Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 4, No. 17, pp. 2117-2122, 1994

0960-894X/94 $7.00+0.00

0960-894X(94)00297-5

N-LINKED ANALOGS OF RETINOID O-GLUCURONIDES: POTENTIAL CANCER CHEMOPREVENTIVE/CHEMOTHERAPEUTIC AGENTS

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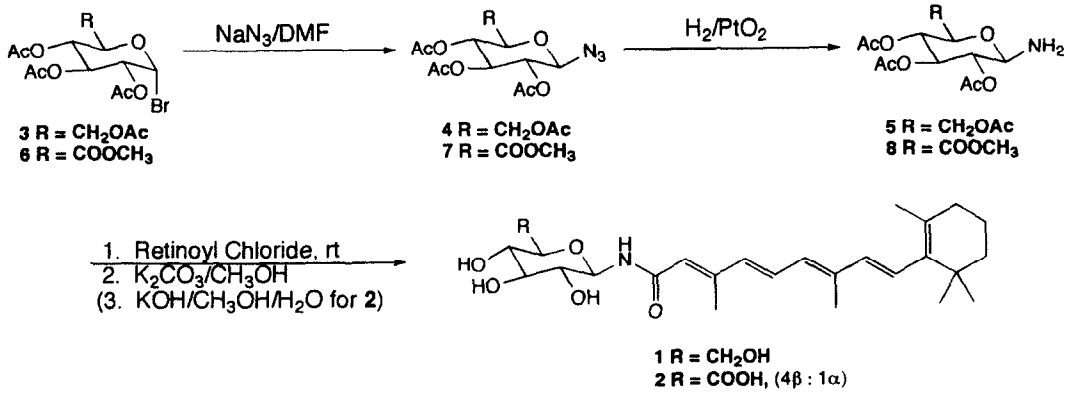
Abstract: Glucuronide metabolites of retinoic acid (RA) and its analogs have been suggested to be active cancer chemopreventive/chemotherapeutic metabolites of the parent molecule. The glucuronide of RA is relatively unstable, thus we prepared N-linked glycoside analogs of RA that are nonsubstrates for ß-glucuronidase in efforts to improve efficacy. The synthesis and biological activity of the analogs are reported.

Recent evidence suggests that the glucuronide conjugates of retinoids may be active metabolites of the parent compounds.[1] The retinoids have generated much interest as dermatologic agents and as cancer chemopreventive or chemotherapeutic compounds.[2a] The retinoids as a class are derived from retinol and its metabolite retinoic acid (RA). The natural retinoids as well as their analogs, are rapidly and extensively metabolized. The majority of these metabolites are the product of ring oxidation. Few of these metabolites retain useful biological activity with the exception of 13-*cis*-RA and the O-acyl glucuronide of RA, retinoyl-ß-glucuronide (RAG).[2b] However, the glucuronide metabolites are susceptible to ß-glucuronidase and acid-catalyzed cleavage and it is not clear whether these carbohydrate conjugates must be hydrolyzed back to the parent molecule to show activity. To aid in understanding the active forms of RA, N-retinoyl glycosides have been synthesized.

We have prepared retinamidoglucoside **1** and retinamidoglucuronide **2** and have found the stability of the N-linked compounds relative to the O-linked glucuronide is substantially increased while retaining many of the physical properties of the parent metabolite. To further assess the properties of these analogs compared to RA, inhibition of carcinogen-induced rat mammary tumors *in vivo* and competitive binding to the nuclear retinoic acid receptors[3] have been evaluated.

The strategy for the synthesis of **1** is shown in scheme 1. Displacement of bromide from the known **3**[4] with two equivalents of sodium azide gave (2,3,4,6-tetra-O-acetyl-ß-D-glucopyranosyl) azide (**4**) in 75% yield. Reduction of azide **4** by catalytic hydrogenation to form amine **5** followed immediately by retinoylation and deprotection gave 1-(ß-D-glucopyranosyl) retinamide (**1**) in 65% overall yield.[5] Routinely, the product obtained before deprotection was treated with diazomethane and chromatographed (SiO_2; 40% EtOAc/C_6H_{14}) to remove traces of methyl retinoate.



Scheme 1. Synthesis of **1** and **2**

The synthesis of **2** is also shown in scheme 1. Displacement of bromide from the known **6**[6] with two equivalents of sodium azide gave methyl (2,3,4-tri-O-acetyl-ß-D-glucopyranuronosyl) azide (**7**) in 70% yield. Reduction of azide **7** by catalytic hydrogenation to form amine **8** followed immediately by retinoylation, chromatography and deprotection as for **1** gave 1-(D-glucopyranosyluronosyl)retinamide (**2**) in 60% overall yield as a 1:1 mixture of anomers. The difficulties producing pure ß-anomer apparently stem from the facile anomerization of amine **8**, facilitated by the electron withdrawing 6-COOH group, which was not detected for amine **5** (Scheme 2). Efforts to discourage the formation of the α-anomer of **8** were successful through conduct of the catalytic hydrogenation and retinoylation at reduced temperatures (-15°C) which resulted in the formation of an inseparable 4ß:1α diastereomeric mixture of **2** as determined by NMR. This mixture was virtually unresolved by analytical HPLC and was used as obtained for further studies.

One of the potential advantages of N-glycosyl compounds in biological systems is their greater hydrolytic stability and their resistance to ß-glucuronidase cleavage to the free sugar and the aglycone. Both of these hydrolytic pathways are available to O-glucuronides. To test the relative stability of the retinamido glycoside linkage toward acidic conditions, samples of **2** and RAG, prepared by a procedure of Barua and Olson[7], were treated with 0.1N HCl at 37°C for 2 h. After this time the O-glucuronide had undergone *ca.* 50% solvolysis to liberate RA as determined by HPLC while **2** remained intact. Thus, it would appear that these analogues show enhanced stability toward acid-catalyzed hydrolysis.

Cleavage of glucuronides to glucuronic acid and the aglycone by ß-glucuronidases appear to be a general reaction for glucuronides, regardless of the structure of the aglycone. To assess the relative stability of the retinamido glycoside linkage toward enzymatic cleavage, samples of **2** and RAG were subjected to a ß-

glucuronidase mediated hydrolysis assay.[8] Within 20 minutes the O-glucuronide, RAG, had undergone complete hydrolysis to liberate RA as determined by HPLC while **2** remained intact. Thus, it would appear that **2** is also relatively stable towards ß-glucuronidase induced hydrolysis.

8a $R_1 = NH_2$, $R_2 = H$
8b $R_1 = H$, $R_2 = NH_2$

Scheme 2. Anomerization of **8**

The results of the effect of feeding 1 mmol/Kg of **1**, **2**, and RA in the diet from 10 days before through 110 days after intubation of female rats with a single 15 mg oral dose of 7,12-dimethylbenz(a)anthracene (DMBA) on the time course of mammary tumor development are summarized in Table 1.[1b] The administration of **1** and **2** greatly prolonged the latency period of mammary cancer. In rats that received DMBA intubation only and AIN-76A rat chow (control group), the first tumors appeared at 42 days post intubation, and 92% of the rats had tumors by 110 days post intubation. In rats that were treated with **1** and **2**, tumor induction was

Table 1. Effects of Retinoids on Progression of DMBA-Induced Rat Mammary Tumors.[a]

compound	tumor latency (days)	tumor incidence (%)	tumor number/rat
control	42	92	1.50
RA	49	83	1.17
1	64	58	0.92
2	64	50	0.83

[a]12 rats/treatment group.

markedly delayed and reduced as opposed to RA. The first tumors were observed at 64 days post DMBA intubation while 58% and 50% respectively had tumors by 110 days post intubation. Retinamide **2** caused the largest reduction in mammary tumor multiplicity. At 110 days post DMBA intubation, the control rats had an average of 1.5 tumors/rat compared to 0.83 tumors/rat in animals receiving **2**. The average number of tumors/rat was 0.92 and 1.17 in animals that received **1** and RA respectively.

In order to gain insight into the possible mechanism(s) by which **1** and **2** show this promising mammary tumor chemopreventive effect, **1** and **2** were tested for their ability to bind to the nuclear retinoic acid receptors (RARs α, ß and γ). Our methods for the generation of RARγ have not previously been described. Thus, the human RARγ *c*DNA was obtained by reverse transcription/PCR amplification using RNA from MCF-7 cells. The coding region of the cDNA was subcloned into the baculovirus vector pVL1393. Production of the receptor protein (hRARγ/BCV) followed standard procedures.[9] hRARγ/BCV exhibits the expected characteristics of a retinoic acid receptor: appropriate molecular size (~50 kD by SDS-PAGE), specific, high-affinity binding to RA (Kd = 0.76 nM), and specific DNA-binding (gel mobility shift of labeled RA response element DNA). Generation of RARs α and ß, and competition binding methodology are described elsewhere.[10] Competition binding analyses were performed using recombinant RAR proteins incubated with [^{3}H]-RA and increasing concentrations of RA, **1** and **2**. As shown in Figure 1, **1** and **2** were ~1500- and ~500-fold less effective than RA in competing for [^{3}H]-RA binding to RARγ (Ki's: RA = 0.5 nM, **1** = 710 nM, **2** = 280 nM). Similar results were observed for binding of **1** and **2** by RARα and RARß (data not shown).

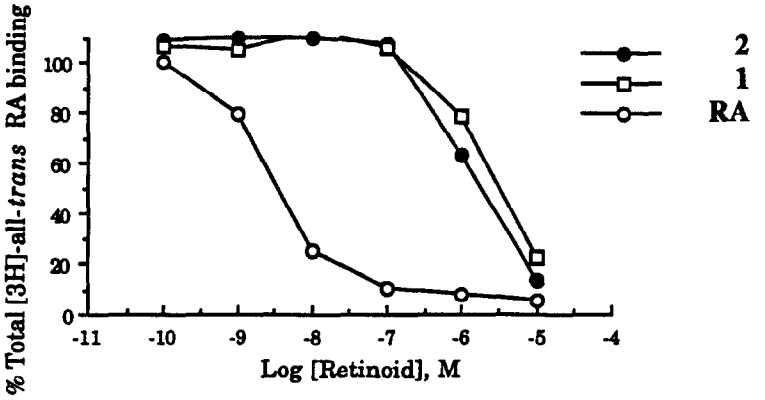


Figure 1. Competition of RA, 1 and 2 for [^{3}H]-RA binding to hRARγ/BCV. Extracts containing 650 fmoles of receptor (1.5 µg total protein) were incubated with 1.3 nM [^{3}H]-RA in the absence or presence of increasing concentrations of unlabelled RA, 1 and 2. Values represent the means of three determinations. (SEMs were less than 4 percent total binding and are not shown)

In summary, the synthesis of the N-glucuronosyl and N-glucosyl analogues of the known RA metabolite RAG have been demonstrated in moderate to good yields. These compounds appear to be relatively hydrolytically stable towards 0.1 N HCl and to ß-glucuronidase cleavage. While **1** and **2** are only moderate (but measurable) competitors of RA binding to the nuclear RARs α, ß, and γ and may bind after conversion of a small amount to RA, the compounds show exciting activity as inhibitors of DMBA-induced mammary tumor development. The chemopreventive effect of **1** and **2** may still be attributable to direct stimulation of RAR(s) provided high enough nuclear concentrations are reached. Alternatively, their activity could still result from slow hydrolysis of the amide bond yielding appropriate concentrations of free RA. The unique activity of **1** and **2** could also result from differences in the efficiency of absorption, distribution or rate of catabolism compared to RA. For example, **1** and **2** may selectively concentrate in the mammary tissue as is also known to occur for the retinoid N-4-hydroxyphenylretinamide. Therefore, further investigations will be required to allow us to explore whether these carbohydrate conjugate metabolites are merely "pro-drugs" or whether they may function as intact molecules.

Acknowledgment. We gratefully acknowledge the financial support of the National Institutes of Health (CA 49837) and the assistance of The Ohio State University Campus Chemical Instrument Center. We also thank Ms. Joan Dandrea for the preparation of this manuscript.

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5. For **1**: mp 166 (dec); IR (KBr) 3369, 2927, 1662 cm^{-1}; UV (CH_3OH) λ_{max} 349 nm (ε 24043); ^1H NMR (DMSO-d_6) δ 1.00 (s, 6H, $C(CH_3)_2$), 1.42 (m, 2H, retinoid H-2)[11], 1.55 (m, 2H, retinoid H-3), 1.70 (s, 3H, retinoid 5-CH_3), 1.95 (br s, 5H, retinoid 9-CH_3 and H-4), 2.28 (s, 3H, retinoid 13-CH_3), 3.15 (br s, 4H, -OH), 3.4-3.7 (m, 6H, pyranose H's), 4.75 (br t, 1H, J-9Hz, pyranose anomeric H), 5.85 (s, 1H, retinoid H-13), 6.04-6.33 (m, 4H, retinoid vinyl H's), 6.99 (dd, 1H, J=11.4, 15 Hz, retinoid H-11), 8.46 (br d), 1H, J=9 Hz, NH); RP-HPLC (CH_3OH/H_2O 9:1, both with 10 mM NH_4OAc, flow rate 1 mL/min) t_R = 6.4 min (96.4% of total peak area); FAB-MS, *m/e* (relative intensity) 484 (M + Na, 53.04).

For **2**: 145° (dec); IR (KBr) 3390, 2925, 1733, 1652 cm^{-1}; UV (CH_3OH) λ_{max} 351 nm (ε 38523); ^1H NMR (DMSO-d_6) δ 1.00 (s, 6H), 1.43 (m, 2H), 1.56 (m, 2H), 1.67 (s, 3H), 1.96 (br s, 5H), 2.28 (s, 3H), 3.21-3.76 (m, 8H), 4.78 (br t, 1H, J=8.9 Hz), 5.83 (s, 1H), 6.07-6.33 (m, 4H), 6.95 (dd, 1H, J=11.7, 14.7 Hz), 8.32 (br d, 0.2H, J= 8.7 Hz, α-NH), 8.53 (br d, 0.8 H, J= 9 Hz, ß-NH); RP-HPLC (CH_3OH/H_2O 9:1, both with 10 mM NH_4OAc, flow rate 1 mL/min) t_R = 4.0 min (96.1% of total peak area); FAB-MS, *m/e* (relative intensity) 552 (M-1 + 2K, 100).

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(*Received in USA* 27 *June* 1994; *accepted* 1 *August* 1994)