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Bioorganic & Medicinal Chemistry 14 (2006) 3038–3048
Bioorganic & Medicinal Chemistry

Synthesis and preliminary chemotherapeutic evaluation of the fully *C*-linked glucuronide of *N*-(4-hydroxyphenyl)retinamide

Joel R. Walker,[a] Galal Alshafie,[b] Nirca Nieves,[c] Jamie Ahrens,[d] Margaret Clagett-Dame,[c,d] Hussein Abou-Issa[b] and Robert W. Curley, Jr.[a,*]

[a]*Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA*
[b]*Department of Surgery, College of Medicine and Public Health, The Ohio State University, Columbus, OH 43210, USA*
[c]*Pharmaceutical Sciences Division, School of Pharmacy, University of Wisconsin-Madison, Madison, WI 53706, USA*
[d]*Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706, USA*

Received 19 August 2005; revised 9 December 2005; accepted 13 December 2005
Available online 10 January 2006

Abstract—All-*trans* retinoic acid analogues such as *N*-(4-hydroxyphenyl)retinamide (4-HPR) are effective chemopreventive and chemotherapeutic agents but their utility has been hampered by dose-limiting side effects. The glucuronide derivatives of 4-HPR, the oxygen-linked 4-HPROG and the carbon-linked 4-HPRCG, have been found to be more effective agents. The synthetic route to the fully *C*-linked analogue of 4-HPROG (4-HBRCG), which employs Suzuki coupling and Umpolung chemistries as key methodologies, is shown. The results of this study show 4-HBRCG to be an effective chemotherapeutic agent in a rat mammary tumor model while being devoid of classical retinoid toxicities.

1. Introduction

Retinol (**1**) and its metabolites (Fig. 1) are involved in regulating many biological processes including vision, reproduction, cell differentiation, and growth. Besides being essential to normal cell function, the retinol metabolite all-*trans* retinoic acid (atRA, **2**) also shows antiproliferative action in cancer.[1] Unfortunately at pharmacologically effective doses, atRA causes severe toxicity. Therefore, the development of retinoid analogues possessing a higher therapeutic index is needed. One of the most investigated synthetic retinoids is *N*-(4-hydroxyphenyl)retinamide (4-HPR; **3**), which has been shown to be effective in numerous types of animal tumor models and has been evaluated in a phase III clinical trial.[2] A possible benefit was reported for the prevention of second breast malignancy in premenopausal women with surgically removed stage I breast cancer or ductal carcinoma in situ (DCIS). Although 4-HPR was generally well tolerated, it resulted in a decrease in plasma retinol levels[3,4] and diminished dark adaptation, and dermatological disorders occurred in a substantial number of subjects.[5]

Glucuronidation of drugs and natural products is a common metabolic pathway that usually facilitates excretion.[6] An important metabolite of **3** is 4-HPR-*O*-glucuronide (4-HPROG; **5**) in which the phenolic hydroxyl group is linked to the sugar. Subsequent to its discovery, **5** was synthesized and evaluated for bioactivity, and was shown to have excellent chemopreventive and chemotherapeutic activities in a rat mammary tumor model.[7] However, it was not determined if the glucuronide **5**, which was shown to be hydrolyzed to **3** via β-glucuronidase,[8] was advantageous due to improved bioavailability of **3** or had activity in its own right as an intact **5**. To study this issue, an enzymatically stable glucuronide analogue was synthesized by replacing the phenolic oxygen with a methylene group. The carbon-linked analogue 4-HPR-*C*-glucuronide (4-HPRCG; **6**) was shown to have excellent chemopreventive[9] and chemotherapeutic[10] properties. Furthermore, much like 4-HPR, **5** and **6** show low affinity relative to atRA for binding to the nuclear retinoic acid receptors (RARs), which mediate most of the actions of natural retinoids.[9] Although 4-HPR causes apoptosis in numerous cancer cell lines,[11] the precise mode of action of these

Keywords: Retinoids; Carbon-linked glucuronide; Chemoprevention; Chemotherapy; Breast cancer; Synthesis.
* Corresponding author. Tel.: +1 614 292 7628; fax: +1 614 292 2588; e-mail: curley.1@osu.edu

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doi:10.1016/j.bmc.2005.12.044

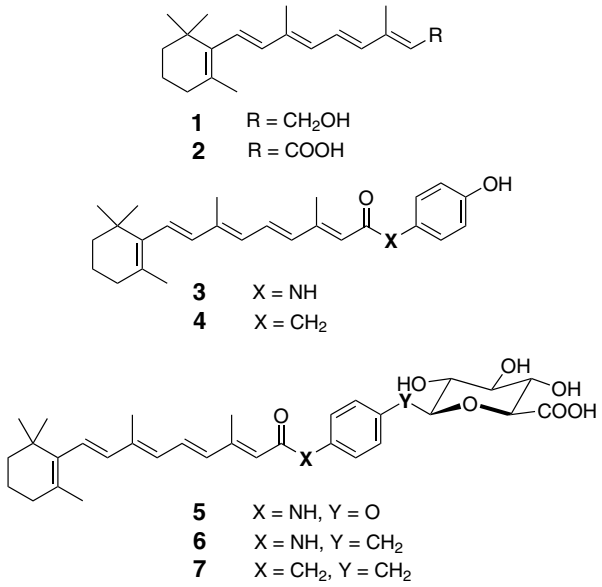


Figure 1. Natural and synthetic retinoids: (**1**) retinol; (**2**) atRA; (**3**) 4-HPR; (**4**) 4-HBR; (**5**) 4-HPROG; (**6**) 4-HPRCG; and (**7**) 4-HBRCG.

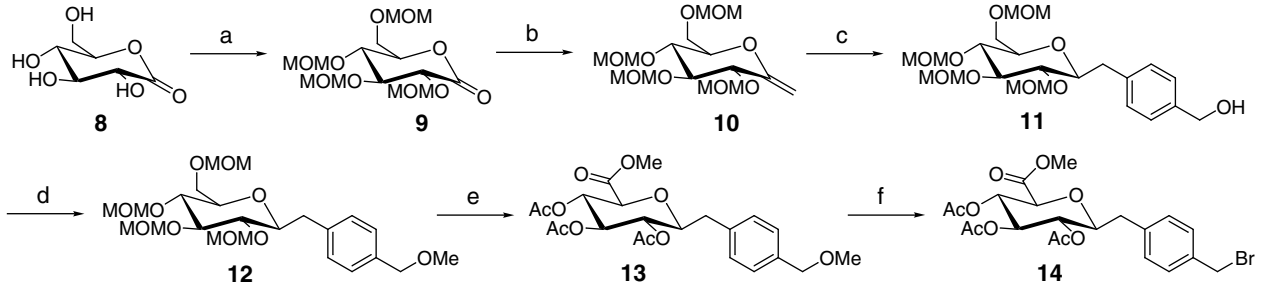
synthetic retinoids remains unclear. While 4-HPR (**3**) has been shown to be an effective chemopreventive and therapeutic agent, some of its effects may be attributed to in vivo hydrolysis of the amide bond, liberating atRA.[12] To investigate this possibility, an unhydrolyzable analogue of 4-HPR, 4-hydroxybenzyl retinone (4-HBR; **4**), was synthesized. Both **3** and **4** were shown to be equipotent chemotherapeutics in the dimethylbenz[*a*]anthracene (DMBA)-induced rat mammary tumor model.[13] In addition, both **3** and **4** cause apoptosis in cultured mammary tumor cells.[12] In vitamin A-deficient rats, **3**, but not **4**, is hydrolyzed to liberate retinoic acid.[12] Furthermore, 4-HPR, but not the *C*-linked analogue **4**, induces CYP26B1 mRNA expression in a RA-like manner in the lungs of vitamin A-deficient rats. Based on the positive chemotherapeutic and apoptotic-inducing activity of **3** and **4**, it appears that hydrolysis of 4-HPR is not required for the therapeutic effect of this retinoid, but rather, the liberation of RA may contribute to its retinoid-based toxic side effects.

4-HPR has been shown to be 100 times less teratogenic than RA and this toxicity may also be caused by the liberation of RA. For the effective antitumor agent 4-HPRCG, amide bond hydrolysis may still occur in vivo liberating retinoic acid by similar mechanisms as for 4-HPR. To eliminate this possibility, the fully *C*-linked analogue (4-HBRCG; **7**) was prepared by replacing the amide bond of 4-HPRCG with a methylene group to give the fully *C*-linked derivative of 4-HPR-*O*-glucuronide, **7** (Fig. 1). While these compounds are technically no longer glucuronides of hydroxyl compounds, abbreviations have been assigned to convey the rationale behind their development and to adhere to conventions in the field. The synthesis and therapeutic evaluation of 4-HBRCG are reported herein.

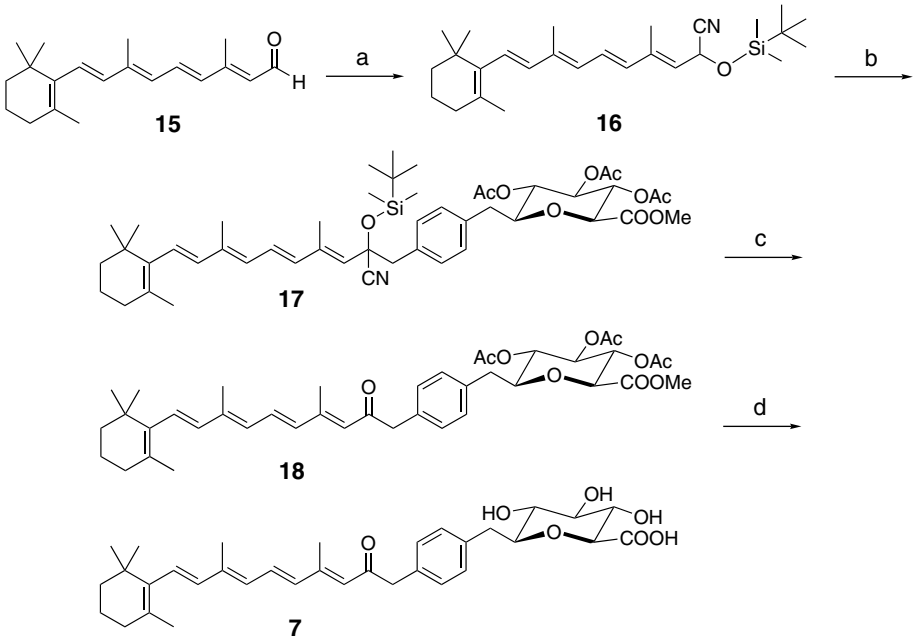
2. Chemistry

The recently reported improved synthetic route to 4-HPRCG employs a Suzuki coupling reaction between an exoanomeric methylene sugar and an aryl bromide.[10] This methodology, originally developed by Johnson and co-workers,[14,15] gives ready access to β-arylmethyl-*C*-glycosides. It was intended to use the same chemistries in the synthetic design of 4-HBRCG, allowing us to obtain the key benzyl bromide **14** (Scheme 1). Using a convergent approach, an Umpolung derivative of retinal was then alkylated with the benzyl bromide to obtain the carbon skeleton of the final target, 4-HBRCG (Scheme 2).

Starting from readily available δ-D-gluconolactone (**8**), hydroxyl protection using mild conditions with MOMCl and diisopropylethylamine gives the protected lactone **9** (Scheme 1). Olefination using Petasis reagent[16,17] gives the known exoanomeric methylene sugar **10** in good yield.[14] Hydroboration of the exocyclic olefin with 9-BBN-H, followed by a Suzuki coupling with *p*-bromobenzyl alcohol, gives exclusively the β-arylmethyl-*C*-glucoside **11**. Previous reports have shown this reaction to be stereoselective.[10,15,18] Due to subsequent planned chemistry, the benzyl alcohol was easily protected to yield the methyl ether **12**. In order to obtain the glucuronide, the MOM groups were cleaved in acid



Scheme 1. Reagents and conditions: (a) MOMCl, (*i*-Pr)$_2$NEt, Bu$_4$NI, CH_2Cl_2, 48 h, 83%; (b) $Cp_2Ti(CH_3)_2$, $PhCH_3$, 70 °C, 18 h, 87%; (c) i—9-BBN-H, THF, reflux, 6 h; ii—$PdCl_2$(dppf), 3 M K_3PO_4, DMF, *p*-bromobenzyl alcohol, 18 h, 67%; (d) i—NaH, THF, 1.5 h; ii—CH_3I, 18 h, 90%; (e) i—6 N HCl, MeOH, 18 h; ii—TEMPO, NaClO, KBr, $NaHCO_3$, 0 °C, 45 min; iii—CH_3I, DMF, 20 h; iv—Ac_2O, pyridine, DMAP, 18 h, 82%; (f) HBr, AcOH, 18 h, 86%.



Scheme 2. Reagents and conditions: (a) TBDSMCN, Et_3N, CH_2Cl_2, 20 h, 78%; (b) i—LiHMDS, THF, −78 °C, 30 min; ii—compound **14**, THF, −78 °C, 3 h, 47%; (c) TBAF, THF, 1 h, 75%; (d) i—K_2CO_3, MeOH, 4 °C, 20 h; ii—KOH, MeOH, 4 °C, 20 h, 82%.

and the primary alcohol was selectively oxidized to the carboxylate using 2,2,6,6-tetramethyl-1-piperidinyloxy free radical (TEMPO).[19,20] Typically, TEMPO is used catalytically. However, these conditions resulted in non-selective oxidation, yielding mixtures of benzylic ketones. Variations in the time, temperature, base, amount of TEMPO, amount of sodium hypochlorite, and order of addition were evaluated without any success in cleanly generating **13**. From past experience in our laboratory, other oxidatively sensitive sugar-type molecules can undergo selective oxidations when excess TEMPO is used.[21] When excess TEMPO, KBr, and NaOCl are premixed in a $NaHCO_3$ solution, deprotected **12** was added and selectively oxidized, efficiently yielding the 6-position carboxylate. Methylation of the carboxylate, followed by acetylation of the remaining alcohols, gives the protected *C*-aryl-glucuronide **13** in good yield over four steps. Benzylic methyl ethers can be displaced by bromide using hydrobromic acid[22,23] and when exposed to HBr in acetic acid, methyl ether **13** smoothly gave the key benzyl bromide *C*-glucuronide intermediate **14**. This surprisingly facile reaction yielded a very stable benzyl bromide, which was isolated by crystallization.

The next step in this route was the key alkylation of electrophile **14** with a retinal anion equivalent (Scheme 2). The most suitable Umpolung strategy for the chemically sensitive retinal is to employ the protected cyanohydrin derivative,[24] more particularly, the silylcyanohydrin[25] of retinal. The trimethylsilylcyanohydrin of retinal was first revealed and used in the synthesis of 4-HBR.[12,26] Retinal (**15**) exposed to *tert*-butyldimethylsilylcyanide (TBDMSCN) with catalytic Et_3N gave chromatographically stable cyanohydrin **16**. In the alkylation reaction, the TBDMS-cyanohydrin was deprotonated with LiHMDS followed by addition of bromide **14**. Subsequent chromatography of the alkylated TBDMS-protected product **17** allowed for recovery of the valuable unreacted bromide. Treatment of the alkylated product with fluoride unmasked the ketone to give the penultimate material **18**. Using model reactions, substantial efforts were done to attempt to improve the yield of the alkylation reaction including comparing TMS- and TBDMS-silylcyanohydrin reactivities, employment of different bases, changes in stoichiometry, temperature, and time. Even though recovery of unreacted bromide **14** was important, yields of **17** remained modest due to sensitivities of the polyene retinoid reactant. Last, careful deprotection of the acetates and saponification of the methyl ester gave the final target, 4-HBRCG (**7**). In the end, more than 2 g of 4-HBRCG was produced to facilitate the animal studies and in vitro assays.

3. Biological results and discussion

Preliminary evaluation of the chemotherapeutic activity of 4-HBRCG (**7**) against mammary tumors was conducted, and its toxicity profile was also assessed. As previously described,[7,13] tumor-bearing female rats (treated ca. 50 days earlier with DMBA) were fed the control or retinoid-containing diets [RA (**2**), 4-HPR (**3**), or 4-HBRCG(**7**)] at 2 mmol/kg diet for 22 days. As shown in Table 1, **7** is as effective as **2** and **3** in reducing tumor volume (30–40%), whereas control group tumor volumes increased nearly 200% by 22 days. Figure 2 shows that the time-course changes in tumor volumes was similar for all three treatment retinoids. Likewise, the data in Table 2 show that for **7**, individual tumors in the

Table 1. Effect of retinoid treatment on DMBA-induced rat mammary tumor volume[a]

Experimental group	Initial tumor volume (cm^3)	Final tumor volume (cm^3)	% Change[b]
Control	0.10 ± 0.05	0.29 ± 0.12	+190[c]
atRA (**2**)	0.08 ± 0.03	0.05 ± 0.02	−38[c]
4-HPR (**3**)	0.12 ± 0.03	0.08 ± 0.02	−33[c]
4-HBRCG (**7**)	0.12 ± 0.06	0.08 ± 0.05	−33[c]

[a] Value = means ± SEM.
[b] Change from baseline.
[c] Denotes statistical significance, $p < 0.05$.

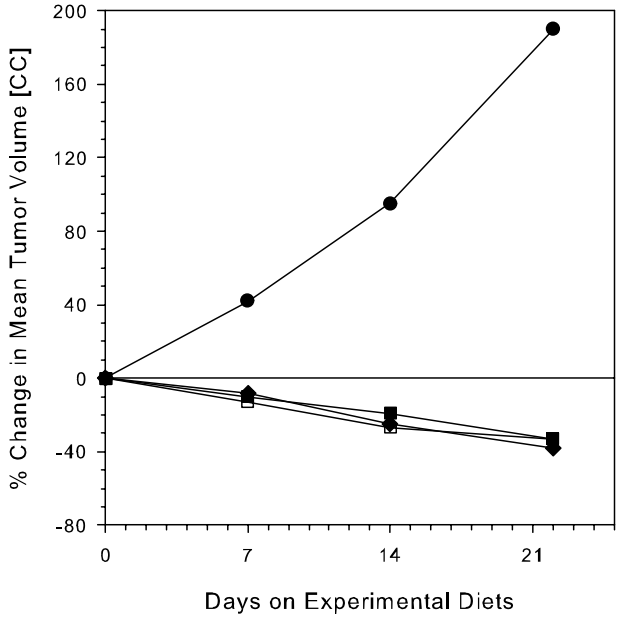


Figure 2. Effect of retinoid treatment on time-course changes in tumor volume. Control (●); atRA (◊); 4-HPR (■); and 4-HBRCG (□).

Table 2. Effect of retinoid treatment on individual tumors

Experimental group	Total number of tumors	Complete regression[a]	Partial regression[b]	New tumors	No effect
Control	15	0	0	2	13
atRA (**2**)	10	1	8	0	1
4-HPR (**3**)	19	1	15	0	3
4-HBRCG (**7**)	10	2	6	0	2

[a] Represents tumors that totally disappeared and could not be palpated.
[b] Represents tumors that showed 25–75% decrease in volume.

group responded similarly to the tumors in the other retinoid-treated groups.

Importantly, 4-HBRCG (**7**) showed evidence of greatly reduced toxicity relative to both RA (**2**) and 4-HPR (**3**). During the feeding period, only RA caused a significant ($p < 0.05$) reduction in normal body weight gain (+0.2% vs +3% for control, 4-HPR, and 4-HBRCG), a common sign of retinoid toxicity. Perhaps of even greater importance, 4-HBRCG caused a much smaller reduction than did either 4-HPR or RA in plasma retinol levels (Fig. 3A). It is well known that both RA[27] and 4-HPR[3] can reduce circulating levels of blood retinol. The 4-HPR-induced reduction in plasma retinol has been shown to produce impaired dark adaptation and is the single-most important factor limiting the doses of 4-HPR that have been used in human clinical trials.[28] Because 4-HBRCG (**7**), fed at 2 mmol/kg diet, did not produce any significant reduction in blood retinol whereas the same amount of 4-HPR (**3**) did, it should be possible to use **7** at relatively higher doses compared to **3** before the risk of night blindness is incurred. The reason that 4-HBRCG (**7**) shows no significant lowering of blood retinol levels may be related to the manner in which it is distributed in vivo (Fig. 3B). 4-HPR (**3**) is known to reduce blood retinol levels by competing for binding to the serum retinol-binding protein (RBP).[3,29] Interestingly, both 4-HPR and the related analogue, 4-HBR (**4**), show equivalent binding to RBP, yet 4-HBR does not lower blood retinol levels.[13] However, it should be noted that 4-HBR circulates in the blood at lower levels compared to 4-HPR when administered in equimolar quantities.[13] Thus, at least with respect to **3** and **4**, blood

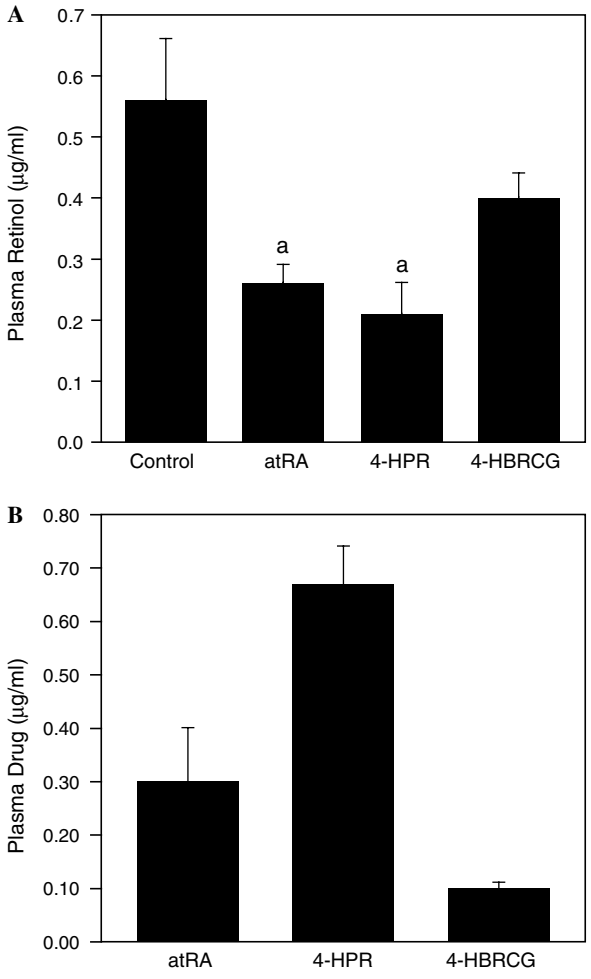


Figure 3. Effect of retinoid treatment on plasma retinol levels (A); the plasma drug levels (B). (A) [a]$p < 0.05$ relative to the control group.

retinol levels are inversely related to the concentration of 4-HPR or its analogue that is present in the blood. In the present study, less 4-HBRCG (**7**) was also present in the plasma at sacrifice compared to 4-HPR, suggesting this may account for the lesser effect of the glucuronide analogue **7** on the circulating blood retinol levels. Thus, future studies of the serum protein binding and tissue distribution of the novel retinoid analogue **7** appear warranted.

As shown in Figure 4A, treatment with RA dramatically increased plasma triglyceride (TG) concentration, whereas 4-HBRCG did not cause this undesirable effect. An increase in plasma TG is a well-known side effect of oral RA administration[30,31] and is mediated by binding to the RAR family.[32] As borne out in human trials, 4-HPR is clearly less potent than RA in producing this side effect.[5] It is possible that hydrolysis of 4-HPR may have accounted for the small but non-significant increase upon feeding of this retinoid in the present study, whereas the non-hydrolyzable 4-HBRCG showed no propensity to increase triglyceride levels.

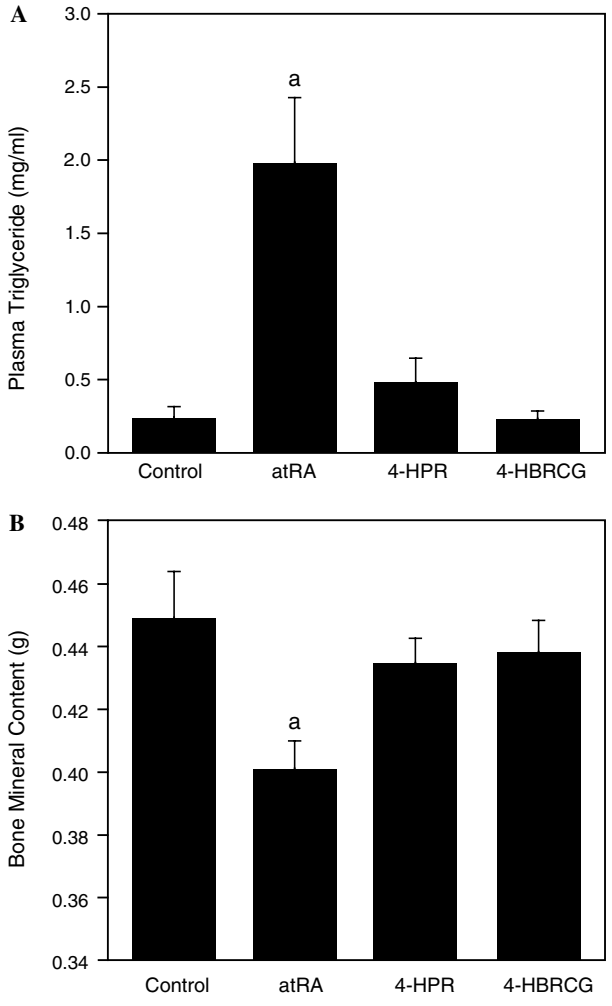


Figure 4. Effect of retinoid treatment on plasma triglyceride level (A) and bone mineral content (B). Values are means ± SEM. (A) $^{a}p < 0.05$ relative to control, 4-HPR, and 4-HBRCG groups. (B) $^{a}p < 0.05$ relative to the control group.

Skeletal abnormalities are another adverse side effect of high-dose retinoid therapy.[33–35] In order to determine the extent of any similar effect for 4-HPR (**3**) and the analogue **7**, we measured the bone mineral content (BMC) of the femur of animals at the end of the feeding study. As expected, atRA produced a significant reduction in the femur BMC compared to control animals (Fig. 4B), whereas the groups receiving **3** or **7** showed no such effect. In an earlier clinical study of women with early breast cancer receiving 4-HPR, a statistically insignificant trend toward an increase in bone resorption markers was noted.[36] This suggests that a 4-HPR analogue such as 4-HBRCG that cannot liberate RA might be advantageous in minimizing the risk of bone complications.

As with 4-HPR and other related analogues (**5** and **6**) we have studied, 4-HBRCG binds poorly to the RARs (Fig. 5). 4-HPR was nearly 3000 times less potent than atRA in competing for [^{3}H]-RA binding to RARβ, and 2500 times less able to compete for binding to the RARγ, confirming earlier work.[9,37] In the present study, 4-HBRCG also showed only weak RAR binding (300 times and 1400 times less potent than atRA in binding to RARβ and RARγ, respectively). Binding of 4-HBRCG was also tested at the RARα, and it was only approximately 20-fold more effective than 4-HPR, which also shows only weak binding at this receptor (data not shown).[9] Furthermore, 4-HBRCG at

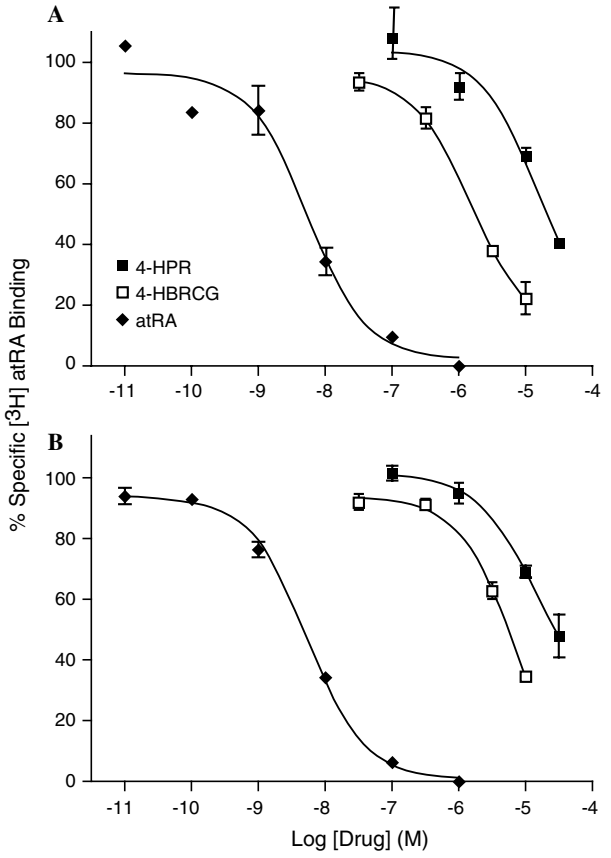


Figure 5. Competition of retinoids for [^{3}H]all-*trans* RA binding to RARβ (A) and RARγ (B); atRA (♦); 4-HPR (■); and 4-HBRCG (□).

concentrations up to $10^{-4.5}$ M showed almost no competition for [^{3}H]-9-*cis* RA binding to the RXRs (data not shown).

In order to evaluate the ability of retinoids to activate RAR-mediated gene transcription in vivo, CYP26 was measured at sacrifice in rat liver and lung. atRA has been shown to induce the atRA-metabolizing CYP26A1 mRNA via binding to RARs and direct interaction of the liganded RAR/RXR heterodimer with retinoic acid response element in the promoter region of this RA-responsive gene.[38,39] The atRA was highly effective in inducing CYP26A1 mRNA in liver (67-fold above control; Fig. 6) and CYP26B1 in the lung (46-fold above control; data not shown). 4-HPR also showed significant activity in inducing CYP26 mRNAs in liver and lung (37- and 20-fold for CYP26A1 and CYP26B1, respectively, compared to control), whereas 4-HBRCG did not induce these cytochrome p450 mRNAs. We showed previously that atRA (**2**), and to a lesser extent 4-HPR (**3**), induces the expression of CYP26B1 mRNA in lungs of vitamin A-deficient rats.[12] The fact that neither **3** nor **7** shows particularly strong binding to the RARs coupled with our finding that 4-HBRCG (**7**) actually shows slightly enhanced affinity compared to 4-HPR for the RARs yet does not induce CYP26 gene expression argues against a direct interaction of 4-HPR with the receptor as a mechanism to explain its ability to induce these mRNAs. Rather, the hydrolysis of 4-HPR to atRA may account for this induction, and as we have shown previously, 4-HPR given orally to vitamin A-deficient rats generates atRA in plasma that is detectable by HPLC.[12] The lack of induction of RAR-mediated gene transcription by 4-HBRCG in vivo supports the conclusion that direct binding of **7** to RARs does not occur at the retinoid levels fed in the present study, and indicates that RA-mediated toxicities should be less of a problem with this improved retinoid, compared to 4-HPR.

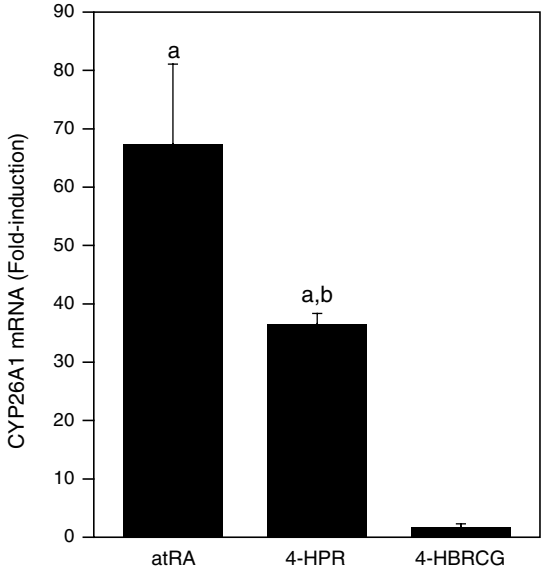


Figure 6. Fold induction of CYP26A1 mRNA in liver of retinoid-fed rats relative to the control group. Values are means ± SEM. [a]$p < 0.05$ relative to control and 4-HBRCG groups; [b]$p < 0.05$ relative to the atRA-fed group.

The toxicity of 4-HPR has been reported to be reduced compared to atRA, however the spectrum of toxicities encountered is similar.[40] 4-HBRCG shows significant improvement when compared to the natural hormone, atRA, for all the measures of toxicity studied here (weight loss, elevation of plasma TG, reduction in BMC, and reduced blood retinol). When compared to 4-HPR, 4-HBRCG also shows advantage as it does not reduce blood retinol to the same extent.

In conclusion, an efficient strategy was developed to synthesize 4-HBRCG which was found to share the ability with 4-HPR and atRA to reduce the size and number of rat mammary tumors. However, a number of toxic effects shown by the parent retinoids are reduced or eliminated with 4-HBRCG. Thus, this fully unhydrolyzable analogue may have a significant advantage as a low toxicity chemotherapeutic agent. Further studies will be required to investigate the chemopreventive potential of 4-HBRCG and to more clearly define its pharmacokinetic profile and mechanism of action.

4. Experimental

4.1. Synthesis

4.1.1. General methods. Anhydrous THF and CH_2Cl_2 were obtained using distillation from sodium benzophenone ketyl and calcium hydride, respectively. Sigma–Aldrich (Milwaukee, WI) supplied starting materials and reagents. Cambridge Isotopes Laboratories (Cambridge, MA) supplied isotope labeled solvents. All reactions and handling of retinoid-containing compounds were done under gold fluorescent lights. TLC was performed on Merck (Gibbstown, NJ) silica gel 60 F_{254} aluminum plates. Column chromatography was performed with Merck silica gel 60 and reverse-phase flash chromatography with Merck Lichroprep® RP-18. Analytical HPLC was done on a Beckman Instruments (San Ramon, CA) unit, with model 127 pump and detector module 166, unless otherwise noted, equipped with a Metachem Polaris (Varian), 5 μm C-18, 250× 4.6 mm column. All retinoids were detected at a wavelength of 350 nm. Melting points were determined with a Thomas–Hoover (Philadelphia, PA) capillary apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer (Wellesley, MA) 241 polarimeter and reported in mol dm^{-1} g^{-1}. Ultraviolet spectra were recorded on a Beckman Instruments DU-40 spectrophotometer. Infrared spectra were recorded as films on silver chloride plates using a Nicolet (Madison, WI) Protégé 460 spectrophotometer. NMR spectra were recorded on a Bruker (Billerica, MA) DRX 400 spectrometer. Mass spectra were recorded on a Micromass (Milford, MA) QTOF Electrospray (ES) mass spectrometer.

4.2. 2,3,4,6-Tetra-*O*-(methoxymethyl)-D-gluconic acid-δ-lactone (9)

To a flame dried flask under argon atmosphere was suspended δ-gluconolactone (**8**) (7.38 g, 41.4 mmol) in CH_2Cl_2 (400 mL). Upon cooling in an ice bath, diisopropylethylamine (57.6 mL, 331 mmol) was added dropwise, followed by careful addition of chloromethyl methyl ether (50 g, 621 mmol). A significant amount of white smoke formed in the reaction vessel. Solid tetrabutylammonium iodide (50 g, 134 mmol) was added and the solution was allowed to warm to rt. The reaction mixture was stirred in the dark for 48 h during which the solution gradually turned red. After cooling to 0 °C, saturated aqueous NH_4Cl (75 mL) was added, the contents diluted with water and the layers separated. The organic layer was washed with brine and the combined aqueous layers were extracted with CH_2Cl_2 (3×). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated. The solids were then triturated with ether (4×) and the ether was concentrated. The resultant oil was chromatographed on silica gel (1:1, hexanes/ethyl acetate) to afford 12.04 g (83%) of clear oil. $[\alpha]_D$ 118.4 (*c* 2.15, CH_2Cl_2); IR (cm^{-1}) 2948 (s), 2885 (s), 1757 (s), 1464 (m), 1443 (m), 1213 (s), 1150 (s), 1035 (s), 912 (m); ^1H NMR ($CDCl_3$) δ 3.36–3.42 (m, 12H), 3.77 (dd, 1H, $J = 3.8$, 11.3 Hz), 3.82 (dd, 1H, $J = 2.8$, 11.3 Hz), 3.99–4.05 (m, 2H), 4.29 (d, 1H, $J = 6.6$ Hz), 4.55–4.56 (m, 1H), 4.65 (s, 2H), 4.69-4.92 (m, 7H); ^{13}C NMR ($CDCl_3$) δ 55.42, 56.05, 56.11, 56.22, 66.12, 73.69, 74.77, 78.43, 96.56, 96.66, 96.78, 96.91, 97.13, 168.70; HRMS (ES) calcd for $C_{14}H_{26}O_{10}$ (M+Na): 377.1424. Found: 377.1408.

4.3. Dimethyl titanocene, $Cp_2Ti(CH_3)_2$ (Petasis' reagent)

To a flame dried flask under argon atmosphere were added titanocene dichloride (14.63 g, 58.8 mmol) and absolute ether (300 mL), which was cooled to 10 °C. Methyl lithium (100 mL, 140 mmol, 1.4 M) was carefully added dropwise in the dark. The cooling bath was removed and the red solution was allowed to stir for 10 min. The solution was then cooled to 0 °C and ice water (25 mL) was carefully added to quench the unreacted methyl lithium. The layers were separated and the aqueous layer was extracted with ether (2×). The combined organic layers were dried (Na_2SO_4) under argon for 1 h and concentrated in the dark at 20 °C to give 12.4 g of orange solid. Dry toluene (100 mL) was added and the reagent was stored at 4 °C and used without characterization.

4.4. 2,6-Anhydro-1-deoxy-3,4,5,7-tetra-*O*-(methoxymethyl)-D-gluco-hept-1-enitol (10)

To a flame dried flask under argon atmosphere was added the sugar lactone **9** (10.05 g, 28.4 mmol) dissolved in dry toluene (140 mL). The toluene solution of dimethyl titanocene (12.4 g, 59 mmol) was then added dropwise to give a red solution. The mixture was then heated to 70 °C and let stir in the dark for 18 h. The resultant black solution was cooled and poured into hexanes (~500 mL). A precipitate formed and was filtered through Celite. The supernatant was concentrated to yield a red oil, which was chromatographed on silica gel (4:1 then 2:1, hexanes/ethyl acetate) to afford 8.66 g (87%) of yellowish oil. $[\alpha]_D$ 46.8 (*c* 2.33, CH_2Cl_2); IR (cm^{-1}) 2940 (m), 2895 (m), 1750 (w), 1440 (w), 1154 (s), 1032 (s), 918 (m); ^1H NMR (DMK-d_6) δ 3.31–3.37 (m, 12H), 3.64–3.71 (m, 2H), 3.78–3.83 (m, 2H), 3.88–3.89 (m, 1H), 4.12 (d, 1H, $J = 5.4$ Hz), 4.35 (s, 1H), 4.51 (s, 1H), 4.62 (s, 2H), 4.66–4.84 (m, 6H); ^{13}C NMR (DMK-d_6) δ 55.15, 55.87, 56.04, 56.19, 67.50, 75.42, 76.68, 77.36, 81.08, 93.43, 95.35, 97.23, 97.64, 97.81, 156.39; HRMS (ES) calcd for $C_{15}H_{28}O_9$ (M+Na): 375.1631. Found: 375.1628.

4.5. 2,6-Anhydro-1-deoxy-1-[4-(hydroxymethyl)phenyl]-3,4,5,7-tetra-*O*-(methoxymethyl)-D-glycero-D-gulo-heptitol (11)

To a flame dried flask under argon atmosphere was added the exocyclic olefin **10** (3.75 g, 10.6 mmol) dissolved in dry THF (100 mL). 9-BBN-H (53.2 mL, 26.6 mmol, 0.5 M) was added dropwise. The mixture was then refluxed for 4.5 h, cooled to rt, then K_3PO_4 (10 mL, 3 M) was added and allowed to stir for 10 min. *p*-Bromobenzyl alcohol (3.98 g, 21.3 mmol) and $PdCl_2$(dppf) (0.686 g, 0.85 mmol) dissolved in DMF (100 mL) were added dropwise and stirred for 18 h. The reaction was diluted with water and ether, and the layers separated. The organic layer was washed with water and brine. The combined aqueous layers were extracted with ether (3×). The organic layers were combined, dried ($MgSO_4$), concentrated, and chromatographed (1:1 then 1:2, hexanes/ethyl acetate) to afford 3.29 g (67%) of orange oil. $[\alpha]_D$ −26.2 (*c* 1.15, DMK); IR (cm^{-1}) 3470 (w), 2932 (m), 2887 (m), 1692 (m), 1444 (w), 1150 (s), 1101 (s), 1024 (s), 918 (m); ^1H NMR (DMK-d_6) δ 2.60 (dd, 1H, $J = 9.4$, 14.4 Hz), 3.18–3.42 (m, 5H), 3.25 (s, 3H), 3.35 (s, 3H), 3.40 (s, 3H), 3.44 (s, 3H), 3.54–3.61 (m, 2H), 3.73 (dd, 1H, $J = 1.8$, 11.3 Hz), 4.51–4.58 (m, 4H), 4.70 (d, 1H, $J = 6.5$ Hz), 4.77–4.85 (m, 4H), 4.93 (d, 1H, $J = 6.5$ Hz), 7.25 (s, 4H); ^{13}C NMR (DMK-d_6) δ 38.35, 55.04, 56.45, 56.55, 64.44, 64.57, 67.42, 77.97, 79.07, 80.32, 81.63, 84.83, 97.20, 99.01, 99.19, 99.32, 127.15, 130.11, 138.75, 141.03; HRMS (ES) calcd for $C_{22}H_{36}O_{10}$ (M+Na): 483.2206. Found: 483.2188.

4.6. 2,6-Anhydro-1-deoxy-1-[4-(methoxymethyl)phenyl]-3,4,5,7-tetra-*O*-(methoxymethyl)-D-glycero-D-gulo-heptitol (12)

To a flame dried flask under argon atmosphere was added the *C*-glycoside benzyl alcohol **11** (2.44 g, 5.3 mmol) dissolved in dry THF (100 mL). Sodium hydride (0.63 g, 26.5 mmol) was added to the flask and the suspension was stirred for 1.5 h. Iodomethane (4.5 g, 31.7 mmol) dissolved in THF (10 mL) was cannulated into the reaction mixture and allowed to stir for 18 h. After cooling in an ice bath, water was added carefully to quench excess NaH. The mixture was extracted with ether (3×), the organic layers combined, washed with brine, dried ($MgSO_4$), concentrated, and then chromatographed (1:1 then 1:2, hexanes/ethyl acetate) to give

2.37 g (94%) of clear oil. $[\alpha]_D$ −27.0 (*c* 4.70, DMK); IR (cm^{-1}) 2981 (s), 2883 (s), 1701 (w), 1513 (m), 1444 (m), 1378 (m), 1301 (m), 1158 (s), 1105 (s), 1028 (s), 918 (s); ^1H NMR (DMK-d_6) δ 2.61 (dd, 1H, J = 9.4, 14.4 Hz), 3.19–3.42 (m, 5H), 3.24 (s, 3H), 3.30 (s, 3H), 3.35 (s, 3H), 3.40 (s, 3H), 3.44 (s, 3H), 3.54–3.64 (m, 2H), 3.73 (dd, 1H, J = 2.6, 13.5 Hz), 4.38 (s, 2H), 4.50 (d, 1H, J = 6.4 Hz), 4.54 (d, 1H, J = 6.4 Hz), 4.70 (d, 1H, J = 6.5 Hz), 4.77–4.85 (m, 4H), 4.93 (d, 1H, J = 6.5 Hz), 7.21 (d, 2H, J = 8.0 Hz), 7.28 (d, 2H, J = 8.0 Hz); ^{13}C NMR (DMK-d_6) δ 33.39, 55.05, 56.47, 56.49, 56.57, 57.97, 67.46, 74.84, 78.00, 79.10, 80.23, 81.66, 84.86, 97.20, 99.01, 99.21, 99.32, 128.15, 130.19, 137.23, 139.45; HRMS (ES) calcd for $C_{23}H_{38}O_{10}$ (M+Na): 497.2363. Found: 497.2384.

4.7. 2,6-Anhydro-7-deoxy-7-[4-(methoxymethyl)phenyl]-3,4,5-tri-*O*-acetyl-L-glycero-L-gulo-heptinoic acid methyl ester (13)

The MOM-protected glucoside **12** (2.43 g, 5.12 mmol) dissolved in methanol (500 mL), aqueous HCl (6 N, 26 mL) was added, and the solution was stirred for 18 h at rt after which the mixture was concentrated to dryness. In a separate flask, KBr (2.42 g, 20.38 mmol) and TEMPO (3.19 g, 20.41 mmol) were added to a saturated $NaHCO_3$ solution (400 mL) and stirred for 20 min at 0 °C. Aqueous NaOCl (11.2 mL, 1.6–2.0 M) was then added and stirred for 10 min. The deprotected sugar from above was dissolved in saturated $NaHCO_3$ solution (100 mL) and added to the flask with the TEMPO mixture. The mixture was stirred for 45 min at 0 °C. The reaction was quenched with EtOH (50 mL) and the contents were washed with ether in a separatory funnel. The aqueous layer was concentrated to dryness and the remaining solid was exhaustively triturated with methanol. The methanol was concentrated and the dried residue was suspended in DMF (180 mL) and then iodomethane (6.4 g) dissolved in DMF (10 mL) was added and allowed to stir for 20 h under argon at rt. Acetic anhydride (40 mL), pyridine (20 mL), and DMAP (15 mg) were then added and allowed to stir for 18 h. The reaction mixture was diluted with water and extracted (3×) with ethyl acetate. The organic layers were washed with water, brine, dried ($MgSO_4$), concentrated, and chromatographed (2:1 then 1:1, hexanes/ethyl acetate) to give 1.90 g (82%) of clear oil that solidified upon standing, mp 84–86 °C. $[\alpha]_D$ −13.04 (*c* 1.15, DMK); IR (cm^{-1}) 2956 (w), 2818 (w), 1750 (s), 1440 (m), 1370 (m), 1211 (s), 1105 (m), 1028 (m); ^1H NMR (DMK-d_6) δ 1.94 (s, 3H), 1.94 (s, 3H), 1.95 (s, 3H), 2.74–2.81 (m, 1H), 2.90 (dd, 1H, J = 3.4, 7.3 Hz), 3.30 (s, 3H), 3.65 (s, 3H), 3.94–3.99 (m, 1H), 4.18 (d, 1H, J = 9.8 Hz), 4.38 (S, 2H), 4.90 (t, 1H, J = 9.8 Hz), 5.05 (t, 1H, J = 9.8 Hz), 5.29 (t, 1H, J = 9.8 Hz), 7.22 (s, 4H); ^{13}C NMR (DMK-d_6) δ 20.39, 20.52, 20.60,38.12, 52.67, 58.03, 70.62, 72.53, 74.09, 74.73, 76.41, 78.62, 128.25, 130.16, 137.43, 137.76, 168.40, 169.89, 170.07, 170.30; HRMS (ES) calcd for $C_{22}H_{28}O_{10}$ (M+Na): 475.1580. Found: 475.1577.

4.8. 2,6-Anhydro-7-deoxy-7-[4-(bromomethyl)phenyl]-3,4,5-tri-*O*-acetyl-L-glycero-L-gulo-heptinoic acid methyl ester (14)

To a dry flask equipped with a $CaSO_4$ drying tube was added the *C*-glucuronide methyl ether **13** (462 mg, 1.02 mmol) along with 30% HBr in acetic acid (5 mL, 25 mmol) at 0 °C. The mixture was stirred for 30 min and then for 18 h at rt. The mixture was diluted with CH_2Cl_2 and then carefully washed with water and saturated $NaHCO_3$ solution. The organic layer was dried ($MgSO_4$), concentrated, and chromatographed (2:1 then 1:1, hexanes/ethyl acetate) to give 440 mg (86%) of white foam, which was crystallized with ether, mp 116–117 °C. $[\alpha]_D$ −12.03 (*c* 5.57, DMK); IR (cm^{-1}) 3026 (w), 2952 (w), 1754 (s), 1440 (m), 1370 (m), 1215 (s), 1101 (m), 1036 (m); ^1H NMR (DMK-d_6) δ 1.93 (s, 3H), 1.94 (s, 3H), 1.95 (s, 3H), 2.76–2.83 (m, 1H), 2.92 (dd, 1H, J = 3.5, 7.3 Hz), 3.64 (s, 3H), 3.96–3.99 (m, 1H), 4.20 (d, 1H, J = 9.7 Hz), 4.62 (s, 2H), 4.90 (t, 1H, J = 9.7 Hz), 5.05 (t, 1H, J = 9.7 Hz), 5.29 (t, 1H, J = 9.7 Hz), 7.25 (d, 2H, J = 8.2 Hz), 7.36 (d, 2H, J = 8.2 Hz); ^{13}C NMR (DMK-d_6) δ 20.40, 20.52, 20.63, 34.37, 38.12, 52.69, 70.58, 72.52, 74.04, 76.35, 78.43, 129.88, 130.68, 137.26, 138.67, 168.39, 169.91, 170.09, 170.29; HRMS (ES) calcd for $C_{21}H_{25}BrO_9$ (M+Na): 523.0580. Found: 523.0602.

4.9. *tert*-Butyl-dimethylsilylcyanohydrin of retinal (16)

To a flame dried flask under argon atmosphere was added retinal (**15**) (1.03 g, 3.62 mmol) dissolved in dry CH_2Cl_2 (50 mL). A catalytic amount of Et_3N (0.1 mL) was added, then *tert*-butyldimethylsilylcyanide (1.0 g, 7.08 mmol) dissolved in CH_2Cl_2 (10 mL) was added by cannulation. The reaction mixture was stirred for 20 h after which the solution was concentrated, chromatographed (95:5, hexanes/ethyl acetate), dried (Na_2SO_4) under argon, and subjected to vacuum overnight to give 1.20 g (78%) of orange oil. UV λ_{max} = 329 nm (ε = 49462); IR (cm^{-1}) 3042 (w), 2960 (s), 2928 (s), 2850 (s), 2239 (w), 1586 (w), 1472 (m), 1358 (m), 1256 (m), 1105 (s), 963 (s), 832 (s), 775 (m); ^1H NMR (DMK-d_6) δ 0.16 (s, 3H), 0.20 (s, 3H), 0.90 (s, 9H), 1.02 (s, 6H), 1.45–1.48 (m, 2H), 1.58–1.63 (m, 2H), 1.70 (s, 3H), 1.99 (s, 6H), 5.57–5.61 (m, 2H), 6.13–6.23 (m, 3H), 6.38 (d, 1H, J = 15.2 Hz), 6.86 (dd, 1H, J = 11.3, 15.2 Hz); HRMS (ES) calcd for $C_{27}H_{43}NOSi$ (M+Na): 448.3012. Found: 448.2982.

4.10. 2,6-Anhydro-7-deoxy-7-[4-(retinoylmethyl)-phenyl]-3,4,5-tri-*O*-acetyl-L-glycero-L-gulo-heptinoic acid methyl ester (18)

To a flame dried flask under argon atmosphere was added THF (40 mL) along with LiHMDS (1.0 M in hexanes, 3.8 mL, 3.8 mmol). The mixture was cooled to −78 °C and the silyl cyanohydrin of retinal **16** (1.08 g, 2.54 mmol) in THF (15 mL) was added by cannulation. The dark red solution was allowed to stir for 30 min at −78 °C. The crystalline bromoglucuronide **14** (2.78 g, 5.56 mmol) in THF (15 mL) was cannulated into the flask and the mixture was stirred for 3 h at −78 °C after

which the solution changed to light red. The reaction was removed from the cold bath and quenched with 1 M NH_4Cl (10 mL). The mixture was extracted with ethyl acetate (3×) and the organic layers were combined, washed with brine, dried (Na_2SO_4), filtered, concentrated, and chromatographed (2:1, hexanes/ethyl acetate) to give 1.0 g (47%) of yellow foam **17** and 1.7 g of recovered bromide **14**. The alkylated product was taken up in 1% aqueous THF (200 mL) and chilled to 0 °C. TBAF (309 mg, 1.18 mmol) was added and the darkened solution was stirred for 1 h. The reaction was diluted with water and extracted with ethyl acetate (3×). The organic layers were combined, washed with brine, dried ($NaSO_4$), filtered, concentrated, and chromatographed (2:1, hexanes/ethyl acetate) to give 628 mg (35% over two steps) of yellow foam. UV $\lambda_{max} = 379$ nm ($\varepsilon = 36182$); HPLC $t_R = 24.0$ min, 1 mL/min (85:15, MeOH/H_2O both with 10 mM NH_4OAc); IR (cm^{-1}) 2956 (w), 2924 (w), 2863 (w), 1754 (s), 1672 (w), 1554 (m), 1436 (w), 1362 (w), 1215 (s), 1081 (w), 1028 (w), 971 (w); ^1H NMR (DMK-d_6) δ 1.02 (s, 6H), 1.45–1.48 (m, 2H), 1.58–1.62 (m, 2H), 1.69 (s, 3H), 1.90 (s, 3H), 1.93 (s, 3H), 1.95 (s, 3H), 2.01 (s, 3H), 2.03–2.05 (m, 2H), 2.28, (s, 3H), 2.75–2.89 (m, 2H), 3.64 (s, 3H), 3.71 (s, 2H), 3.95–3.98 (m, 1H), 4.19 (d, 1H, $J = 9.8$ Hz), 4.90 (t, 1H, $J = 9.8$ Hz), 5.05 (t, 1H, $J = 9.8$ Hz), 5.29 (t, 1H, $J = 9.8$ Hz), 6.15–6.35 (m, 5H), 7.13–7.20 (m, 5H); ^{13}C NMR (DMK-d_6) δ 13.45, 14.68, 20.41, 20.96, 21.08, 21.15, 22.47, 34.15, 35.41, 38.76, 40.86, 52.25, 53.23, 71.18, 73.09, 74.63, 76.93, 79.13, 126.89, 129.86, 130.73, 130.93, 131.01, 131.47, 133.69, 134.89, 135.11, 137.14, 137.26, 138.95, 139.09, 140.96, 152.68, 168.97, 170.45, 170.64, 170.84, 198.78; HRMS (ES) calcd for $C_{41}H_{52}O_{10}$ (M+Na): 727.3458. Found: 727.3456.

4.11. 2,6-Anhydro-7-deoxy-7-[4-(retinoylmethyl)-phenyl]-L-glycero-L-gulo-heptinoic acid (7)

To a flask was added protected **18** (1.15 g, 1.64 mmol) dissolved in methanol (500 mL) and chilled to 4 °C. Potassium carbonate (136 mg, 0.98 mmol) was added and allowed to stir for 20 h. The reaction mixture was concentrated at 25–30 °C to ~200 mL. Adjustment to the original volume with methanol was followed by addition of 1 N KOH (14 mL, 14 mmol). After stirring for 20 h at 4 °C, the reaction mixture was warmed and allowed to stir for 5 h at rt. It was then cooled to 0 °C and carefully adjusted to pH 7 with 4 N HCl. The reaction mixture was concentrated at 25–30 °C to ~100 mL, cooled back to 0 °C, and the pH carefully adjusted to 3 with 1 N HCl. The suspension was extracted with ethyl acetate and the organic layers were combined, dried (Na_2SO_4) under argon for 2 h, and carefully concentrated. The residue was chromatographed on reverse-phase silica gel (gradient 70:30 to 85:15, methanol/water) to yield 759 mg (82%) of yellow foam, which was stored at −80 °C until needed. UV $\lambda_{max} = 382$ nm ($\varepsilon = 30019$); HPLC $t_R = 9.2$ min (1 mL/min, 85:15 MeOH:H_2O both with 10 mM NH_4OAc); IR (cm^{-1}) 3384 (br), 2920 (s), 1721 (m), 1664 (s), 1550 (s), 1427 (m), 1362 (m), 1232 (w), 1089 (m), 1052 (m), 1102 (s), 967 (w); ^1H NMR (MeOH-d_4) δ 0.94 (s, 6H), 1.38–1.41 (m, 2H), 1.54–1.58 (m, 2H), 1.61 (s, 3H), 1.91 (s, 3H), 1.93–1.96 (m, 2H), 2.20 (s, 3H), 2.60 (dd, 1H, $J = 8.7$, 14.4 Hz), 3.03–3.08 (m, 2H), 3.21–3.29 (m, 2H), 3.37 (t, 1H, $J = 9.5$ Hz), 3.53 (d, 1H, $J = 9.5$ Hz), 3.62 (s, 2H), 6.03–6.25 (m, 5H), 7.02–7.16 (m, 5H); ^{13}C NMR (MeOH-d_4) δ 12.88, 14.45, 20.29, 21.04, 21.93, 29.40, 34.00, 35.25, 38.34, 40.76, 52.11, 73.44, 74.63, 79.28, 80.36, 82.36, 124.37, 126.26, 129.92, 130.19, 130.86, 131.01, 131.12, 134.00, 134.23, 136.82, 138.70, 138.91, 139.04, 141.12, 154.22, 173.26, 201.21; HRMS (ES) calcd for $C_{34}H_{44}O_7$ (M+Na): 587.2985. Found: 587.2989.

5. Biology

5.1. Animal studies

Mammary tumors were induced by intragastric intubation of 50-day-old female Sprague–Dawley rats (Harlan Industries, Indianapolis, IN) with a single dose of 15 mg DMBA in 1.0 mL of sesame oil per rat. The rats were then maintained on a powdered Teklad 22/5 rodent chow diet (W): 8640 (Harlan Industries, Indianapolis, IN), and allowed food and water ad libitum. Four months later, rats which had developed palpable tumors were randomly assigned to the experimental groups (four rats/group). The retinoid-treated groups were fed diets supplemented with 2 mmol/kg diet atRA, 4-HPR, or 4-HBRCG, respectively. The retinoids were added to the diet in a vehicle consisting of 25 mL of ethanol–tricaprylin (1:4, v/v) plus 2% (w/v) of α-tocopherol as previously described.[9] This vehicle was also added to the control diet. The additives were blended into the chow diets using a Hobart food mixer. The diets were fed in stainless steel feeders designed with food hoppers. The food was replaced weekly with freshly prepared diets. Food consumption was determined once weekly, and from that the average daily consumption/rat was estimated. These diets were continuously fed for 22 days. Animals were also weighed weekly and monitored for general health status and signs of possible toxicity due to treatment.

Baseline measurement of initial tumor sizes, numbers, and rat body weights was determined immediately before commencement of treatments, and final measurements were recorded just prior to sacrifice of the animals. Animals were palpated for tumors twice weekly and tumor diameters were measured weekly by a micrometer caliper. Tumor volumes were calculated using the formula $[V = \frac{4}{3}\pi r^3]$, where r is one-half the mean of the sum of the largest diameter and the axis at right angle to it. All tumors as well as lungs, liver, and femur were excised at the end of the experiment for chemical and histopathological evaluation. Blood samples were also taken from each animal for determination of plasma retinol and triglyceride levels.

5.2. Plasma triglyceride measurement

Bloods were drawn from anesthetized animals in the presence of EDTA as an anticoagulant, and the

resulting plasma was used for the measurement of plasma 'true' triglyceride levels using a kit from Sigma–Aldrich (Saint Louis, MO). Briefly, the total plasma triglyceride and glycerol concentrations were determined, and the glycerol component was subtracted from the total plasma triglyceride measurement to obtain the 'true' serum triglyceride concentration.

5.3. Plasma retinoid assay

To 500 μL of plasma was added 150 μL of ethanol containing 0.75 μg of internal standard (*N*-(4-chlorophenyl)retinamide). After mixing 30 s, 500 μL of ethyl acetate was added followed by 1 min of mixing and centrifugation for 5 min at 1000 rpm in an IEC CL centrifuge. The ethyl acetate layer was removed and syringe filtered through a 0.45 μm filter. The ethyl acetate extraction was repeated two more times. The combined extracts were evaporated and the residue reconstituted in 100 μL of methanol. The methanol extract (20 μL) was analyzed by HPLC. Chromatography was done on a precolumn equipped 250× 4.6 mm Beckman Ultrasphere ODS column with an 85% methanol/water mobile phase (both containing 10 mM ammonium acetate) flowing at 1 mL/min. Analysis for both internal standard and retinol was conducted at 350 nm, and internal standard recoveries and retinol levels were determined by comparison with standard curves, with adjustment of the retinol level based on recovery. Recoveries of internal standard averaged ca. 78%. Previous extraction of plasma from vitamin A deficient rats showed no substances interfering with the elution position of the retinol or internal standard. In the 4-HPR-treated group, plasma levels of this retinoid were evaluated simultaneously in the same samples as above. In order to avoid interfering substances, plasma treatment retinoid levels for RA and 4-HBRCG were measured using the above system and a step gradient of 75% methanol for 15 min followed by 85% methanol for 40 min.

5.4. Bone mineral content measurement

The femur was disarticulated from the leg, and the adhering soft tissue was removed by dissection. Femurs were scanned using the Lunar PIXImus 2 system (Model X2608, General Electric using LUNAR software version 1.45), and control measurements were made using the small animal quality control phantom. Femurs were scanned five times each with repositioning at each measure. The average value of the bone mineral content (BMC) in grams for each animal is reported as one independent measure.

5.5. Nuclear retinoid receptor binding assay

Competition of **3** and **7** with [^{3}H]-all-*trans*-RA (4.2–4.6 nM) for binding to RARβ and RARγ, and with [^{3}H]-9-*cis* RA (1.9 nM) for binding to RXRγ was determined using an in vitro ligand binding assay.[41,42] [^{3}H]-All-*trans*-RA (40.5 Ci/mmol) or [^{3}H]-9-*cis* RA (69.4 Ci/mmol) was added to receptor containing extracts in the absence and presence of increasing concentrations of competing ligands at 4 °C for 3 h. A hydroxylapatite (HAP) assay was used to separate ligand bound to receptor from that free in solution, and the radioactivity associated with the HAP pellet was measured by scintillation counting.

5.6. Isolation of RNA and quantitative PCR

Total and poly(A)$^+$ RNA was isolated as described.[43] Briefly, lung and liver tissue was collected and flash-frozen in liquid nitrogen until use. Tissue (0.5–1.0 g) was homogenized in buffer (1:10, w/v), and total RNA was isolated according to the method of Chomczynski and Sacchi.[44] A rat CYP26A1 partial cDNA was generated by PCR amplification from E10.5 day rat embryo cDNA. The upstream (5′-GCA GAT GAA GCG CAG GAA ATA CG-3′) and downstream (5′-CCC ACG AGT GCT CAA TCA GGA-3′) primers were designed based on the murine cDNA (gi: 668110). The 635 bp cDNA was subcloned into pGEM-Teasy (Promega, Madison, WI) and sequenced. Similarly, a rat CYP26B1 partial cDNA was generated by PCR amplification from E11.5 day rat embryo cDNA. The upstream (5′-GCT ACA GGG TTC CGG CTT CCA GTC-3′) and downstream (5′-TCC AGG GCG TCC GAG TAG TCT TTG-3′) primers were designed based on the murine cDNA (gi: 31341987), and the 606 bp control cDNA was subcloned and sequenced. The quantitative polymerase chain reaction (Q-PCR) assay was performed using the real-time LightCycler system (Roche, Indianapolis, IN, USA) with LightCycler faststart DNA master SYBR green1 kit (Roche, Indianapolis, IN, USA) according to the manufacturer's protocols. Poly(A)$^+$ RNA (0.5–1.0 μg) was reverse transcribed (RT) using AMV enzyme (Promega, Madison, WI, USA) and random hexamers. The following primer sets were used for Q-PCR: CYP26A1, upstream 5′-ATG ATT CCT CGC ACA AGC AG-3′, downstream 5′-GCT CCA GAC AAC CGC TCA CT-3′; CYP2B1, upstream 5′-AGG CCC AGC GAC TTA CCT TC-3′, downstream 5′-AGG GCG TCC GAG TAG TCT TT-3′; and GAPDH, upstream 5′-TGA AGG TCG GTG TGA ACG GAT TTG GC-3′, downstream 5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′. The primer sets for CYP26A1, CYP26B1, and GAPDH amplify 409–519 bp (gi: 18426827), 708–957 bp (gi: 31220748), and 854–1836 bp (gi: 31377487), respectively.

5.7. Statistical analysis

Descriptive statistics on tumor volumes, tumor numbers, retinol, triglyceride levels, and BMC were examined and compared among the experimental groups. The statistical significance of the groups' comparisons was obtained using analysis of variance (ANOVA), ANOVA with repeated measures, and nonparametric tests.[45–47] Values were considered significant when the $p < 0.05$.

Acknowledgments

This work was supported by Grant CA49837 from the National Cancer Institute, which is gratefully

acknowledged. We thank P. Schweiterman and A. Zdawczyk for assistance in measuring plasma retinoid levels, M. Highland for analysis of triglyceride levels, and W. Zhang for BMC measurements.

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