP1

enzymes. One such molecule, *DMU-135* (3,4-methylenedioxy-3′,4′,5′-trimethoxy chalcone; Fig. 11c) has recently been described.[143] *DMU-135* is converted by CYP1B1 within tumors to form its active metabolite, *DMU-117*, a potent non-selective tyrosine kinase inhibitor (and potentially a COX inhibitor as well). *DMU-135* is being developed as a chemopreventative agent and has shown the ability to prevent tumor gastrointestinal formation in the Apc^{Min+} mouse model without any sign of toxicity. *DMU-135* represents the first prodrug specifically targeted for activation by CYP1B1.

4.2. CYP2

Many members of the CYP2 family have been identified in extrahepatic tissue.[12,14,113] Of note, four are novel CYPs discovered by the Human Genome Project: CYP2S1, CYP2R1, CYP2U1, and CYP2W1.[144] These enzymes play an important role in xenobiotic metabolism, but are also important in endogenous metabolism as well. For example, CYP2S1 can metabolize ATRA, and CYP2R1 is a vitamin D hydroxylase.[144] The potential role of these enzymes in tumor development and progression remains unclear.

CYP2W1 is of particular interest, however, because it has recently been demonstrated as a tumor-specific CYP,[24,25] especially in gastric and adrenal cancers. Little is known about CYP2W1, but arachidonic acid and indole have recently been identified as potential substrates.[144,145] Despite our limited knowledge of the enzyme's function, its apparent tumor-specific expression is intriguing. Further research is needed to determine if this enzyme may be a potential target (either through inhibition or activation of prodrugs) for the prevention or treatment of gastric and adrenal cancers.

4.3. Bioreductive prodrug AQ4N

The topoisomerase inhibitor prodrug *AQ4N* (1,4-bisan5,8-dihydroxyanthracene-9,10-dione; Novacea®, Fig. 11d) exploits the expression of exogenous metabolizing CYPs in tumor cells in a unique manner. Though it does not target a specific CYP enzyme, the local expression of CYPs is required for its activation making it a notable strategy in CYP-based cancer therapy.

It is well established that hypoxic conditions often exist within the tumor microenvironment.[146] *AQ4N* is activated to its basic amine, *AQ4*, only in the hypoxic tumor microenvironment by CYP3A4, CYP1A1, and CYP1B1.[147] *AQ4N* is a weak DNA binder, but *AQ4* interacts tightly with DNA and can penetrate surrounding cells increasing its efficacy within a tumor.[147,148] The bioreduction of *AQ4N* is strongly inhibited by oxygen, ensuring its activation only occurs under hypoxic conditions. Because hypoxic cells are resistant to chemo- and radiotherapies, *AQ4N* is being developed mainly as an adjuvant treatment option.[149–151] *AQ4N* is entering phase Ib/IIa clinical trials.[152]

5. Conclusions

Great progress has already been made in the targeting of CYP enzymes in cancer therapy. For example, aromatase inhibitors have changed the way estrogen dependent cancers such as BCa are treated. This success has paved the way for similar strategies in inhibiting androgen production to combat AD prostate cancer. Molecules designed to block the CYPs responsible for 1,25-D_3 and ATRA metabolism (i.e., RAMBAs) offer advantages in vitamin therapy to fight several forms of cancer. The potential role of xenobiotic metabolizers CYP1A1 and CYP1B1 in the activation of carcinogens and inactivation of chemotherapeutics suggests a potential therapeutic benefits in inhibiting these enzymes. Furthermore, CYP expression in tumor cells is being exploited with prodrugs such as *phortress, aminoflavone, DMU-135*, and *AQ4N* that are activated by these enzymes within the tumor.

Of the drugs discussed, only aromatase inhibitors have achieved clinical success. It remains to be seen whether or not the pre-clinical excitement surrounding other CYP directed therapeutics will resonate in the clinic. Still, CYP directed drugs offer a desperately needed new approach in cancer chemotherapy.

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Vincent C.O. Njar completed his B.Sc. (Hons) in chemistry at University of Ibadan (Nigeria) in 1976 and went on to obtain a Ph.D. in organic chemistry in 1980 from University College London (UK) under the supervision of Derek V. Banthorpe. Following 2 years of postdoctoral research with the late Eliahu Caspi at Worcester Foundation for Experimental Biology, Shrewsbury, Mass (USA), he joined the Department of Chemistry at the University of Ibadan (Nigeria) as Lecturer II. He was promoted through the ranks and became Professor of Organic Chemistry in 1996. He is currently Associate Professor of Pharmacology & Experimental Therapeutics at University of Maryland School of Medicine, Baltimore, MD, USA. His research focus is on medicinal chemistry, pharmacology and development of inhibitors of androgen synthesis (CYP17 inhibitors), anti-androgens, androgen receptor down-regulating agents (ARDAs) and retinoic acid metabolism blocking agents (RAMBAs) as potential anti-cancer agents. Research on the combination of retinoids and/or RAMBAs with histone deacetylase inhibitors (HDACIs) and DNA methylation inhibitors (DMIs) is also being pursued. Our Technology on "CYP17 inhibitors/anti-androgens for prostate cancer" has recently been licensed by our University to Tokai Pharmaceuticals Inc., Boston, MA, USA. His research is interdisciplinary and has strong therapeutics translational potentials. Overall, his research is at the interface of medicinal chemistry and pharmacology/oncology aimed at discovery and development of new drugs for treatments of breast and prostate cancers.

Robert D. Bruno received a B.S. degree in Biology from James Madison University (Virginia, USA) in 2004. He is currently a Ph.D. student in the Molecular Medicine graduate program at the University of Maryland, Baltimore where he works with Dr. Vincent Njar. His research relates to the development of new therapeutic strategies for advanced prostate cancer using novel CYP17 (lyase) inhibitors/anti-androgens in combination with androgen receptor down-regulating agents.

Design, Synthesis, and Evaluation of Novel Mutual Prodrugs (Hybrid Drugs) of All-*trans*-Retinoic Acid and Histone Deacetylase Inhibitors with Enhanced Anticancer Activities in Breast and Prostate Cancer Cells in Vitro

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Novel mutual prodrugs (MPs) of ATRA (all-*trans*-retinoic acid) and HDIs (histone deacetylase inhibitors) (**10**, **13**, **17**−**19**) connected via glycine acyloxyalkyl carbamate linker (AC linker) or through a benzyl ester linker (1,6-elimination linker) were rationally designed and synthesized. Most of our novel MPs were potent inhibitors of growth of several hormone-insensitive/drug resistant breast cancer cell lines and the hormone-insensitive PC-3 prostate cancer cell line. The novel MPs exhibited differential antiproliferative potencies in both MDA-MB-231 and PC-3 cell lines. Whereas **19** (VNLG/124) [4-(butanoyloxymethyl)phenyl(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate] with a GI_{50} of 10 nM was the most potent MP versus the MDA-MB-231 cells, **13** (VNLG/66) [{*N*-[*N*-{2-[4-{[3-pyridylmethoxy)carbonyamino]-methyl}phenyl) carbonylamino]phenyl} carbamoylcarbamoyloxy}methyl(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethyl cyclohex-1-enyl)nona-2,4,6,8-tetraenoate] with a GI_{50} = 40 nM was the most potent versus the PC-3 cells. MP **19** exhibited the most benefit because its GI_{50} of 10 nM versus MDA-MB-231 cells was remarkably 1085-fold lower than that of parent ATRA and over 100000-fold lower than butyric acid (BA).

Introduction

Cancer cells show various degrees of differentiation, and there is normally an inverse relation between the degree of cell differentiation and the clinical aggressiveness of cancer.[1] Differentiation induction of malignant cells is defined by the ability of an agent to induce a more normal or benign phenotype in these cells. Certain retinoids (e.g., all-*trans*-retinoic acid (ATRA)[a] and its isomers) are among the better known differentiation-inducing agents. These retinoids binds with RAR and RXR receptors, and the ligand−receptor complex interaction with retinoid responsive DNA sequence leads to activation of target genes: transcription.[2] Retinoids are currently in clinical use for the treatment of cancers such as acute promyelocytic leukemia (APL) and neuroblastoma.[3,4] The clinical development of retinoids in the treatment of epithelial tumors has been hampered by the development of resistance.[5] Loss of retinoids sensitivity has been associated with lack of RARβ2 expression.[6] Recently, it is reported that lack of RARβ2 expressions in retinoid resistant tumors is associated with RARβ2 promoter hypermethylation and histone deacetylation.[7,8]

The discovery of recruitment of histone deacetylase (HDAC) enzyme by nuclear receptors in cancer has provided a rationale for using inhibition of HDAC activity to release transcriptional repression as a viable option toward achieving eventual therapeutic benefit.[9] Histone deacetylase inhibitors (HDIs) block deacetylation function, causing cell cycle arrest, differentiation, and/or apoptosis of many tumors.[10] Silencing of genes that affect growth and differentiation has been shown to occur by aberrant DNA methylation in promoter region and by changes in chromatin structure that involve histone deacetylation.[11,12] Recent studies have established a link between oncogene-mediated suppression of transcription and recruitment of HDAC into the nuclear complex.[13–15] Several laboratories have reported that the translocation-generated fusion oncogenes (PML-RAR and PLZF-RAR) in APL suppress transcription as a result of sequestering HDAC enzyme.[16,17] Resistance to ATRA of human APL cell lines could be overcome by addition of HDAC inhibitors (HDIs).[16,17] Of particular importance is the observation that an APL patient, who failed multiple therapies and was highly resistant to ATRA, responded to the combination treatment of ATRA and phenylbutyric acid.[18]

Furthermore, in the presence of retinoids, HDIs induce acetylation in RARβ2 hypermethylated promoters leading to the re-expression of RARβ2 in RARβ2-negative retinoid-resistant tumor cells resulting in an additive inhibitory effect on tumor cell growth in vitro and in vivo.[7,19,20] Our group recently reported that the combination of several HDIs with either retinoids or our atypical retinoic acid metabolism blocking agents (RAMBAs) resulted in additive/synergistic growth inhibition of human prostate cancer cells and tumor xenografts.[21,22] Combination treatment with **2** (MS-275) [(*N*-(2-aminophenyl)4-[*N*-(pyridine-3-ylmethoxycarbonyl)aminomethyl]benzamide, a HDI in several phase 2 clinical trials[23] and 13-*cis*-retinoic acid restored retinoid sensitivity in human prostate carcinoma cell

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[a] Abbreviations: APL, acute promyelocytic leukemia; ATRA, all-*trans*-retinoic acid; BA, butyric acid; FDA, Food and Drug Administration; GI_{50}, concentration of compound that cause 50% growth inhibition; HDAC, histone deacetylase; HDI, histone deacetylase inhibitor; MP, mutual prodrug; PD, prodrug; RAMBAs, retinoic acid metabolism blocking agents; SAHA, suberoyl hydroxamic acid.

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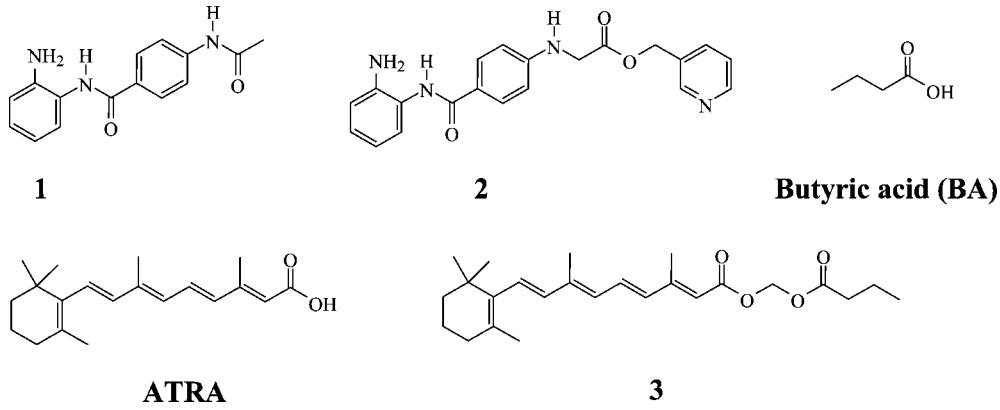
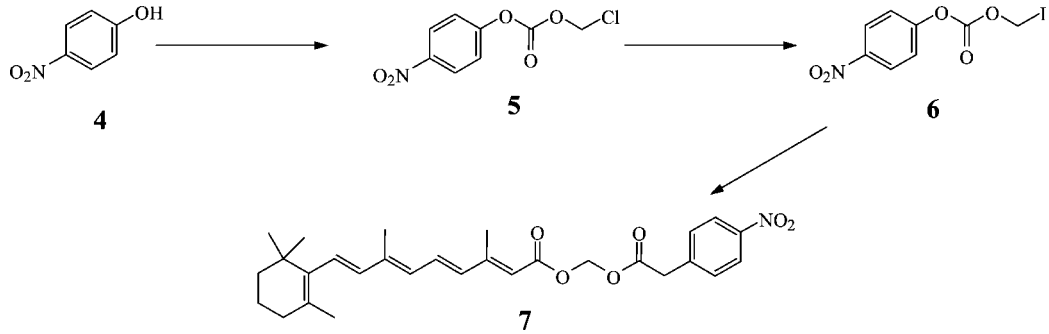
Chart 1. Chemical Structures of **1**−**3**, Butyric Acid (BA), and All-*trans*-Retinoic Acid (ATRA)



Scheme 1. Synthesis of *p*-Nitrophenyl Retinoyloxymethyl Carbonate[a]



[a] Reagents and conditions: (i) $ClCO_2CH_2Cl$, py, $CHCl_3$, rt, 16 h; (ii) NaI, acetone, rt, 24 h; (iii) ATRA, $AgCO_3$, acetone, reflux, 6 h.

lines and had a greater inhibitory effect on tumor cell growth than single agents in vitro and in vivo.[24] Indeed, on the basis of this preclinical study, a phase 1 clinical trial has now been initiated by Pili and colleagues.[25] It should be pointed out that several HDIs are at various stages of clinical development,[10,26] and one of the early HDIs, *N*-hydroxy-N^1-phenylacetanediamide, also called suberoylanilide hydroxamic acid (SAHA),[27,28] was recently (2006) approved by the U.S. Food and Drug Administration (FDA) for the treatment of advanced cutaneous T-cell-lymphoma.[10]

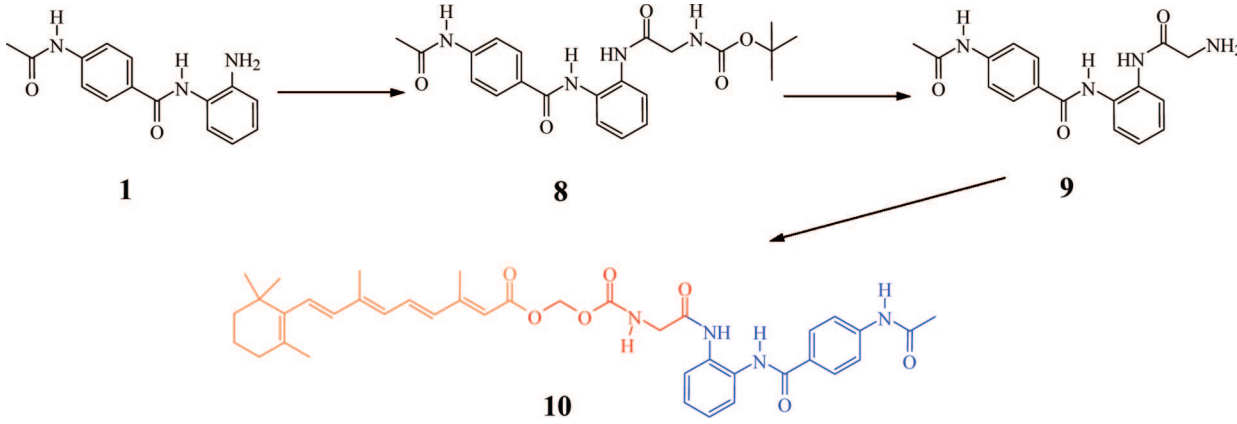
The putative synergistic interaction between retinoids and HDIs has provided the impetus for synthesis and evaluation of mutual prodrugs (MPs) of these agents with hopes of attainment of superior therapeutic efficacy. It is relevant to state here that a mutual prodrug (hybrid drug) is a type of carrier linked prodrug where the carrier used is another pharmacologically active compound instead of some inert molecule. Typically, when two synergistic agents are administered individually but simultaneously, they will be transported to the site of action with different efficiencies. However, when it is desirable to have the two agents reach a site simultaneously, the MP strategy may be used to an advantage. Indeed, the MP of ATRA and butyric acid (BA) called retinoyloxymethyl butyrate, **3** (RN1) has been shown to function at lower concentrations than ATRA or BA alone in the ATRA-sensitive leukemia cell line HL-60.[29] In a recent study, RN1 was found to exhibit significant growth inhibitory activity in both ATRA-sensitive and -resistant APL cells.[30] The chemical structures of **1** (CI-994) [*N*-(2aminophenyl)-4-acetylaminobenzamide], **2**, BA, ATRA, and **3** are presented in Chart 1.

In continuation of our research in this area, we have designed and synthesized two classes of novel MPs of ATRA and HDIs **1**, **2**, or BA. One class is glycine linked **1** or **2** with ATRA connected via an acyloxyalkyl carbamate (**10** and **12**), and the second class is based on the 1,6-elimination concept (**17**−**19**). The new compounds were evaluated against several breast (MCF-7, MCF-7_{TAMR}, MCF-7_{HOXB7} LTLC, LTLT-Ca, and MDA-MB-231) and prostate (PC-3) cancer cell lines, most of which are generally resistant to most therapeutic agents and were found to be potent antineoplastic agents. Importantly, the MPs possess enhanced anticancer activities compared to the parent compounds or their combinations. A preliminary account of part of this work has been presented.[31]

Chemistry. (Acyloxy)alkyl Carbamate Mutual Prodrugs. The (acyloxy) alkyl ester linker has been successfully used for carboxylic acid containing agents to prepare prodrugs (PDs) and MPs, as this linker is very labile and is cleaved by the esterase enzyme. MP of ATRA and butyric acid (**3**) has been prepared using this concept by Nudelman and colleagues.[29,32,33] In addition, amine containing drugs have also been converted into their corresponding acyloxyalkyl carbamates and found to be excellent bioreversible prodrugs.[34,35]

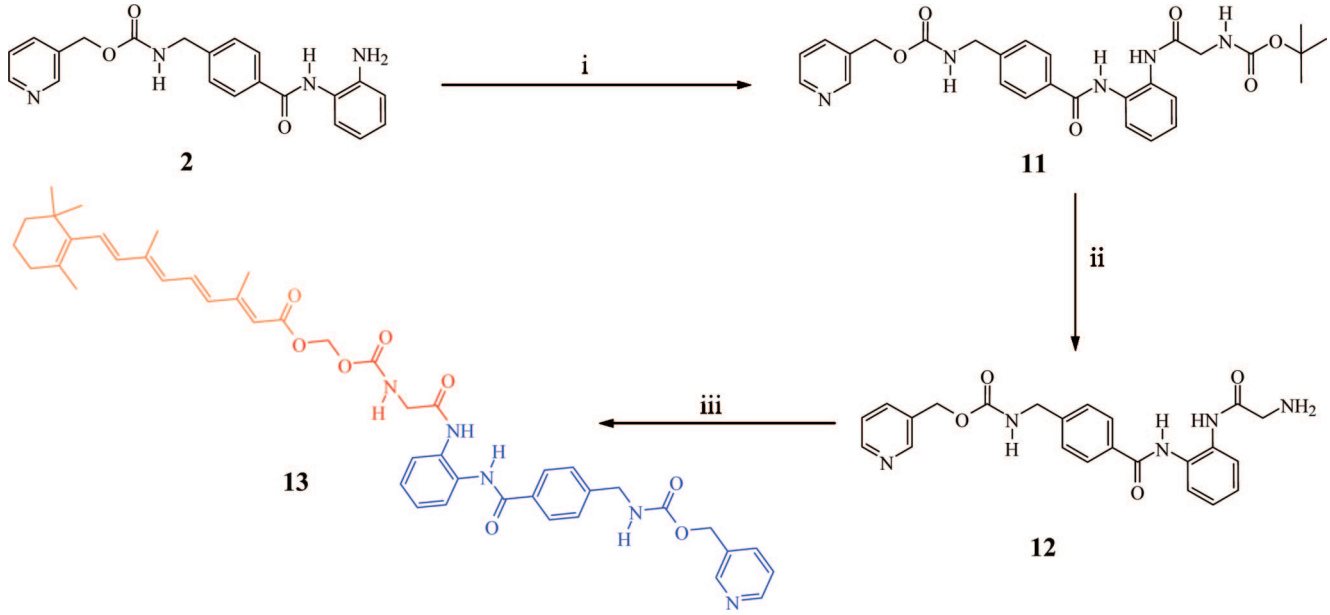
To prepare mutual prodrugs of ATRA and **1** or **2** using this strategy, we first synthesized *p*-nitrophenyl retinoyloxymethyl (**7**) carbonate in three steps (Scheme 1). Thus, treatment of *p*-nitrophenol (**4**) with chloromethyl chloroformate in the presence of pyridine as base gave chloromethyl-*p*-nitrophenyl carbonate (**5**) in good yield (52%). Compound **5** was converted to the corresponding iodomethyl *p*-nitrophenyl carbonate (**6**) following treatment with NaI. Treatment of **6** with ATRA in the presence of silver carbonate as base in acetone afforded the desired *p*-nitrophenyl retinoyloxymethyl carbonate (**7**) in 26% yield. Attempts to condense aromatic amino group of **1** or **2** with compound **7** were unsuccessful. The reason for this is unknown at this time but may be due to low nucleophilicity/steric hindrance of the aromatic amine moiety in **1** and **2**. On

Scheme 2. Synthesis of Mutual Prodrug of ATRA and **1** with (Acyloxy)alkyl Carbamate Linker[a]



[a] Reagents and conditions: (i) Boc-Gly-OH, DCC, HOBt, DMF, rt, 18 h; (ii) TFA, CH_2Cl_2, 2 h; (iii) **7**, HMPA, TEA, rt, 24 h.

Scheme 3. Synthesis of Mutual Prodrug of ATRA and **2** with (Acyloxy)alkyl Carbamate Linker[a]

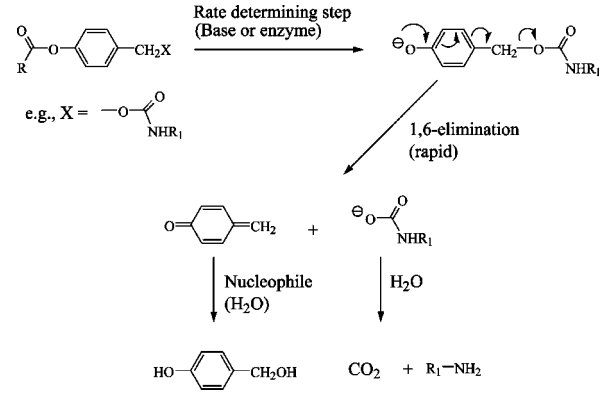


[a] Reagents and conditions: (i) Boc-Gly-OH, DCC, HOBt, DMF, rt, 18 h; (ii) TFA, CH_2Cl_2, 2 h; (iii) **7**, HMPA, TEA, rt, 24 h.

the basis of previous studies,[36] we decided to prepare amino acid derivative (prodrugs) of CI-994 and MS-275 for coupling with compound **7**. First, we prepared a glycine derivative of compound **1** (**9**) in two steps. *N*-Boc-glycine was coupled to **1** to give **8** (70% yield) by the active ester method using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) followed by deprotection with trifluroacetic acid (TFA) to give the glycine derivative of compound **1** (**9**). Treatment of **9** with *p*-nitrophenyl retinoyloxymethyl carbonate (**7**) afforded the desired MP **10** in good yield (60%) (Scheme 2). A similar synthetic procedure was used to synthesize the MP of ATRA and **2**, compound **13**, as outlined in Scheme 3.

1,6-Elimination Based Prodrugs. Generally, 1,4- or 1,6-elimination of HX (where X is a good leaving group like halide, functionalized oxygen derivatives such as carboxylates, or a carbamic acid anion) from benzyl compounds bearing strong electron-releasing *o*- or *p*-hydroxy or -amino substituents is a fast reaction that occurs under mildly basic conditions. Concomitantly, quinone methides and quinonimine methides are produced (Scheme 4).[37] Although many prodrugs have been prepared using the 1,6-elimination concept, especially for tumor

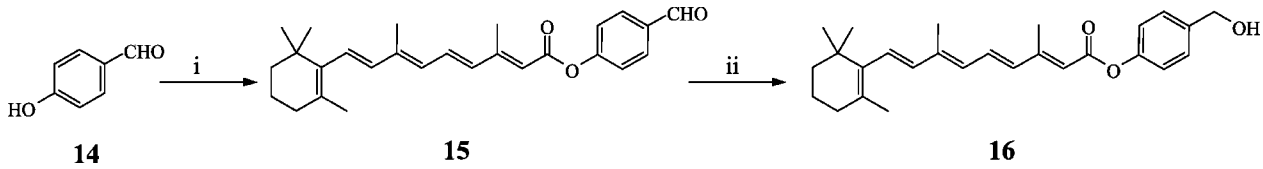
Scheme 4. Drug Release from Prodrug via 1,6-Elimination Mechanism



targeted drug delivery,[38] there are *very few* examples of MPs based on this concept.[39,40]

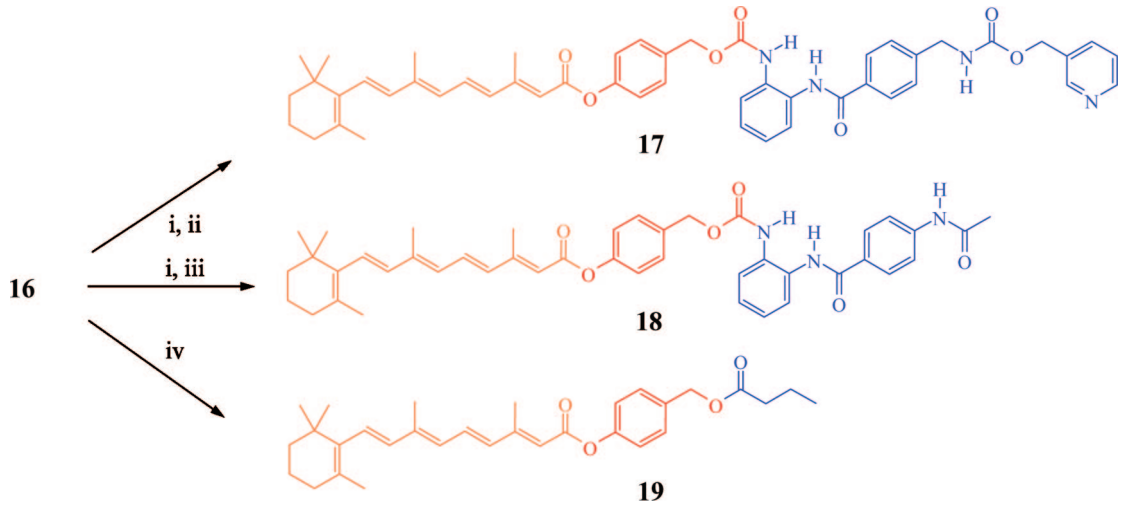
First, we prepared all-*trans*-retinoic acid benzyl alcohol ester (**16**), a key intermediate for the synthesis of MPs of various HDIs based on the 1,6-elimination concept (Scheme 5). *p*-

Scheme 5. Synthesis of Retinoic Acid Benzyl Alcohol Ester[a]



[a] Reagents and conditions: (i) ATRA, DCC, DMAP, DMF, rt, 24 h: (ii) $NaBH_4$, $CHCl_3$: IPA [1:5], 0 °C, 1 h.

Scheme 6. Synthesis of Mutual Prodrugs of ATRA and HDIs based on 1,6-Elimination Concept[a]



[a] Reagents and conditions: (i) triphosgene, Na_2CO_3, $PhCH_3$, 0 °C, 6 h; (ii) **2**, TEA, THF, 0 °C, rt, 16h; (iii) **1**, TEA, THF, 0 °C, rt, 16 h; (iv) butyric acid, DCC, DMAP, rt, 24 h.

Hydroxybenzaldehyde was coupled with ATRA using DCC/DMAP in DMF to yield benzaldehyde ester of ATRA (**15**). Compound **12** was readily reduced with $NaBH_4$ to the corresponding alcohol (**16**), which was then converted to the corresponding chloroformate intermediate by treatment with triphosgene and Na_2CO_3 in toluene. This chloroformate was used in the subsequent step without purification. Reaction of the chloroformate with **2** using triethylamine (TEA) as base in THF produced the desired MP **17**. A similar reaction of the chloroformate derivative with **1** gave MP **18**. Finally, reaction of alcohol (**16**) with butyric acid in the presence of DCC/DMAP yielded MP **19**.

To be effective MPs, these modified novel compounds must revert rapidly and quantitatively to ATRA and the corresponding HDIs in animal tissues and cell cultures. Thus, we developed HPLC methods (see Experimental Section) to briefly study the cleavage of compounds **10**, **12**, and **17**−**19** in fresh mouse plasma. We observed that all MPs were completely cleaved to their parent compounds within 1 h of incubations at 37 °C. Furthermore, the stability of mutual prodrugs was studied in 0.02 M phosphate buffer (pH = 7.2) and also in 80% human serum (obtained from Sigma) containing 20% 0.02 M phosphate buffer as previously described.[41] The MPs were each incubated at 37 °C for 24 h, extracted and analyzed by HPLC. We found that all the MPs were stable under these conditions. The difference in stabilities of the MPs in fresh mouse plasma and commercial human serum may be due to deactivation of certain enzymes such as esterases and peptidases required for cleavage in human serum.

Biological Studies. Most retinoids, including ATRA, are generally weak inhibitors of proliferation of hormone-insensitive breast and prostate cancer cells.[1,3] We[22,31,42] and others[19,23,24,29,30] have demonstrated that cotreatment of combinations of retinoids with some HDIs cause additive/synergistic inhibition of growth of these cancer cell lines. In an effort to improve delivery (efficacy) of each agent, we have designed and synthesized novel MPs of ATRA and three HDIs, including BA, **1**, and **2** as described above. We hypothesize that the MPs might exert stronger antiproliferative activity in cancer cells than cotreatments of the two agents that comprise the MPs, or than each of the two agents alone.

Table 1. GI_{50} (Growth Inhibitory Activity) of ATRA and HDIs and Mutual Prodrugs from Dose−Response Curves in PC-3 and MDA-MB-231 Cell Lines

compounds	GI_{50} values (μM)[a]	
	PC-3 prostate cancer cells	MDA-MB-231 breast cancer cells
ATRA	7.6	10.85
1	0.29	0.17
2	0.19	0.009
sodium butyrate (BA)	72.44	>1000
10, (VNLG/60), ATRA-**1** (AC linker)[b]	4.27	0.63
13, (VNLG/66), ATRA-**2** (AC linker)[b]	0.04	0.94
17, (VNLG/114) ATRA-**2** (1,6-E linker)[b]	0.18	0.17
18, (VNLG/122), ATRA-**1** (1,6-E linker)[b]	0.87	0.02
19, (VNLG/124), ATRA-BA (1,6-E linker)[b]	1.02	0.01

[a] The GI_{50} values were determined from dose−response curves (by nonlinear regression analysis using GraphPad Prism) compiled from at least three independent experiments, SEM < 10%, and represents the compound concentration (μM) required to inhibit cell growth by 50%. [b] AC linker = acyloxymethylcarbamate linker and 1,6-*E* linker = 1,6-elimination linker.

MPs Inhibit Proliferation of MDA-MB-231 Breast and PC-3 Prostate Cancer Cells. First, the growth inhibitory effect of ATRA and different HDIs were evaluated and GI_{50} values (concentrations that cause 50% growth inhibition) were obtained from the dose−response curve and are presented in Table 1. The cell growth inhibitory potencies elicited by ATRA were similar in both MDA-MB-231 (GI_{50} = 10.8 μM) and PC-3 (GI_{50}

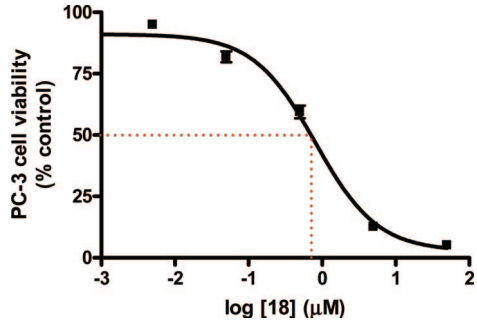


Figure 1. Concentration-dependent curve showing the antiproliferative effect of MP **18** on human prostate cancer PC-3 cells. Data are means (± SEM) of at least three independent experiments. The experiments with the other compounds gave plots that were similar to that shown above.

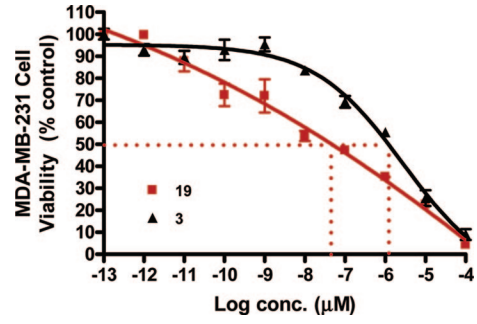


Figure 2. Growth inhibitory effect of **3** and MP **19** in MDA-MB-231 breast cancer cell line. Data are means (± SEM) of at least three independent experiments.

= 7.6 μM) cells. With respect to the HDIs, both cell lines were sensitive to **2**, and as expected, BA was a very weak inhibitor of cell growth. In both cell lines, the same order of potency, **2** > **1** > ATRA ≫ BA, was observed. In addition, significant differences between the two cell lines were (i) the gigantic difference between the potencies of BA in PC-3 (GI_{50} = 72.44 μM) versus MDA-MB-231 (GI_{50} > 1 mM) and (ii) sensitivity of MDA-MB-231 to **2** (GI_{50} = 9.0 nM) compared to PC-3 (GI_{50} = 190 nM), a 21-fold difference.

To assess the effect of MPs on cell growth, PC-3 and MDA-MB-231 cells were treated with MPs for 4 or 6 days, respectively. A typical dose response curve for the antiproliferative effect of MP **18** is presented in Figure 1. Relative to other MPs, **13** (ATRA-**2** with AC linker) was the most potent at inhibiting PC-3 cell growth (GI_{50} = 40.0 nM) while **10** (ATRA-**1** with AC linker) was the least potent (GI_{50} = 4.27 μM). In this cell line, the order of potency was **13** > **17** > **18** > **19** > **10**. The efficacies of MPs were compared to the efficacies of ATRA or HDIs alone in PC-3 prostate cancer cells. In general, the GI_{50} values of all MPs were 1.8- to 190-fold lower than that of ATRA and 17- to 1811-fold lower that of BA. Comparing the efficacies of MPs with either of the HDIs, MP **16** (ATRA-BA, with 1,6-elimination linker) exhibited the most benefit because its GI_{50} of 1.02 μM was 74-fold lower than BA. Given the potent cell growth inhibition (GI_{50} = 190 nM) caused by compound **2**, it is remarkable that MP **13** (ATRA-**2** with AC linker) was still very potent with a GI_{50} of 40 nM, 4.75-fold lower than **2**. In contrast, MPs with HDI **1**, **10**, and **18**, with GI_{50} values of 4.27 and 0.87 μM, respectively, were each less potent than **1** (GI_{50} = 0.29 μM). The reason(s) for these differential potencies of the different MPs in PC-3 cells are unknown at this time, but may be idiosyncratic, possibly due to extents and efficiencies of cell membrane penetration and/or intracellular cleavage of MPs.

Compared to their efficacies in PC-3 cells, the MPs exhibited different potencies in the MDA-MB-231 cells. Relative to other MPs, **19** (ATRA-BA with 1,6-elimination linker) was the most potent at inhibiting MDA-MB-231 cell growth (GI_{50} = 10.0 nM), while **13** (ATRA-**2** with AC linker) was the least potent (GI_{50} = 940 nM). In this cell line, the order of potency was **19** > **18** > **17** > **10** > **13**. Other notable observations on the antiproliferative effects of ATRA, HDIs, and MPs in this cell line were: (i) that MP **19** exhibited the most benefit because its GI_{50} of 10 nM was remarkably 1085-fold lower that that of ATRA and over 100000-fold lower than BA, (ii) MP **18** (ATRA-**1** with 1,6-E linker) with GI_{50} = 20 nM is superior to related MP **10** (ATRA-**1** with AC linker), GI_{50} = 630 nM, a robust 31.5-fold difference, and (iii) **18** is also more potent than either ATRA (543-fold lower) or **1** (8.5-fold lower). Indeed, this gain in function of **19** in this cell line is by far superior to that previously reported by Nudelman and Rephaeli[29] for retinoyloxymethyl butyrate (**3**, an MP derived from ATRA and BA with an acyloxyalkyl linker) in myeloid leukemia cell line HL-60. It might be unexpected that the coupling of ATRA to BA would cause such a large increase in activity, considering the low potency of BA. As previously reported,[29] the results may be explained by a combination of two factors: (i) the ATRA fragment of **19** imparts lipophilicity and facilitates the penetration of BA to the cellular target site, and (ii) the intracellularly released ATRA and BA affect the cells synergistically.

Furthermore, in our desire to compare the efficacy of **3** with that of our closely related MP **19**, we synthesized compound **3** as previously described[29] and assessed their antiproliferative activities head-to-head in MDA-MB-231 cells. As shown in Figure 2, the GI_{50} of **19** for inhibition of growth of MDA-MB-231 cells was 48 nM, 25-fold lower than that of **3** (GI_{50} = 1.18 μM). Together, these data suggest that the acyloxymethycarbamate linker is superior to the acyloxyalkyl linker. On the basis of the mean GI_{50} values of all MPs obtained for the two cell lines, it tempting to suggest that that the 1,6-elimination linker with mean GI_{50} = 0.035 μM (n = 6) is superior to AC linker with mean GI_{50} = 1.47 μM (n = 4) (see Table 1). Validation of this assertion would probably require analysis of larger data set. It should be stated that some byproduct resulting from intracellular cleavage of MPs such as formaldehyde (generated from MPs with acyloxylalkyl linker)[29,43] or quinine methide (generated from MPs with 1,6-elimination type linker)[39] have also been implicated in the anticancer activities of the two components of the MPs. Experiments to assess the possible involvement of byproduct of our MPs are envisioned in future mechanistic studies.

Based on these encouraging results with the novel MPs, we wished to assess further possible advantage of the MPs over simultaneous treatment of components of the MPs. Using representative MPs (**17**−**19**), we observed that the antiproliferative activities in both cell lines elicited by the MPs were each greater than those of the combined parent ATRA and HDIs (Figure 3a−e). Treatment of PC-3 cells with 20 μM **19** resulted in significantly potent growth inhibition (~80%) compared to a mixture of 10 μM ATRA and 10 μM BA (Figure 3a). Furthermore, using dose−response curves, the antiproliferative activity elicited by **19** (GI_{50} = 1.7 μM) was 15-fold lower than the combination of increasing concentrations of ATRA and BA (10.0 μM) (GI_{50} = 25.7 μM) (Figure 3b). Similar results were also obtained for **18** versus parent ATRA (increasing concentrations) and **1** (0.2 μM) (Figure 3c) and **17** versus parent ATRA

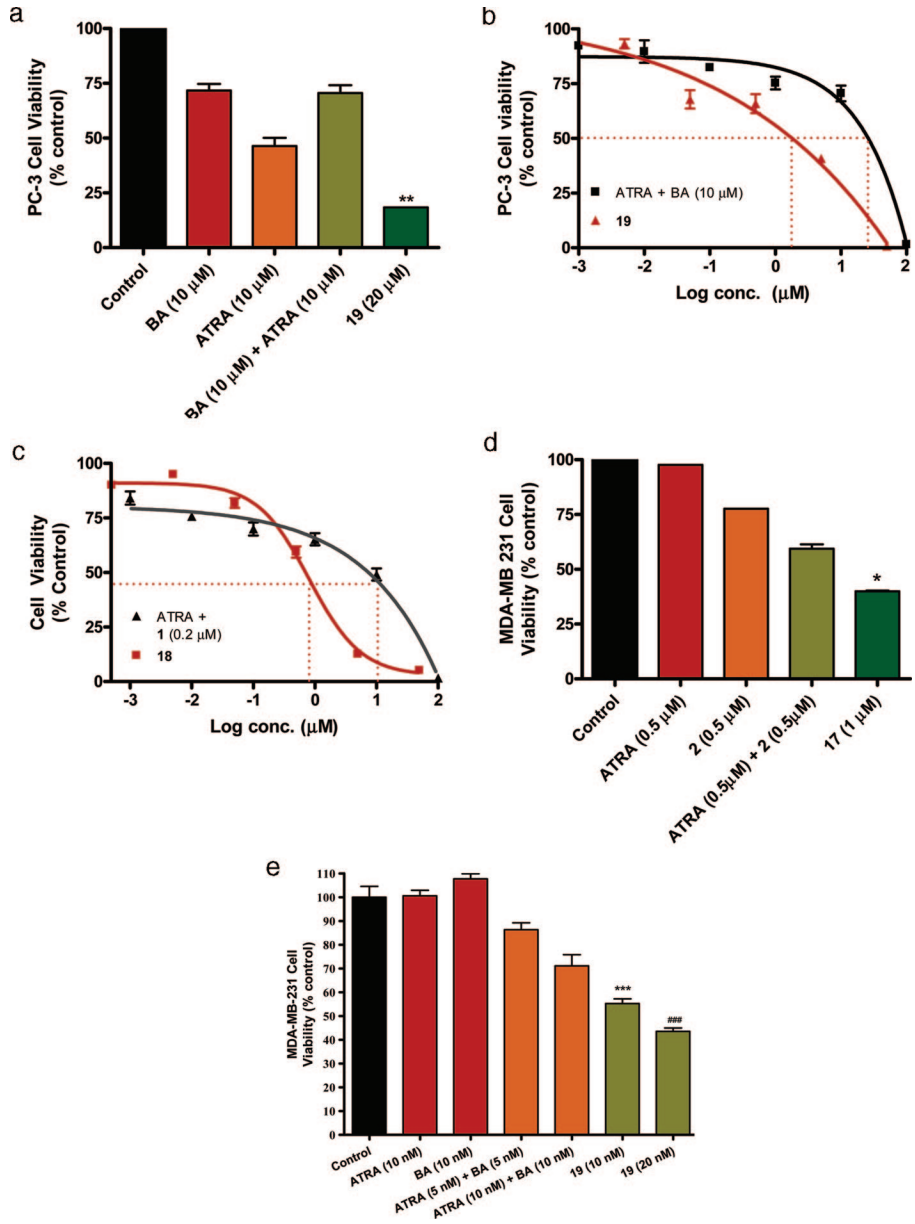


Figure 3. (a) Effect of ATRA and butyric acid administered alone or in combination and of mutual prodrug **19** on PC-3 cell growth. ** indicates a significant increase from control and BA, ATRA, or BA + ATRA treatments ($P < 0.001$). (b) Growth inhibitory effect of ATRA and sodium butyrate in combination and of corresponding mutual prodrug **19** in PC-3 cell line. Data are means (± SEM) of at least three independent experiments. (c) Effect of ATRA and **1** administered alone or in combination and of corresponding mutual prodrug **18** on PC3 cell growth. Data are means (SEM < 10%) of at least three independent experiments. (d) Effect of ATRA and **2** administered alone or in combination and of corresponding mutual prodrug **17** on MDA-MB-231 cell growth. * indicates a significant increase from control and **2**, ATRA, or **2** + ATRA treatments ($P < 0.01$). (e) Effect of ATRA and butyric acid administered alone or in combination and of mutual prodrug **19** on MDA-MB-231 cell growth. *** indicates a significant increase from ATRA (5 nM) + BA (5 nM) treatment ($P < 0.0001$). ### indicates a significant increase from ATRA (10 nM) + BA (10 nM) treatment ($P < 0.0001$).

and **2** (Figure 3d) in PC-3 cells, and also for **19** versus parent ATRA and BA in MDA-MB-231 (Figures 3e).

MPs Also Inhibit Proliferation of Drug-Resistant Breast Cancer Cell Lines. Given the exquisite potency of most of our novel MPs in MDA-MB-231 and PC-3 cell lines, it seemed logical to investigate their effects on the growth of some known drug-resistant breast cancer cell lines, including MCF-7_{TAMR}, MCF-$7_{HOX\text{-}B7}$, LTLC, and LTLT-Ca (see Experimental Section for description of cell lines phenotypes) compared to parental MCF-7 cells. As presented in Table 2, most of the MPs tested, including **10**, **13**, **17**, and **18** resulted in potent inhibition of these resistant cell lines, with GI_{50} values in the low nanomolar range.

Conclusion

We have developed rationale strategies that allowed us to synthesize novel mutual prodrugs (MPs) of ATRA and three promising HDIs, BA, **1**, and **2**. Most of these novel MPs were shown to possess potent antiproliferative activity versus hormone/drug-resistant breast and prostate cancer cell lines. The uniqueness of these novel MPs stem from the combination of two moieties, ATRA and HDI (BA or **1** or **2**), each affecting

Table 2. GI_{50} Values Obtained from Dose−Response Curves in Other Breast Cancer Cell Lines

	GI_{50} values (μM)[a]				
compounds	MCF-7	LTLC	LTLT-Ca	MCF-7_{TAMR}	MCF-$7_{HOX\text{-}B7}$
10	0.25	n/d	n/d	0.17	1.20
13	0.15	0.02	0.65	0.02	0.006
17	0.06	n/d	n/d	0.21	0.72
18	0.52	n/d	n/d	0.08	8.13

[a] The GI_{50} values were determined from dose−response curves (by nonlinear regression analysis using GraphPad Prism) compiled from at least three independent experiments, SEM < 10%, and represent the compound concentration (μM) required to inhibit cell growth by 50%. n/d = not determined.

distinctive cellular targets and when released simultaneously inside the cancer cells probably act synergistically. Evaluation of their mechanisms of action and in vivo antitumor efficacies of some of these novel agents in breast and prostate tumor xenograft models are currently underway in our laboratory.

Experimental Section

Chemistry. General procedures and techniques were identical with those previously reported.[22] ^{1}H NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 at 500 MHz with Me_4Si as an internal standard using a Varian Inova 500 MHz spectrometer. High-resolution mass spectra (HRMS) were determined on a Bruker 12Tesla APEX-Qe FTICR-MS by positive ion ESI mode by Susan A. Hatcher, Facility Director, College of Sciences Major Instrumentation Cluster, Old Dominion University, Norfolk, VA. Retinoids (all-*trans*-retinoic acid from LKT Laboratories, Inc., St. Paul, MN). Compounds **1** and **2** were synthesized in our laboratory as previously reported.[21] All other reagents were purchased from Sigma-Aldrich. Although the retinoidal intermediates and final products appeared to be relatively stable to light, precautions were taken to minimize exposure to any light source and to the atmosphere. Thus, all operations were performed in dim light, with reaction vessels wrapped with aluminum foil. All compounds were stored in an atmosphere of argon and in the cold (−20 or −80 °C).

2-Chloromethyl-*p*-nitrophenyl Carbonate (5). To an ice-cold mixture of *p*-nitrophenol (**4**, 1.39 g, 10 mmol) and pyridine (0.8 g, 10 mmol) in $CHCl_3$ (50 mL) was added chloromethyl chloroformate (1.41 g, 11 mmol). After approximately 30 min at 0−4 °C, the reaction mixture was stirred further for 16 h at rt. Following successive washing with 0.5% aq NaOH and water, the $CHCl_3$ layer was dried over anhydrous Na_2SO_4 and evaporated to give thick yellow oil. This crude product was purified using flash column chromatography [FCC, pet. ether/EtOAc, (9:1)] to obtain **5** (1.2 g, 52%); mp 44−45 °C. ^{1}H NMR ($CDCl_3$): δ 5.85 (s, 2H, CH_2), 7.43 (d, 2H, J = 7.5 Hz, Ar-Hs), 8.31 (d, 2H, J = 9 Hz, Ar-Hs).

2-Iodomethyl-*p*-nitrophenyl Carbonate (6). Compound **5** (2.0 g, 8.63 mmol) dissolved in acetone was treated with NaI (2.16 g, 14.42 mmol) and then stirred at rt for 24 h. The reaction mixture was evaporated and the residue was dissolved in CH_2Cl_2, followed by washing with saturated solution of sodium bisulfite and water. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to obtain thick brown oil **6** (2.3 g). The crude product was used as such without further purification. ^{1}H NMR: δ 6.07 (s, 2H, CH_2), 7.43 (d, 2H, J = 8.0 Hz, Ar-Hs), 8.31 (d, 2H, J = 8.5 Hz, Ar-Hs).

(4-Nitrophenoxycarbonyloxy)methyl(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate (7). ATRA (0.3 g, 1 mmol) was dissolved in acetone (15 mL) and to this was added Ag_2CO_3 (303 mg, 1.1 mmol) and refluxed for 1 h. The reaction mixture was cooled to rt (mixture A). Crude compound **6** (0.388 g) was dissolved in acetone (10 mL) separately and stirred at rt. Mixture A was added slowly to the solution of **6** followed by refluxing for 6 h. The reaction mixture was cooled to rt, filtered, and the filtrate was evaporated to dryness. The crude product was purified by FCC [pet. ether/EtOAc, (9.5:0.5)] to obtain pure **7** (129 mg, 26%); mp: 35−36 °C. ^{1}H NMR ($CDCl_3$): δ 1.03 (s, 6H, 16 and 17-CH_3), 1.46 (m, 2H, CH_2), 1.60 (m, 2H, CH_2), 1.71 (s, 3H, 18-CH_3), 2.02 (s, 3H, 19-CH_3), 2.04 (m, 2H, CH_2), 2.40 (s, 3H, 20-CH_3), 5.82 (s, 1H, 14-H), 5.94 (s, 2H, CH_2), 6.23 (m, 4H, 7, 8, 10 and 12-Hs), 7.09 (dd, 1H, J = 13.5 Hz, 11H), 7.42 (d, 2H, J = 9.5 Hz, Ar-Hs), 8.29 (d, 2H, J = 9 Hz, Ar-Hs).

***N*-(2-{[4-(Acetylamino)phenyl]carbonylamino}phenyl)-2-[(*tert*-butoxy)carbonylamino] acetamide (8).** Boc-glycine (0.210 g, 1.2 mmol) and 1-hydroxybenzotriazole (HOBt) (0.162 g, 1.2 mmol) were dissolved in DMF (5 mL) and stirred at 0−5 °C. To this added solution was added **1** (0.269 g, 1 mmol), followed by dicyclohexylcarbodiimide (DCC) (0.248 g, 1.2 mmol). The cooling bath was removed after 30 min, and the reaction mixture was stirred at rt for 18 h. The reaction mixture was poured into ice-cold water and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude product was purified by FCC [CH_2Cl_2/EtOH, (9:1)] to afford 300 mg pure compound **8** (70%); mp: 125−126 °C. ^{1}H NMR: δ 1.34 (s, 9H, CH_3), 2.08 (s, 3H, CH_3), 3.73 (s, 2H, CH_2) 7.21 (s, 2H, Ar-Hs), 7.59 (s, 1H, Ar-H), 7.63 (s, 1H, Ar-H), 7.72 (d, 2H, J = 7.5 Hz, Ar-Hs), 7.94 (d, 2H, J = 8.5 Hz, Ar-Hs), 9.51 (s, 1H, NH), 9.82 (s, 1H, NH), 10.22 (s, 1H, NH).

***N*-(2-{[4-(Acetylamino)phenyl]carbonylamino}phenyl)-2-aminoacetamide (9).** To an ice-cold solution of compound **8** (250 mg, 0.586 mmol) in CH_2Cl_2 (4 mL) was added TFA (4 mL), followed by stirring at 0−5 0 °C for 2 h. The reaction mixture was evaporated to dryness; acetone was added and stirred for 30 min. The white precipitate that formed was filtered and dried under vacuum to give pure compound **9** (148 mg, 77%); mp: 215−218 °C. ^{1}H NMR: δ 2.13 (s, 3H, CH_3), 3.42 (s, 1H, NH_2), 3.86 (s, 2H, CH_2), 7.28 (s, 1H, Ar-H), 7.68 (s, 1H, Ar-H), 7.77 (d, 2H, J = 9 Hz, Ar-Hs), 7.99 (d, 2H, J = 9 Hz, Ar-Hs), 8.17 (s, 2H, Ar-Hs), 9.71 (s, 1H, NH), 9.80 (s, 1H, NH), 10.30 (s, 1H, NH).

(*N*-{[*N*-(2-{[4-(Acetylamino)phenyl]carbonylamino}phenyl)-carbamoyl]methyl}carbamoyloxy) methyl (2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate (10). To the solution of compound **7** (50 mg, 0.101 mmol) in hexamethylphosphoramide (HMPA) (1 mL) was added compound **9** (49 mg, 0.15 mmol) and Et_3N (210 μL, 0.15 mmol), and the reaction mixture was stirred at rt for 24 h. The reaction mixture was poured into ice-cold water and extracted with CH_2Cl_2. The organic layer was dried with anhydrous Na_2SO_4 and evaporated to dryness. The crude product was purified using FCC [CH_2Cl_2/EtOH, (20:1)] to give pure **10** (42 mg, 60%); mp: 32−33 °C. IR ($CHCl_3$): 3429, 1734, 1676, 1599, 1508, 1457, 1335, 1297, 1214, 1066, 986, 754, 668 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 1.03 (s, 6H, 16,17-CH_3), 1.71 (s, 3H, 18-CH_3), 2.02 (s, 3H, 19-CH_3), 2.13 (s, 3H, CH_3), 2.40 (s, 3H, 20-CH_3), 3.90 (s, 2H, CH_2), 5.59 (s, 2H, CH_2), 5.80 (s, 1H, 14-H), 6.28 (m, 4H, 7-, 8-, 10- and 12-Hs), 7.20 (dd, 1H, J = 14.7 Hz, 11-H), 7.23 (s, 2H, Ar-H), 7.61 (s, 1H, Ar-H), 7.63 (s, 1H, 9Ar-H), 7.72 (d, 2H, J = 7.5 Hz, Ar-Hs), 7.94 (d, 2H, J = 8.5 Hz, Ar-Hs), 8.1 (s, 1H, NH), 9.817 (s, 1H, NH), 10.3 (s, 1H, NH). HRMS calcd 683.3439 ($C_{39}H_{46}N_4O_7H^+$), found 683.3448.

2-[(*tert*-Butoxy)carbonylamino]-*N*-{2-[(4-{[(3-pyridylmethoxy)-carbonylamino]methyl} phenyl)carbonylamino]phenyl}acetamide (11). Boc-glycine (0.210 g, 1.2 mmol) and HOBt (0.162 g, 1.2 mmol) was dissolved in DMF (5 mL) and stirred at 0−5 °C. To this was added **2** (0.376 g, 1 mmol), followed by DCC (0.247 g, 1.2 mmol). The cooling bath was removed after 30 min and reaction mixture was stirred at rt for 18 h. The reaction mixture was poured into ice-cold water and extracted with EtOAC. The organic layer was washed with brine and dried over anhydrous Na_2SO_4 and then evaporated to dryness. The crude product was purified by FCC [CH_2Cl_2/EtOH (9:1)] to afford 380 mg (71%) of pure compound **11** as a low melting solid. ^{1}H NMR ($CDCl_3$): δ 1.23 (s, 9H, CH_3), 3.87 (s, 2H, CH_2), 4.39 (d, 2H, J = 5.5, CH_2), 5.14 (s, 2H, CH_2), 7.15 (m, 2H, Ar-Hs), 7.29 (d, 2H, J = 7.5 Hz, Ar-Hs), 7.61 (d, H, J = 8 Hz, Ar-Hs), 7.72 (d, 1H, J = 7 Hz, Ar-Hs), 7.79 (m, 1H, Ar-H), 7.83 (d, 2H, J = 8.5 Hz, Ar-Hs), 8.52 (s, 1H, NH), 8.58 (s, 1H, NH_2), 8.92 (s, 1H, NH), 9.24 (s, 1H, NH).

2-Amino-*N*-{2-[(4{[(3-pyridylmethoxy)carbonylamino]methyl}phenyl)carbonylamino]phenyl} acetamide (12). To an ice-cold solution of compound **11** (300 mg, 0.562 mmol) dissolved in

CH_2Cl_2 (4 mL) was added TFA (4 mL) and stirred at 0−5 °C for 2 h. The reaction mixture was evaporated to dryness and to this was added acetone followed by stirring for 30 min. The white precipitate was filtered and dried under vacuum to give pure **12** as a low melting solid (169 mg, 69%). ^{1}H NMR (DMSO-d_6): δ 3.82 (s, 2H, CH_2), 4.28 (d, 2H, J = 6 Hz, CH_2), 5.11 (s, 2H, CH_2), 7.24 (d, 1H, J = 7 Hz, Ar-Hs), 7.39 (d, 1H, J = 8 Hz, Ar-H), 7.45 (m, 1H, Ar-H), 7.65 (s, H, Ar-H), 7. 83 (s, 1H, Ar), 7.95 (d, 2H, J = 8 Hz, Ar-Hs), 8.08 (s, 1H, Ar−H), 8.25 (s, 1H, Ar-H), 8.623 (s,1H, Ar-H), 8.563 (s,1H, Ar-H), 9.55 (s, 1H, NH), 9.77 (s, 1H, NH), 10.02 (s, 1H, NH).

{*N*-[*N*-{2-[4-{[3-Pyridylmethoxy)carbonyamino]methyl}phenyl)carbonylamino]phenyl}carbamoyloxy}methyl(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate (13). To the solution of **7** (50 mg, 0.101 mmol) in HMPA was added **12** (64.98 mg, 0.15 mmol) and Et_3N (210 μL, 0.15 mmol) and the reaction mixture was stirred at rt for 24 h. The reaction mixture was poured into ice-cold water and extracted with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude product was purified using FCC [CH_2Cl_2/EtOH, (20:1)] to yield compound **13** (60 mg, 65%); mp: 56−58 °C. IR ($CHCl_3$): 3684, 1715, 1651, 1592, 1519, 1477, 1336, 1296, 1214, 1123, 988, 754, 668 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.02 (s, 6H, 16,17-CH_3), 1.47 (m, 2H, CH_2), 1.61 (m, 2H, CH_2), 1.70 (s, 3H, 18-CH_3), 1.99 (s, 3H, 19-CH_3), 2.29 (s, 3H, 20-CH_3), 4.0 (s, 2H, CH_2), 4.43 (s, 2H, CH_2), 5.16 (s, 2H, CH_2), 5.71 (s, 1H, 4-H), 5.58 (s, 2H, CH_2), 5.80 (s, 1H, 14-H), 5.94 (s, 2H, CH_2), 6.23 (m, 4H, 7-, 8-, 10- and 12-Hs), 7.09 (dd, 1H, J = 14.7 Hz, 11-H), 7.33 (m, 4H, Ar-Hs), 7.63 (d, 2H, J = 7 Hz, Ar-Hs), 7.72 (s, 1H, Ar-−H), 7.87 (s, 2H, Ar-Hs), 8.11 (s, 1H, NH), 8.09 (s, 1H, NH), 8.63 (s, 1H, NH), 9.51 (s, 1H, NH), 9.81 (s, 1H, NH), 10.21 (s, 1H, NH). HRMS calcd 790.3810 ($C_{45}H_{51}N_5O_8Na^+$), found 790.3810.

4-Formylphenyl(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate (15). ATRA (0.6 g, 2 mmol), 4-hydroxybenzaldehyde (**14**) (0.293 g, 2.4 mmol), and DMAP (0.293 g, 2.4 mmol) were dissolved in dry DMF and to this solution was added DCC (0.5 g, 2.4 mmol) at 0−10 °C. The reaction mixture was stirred for 24 h at rt. The reaction mixture was filtered, poured into ice-cold water and extracted with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give a crude product that was purified by FCC [CH_2Cl_2/EtOH, 9.5:0.5] to give the desired pure **15** (0.37 g, 91%); mp: 118−119 °C. ^{1}H NMR ($CDCl_3$): δ 1.04 (s, 6H, 16, 17-CH_3), 1.32 (m, 2H, CH_2), 1.72 (s, 3H, 18-CH_3), 1.90 (m, 2H, CH_2), 2.03 (s, 3H, 19-CH_3), 2.42 (s, 3H, 20-CH_3), 5.99 (s, 1H, 14-H), 6.27 (m, 4H, 7,8,10,12-Hs), 7.10 (dd, 1H, 11-H), 7.31 (d, 2H, J = 8.5 Hz, Ar-Hs), 7.92 (d, 2H, J = 8.5 Hz, Ar-Hs), 9.99 (s, 1H, CHO).

4-(Hydroxymethyl)phenyl (2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate (16). Compound **15** (0.35 g, 0.86 mmol) was dissolved in IPA:$CHCl_3$ (1:5, 50 mL)) and cooled to 0 °C. $NaBH_4$ (0.037 g) was then added to this, and the reaction mixture was stirred for 1 h at 0 °C. The reaction was quenched by addition of acetone (1 mL), evaporated to dryness, and purified by FCC [CH_2Cl_2/EtOH, (9:1)] to give pure **16** (0.32 g, 78.8%); mp: 89−90 °C. ^{1}H NMR ($CDCl_3$): δ 1.03 (s, 6H, 16, 17-CH_3), 1.48 (m, 2H, CH_2), 1.62 (m, 2H, CH_2), 1.72 (s, 3H, 18-CH_3), 2.02 (s, 3H, 19-CH_3), 2.40 (s, 3H, 20-CH_3), 4.69 (s, 2H, CH_2), 5.99 (s, 1H, 14-H), 6.26 (m, 4H, 7,8,10,12-Hs), 7.08 (dd, 1H, 11-H), 7.11 (d, 2H, J = 8.5 Hz, Ar-Hs), 7.38 (d, 2H, J = 8.0 Hz, Ar-Hs).

4-{[*N*-(2-{[4-({[(3-Pyridylmethyl)oxycarbonyl]methyl}amino)-phenyl]carbonylamino}phenyl)carbamoyloxy]methyl}phenyl(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate (17). To a solution of triphosgene (118 mg, 0.39 mmol) in toluene (10 mL) at 0 °C was added $NaHCO_3$ (42 mg, 0.39 mmol), and the reaction mixture was stirred for an 1 h. Compound **16** (135.0 mg, 0.32 mmol) dissolved in dry toluene (5 mL) was added dropwise over 30 min, and the resulting reaction mixture was further stirred at 0 °C for 16 h. The reaction mixture was filtered, and the filtrate was evaporated to obtain dark-brown oil that was reconstituted in THF (5 mL). This THF solution was added to the solution of **2** (124 mg, 0.33 mmol) and TEA (55 μL, 0.39 mmol) in THF (5 mL) at 0 °C and then stirred further at rt for 16 h. The reaction mixture was evaporated and purified by FCC [CH_2Cl_2/EtOH, 9:1] to give compound **17** (110 mg, 40%); mp: 128−130 °C. IR ($CHCl_3$): 3306, 1725, 1694, 1555, 1458, 1324, 1259, 1213, 1129, 1073, 749 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.02 (s, 6H, 16, 17-CH_3), 1.48 (m, 2H, CH_2), 1.62 (m, 2H, CH_2), 1.70 (s, 3H, 18-CH_3), 2.01 (s, 3H, 19-CH_3), 2.34 (s, 3H, 20-CH_3), 4.28 (d, 2H, J = 6 Hz, CH_2), 5.09 (s, 2H, CH_2), 5.14 (s, 2H, CH_2), 6.04 (s, 1H, 14-H), 6.25 (m, 4H, 7,8,10,12-Hs), 6.537 (s,1H, Ar-H), 6.507 (s, 1H, Ar-H), 7.16 (m, 4H, 11-H and Ar-Hs), 7.39 (d, 2H, J = 7 Hz, Ar-Hs), 7.44 (d, 2H, J = 8.5 Hz, Ar-Hs), 7.53 (d, 1H, J = 8 Hz, Ar-Hs), 7.61 (d, 1H, J = 8 Hz, Ar-Hs), 7.76 (d, 1H, J = 7 Hz, Ar-Hs), 7.90 (d, 2H, J = 7.5 Hz, Ar-Hs), 7.95 (s, 1H, Ar-H), 8.31 (s, 1H, Ar-H), 8.53 (s, 1H, Ar-H), 8.59 (s, 1H, NH), 9.05 (s, 1H, NH), 9.78 (s, 1H, NH). HRMS calcd 809.3908 ($C_{49}H_{52}N_4O_7H^+$), found 809.3898.

4-{[*N*-2-{[4-(Acetylamino)phenyl]carbonylamino}phenylcarbamoyloxy]methyl}phenyl (2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate (18). To a solution of triphosgene (118 mg, 0.39 mmol) in dry toluene (10 mL) at 0 °C was added $NaHCO_3$ (42 mg, 0.39 mmol), and the reaction mixture was stirred for 1 h. Compound **16** (135 mg, 0.32 mmol) dissolved in dry toluene (5 mL) was added dropwise over 30 min, and the resulting reaction mixture was further stirred at 0 °C for 16 h. The reaction mixture was filtered, and filtrate was evaporated to obtain dark-brown oil, which was reconstituted in THF (5 mL). This THF solution was added to the solution of **1** (89 mg, 0.33 mmol) and TEA (55 μL, 0.39 mmol) in THF (5 mL) at 0 °C and then stirred further at rt for 16 h. The reaction mixture was evaporated and purified by FCC [CH_2Cl_2/EtOH, 9:1] to give compound **18** (98 mg, 42%); mp: 123−124 °C. IR ($CHCl_3$): 3310, 1718, 1654, 1600, 1508, 1312, 1215, 1125, 758 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.02 (s, 6H, 16,17-CH_3), 1.45 (m, 2H, CH), 1.57 (m, 2H, CH_2), 1.70 (s, 3H, 18-CH_3), 2.01 (s, 3H, 19-CH_3), 2.08 (s, 3H, CH_3), 2.35 (s, 3H, 20-CH_3), 5.14 (s, 4H, CH_2), 6.09 (s, 1H, 14-H), 6.26 (m, 4H, 7-, 8-, 10- and 12-Hs), 6.54 (s, 1H, CH), 6.54 (s, 1H, CH), 7.11 (d, 2H, J = 8 Hz, Ar-Hs), 7.17 (m, 2H, Ar-Hs), 7.43 (d, 2H, J = 8 Hz, Ar-Hs), 7.51 (d, 1H, J = 7.5 Hz, Ar-Hs), 7.60 (d, 1H, J = 7.5 Hz, Ar-Hs), 7.91 (d, 2H, J = 8.0 Hz, Ar-Hs), 8.31 (s, 2H, Ar), 9.03 (s, 1H, NH), 9.73 (s,1H, NH), 10.22 (s, 1H, NH). HRMS calcd 702.3537 ($C_{43}H_{47}N_3O_6H^+$), found 702.3541.

4-(Butanoyloxymethyl)phenyl(2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate (19). To a solution of butyric acid (42 mg, 0.47 mmol) in DMF (5 mL) was added compound **16** (200 mg, 0.47 mmol), DCC (108 mg, 0.52 mmol), and DMAP (63.75, 0.52 mmol), and the reaction mixture was stirred at rt for 24 h. The reaction mixture was poured into ice-cold water (50 mL) and extracted with CH_2Cl_2 (25 mL × 3), and the organic layer was dried over anhydrous Na_2SO4 and evaporated to dryness. The crude product was purified by FCC [pet. ether/EtOAc, (50:1)] pure **19** as a yellow oil (123 mg, 52%); mp: 38−40 °C. IR ($CHCl_3$): 1723, 1577, 1353, 1234, 1214, 1123, 966, 753 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.934 (t, 3H, J = 7. 11^1-CH_3), 1.03 (s, 6H, 16,17-CH_3), 1.11 (m, 2H, CH_2), 1. 53 (m, 2H, CH_2), 1.68 (m, 2H, 10^1-CH_2), 1.72 (s, 3H, 18-CH_3), 2.02 (s, 3H, 19-CH_3), 2.33 (s, 3H, 20-CH_3), 2.46 (m, 2H, 9^1-CH_2), 5.10 (s, 2H, CH_2), 6.00 (s, 1H, 14-H), 6.27 (m, 5H, 7-, 8-, 10-, 11- and 12-Hs), 7.11 (d, 2H, J = 8 Hz, Ar-Hs), 7.37 (d, 2H, J = 8.5, Ar). HRMS calcd 499.2818 ($C_{31}H_{40}O_4Na^+$), found 499.2822.

Kinetics of Hydrolysis in Plasma. Blood was collected from male SCID mice (n = 10) via cardiac puncture and centrifuged to obtain plasma. Plasma (0.8 mL) was mixed with 0.2 mL of 0.02 M phosphate buffer (pH = 7.2). Incubations were performed at 37 °C using a shaking water bath. The reaction was initiated by adding 25 μL of stock solution of mutual prodrugs (1 mg/mL in CH_3CN) to preincubated plasma, and aliquots were taken after 1 h for processing and analyses. Plasma samples underwent solid-phase extraction using 3 mL of C18 Bond Elut columns (Varian, Harbor

City, CA), which had previously been rinsed with methanol (3 mL) and distilled water (3 mL). Then 500 μL of the sample were loaded, the column was washed with 3 mL of distilled water, and the drug was eluted with 2 mL of acetonitrile (in the case of MPs of CI-994 1:1 MeOH:CH_3CN was used). Eluates were evaporated to dryness. Samples were then reconstituted in 400 μL of acetonitrile and filtered through a 0.45 μm filter (Ultrafree-MC; Millipore Corporation, Bedford, MA) before analyses by HPLC. Chromatographic analysis was achieved by a reverse-phase HPLC method on a Waters Novapak C18 column (3.9 mm × 150 mm) protected by Waters guard cartridge packed with C18 as previously described. The HPLC system used in this study consisted of a Waters solvent delivery system, a Waters controller (Milford, MA) coupled to a Waters 717plus autosampler, and a Waters 996 photodiode array detector operating at 240.0 and 350 nm. A multilinear gradient solvent system, (i) 20 mM aqueous ammonium acetate buffer/methanol (50:50, v/v) (100−0%), and (ii) methanol (0−100%) at a flow rate of 0.8 mL/min, was used. Retention times (mins) for ATRA, HDIs, and prodrugs were as follows: ATRA (21.85), **2** (3.037), **1** (6.75), **10**, **13**, **17**, **18**, and **19** (23.97, 24.45, 25.63, 26.28, and 26.93, respectively). ATRA and the mutual prodrugs were detected at 350 nm, while HDIs were detected at 245 nm.

The stability of the mutual prodrugs were assessed in 0.02 M phosphate buffer (pH = 7.2) by incubations of each agent for 48 h at 37 °C. Samples were processed and analyzed as described above.

Cell Culture. PC-3 (androgen receptor negative, AR -ve) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin. Cells were grown as a monolayer in T75 tissue culture flasks in a humidified incubator (5% CO_2, 95% air) at 37 °C.

Other cell lines used in this study including MDA-MB-231 (estrogen receptor negative, ER −ve) and MCF-7 (ER +ve) were also purchased from ATCC and were cultured as previously described.[44,45] LTLC and LTLT-Ca were kindly provided by Dr. Angela Brodie, University of Maryland, Baltimore, and details of their phenotypes and culturing conditions are as previously reported.[46,47] MCF-7_{TAM} and MCF-$7_{HOXB\text{-}7}$ were provided by Dr. Saraswati Sukumar of Johns Hopkins University, Baltimore, and details of their phenotypes and culturing conditions are as previously reported.[48] Except for MCF-7 breast cancer cell, all other breast cancer cells used in this study are insensitive to endocrine therapeutic agents and to most anticancer agents.

Cell Growth Inhibition (MTT Colorimetric Assay). PC-3 cells were seeded in 24-well plates (Corning Costar) at a density of 2 × 10^4 cells per well per 1 mL of medium. Cells were allowed to adhere to the plate for 24 h and then treated with different concentrations of ATRA, HDIs, or MPs dissolved in 10% DMSO, 90% ethanol. Cells were treated for five days with renewal of prodrug and media on day 3. On the fifth day, medium was renewed and 100 μL of MTT (3-(4,5-dimethylthiazol-2-*yl*)-2,5-diphenyl-2*H*-tetrazolium bromide from Sigma) solution (0.5 mg MTT/mL of media) was added to the medium such that the ratio of MTT:medium was 1:10. The cells were incubated with MTT for 2 h. The medium was then aspirated and 500 μL of DMSO was added to solubilize the violet MTT-formazan product. The absorbance at 560 nm was measured by spectrophotometry (Victor 1420 multilabel counted, Wallac). For each concentration of agent or MPs, there were triplicate wells in each independent experiment. GI_{50} values were calculated by nonlinear regression analysis using GraphPad Prism software.

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Supporting Information Available: HPLC chromatograms and high-resolution mass spectral data of MPs **10**, **13**, **17**, **18**, and **19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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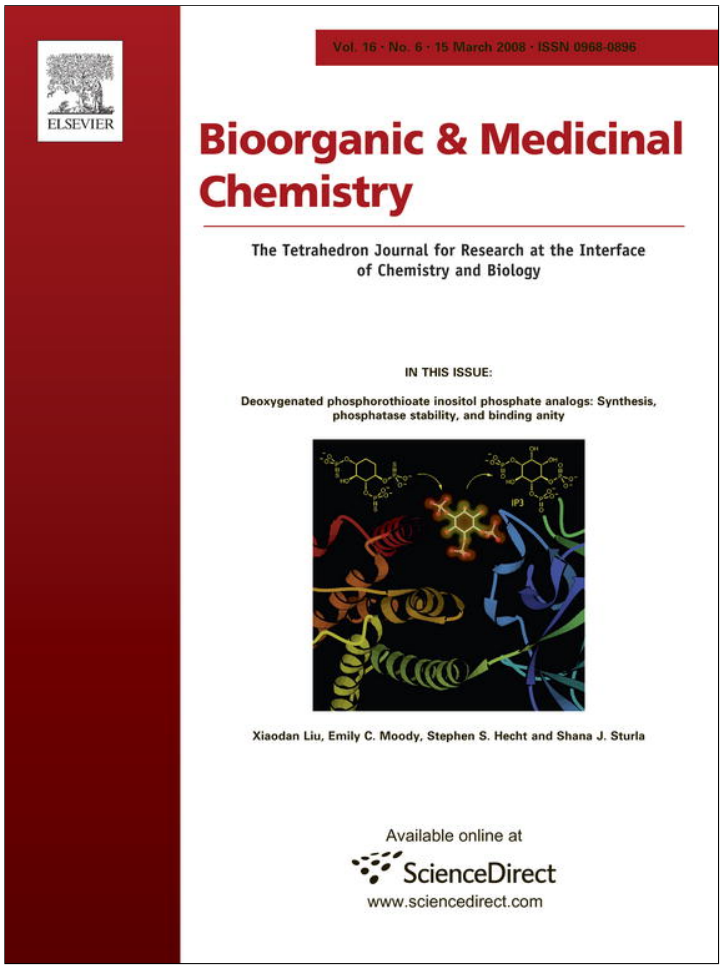

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Improved synthesis of histone deacetylase inhibitors (HDIs) (MS-275 and CI-994) and inhibitory effects of HDIs alone or in combination with RAMBAs or retinoids on growth of human LNCaP prostate cancer cells and tumor xenografts

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Abstract—We have developed new, simple, and efficient procedures for the synthesis of two promising histone deacetylase inhibitors (HDIs), CI-994, (*N*-(2-aminophenyl)-4-acetylaminobenzamide), and MS-275 (*N*-(2-aminophenyl)4-[*N*-(pyridine-3-yl-methoxycarbonyl)aminomethyl]benzamide) from commercially available acetamidobenzoic acid and 3-(hydroxymethyl)pyridine, respectively. The procedures provide CI-994 and MS-275 in 80% and 72% overall yields, respectively. We found that the combination of four HDIs (CI-994, MS-275, SAHA, and TSA) with retinoids all-*trans*-retinoic acid (ATRA) or 13-*cis*-retinoic acid (13-CRA) or our atypical retinoic acid metabolism blocking agents (RAMBAs) **1** (VN/14-1) or **2** (VN/66-1) produced synergistic anti-neoplastic activity on human LNCaP prostate cancer cells. The combination of **2** and SAHA induced G1 and G2/M cell cycle arrest and a decrease in the S phase in LNCaP cells. **2** + SAHA treatment effectively down-regulated cyclin D1 and cdk4, and up-regulated pro-differentiation markers cytokeratins 8/18 and pro-apoptotic Bad and Bax. Following subcutaneous administration, **2**, SAHA or **2** + SAHA were well tolerated and caused significant suppression/regression of tumor growth compared with control. These results demonstrate that compound **2** and its combination with SAHA are potentially useful agents that warrant further preclinical development for treatment of prostate cancer.

1. Introduction

Prostate cancer (PCA) is the most common malignancy and age-related cause of cancer death worldwide. Apart from lung cancer, PCA is the most common form of cancer in men and the second leading cause of death in American men. In the United States in 2007, an estimated 218,890 new cases of prostate cancer will be diagnosed and about 27,050 men will die of this disease.[1] The growth of most prostate tumors depends on androgens during the initial stages of tumor development, and thus, anti-hormonal therapy by surgical or medical suppression of androgen action remains a major treatment option of the disease.[2] Although this treatment may be initially successful, most tumors eventually recur due to the expansion of an androgen-refractory population of PCA cells.[3] Metastatic disease that develops even after potentially curative surgery remains a major clinical challenge. Therapeutic treatments for patients with metastatic PCA are limited because current chemotherapeutic and radiotherapeutic regimens are largely ineffective.[4] Hence, there is urgent need to develop new therapeutic agents with defined targets to prevent and treat this disease.

PCA tumors that arise after anti-hormonal therapy generally are less differentiated and it is believed that agents that can induce the cells to differentiate would represent a new therapeutic strategy.[5] Hence, the goal of differentiation therapy is to induce malignant cells to pass the

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block to maturation by allowing them to progress to more differentiated cell types with less proliferative ability. Breslow and colleagues[6] have led the way in the discovery of agents that inhibit the enzyme histone deacetylase (HDAC), thereby altering chromatin structure and changing gene expression patterns. Histone deacetylase inhibitors (HDIs) are potent differentiating agents toward a variety of neoplasms, including leukemia, and breast and prostate cancers. Combinations of HDIs with other known therapies including retinoic acids (RAs) have been investigated. RAs exert their effects via a nuclear receptor complex that interacts with promoters of RA-responsive genes.[7] An HDAC subunit is an integral part of this co-repressor complex, which is involved in transcriptional silencing in the absence of ligand.[8] This association provides a rationale for combining HDIs and RAs/retinoids therapeutically. One of the early HDIs discovered by Breslow and colleagues is *N*-hydroxy-N^1-phenylactanediamide, also called suberoylanilide hydroxamic acid (SAHA).[9,10] This compound (trade name: Vorinostat®) was recently (2006) approved by the U.S. Food and Drug Administration (FDA) for the treatment of advanced cutaneous T-cell-lymphoma.[11]

Recently, we reported on a family of compounds that inhibit the P450 enzyme(s) responsible for the metabolism of all-*trans*-retinoic acid (ATRA).[12] These compounds also referred to as retinoic acid metabolism blocking agents (RAMBAs) are able to enhance the antiproliferative effects of ATRA in breast and prostate cancer cells in vitro.[13] In addition, the RAMBAs were shown to induce differentiation and apoptosis in these cancer cell lines. However, we also observed that the breast cancer cell lines were exquisitely more sensitive to the RAMBAs.[14,15] We also reported recently that combination of SAHA with either retinoids or RAMBAs resulted in additive/synergistic PCA (LNCaP and PC-3 cell lines) growth inhibition in vitro.[16]

In continuation of our research in this area, we have discovered improved syntheses of two promising HDIs,[17–19] CI-994, (*N*-(2-aminophenyl)-4-acetylaminobenzamide), and MS-275 (*N*-(2-aminophenyl)4-[*N*-(pyridine-3-yl-methoxycarbonyl)aminomethyl]benzamide). Furthermore, we assessed the effects of our novel RAMBAs and retinoids (see Chart 1) in combination with some HDIs in human prostate cancer model systems in vitro and in vivo. The molecular effects of compound **2** + SAHA in prostate cancer cells include inhibition of proliferation, regulation of cell cycle, and induction of differentiation and apoptosis.

2. Results and discussion

2.1. Chemistry

To the best of our knowledge only one method has been reported for synthesis of CI-994. This synthesis of CI-994 by Weiershausen et al.[20] is outlined in Scheme 1. The three-step procedure involves the reaction of oxalyl chloride with 4-acetamidobenzoic acid to give the corresponding acid chloride that was coupled with 2-nitroaniline in situ to afford *N*-(2^1-nitrophenyl)-4-acetylaminobenzamide in 20.0% yield. This was then hydrogenated in THF using 10% palladium on activated charcoal to produce CI-994 (**3**) in 69.0% yield. The overall yield was only 13.8%. Apart from the low yield, this method is tedious and requires special hydrogenation conditions.

We have developed a simple and efficient *one-step* procedure for the synthesis of CI-994 (Scheme 2). The readily available acetamidobenzoic acid (**6**) was converted into its imidazolide derivative by reaction with *N*, N^1-carbonyldiimidazole (CDI) in THF at room temperature. This was further reacted with 1,2-phenylenediamine in the presence of TFA to obtain CI-994 (**3**) in 85.0% yield. The product was recrystallized from THF/methanol to give pure CI-994 (**2**) in 80.0% yield.

Another promising HDI *N*-(2-aminophenyl)4-[*N*-(pyridine-3-yl-methoxycarbonyl)aminomethyl]benzamide (MS-275, **4**) is currently in several phase I/II clinical trials for various solid tumors and hematological malignancies.[21] MS-275 was previously synthesized by Suzuki et al. via a three-step procedure in 50.96% overall yield (Scheme 3).[22] In addition to the modest overall yield, this procedure has other disadvantages such as a tedious method for the preparation of an acid chloride using oxalyl chloride and also it requires the use of column chromatography for purification of MS-275.

Here again, we have developed a new *two-step* procedure for preparation of MS-275 as outlined in Scheme 4. Condensation of 3-(hydroxymethyl)pyridine (**7**) and 4-(aminomethyl)benzoic in the presence of CDI gave 4-[*N*-(pyridin-3-yl-methoxycarbonyl) aminomethyl]benzoic acid (**8**) in 91.0% yield. In the previous method of Suzuki et al., the carboxylic acid derivative **8** was first converted into acyl chloride hydrochloride by treatment of oxalyl chloride in toluene and then reacted with imidazole to form the acylimidazole intermediate.[22] However, we synthesized the imidazolide of intermediate **8** by treatment with CDI at 55–60 °C in THF. The imidazolide was then further reacted in situ with 1,2-phenylenediamine in the presence of TFA at room temperature to afford MS-275 (**4**). Furthermore, we developed a simpler process for large scale purification of crude MS-275 instead of using conventional column chromatography. Thus, after the completion of reaction, the solvent was evaporated and to the concentrate we added the mixture of hexane and water (2:5, v/v) and stirred for 1 h. The resulting precipitate was filtered, washed with hexane and dried. The crude product was further stirred twice in dichloromethane to remove excess of 1,2-phenylenediamine, filtered, and washed with hexane to give pure MS-275 in 80.0% yield (>99% as determined by HPLC). The overall yield for our simple and efficient production of MS-275 (**4**) was 72.8%. Finally, the methods described here for the synthesis of CI-994 and MS-275 are green chemistry methods because of the greatly increased yields and fewer number of reaction steps.

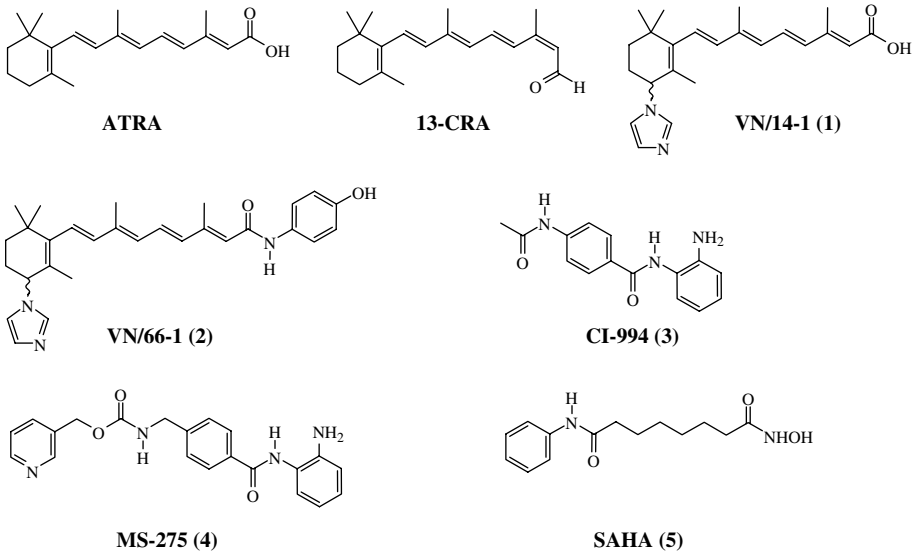
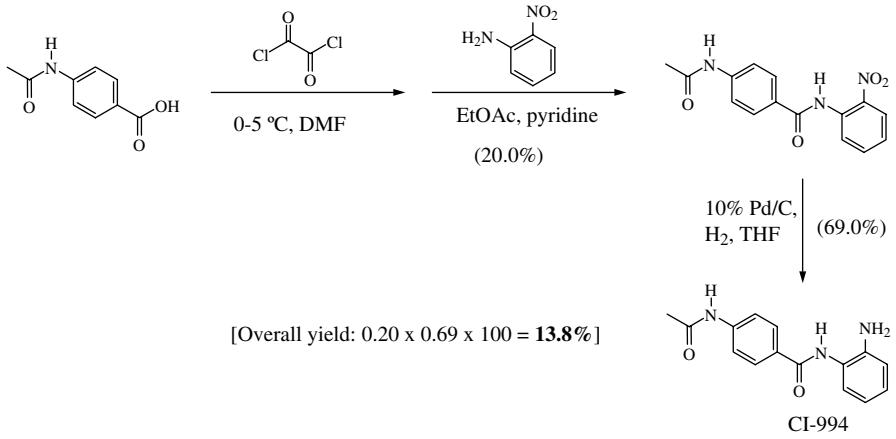
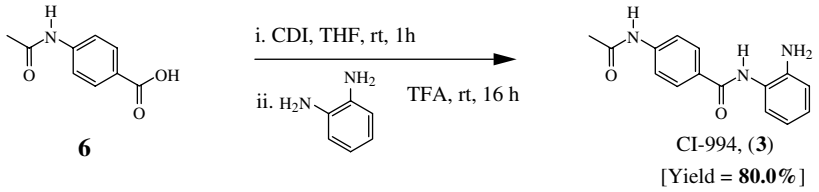


Chart 1. Structures of retinoids, RAMBAs, and HDIs.



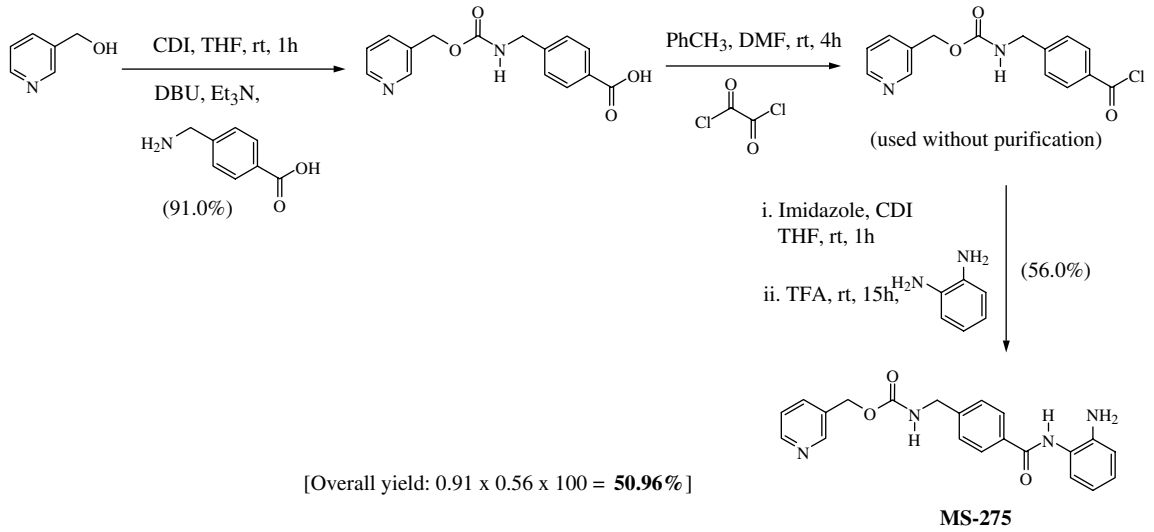
Scheme 1. Previous procedure for synthesis of CI-994 (**3**).



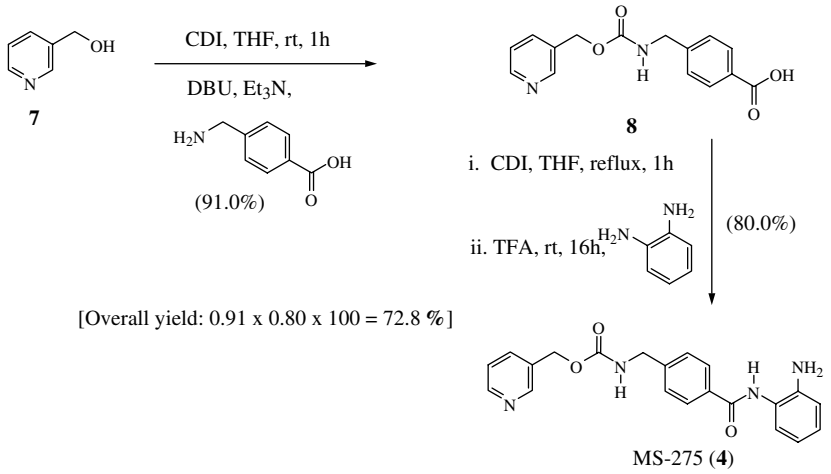
Scheme 2. New synthesis of CI-994 (**3**).

2.2. Biological studies

2.2.1. Effects of retinoids or RAMBAs alone or in combination with HDIs on LNCaP cell proliferation. We first studied the effects of retinoids, RAMBAs, and HDIs as single agents on LNCaP cell viability using the MTT assay and the IC_{50} values were determined from dose–response curves as shown for MS-275 (Fig. 1). The growth inhibitory experiments with the other compounds gave plots that were essentially the same as in Figure 1. The HDIs, MS-275, SAHA, and CI-994, and RAMBA **2** were efficacious with IC_{50} values of 0.36, 1.0, 7.4, and 5.5 μM, respectively. In contrast, the retinoids, ATRA and 13-CRA, or RAMBA **1** were less efficacious, since each of the compounds did not significantly inhibit cell growth even at concentrations as high as 10 μM. However, the combinations of retinoids or RAMBAs with the HDIs caused dramatic, *synergistic* inhibitory effects as shown in Figure 2a–e. Of significance is our observation that the combination of exceptionally low doses of ATRA (0.1 nM) or **1** (0.1 nM) or **2** (0.1 nM) with MS-275 (0.1 nM) resulted in a growth inhibition of 65.0%, 67.0%, and 70.0%, respectively (Fig. 2c–e). As we reported previously,[16] treatment with



Scheme 3. Previous procedure for synthesis of MS-275 (**4**).



Scheme 4. New synthesis of MS-275 (**4**).

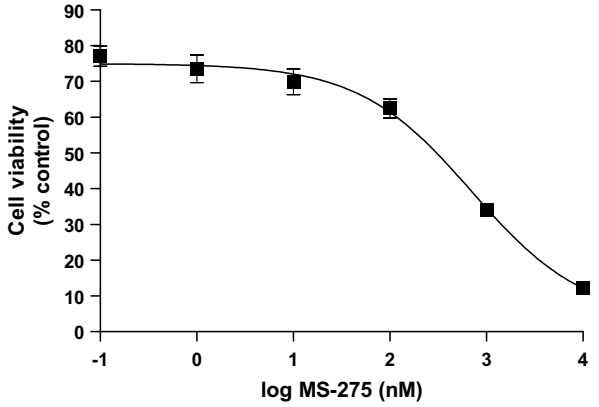


Figure 1. Antiproliferative effect of MS-275. LNCaP cell proliferation was measured after 6 days of treatment using a MTT assay as described in Section 4. Data are means (SEM < ±10%) of at least three independent experiments. The experiments with the other compounds gave dose–response plots that were essentially the same as shown above.

2 (5.0 μM) + SAHA (1.0 μM) resulted in >95.0% growth inhibition of LNCaP cells (data not shown). We also found that trichostatin A (TSA), a first generation and an experimental HDI, in combination with **2** caused a synergistic inhibitory effect in LNCaP cells (data not shown). It is pertinent to state here that although we had previously found that the combination of **2** + SAHA resulted in *additive* growth inhibition, the effects observed in the present study with the various combinations are clearly *synergistic* (refer Fig. 2a–e). This assertion is based on our findings that the inhibition of cell growth by each of the combinations was greater than the sums of each of the two compounds separately; using the Valeriote and Lin analysis.[23] These results clearly show that HDIs can effectively enhance the growth inhibitory activities of both retinoids and RAMBAs. It is well known that although the RAR/RXR receptors interact with HDACs, the ligands that interact with either of these proteins, i.e., the retinoids/RAMBAs and HDIs, have other mechanisms of action, that are

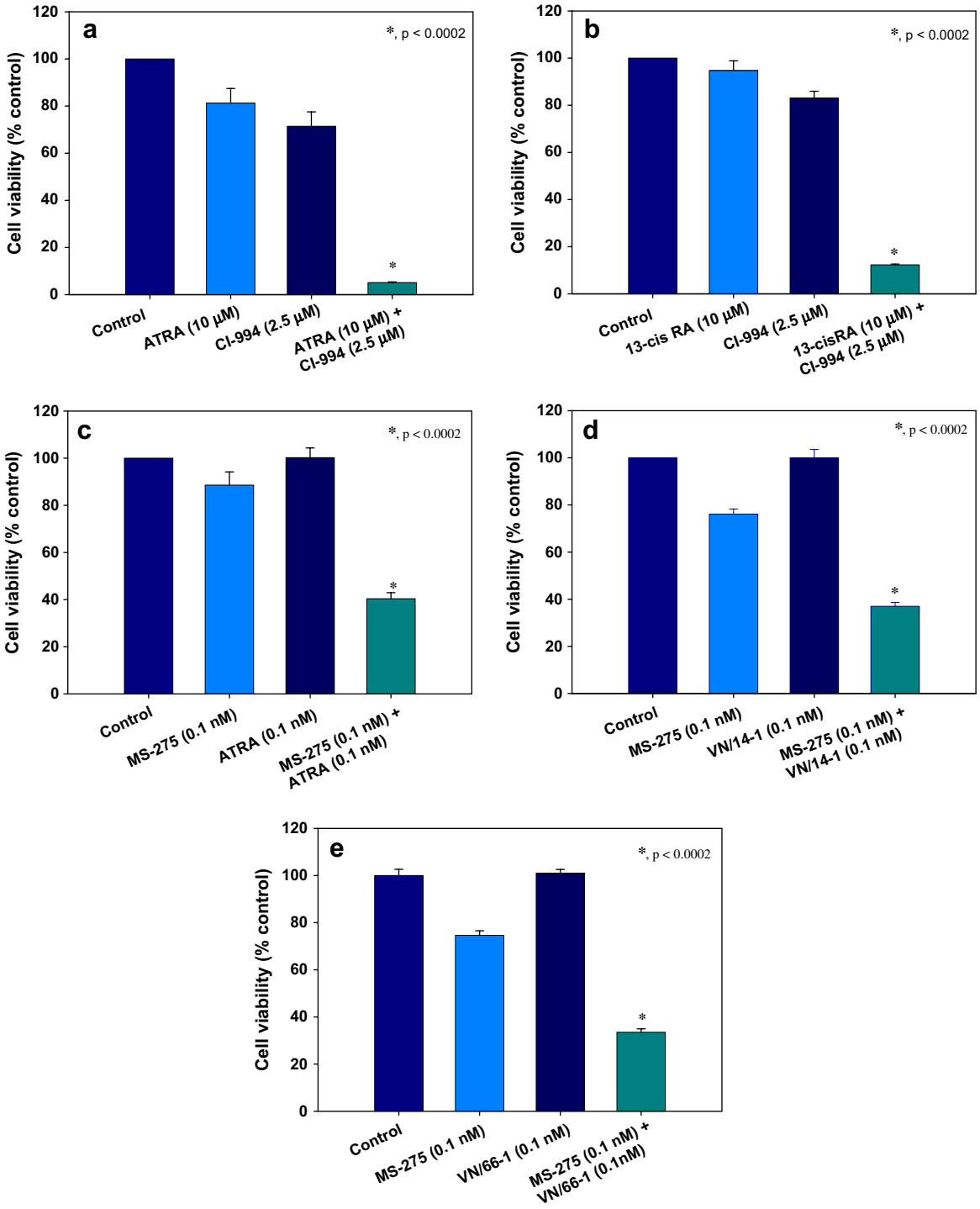


Figure 2. The effect of various HDACIs in combination with retinoids or RAMBAs on LNCaP cell viability as assessed by the MTT assay. (a) The effect of ATRA, CI-994, and the combination of the two at fixed concentrations. (b) The effect of 13-cis RA and CI-994. (c) The effect of ATRA and MS-275. (d) The effect of VN/14-1 and MS-275, (d) The effect of VN/66-1 and MS-275. (e) The effect of VN/66-1 and SAHA, $^{*}P < 0.0002$ (*t*-test) for all graphs. Data are means (±SE) of at least three independent experiments.

likely to cause synergistic cell growth inhibitory effects. It should also be stated that retinoid-specific signaling alone does not explain the differences in sensitivity of different cell lines to the different retinoids.[24] In addition, although the HDIs are noted to resensitize certain genes that are silenced in cancer cells, thereby enhancing the functional activity of RARs, they also show other anticancer activities, such as cell cycle arrest and apoptosis.[25]

A recent study by Faller and colleagues demonstrated enhanced (synergistic) suppression of androgen sensitive LNCaP and CWR22-rv1 cells when retinoids were combined with several HDIs.[26] However, Pili and colleagues

reported that phenylbutyrate in combination with 13-CRA has an additive inhibitory effect in LNCaP cells in vitro.[27] These differences may be due to the phenotypes/genotypes (number of passages in culture) of the cell lines utilized.

2.2.2. Effects of 2 and SAHA on cell cycle and pro-differentiation and pro-apoptotic proteins. Treatments of **2** (1 μM) + SAHA (10 nM) on LNCaP cells were used to further investigate the mechanisms of action. We elected to use SAHA in these studies because the agent is currently in clinical use.[11] The effects of **2**, SAHA or the combination of both agents on cell cycle profile of LNCaP cells were examined by flow cytometry as we have previously described.[12,15,28] Compared to control, **2** and SAHA alone induced G1 cell cycle arrest, while simultaneous treatment of both agents induced both G1 and G2/M cell cycle arrest with concomitant and significant decreases in percentage of cells in S phase (Table 1). The effects of these agents on protein expression of G1 phase cell cycle regulatory proteins, differentiation, and apoptosis were also examined in LNCaP cells by Western blot analysis following treatment for 24 h. As can be seen in Figure 3, treatment with **2** + SAHA caused significant up-modulation of cytokeratins 8/18 (4.5-fold), Bad (2.4-fold), Bax (5.4-fold) and down-modulation of cyclin D1 (>10-fold) and cdk4 (10-fold). Clearly, this treatment resulted in induction of differentiation, apoptosis, and cell cycle arrest. A lack of cellular differentiation, uncontrolled cell cycle progression, and evasion of apoptosis are hallmarks of many human cancers.[29,30] Therefore, therapeutic agents such as **2** + SAHA that can simultaneously impede cell cycle progression and promote differentiation and apoptosis in cancer cells are highly desirable. The cytostatic and cytotoxic properties of **2** + SAHA in prostate cancer indicate that the biological mechanisms of action of these two agents are diverse, thus rendering them more attractive for preclinical and clinical development for prostate cancer therapy.

2.2.3. Inhibition of human LNCaP prostate cancer xenografts by 2 and SAHA in SCID mice. To confirm our in vitro findings, we evaluated the in vivo anticancer efficacy of **2** and SAHA in a human LNCaP tumor xenograft model. LNCaP xenografts were grown in SCID mice and treated (subcutaneous administration) with **2** (10 → 20 mg/kg/day) or SAHA (20 → 10 mg/kg/day) or the combination of the two agents. As shown in Figure 4, growth of tumors was significantly inhibited with either **2** or SAHA as compared to control (81.6% and 97.1% inhibition, respectively), and tumors in the group treated with **2** + SAHA actually *regressed* by 22% over the duration of the experiment. In addition, these treatments did not cause any overt toxicity as evaluated by weight gain. A recent study by Pili and colleagues[27] reported that treatment of LNCaP tumor xenografts with phenylbutyrate (PB) or 13-CRA resulted in modest tumor growth inhibition that was generally not statistically significant as compared with control, but that the combination of PB and 13-CRA resulted in a significant additive inhibitory effect (up to 90% growth inhibition as compared with control). Given the excellent efficacies of **2** or SAHA and their combination reported in this study, it seems probable that **2** or its combination with SAHA may be more effective anticancer agents. In addition, the anti-tumor efficacies of **2** and SAHA appear to be superior to the reported efficacy of SAHA on related androgen-dependent CWR22 prostate tumors.[31] This is the first report of combination of a RAMBA/retinoid with an HDI that causes LNCaP tumor regression.

Table 1. Cell cycle analysis of effects of VN/66-1, SAHA or their combination on LNCaP cells

Cell cycle distribution in LNCaP cells[a]			
Treatment	G1 (%)	S (%)	G2/M (%)
Control	72.61	27.39	0
VN/66-1 (5 μM)	89.14	10.86	0
SAHA (1 μM)	81.90	18.10	0
VN/66-1 + SAHA	87.34	4.94	7.72

Percentage distribution of cells in each of the cell cycle phase is the mean obtained from experiments performed in triplicate of at least two independent experiments, SEM < ±10%.

[a] Percent of cells in each phase of the cell cycle after an 18 h treatment of LNCaP cells with either VN/66-1 (5.0 μM), SAHA (1.0 μM) or VN/66-1 + SAHA (5.0 + 1.0 μM), respectively.

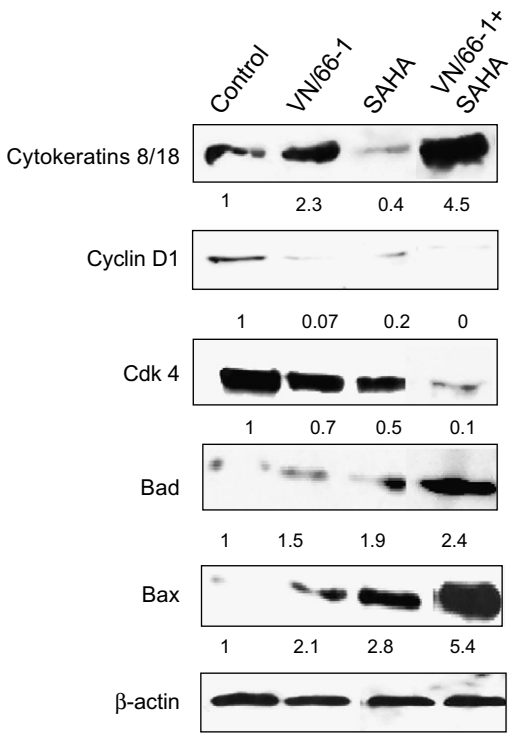


Figure 3. Western blot analysis for various proteins in LNCaP whole cell lysates after a 24 h treatment with VN/66-1 (1.0 μM), SAHA (10 nM) or their combination. Densitometry was carried out relative to loading control (β-actin). Blots are representative of at least three experiments.

3. Conclusions

We have developed new methods that enabled us to synthesize two promising HDIs, CI-994 and MS-275, in excellent and improved overall yields. In addition, we

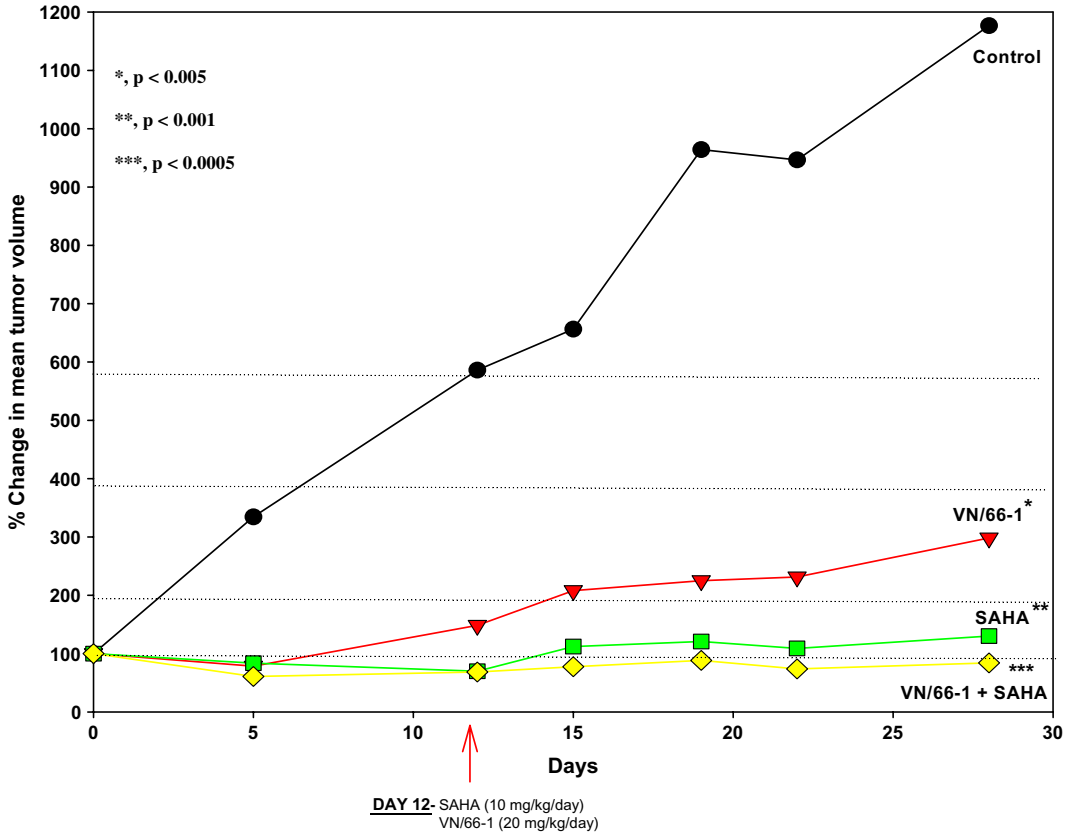


Figure 4. The effect of VN/66-1 (10 mg/kg), SAHA (20 mg/kg), and the combination (10 + 20 mg/kg, respectively) in a LNCaP xenograft model in male SCID mice. Doses were switched on day 12 to VN/66-1 (20 mg/kg), SAHA (10 mg/kg), and the combination (20 + 10 mg/kg, respectively) as indicated by the red arrow. Mice ($n = 7$) were injected subcutaneously QD, tumors were measured twice a week. Statistical significance was determined by the *t*-test. $^{*}P < 0.005$ and $^{**}P < 0.001$; $^{***}P < 0.0005$ versus control. Not shown on graph: $p < 0.05$ for VN/66-1 versus SAHA; $p < 0.01$ for VN/66-1 versus VN/66-1 + SAHA; $p < 0.05$ for SAHA versus VN/66-1 + SAHA.

have shown that retinoids and RAMBAs interact with HDIs to cause synergistic inhibition of growth of LNCaP prostate cancer cells. These studies are the first to specifically explore the biological mechanisms of action of a RAMBA in combination with HDACI. The combination of **2** and SAHA inhibits the growth of LNCaP cancer cells by inducing G1 and G2/M cell cycle arrest and induction of differentiation and apoptosis. Compound **2**, SAHA, and the combination exhibited potent anti-tumor efficacy in vivo. On the basis of these impressive results, further preclinical studies are warranted to develop RAMBAs and HDIs such as **2** and SAHA for prostate cancer treatment.

4. Experimental

4.1. Chemistry

General procedures and techniques were identical to those previously reported.[12,16] Infrared spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer using Nujol paste or KBr pellets. High-resolution mass spectra (HRMS) were determined on a Bruker 12T APEX-Qe FTICR-MS with an Apollo II ion source (College of Sciences Major Instrumentation Cluster, Old Dominion University, Norfolk, VA). ^{1}H NMR spectra were recorded in $CDCl_3$ and DMSO-d_6 at 500 MHz with Me_4Si as an internal standard using a Varian Inova 500 MHz spectrometer. Melting points (mp) were determined with a Fischer Johns melting point apparatus and are uncorrected.

4.1.1. *N*-(2-Aminophenyl)-4-acetylaminobenzamide (3, CI-994). To the suspension of acetamido benzoic acid (**6**, 7.5 g, 0.041 mol) in 75 ml THF was added CDI (7.5 g, 0.046 mol) portionwise at room temperature. The reaction mixture was stirred for 1 h to form acylimidazole followed by addition of 1,2-phenylenediamine (36.2 g, 0.335 mol) and TFA (4.19 g, 0.036 mol, 2.8 ml) and stirring for 16 h. The reaction mixture was then filtered to afford crude CI-994 which was re-crystallized from THF/methanol to give 9.0 g of CI-994 (**3**) as white crystals (yield, 80%). HPLC analysis showed purity > 99%. Mp 207–208 °C; IR (Nujol): 3289, 1646, 1540, 1456, 1297, 739 cm^{-1}; ^{1}H NMR: δ 2.08 (s, 3H, CH_3), 4.86 (s, 2H, NH_2), 6.59 (s, 1H, Ar-H), 6.78 (d, 1H, $J = 7.5$ Hz, Ar-H), 6.96 (s, 1H, Ar-H), 7.16 (d, 1H, $J = 7.5$ Hz, Ar-H), 7.69 (d, 2H, $J = 7.5$, Ar), 7.94 (d, 2H, $J = 8.0$, Ar-Hs), 9.55 (s, 1H, NH), 10.188 (s, 1H, NH). HRMS Calcd 269.1164 ($C_{15}H_{15}N_3O_2$), found 269.1161. These spectral and analytical data are as previously reported.[20]

4.1.2. 4-[*N*-(Pyridin-3-yl-methoxycarbonyl)aminomethyl]benzoic acid (8). To a suspension of 1,1′-carbonyldiimidazole (CDI, 25.6 g, 158 mmol) in THF (120 mL) was added 3-pyridinemethanol (**7**, 17.3 g, 158 mmol) in THF (50 mL) at 10 °C, and the mixture was stirred for 1 h at rt. The resulting solution was added to a suspension of 4-(aminomethyl)benzoic acid (22.6 g, 158 mmol), DBU (24.3 g, 158 mmol), and triethylamine (22.2 mL, 158 mmol) in THF (250 mL). After stirring for 5 h at rt, the mixture was evaporated to remove THF and then dissolved in water (300 mL). The solution was acidified with HCl (pH 5) to precipitate a white solid which was collected by filtration, washed with water (300 mL) and methanol (50 mL), respectively, and dried to give pure **8** (41.1 g, 91% yield): mp 207–208 °C; IR (KBr) 3043, 1718, 1568, 1434, 1266, 1108, 1037, 984, 756 cm^{-1}; ^1H NMR (DMSO-d_6) δ 4.28 (d, 2H, $J = 5.9$ Hz), 5.10 (s, 2H), 7.3–7.5 (m, 3H), 7.7–8.1 (m, 4H), 8.5–8.7 (m, 2H). These spectral and analytical data are as previously reported.[22]

4.1.3. *N*-(2-Aminophenyl)-4-[*N*-(pyridin-3-yl-methoxycarbonyl)aminomethyl]benzamide (4, MS-275). To a suspension of **8** (5.0 g, 0.017 mol) in THF (100 mL) was added CDI (3.12 g, 0.019 mol), and the mixture was stirred for 3 h at 60 °C. After formation of acylimidazole the clear solution was cooled to rt. To this were added 1,2-phenylenediamine (15.11 g, 0.14 mmol) and trifluoroacetic acid (1.2 mL, 0.015 mol) and then stirred for 16 h. The reaction mixture was evaporated to remove THF and the crude product was stirred in a mixture of hexane and water (2:5, v/v) for 1 h and filtered and dried. The residue was triturated in dichloromethane twice to afford pure MS-275 (**4**) as off- white powder 5.25 g, 80% yield: mp 159–160 °C; IR (KBr) 3295, 1648, 1541, 1508, 1457, 1309, 1183, 742 cm^{-1}. ^1H NMR (DMSO-d_6) δ 4.28 (d, 2H, $J = 5.9$ Hz), 4.86 (s, 2H), 5.10 (s, 2H), 6.60 (t, 1H, $J = 7.3$ Hz), 6.78 (d, 1H, $J = 7$ Hz), 6.97 (t, 1H, $J = 7$ Hz), 7.17 (d, 1H, $J = 8$ Hz), 7.3–7.5(m, 3H), 7.78 (d, 1H, $J = 8$ Hz), 7.93 (d, 2H, $J = 8$ Hz), 8.53 (d, 1H, $J = 3.7$ Hz), 8.59 (s, 1H), 9.61 (s, 1H); HRMS Calcd 376.1560 ($C_{21}H_{20}N_4O_3$), found 376.1558. These spectral and analytical data are as previously reported.[22]

4.2. Biology

4.2.1. Cell growth inhibition assay (MTT colorimetric assay). LNCaP cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin and streptomycin, as the complete culture medium. Cells (2×10^4) were seeded in 24-well plates and incubated in a 5% CO_2 incubator at 37 °C for 1 day. Cultures were treated with various compounds as listed, alone and in combination on day 2 and 4. Cells were washed on day 2 and 4 and media were changed. Mitochondrial metabolism was measured as a marker for cell growth by adding 100 μl/well MTT (5 mg/ml in medium) with 2- h incubation at 37 °C on Day 6. Crystals formed were dissolved in 500μl of DMSO. The absorbance was determined using a microplate reader at 560 nm. The absorbance data were converted into cell proliferation percentage. Each assay was performed in triplicate.

4.2.2. Western immunoblotting. LNCaP cells were treated for 24 h and harvested thereafter. The cells were washed with ice-cold DPBS, scraped, processed, and the supernatant separated and stored at −80 °C. Western blotting was carried out as described previously.[32] Antibodies against cytokeratins 8/18 and cyclin D1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and Cdk4, Bad, and Bax were purchased from Cell Signaling Technology, Inc. (Danvers, MA).

4.2.3. Cell cycle analysis. Cells were plated in T-75 flasks containing complete RPMI 1640 medium for 24 h. Cells were serum, starved after washing with phosphate-buffered saline and incubating with RPMI 1640 (minus phenol red) with 0.2% FBS for 48 h. Under these conditions cells were arrested in the G0/G1 phase as determined by flow cytometry. Cells were then stimulated by the addition of complete RMPI 1640 medium containing 10% FBS. Cells were treated with VN/66-1 or SAHA for various times. Cells were washed with PBS, trypsinized, resuspended in 10 ml PBS, and counted. They were then centrifuged (10 min, 2500 rpm at 4 °C), resuspended in PBS fixed in 70% ice-cold ethanol, and stored in −20 °C until staining. Cells were stained for at least 1 h in the dark with a solution containing 20 μg/ml propidium iodide (Sigma), 0.02 μg/ml RNAse, and 1% Triton X-100 (Sigma). The DNA content in the treated and mock-treated groups was measured by flow cytometry analysis using a FACSort flow cytometer (Becton–Dickinson, San Jose, CA); 15,000 events were analyzed for each sample. ModFit LT version 3.1 (Verity Software House Ind., ME) was used to analyze cell cycle distribution. The mean of two independent experiments is reported.

4.2.4. Animal studies. All animal studies were performed according to the guidelines approved by the Institution of Animal Care and Use Committee (IACUC) of the University of Maryland School of Medicine. Male SCID mice 4–6 weeks of age were obtained from the National Cancer Institute (Fredrick, MD). The animals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water ad libitum. Subconfluent cells were scraped into Dulbecco's phosphate-buffered saline, collected by centrifugation, and resuspended in Matrigel (10 mg/mL) at 5.0×10^7 cells/ml. Each animal received subcutaneous inoculations in one site per flank with 100 μl of cell suspension. Animals were randomly grouped with 7 mice per group. Tumors were measured twice weekly with calipers, and tumor volume was calculated by the formula ($4/3\ \pi r_1^2 \times r_2$), where r_1 is the smaller radius and r_2 is the larger radius. Treatments began when the tumors reached a measurable size (approximately 100 mm^3). VN/66-1 (**2**) and SAHA were prepared in sterile conditions as suspensions in 0.3% hydroxypropyl cellulose (HPC).

4.2.5. Statistical analysis. All experiments were carried out in at least triplicate and data are expressed as means ± SE where applicable. Treatments were compared to controls using Student's *t*-test with either GraphPad Prism or Sigma Plot. Various treatment groups were

compared using the analysis of variance (ANOVA). *P* values less than 0.05 were considered to be statistically significant.

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