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Potent Inhibition of Retinoic Acid Metabolism Enzyme(s) by Novel Azolyl Retinoids

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Abstract—Novel (±)-4-azolyl retinoic acid analogues **4**, **5**, **7** and **8** have been designed and synthesized and have been shown to be powerful inhibitors of hamster microsomal all-*trans*-retinoic acid 4-hydroxylase enzyme(s). (±)-4-(1*H*-Imidazol-1-yl)retinoic acid (**4**) is the most potent inhibitor of this enzyme reported to date.

Introduction

Retinoids, natural and synthetic analogues of all-*trans* retinoic acid (ATRA) play key roles in many biological functions, including induction of cellular proliferation, differentiation and apoptosis as well as developmental changes.[1] ATRA is being used in differentiation therapy of cancer, in cancer chemoprevention, and for the treatment of acne. In spite of these encouraging results, the effects of ATRA therapy on human prostate cancer in the clinic has been scarce and disappointing.[2] It has been suggested that the therapeutic effects of ATRA are undermined by its rapid in vivo metabolism and catabolism by cytochrome P450-dependent enzyme(s).[3]

One of the strategies for preventing in vivo catabolism of ATRA is to inhibit the P450 enzyme(s) responsible for this process. Indeed, this seems to be an emerging approach that may yield effective agents for the chemoprevention and/or treatment of prostate and other kinds of cancer.[4] The major pathway of metabolic deactivation of ATRA starts with hydroxylation at C-4 to form 4-hydroxy-ATRA that is oxidized into 4-oxo-ATRA, which is further transformed into more polar metabolites.[5] The first and rate-limiting step in the process is catalyzed by a cytochrome P450-dependent 4-hydroxylase enzyme. Although the exact nature of this enzyme remains to be elucidated, a novel P450 enzyme (designated CPY26) with specific ATRA 4-hydroxylase activity, which is also rapidly induced by ATRA has recently been cloned from zebra fish,[6a] mouse,[6b] and man.[6c]

ATRA 4-hydroxylase activity was previously thought to mainly reside in the liver, but its presence has now been unequivocally demonstrated in a number of organs, skin, and tumor cells.[7] In principle, inhibitors of ATRA 4-hydroxylase (also referred to as retinoic acid metabolism blocking agents [RAMBAs]) should delay in vivo ATRA catabolism resulting in increased endogenous levels. This effect should improve control of neoplastic differentiation and growth and possibly exhibit antitumor activity. This rationale has been extensively tested with liarozole fumarate (Liazal™) in vitro, in vivo as well as in the clinic by researchers of Janssen Pharmaceutica NV and collaborators.[4,8] Laizal may soon become available for the treatment of a number of cancers and also of dermatological diseases.[9] However, this compound is not specific and inhibits ATRA 4-hydroxylase only at micromolar concentration.

Because of the potential of RAMBAs in cancer therapy and also the need to design more potent and specific inhibitors of the enzyme, we recently became interested in this area. Given the significance of azole groupings of many drugs,[10] which are inhibitors of P450 enzymes, we reasoned that introducing azole grouping at C-4 of ATRA should yield specific and potent inhibitors of this enzyme. With this design, it may be possible to produce substrate-based compounds that will not only interact with

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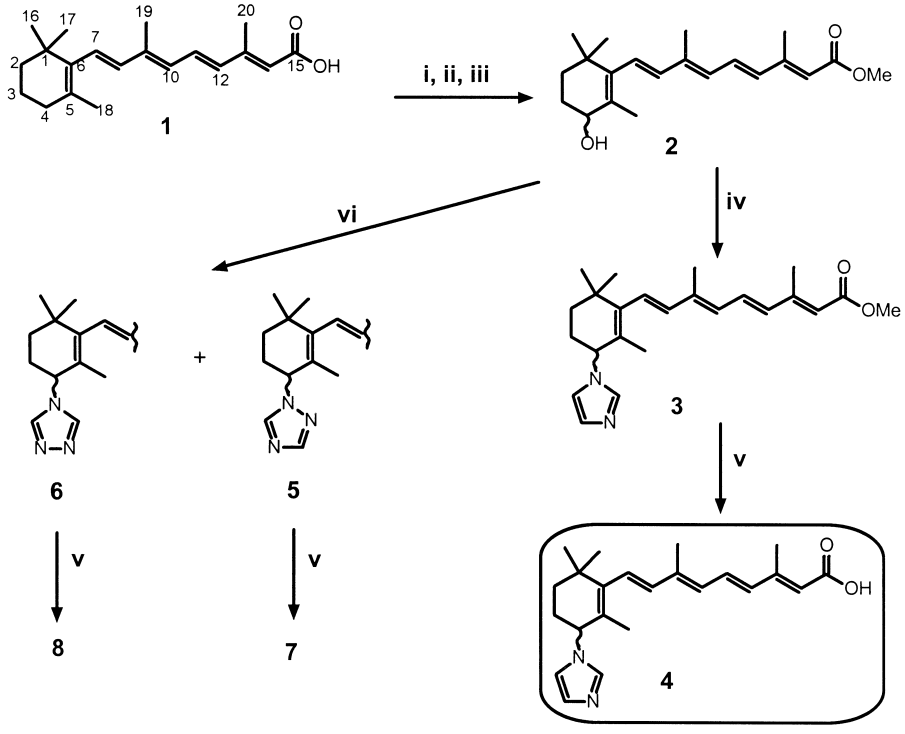
the retinoid-binding site of the enzyme, thus introducing high specificity, but also provide a sixth ligand to the enzyme's heme iron resulting in tight binding. We have successfully used this approach in preparing inhibitors with K_i values in the nanomolar range against 17α-hydroxy/17,20-lyase (CYP17), another cytochrome P450-dependent enzyme that catalyzes the conversion of pregnane steroids into androgens.[11] In this communication, we wish to report the synthesis and biological activity of novel and highly potent substrate-based inhibitors of ATRA metabolism enzyme(s). One of our compounds, (±)-4-(1*H*-imidazol-1-yl)retinoic acid (**4**) is the most potent RAMBA reported to date.[12]

Chemistry

Although a number of azoles have been synthesized directly from alcohols or from their corresponding halides or sulfonates, we expected difficulties in the present case because of the allylic nature of the 4-hydroxy group of 4-hydroxy methyl retinoate (4-HMR, **2**), a key intermediate in our projected synthetic scheme. Literature precedent for the conversion of benzylic alcohols into the corresponding imidazole involves treatment of the alcohol with *N,N′*-carbonyldiimidazole, CDI) in refluxing $CHCl_3$ for 1 h.[13] Only a trace (~3%) of the desired product together with many intractable by-products were obtained on applying these conditions to **2**. Furthermore, attempts to transform **2** to the corresponding sulfonate or halide failed. We eventually synthesized the desired 4-azolyl retinoids as outlined in Scheme 1. A key step in the preparation of our new inhibitors involves the reaction of **2** with appropriate *N,N′*-carbonyldiazole reagents in dry CH_3CN at rt to give the corresponding 4-azolyl retinoids in almost quantitative yields. Indeed, this smooth allylic nucleophilic substitution reaction is remarkable given that such reactions are usually plagued with rearrangement reactions.[14]

The synthesis of our most potent inhibitor, (±)-4-(1*H*-imidazol-1-yl)retinoic acid **4**, is described in detail below. Compound **2** was synthesized in three steps from the commercially available ATRA (**1**) following established procedure.[15] The hydroxy compound **2** in dry CH_3CN was treated with CDI at rt to give the 4-imidazole derivative **3** (95% yield). Alkaline hydrolysis of **3** in refluxing methanol under N_2 gave the desired (±)-4-(1*H*-imidazol-1-yl)retinoic acid (**4**, 80% yield).

Reaction of **2** with *N,N′*-carbonyldi(1,2,4-triazole) (CDT, Scheme 1) as described above gave a mixture of two regioisomers, which were readily separated by flash column chromatography (FCC, silica gel) into the less polar 1*H*-1,2,4-triazole (**5**) and the more polar 4*H*-1,2,4-triazole (**6**) in the ratio of 1.8:1, respectively. These isomers were easily identified by their proton NMR spectra as the two nonequivalent protons of the triazole moiety in **5** appeared as two singlets at δ 7.98 and 8.02 while the two equivalent protons in **6** appeared as a singlet at δ 8.15. These assignments are in agreement with data of 1,2,4-triazole regioisomers of established structure.[11] These triazoles were each hydrolyzed to give the desired (±)-4-(1*H*-1,2,4-triazol-1-yl)retinoic acid (**7**) and (±)-4-(4*H*-1,2,4-triazol-1-yl)retinoic acid (**8**). All compounds gave satisfactory analytical and spectroscopic data.[16]



Scheme 1. Reagents and conditions: (i) $TMSCHN_2$/benzene:MeOH, N_2, rt; (ii) Activated MnO_2/CH_2Cl_2, rt; (iii) $NaBH_4$/MeOH, rt; (iv) CDI/CH_3CN, rt; (v) 10% KOH/MeOH, N_2, reflux; (vi) CDT/CH_3CN, rt.

Proton NMR chemical shifts for these compounds were assigned by comparison with reported values of closely related compounds.[11,15]

Biological Results and Discussions

The prospective inhibitors were evaluated using microsomal preparations of male hamster liver fortified with NADPH. Using [11,12-^{3}H]retinoic acid (7.72 pmol) as substrate, the activity of ATRA 4-hydroxylase corresponds to production of polar metabolites, including 4-hydroxy- and 4-oxo-ATRA.[17] The products were analyzed and quantified by reverse phase HPLC, the radioactivity being measured by an on-line radio-detector. Figure 1A shows a representative chromatogram of the radioactivity profile obtained by HPLC analysis of metabolites of ATRA metabolism.

Thus, ATRA was converted into at least four highly polar metabolites (HPM, retention time, $R_t = 6$–10 min), two prominent metabolites of medium polarity (MMP, $R_t = 14$ and 16 min, respectively), which eluted at the same positions as authentic 4-oxo-and (±)-4-hydroxy-ATRA, respectively. In the presence of 1.0 μM **4** (Fig. 1B), the HPMs were completely abolished and the MMPs were significantly suppressed. This result indicates that the two prominent MMP, most probably, 4-hydroxy- and 4-oxo-ATRA are the obligatory precursors of the HPMs, in agreement with current consensus.[17]

IC_{50} values for inhibition of ATRA catabolism by our novel compounds, liarozole fumarate and ketoconazole were determined using 7.72 pmol [11,12-^{3}H]-ATRA as the substrate. The results are presented in Table 1.

All the novel azolyl compounds were more potent than either ketoconazole or liarozole. The results suggest that the nature of azole is important in determining the affinity for the enzyme. The most active compound, **4** with an IC_{50} value of 0.1 μM is 60-fold more potent than liarozole ($IC_{50} = 6.0$ μM) the only RAMBA to undergo phase III clinical studies in prostate cancer and in psoriasis.[9,13] Compound **4** is by far more potent than any

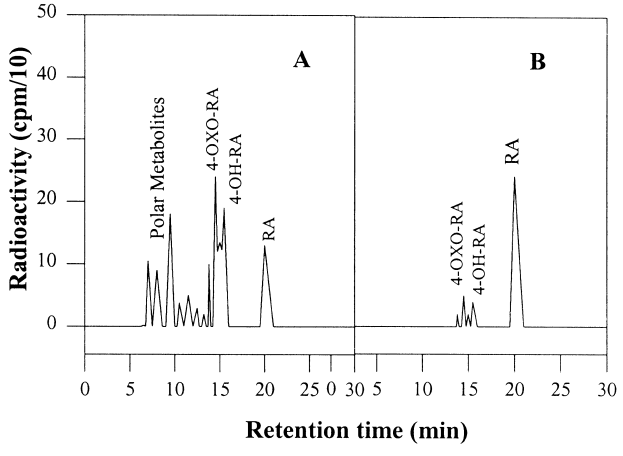


Figure 1. Reverse-phase HPLC chromatograms showing the metabolism of [11,12-^{3}H] in the absence (A) or presence of 1 μM **4** (B).[18]

Table 1. Inhibition of ATRA catabolism

Compound	IC_{50}, μM[a]
4	0.10 (±0.001)
5	0.68 (±0.003)
7	0.88 (±0.005)
8	1.62 (±0.008)
Liazal	6.00 (±0.008)
Ketoconazole	34.00 (±0.008)

[a]Values are means of three experiments, standard deviation is given in parentheses. IC_{50} values for inhibition of ATRA catabolism were measured using 7.27 pmol [11,12-^{3}H]-ATRA as substrate and were determined from dose–response curves.

inhibitor of this enzyme previously described. The inhibitory potencies of these compounds versus the novel cytochrome P450 enzyme (CYP26) with specific ATRA 4-hydroxylase activity, are warranted and should be of utmost interest. Furthermore, it would be expected that these compounds will inhibit ATRA metabolism in vivo resulting in enhanced basal ATRA levels, given that their inhibitory potencies are in the range that could potentially be useful for physiological studies.

In summary, we have developed a new method that enabled us to synthesize novel 4-azolyl retinoids that proved to be powerful inhibitors of hamster microsomal ATRA metabolism enzyme(s). Compound **4** is the most potent RAMBA known to date. These compounds may have therapeutic potential for the treatment of cancers and dermatological diseases. Further work in this direction is being pursued.[19]

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Note Added in Proof

We recently became aware of a report by L. Nadin and M. Murray which suggests that CYP2C8 is a major contributor to ATRA 4-hydroxylation in human liver (*Biochem. Pharmacol.* **1999**, *58*, 1201).

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16. Data for **4**: mp 128–130 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.13 (s, 6H, 16- and 17-CH_3s), 1.56 (m, 2H, 3-Hs), 1.67 (s, 3H, 18-CH_3), 1.87 (m, 2H, 2-Hs), 2.02 (s, 3H, 19-CH_3), 2.32 (s, 3H, 20-CH_3), 4.84 (s, 1H, 4-H), 5.85 (s, 1H, 14-H), 6.21 (s, 3H, 7-, 10- and 12-Hs), 6.33 (d, J = 15.0 Hz, 1H, 8-H), 7.00 (t, J = 14.0 Hz, 1H, 11-H), 7.16 (s, 1H, 4′-H), 7.26 (s, 1H, 5′-H), 7.46, (s, 1H, 2′-H), 8.75 (brs, 1H,-COOH). Anal. calcd for $C_{23}H_{30}O_2N_2$: C, 75.38; H, 8.25; N. 7.64. Found: C, 75.72; H, 8.65; N, 7.67. HRMS calcd for $C_{23}H_{30}O_2N_2$ 366.3061, found 366.305. Data for **7**: mp 95–97 °C. ^{1}H NMR (300 MHz) δ 1.10 and 1.13 (2s, 6H, 16- and 17-CH_3s), 1.50 (brs, 2H, 3-Hs), 1.65 (s, 3H, 18-CH_3), 1.97 (m, 2H, 2-Hs), 2.03 (s, 3H, 19-CH_3), 2.36 (s, 3H, 20-CH_3), 4.86 (s, 1H, 4-H), 5.84 (s, 1H, 14-H), 6.32 (m, 4H, 7-, 8-, 10- and 12-Hs), 7.01 (t, J = 14.5 Hz, 1H, 11-H), 8.10 (s, 1H, 3′-H), 8.31 (s, 1H, 5′-H), 8.46 (brs, 1H,-COOH). Anal. calcd for $C_{22}H_{29}O_2N_3$: C, 71.90; H, 7.95; N, 11.43. Found: C, 71.70; H, 8.11; N, 11.53. HRMS calcd for $C_{22}H_{29}O_2N_3$ 367.4938, found 367.4935. Data for **8**: mp 105–108 °C. ^{1}H NMR (300 MHz) δ 1.11 and 1.14 (2s, 6H, 16- and 17-CH_3s), 1.52 (m, 2H, 3-Hs), 1.65 (s, 3H, 18-CH_3), 1.96 (m, 2H, 2-Hs), 2.03 (s, 3H, 19-CH_3), 2.37 (s, 3H, 20-CH_3), 4.78 (s, 1H, 4-H), 5.85 (s, 1H, 14-H), 6.19 (m, 4H, 7-, 8-, 10- and 12-Hs), 7.01 (t, J = 14.2 Hz, 1H, 11-H), 8.46 (s, 2H, 3′- and 5′-Hs), 8.65 (brs, 1H,-COOH). Anal. calcd for $C_{22}H_{29}O_2N_3$: C, 71.90; H, 7.95; N, 11.43. Found: C, 71.90; H, 7.79; N, 11.30. HRMS calcd for $C_{22}H_{29}O_2N_3$ 367.4938, found 367.4939.
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18. Hamster liver microsomes (500 μg of protein) were incubated for 30 min at 37 °C with [11,12-^{3}H]ATRA (0.4 μCi, 7.72 pmol) and NADPH. The extracts were analyzed with C_{18} Bondapak column eluted with a multi-linear gradient solvent system: I, MeOH:H_2O:HCOOH (60:40:0.05) containing 10 mM NH_4OAc (100→0%) and ii, MeOH (0→100%) at 2 mL/min. The R_ts of ATRA, 4-hydroxy-ATRA and 4-oxo-ATRA were determined by UV absorbance at 350 nm. Typically, 80±5% of [11,12-^{3}H]ATRA was converted into the metabolites.
19. Detailed experiments with these novel RAMBAs involving enzyme specificity, retinoid receptor binding, and in vivo antitumor studies are in progress.