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

Lalji K. Gediya,[†,§] Aakanksha Khandelwal,[†,§] Jyoti Patel,[†,||] Aashvini Belosay,[†,⊥] Gauri Sabnis,[†] Jhalak Mehta,[†] Puranik Purushottamachar,[†] and Vincent C. O. Njar*[,†]

Department of Pharmacology & Experimental Therapeutics, University of Maryland School of Medicine, 685 West Baltimore Street, Baltimore, Maryland 21201-1559, The University of Maryland Marlene and Stewart Greenebaum Cancer Center, School of Medicine, Baltimore, Maryland 21201-1559

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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

* To whom correspondence should be addressed. Phone: (410) 706 5885. Fax: (410) 706 0032. E-mail: vnjar001@umaryland.edu.

† University of Maryland School of Medicine, Baltimore.

§ These authors contributed equally to this work.

|| Present Address: Laboratory of Cancer Biology and Genetics, National Institutes of Health, Bethesda, Maryland.

⊥ Present Address: Department of Food Science and Human Nutrition, University of Illinois, 905 South Goodwin Avenue, Urbana−Champaign, Illinois 61801.

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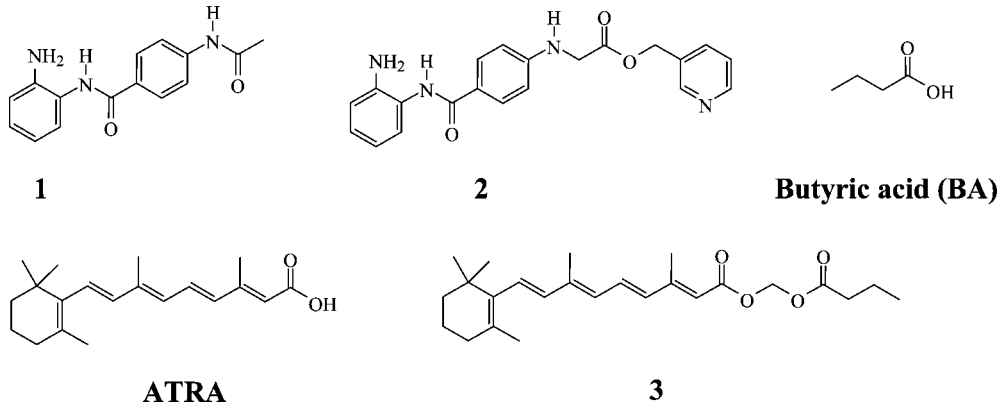
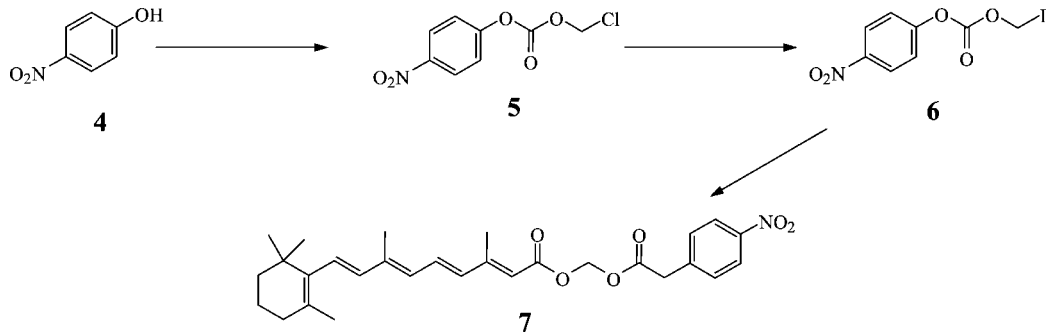
Chart 1. Chemical Structures of **1**−**3**, Butyric Acid (BA), and All-*trans*-Retinoic Acid (ATRA)



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]

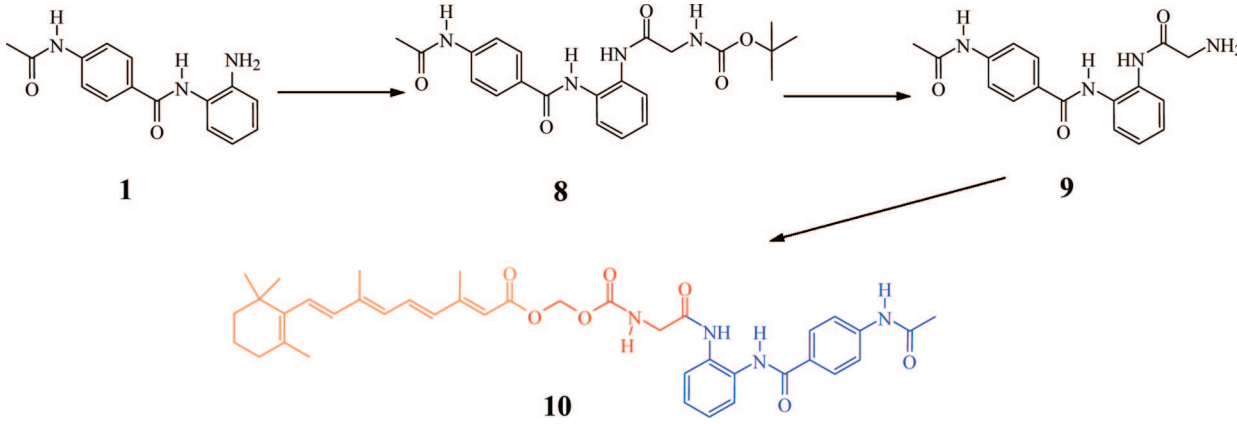
The putative synergistic interaction between retinoids and HDIs has provided the impetus for synthesis and evaluation of mutual prodrugs (MPs) of these agents with hopes of attainment of superior therapeutic efficacy. It is relevant to state here that a mutual prodrug (hybrid drug) is a type of carrier 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]

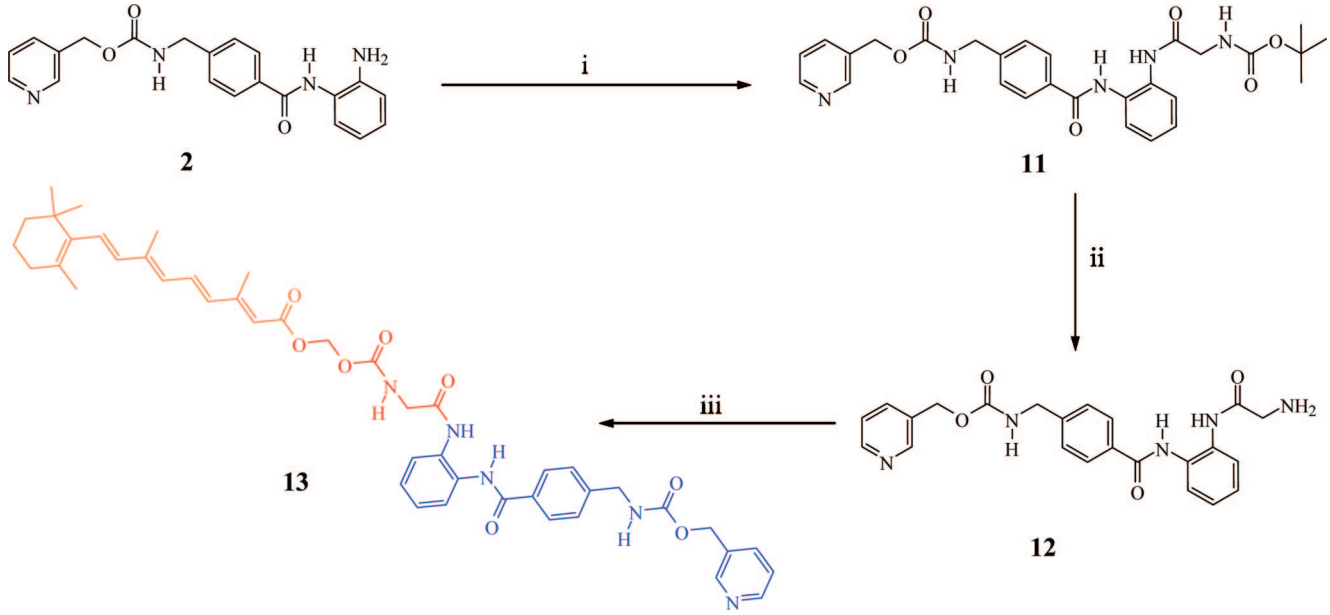
To prepare mutual prodrugs of ATRA and **1** or **2** using this strategy, we first synthesized *p*-nitrophenyl retinoyloxymethyl (**7**) carbonate in three steps (Scheme 1). Thus, treatment of *p*-nitrophenol (**4**) with chloromethyl chloroformate in the presence of pyridine as base gave chloromethyl-*p*-nitrophenyl 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]

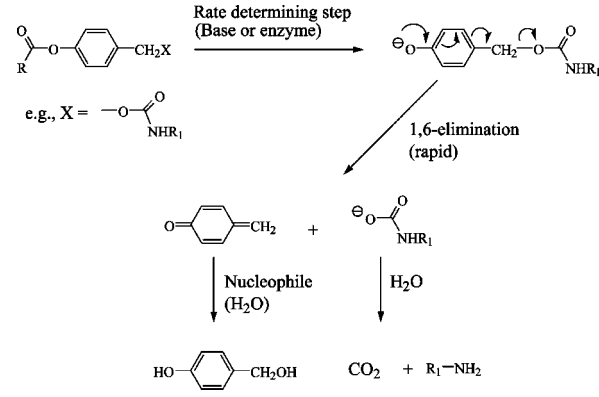


[a] Reagents and conditions: (i) Boc-Gly-OH, DCC, HOBt, DMF, rt, 18 h; (ii) TFA, CH_2Cl_2, 2 h; (iii) **7**, HMPA, TEA, rt, 24 h.

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

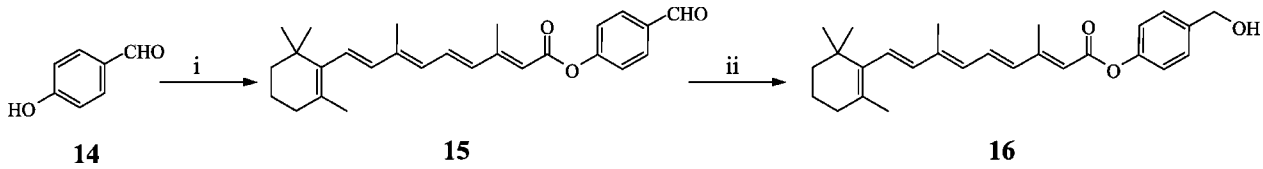
Scheme 4. Drug Release from Prodrug via 1,6-Elimination Mechanism



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

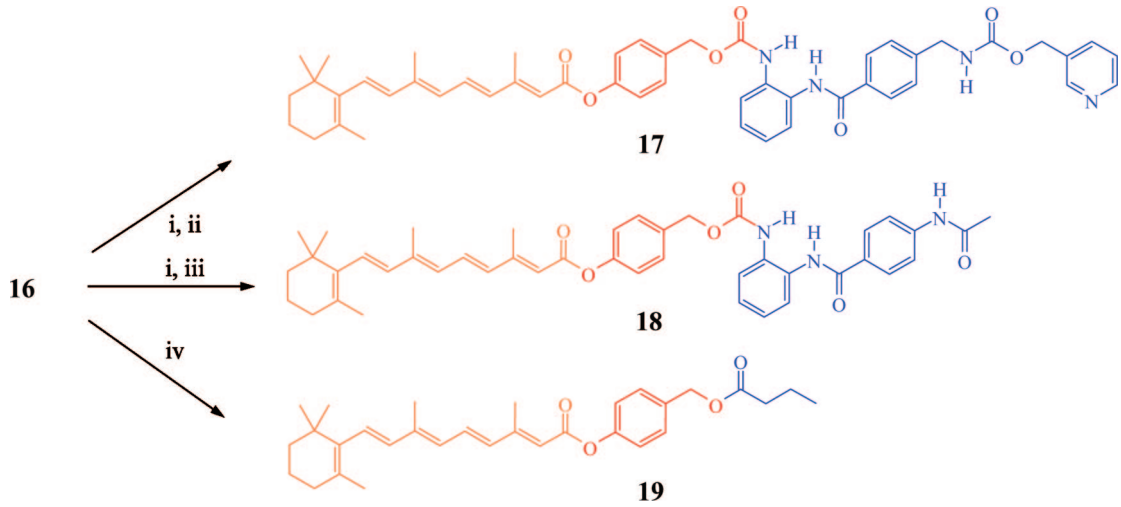
First, we prepared all-*trans*-retinoic acid benzyl alcohol ester (**16**), a key intermediate for the synthesis of MPs of various HDIs based on the 1,6-elimination concept (Scheme 5). *p*-

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}

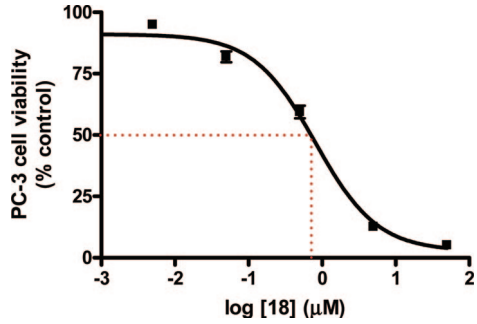


Figure 1. Concentration-dependent curve showing the antiproliferative effect of MP **18** on human prostate cancer PC-3 cells. Data are means (± SEM) of at least three independent experiments. The experiments with the other compounds gave plots that were similar to that shown above.

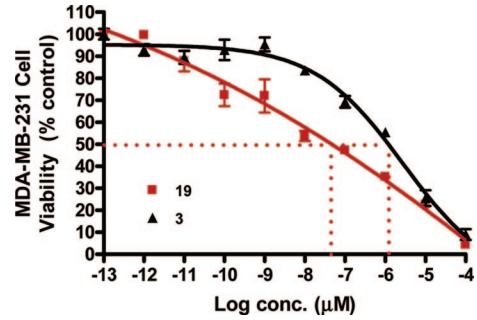


Figure 2. Growth inhibitory effect of **3** and MP **19** in MDA-MB-231 breast cancer cell line. Data are means (± SEM) of at least three independent experiments.

= 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

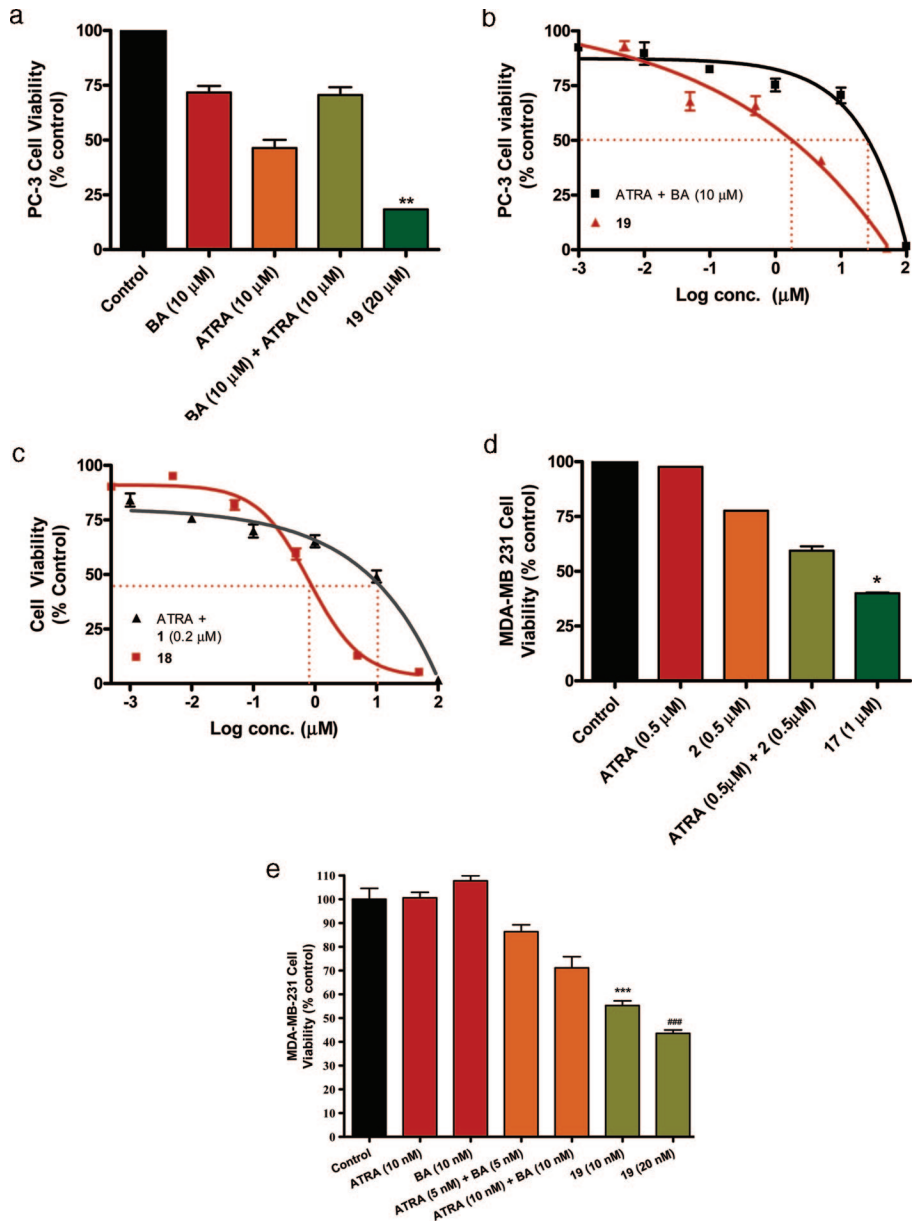


Figure 3. (a) Effect of ATRA and butyric acid administered alone or in combination and of mutual prodrug **19** on PC-3 cell growth. ** indicates a significant increase from control and BA, ATRA, or BA + ATRA treatments ($P < 0.001$). (b) Growth inhibitory effect of ATRA and sodium butyrate in combination 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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