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RAR–RXR Selectivity and Biological Activity of New Retinoic Acid Analogues with Heterocyclic or Polycyclic Aromatic Systems

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Abstract—The cell biological activity of novel retinoids and rexinoids is described. The stereochemistry of the new compounds was analyzed and ligand docking experiments revealed the structural basis of their RAR binding characteristics. The new ligands activate nuclear retinoic acid receptors (RAR, RXR) with distinct selectivity patterns, as determined in genetically engineered 'reporter' cells. The biological activity of the novel retinoids was assessed by differentiation of NB4 acute promyelocytic leukemia cells.

Retinoids interact with a great diversity of proteins, such as cytoplasmic retinoid binding proteins (e.g., CRABP), nuclear retinoic acid receptors (RARα, β, γ and RXRα, β, γ), and rhodopsins, which control multiple biological processes comprising such diverse phenomena as retinoid transport, gene expression, immunomodulation, and vision.[1]

The genetic action of retinoids is largely, if not exclusively, due to their function as ligands for two classes of receptors — the retinoid (RARα, RARβ, RARγ) and rexinoid (RXRα, RXRβ, RXRγ) receptors which are members of the nuclear receptor superfamily.[2,3] Biologically active retinoid analogues interact with nuclear retinoic acid receptors thereby initiating specific allosteric conformational changes,[4] that generate a transcriptionally competent receptor which is capable of binding to specific DNA sequences (Retinoic Acid Response Elements, RAREs) found in the promoter regions of target genes. The coordinate regulation of cognate gene networks triggers phenomena like cell differentiation, proliferation, survival and apoptosis.

The synthesis of stilbene-4-carboxylic acid analogues attained intensive interest in anticancer research in connection with the biological experiments, establishing that derivatives of TTNPB {(E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propen-1-yl)]benzoic acid} are more active in antipapilloma tests than retinoic acid but have increased toxicity.

The high toxicity of great doses of *all trans* retinoic acid (ATRA) and the short duration time of the remission during the therapy of acute promyelocytic leukemia (APL) with ATRA challenged the chemists to synthesize new retinoids and study their mechanism of action. Recent findings reveal that differential RAR/RXR activity leads to different therapeutic and teratogenic profile of the retinoid analogues.[5]

Several scientific groups have synthesized 1,2-diarylethene arotinoids (poly*aro*matic re*tinoids* or retinobenzoic acids), including 4-methoxy-2,3,6-trimethylphenyl,[6a] alkyl,[6b] trimethylsilyl-phenyl,[6c] substituted azulenyl[6d] ethenyl benzoic acids and studied their biological activity. In addition to these structures, chromane derivatives have been synthesized and found to exert decreased toxicity in anticancer investigations.[6e]

Finally, also coumarin derivatives such as *cis* 7-[(3-ethoxycarbonyl)phenylethenyl]-4-methylcoumarin are interesting compounds with antitumor activity.[6f]

Here, we report on the receptor selectivity and cell differentiative activity of the arotinoid acids **1**–**4**[7] and reti-

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noic acid analogues **5**–**7**. (Figs 1 and 2).[8] The new analogues contain coumarin or bulky planar polycyclic groups (condensed aromatic ring systems) in the hydrophobic moieties of their molecules and have modified side chains (**1**–**4**, **7**) in comparison to the natural retinoic acid. The new compounds were used to explore specificity and flexibility of the binding pocket of the retinoic acid receptors RARα, β, γ and RXRβ and to define their differentiation potential on NB4 t(15;17) leukemia cells.

The new retinoic acid analogues **1**–**7** were obtained by reaction sequences including highly stereoselective Horner–Wadsworth–Emmons (HWE) olefinations and subsequent basic hydrolysis of the esters (Figs 1 and 2).

The HWE reactions by the synthesis of compounds **1**–**4** followed by hydrolysis lead to products having only *E*-configuration of the newly formed double bond. The coupling constants of the vinyl hydrogens in the ^{1}H NMR spectra for compounds **1**–**4** and for the preceding esters corresponded to *E*-olefin geometry (16–16.5 Hz).

The synthesis of coumarin compounds in the presence of strong bases (NaOH, NaH, etc.) is complicated because of lactone ring opening. Therefore, olefination reaction and basic hydrolysis of coumarin compounds were carried out under dark (dim red light) to avoid the light-induced isomerization of the intermediate *cis* *o*-hydroxycinnamic acid derivatives. Under these conditions the re-cyclization of the coumarin lactone was possible by acidification of the reaction mixture to pH 1–2 with diluted HCl (1:1) before extraction of the product with ethylacetate.

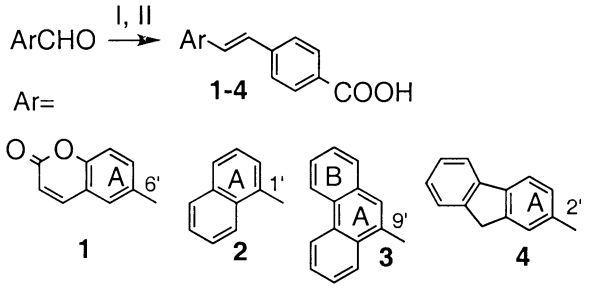

Figure 1. Synthesis of coumarin[9] and polycyclic arotinoids **1**–**4**: (I) NaH, THF, −30, −40 °C, $(EtO)_2P(O)CH_2C_6H_4COOCH_3$; (II) 5 N NaOH, MeOH, Δ.

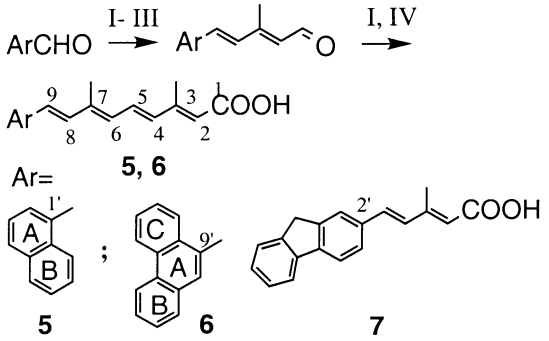

Figure 2. Synthesis of the retinoids **5**–**7** (I) NaH, THF, C_5-phosphonate $(EtO)_2P(O)CH_2C(CH_3)$=CH–$COOCH_3$, −30, −40 °C; (II) DIBAL; (III) MnO_2; (IV) 5 N NaOH, MeOH, reflux.

The new retinoic acid analogues **5**–**7** (Fig. 2) have in the hydrophobic moiety of their molecules bulky planar aromatic systems (1′-naphthyl, 9′-phenanthryl or 2′-fluorenyl aromatic rings) instead of the trimethylcyclohexyl ring of the natural retinoic acid. The 1′-naphthyl and 9′-phenanthryl analogues **5** and **6** were synthesized by multistage procedures, using two C_5-chain extensions, and for the 2′-fluorenyl analogue **7** (Fig. 2) only one C_5-extension procedure was used.

The analysis of the ^{1}H NMR spectra of the reaction mixtures after the HWE reactions showed that the amount of *all-E* isomer in the reaction mixtures after the first C_5-extensions was in the range of 87–89%. The small quantities of 2*Z* isomers (11–13%) of the corresponding esters are due to partial stereomutation (in the presence of the base) of the conjugated double bond available in the starting C_5-phosphonate and are independent on the starting *E*/*Z* ratio.

The stereochemical structure of the tetraenoic acids **5** and **6** was determined by Nuclear Overhauser Effect Spectroscopy (NOESY) and by analysis of the coupling constant values of the olefinic protons.

The chemical shift values of the 3-CH_3-groups of the both analogues were shifted to lower field, compared to the corresponding values of the 7-CH_3-groups. Irradiation of the 7-CH_3-protons gave NOEs on H-5 and H-9, establishing the 6*E*, 8*E*-configurations of the polyenic side chains.

The coupling constant values of H-8 and H-9 (15.7 Hz for both analogues **5** and **6**) were consistent with the proposed 8*E* configuration of the C8–C9 double bonds.

The *E*-structure around the C4–C5 double bond of the polyenic side chains was assigned in correspondence with the *J*-constants of the double doublet of H-5 ($J_{H5\text{-}H6}$ 11.5 Hz for **5**; $J_{H5\text{-}H6}$ 11 Hz for **6**; $J_{H4\text{-}H5}$ 15 Hz for both retinoids **5** and **6**).

A NOE was observed between H-4 and H-2, establishing the *E*-configuration of the C2–C3 double bond for the naphthyl analogue **5**. A twisted conformation at the end of the polyenic side chain of the phenanthryl analogue **6** was proposed in connection with the absence of NOE-interactions of the 3-CH_3 and H-2 with olefinic protons.

The preferred conformations of the polyenic compounds **5** and **6** at the hydrophobic polycyclic terminus of the molecules were determined by NOE-analysis of the steric interactions of the olefinic protons H-8 and H-9 with the neighboring aromatic protons. The interactions through the space between the olefinic proton H-8 and the aromatic proton H-10′ for the phenanthryl analogue **6** established the 9-*s-trans* structure around the single bond, connecting the polyenic side chain at C-9 with the phenanthrene aromatic system. For the naphthyl analogue **5** the clarification by NOESY of the preferred conformation of the cycle to the polyenic chain is difficult because of the close chemical shifts' values of α- and β-naphthyl protons.

The RARα, β, γ and RXRβ selectivity of the newly synthesized retinoids and arotinoids was studied by a reporter cell assays. The 'reporter' cell lines consist of stably integrated (1) luciferase reporter genes driven by five Gal4 response elements in front of a β-globin promoter and (2) co-integrated expression cassettes for recombinant chimeric receptors comprising the DNA binding domain of the yeast transcriptional activator Gal4 fused to the ligand binding domain (comprising the AF-2 transcription activation function) of the corresponding nuclear receptors. The results of the transactivation assays for the synthetic retinoids described above are summarized in Table 1.

Compounds **1** and **4** have week agonist activity for all receptors studied. Compound **4** has some RARα, β and residual RXRβ activity.

Compound **3** is a strong selective agonist for both RARα and RARβ. Also the retinoids **2** and **6** are selective RARα,β agonists and display very little, if any, agonist activity for RARγ (cf., the induction seen with the positive control, ATRA). Residual RXRβ agonistic activity was seen with **2** and **6** but was negligible for compound **3**.

The retinoic acid analogue **5** is a pan-agonist for RAR α, β, γ and RXRβ. Its RXRβ activity is the strongest of the seven compounds tested and is almost equivalent to 9 *cis* retinoic acid. The fluorenyl retinoid **7** (2*E*/2*Z* = 76/24) is a pan-RAR agonist with the strongest RARα activity of the seven tested compounds.

Comparison of the various novel retinoids reveals that compounds **5** and **7** exhibits the lowest RAR isotype selectivity (Table 1). Ligand docking experiments provide a rational for most of the observed receptor selectivities (Table 2).

In comparison, compound **6** has common RARα, β but strongly reduced RARγ activity which is due to its bulky phenanthryl moiety that is oriented nearly perpendicular to the side chain anchoring the ligand in the pocket by its carboxylate. In **7**, the similarly bulky fluorenyl group is oriented in the same direction to the side chain. We have previously shown that three residues Ala-234, Met-272 and Ala-397 (Fig. 3) in the ligand-binding pockets of the RAR isotypes define the RAR selectivity of synthetic retinoids.[10]

It is most likely that Met-272 that restricts the ligand-binding pocket of RARγ[10–12] and is replaced by Ile in RARα and RARβ is responsible for the discrimination between the retinoid **6** and **5** or **7**. However, the three compounds are still agonists because they do not contain moieties as bulky as the quinolyl group of BMS614 which interferes with the positioning of helix H12 that is important for coactivator interaction.[10,13] The compounds **2**, **3**, **5**, **6**, **7** exhibit similar RARα and RARβ selectivity because the ligand-binding pockets of these receptors differ only by one residue (RARα Ser-232 corresponds to RARβ Ala-225) and the present retinoids have not been designed to distinguish between these two residues.

To assess the biological activity of the novel retinoids we have studied their potential to induce differentiation of NB4 cells,[14] the prototypic cellular model for retinoic acid-based differentiation therapy of APL. FACS analysis of the granulocyte differentiation marker CD11c revealed that all retinoids (**2**, **3**, **6**, **7**) with RARα agonist activity could induce NB4 cell maturation (Table 3) which is in correspondence with the molecular basis of the disease.[15]

Table 1. Differential RAR and RXR activity for compounds **1**–**7**

Compound	Receptor selectivity			
	RARα	RARβ	RARγ	RXRβ
DMSO	0.8	0.9	0.9	0.9
AT-RA	9.4	4.2	11.3	—
9cis-RA	—	—	—	6.4
1	2.0	1.7	1.7	1.9
2	4.0	3.5	1.6	2.5
3	4.8	4.5	1.8	1.8
4	2.5	3.4	1.7	2.4
5	4.7	3.6	4.8	5.2
6	4.1	3.0	1.3	2.3
7	5.4	4.1	4.3	2.4

Engineered Hela 'reporter' cell lines were exposed to the various ligands indicated at a concentration of 10^{-6} M. The bioluminescence emanating from lysed cells was quantitated by single-photon counting. The values indicated represent the ratio of the numbers of photons counted in the presence over the photons counted in the absence of ligand. Thus, the higher the values the higher is the transcriptional activity of the corresponding receptor induced by the ligand.

Table 2. Results of the ligand docking for **1**–**7**

Compd	Comments
1	Hydrophobic environment for the lactone ring. No hydrogen bond partner.
2	Short molecule. The part corresponding to the β-ionone ring of RA does not exist. Weak interactions with RARα V395 and RARβ V388 (H11). Too far from RARγ A397 (H11). Ring A has too close contacts with RARγ M272 (H5).
3	Similar to **2** with additional steric hindrance between ring B and RARγ M272 (H5).
4	Long molecule with very few possibilities to bend. Very close contacts with helix H11 residues. Difficult to dock into binding pockets. Too close to RARγ M272 (H5).
5	Resembles RA. Ring A can be superposed on the β-ionone ring, ring B being oriented as the methyl group of RA. No discrimination between receptor isotypes.
6	Long molecule. Can be bent like RA. Ring C too close to RARγ M272 (H5), ring B too close to A397 (H11), M408 (H12).
7	Fits easily into all pockets.

The RARγ LBD forces ligands to adapt to the ligand-binding pocket (LBP) which remains essentially unchanged.[12a,12b] Therefore, the molecular graphics program O was used to manually dock compounds **1**–**7** into RARα and RARγ LBPs (1DKF, 1FCY; Protein Data Bank codes), respectively, as models.[12b,13] The torsion option in O was used to generate compounds with a geometry similar to that of the ligands in the crystal structures. If necessary, the conformations of some LBP side-chains were modified with O in order to maximize protein-ligand interaction. Ligands are differently positioned into RARα(and most likely RARβ) when compared to RARγ LBPs. Due to the presence of M272 in helix H5 of RARγ (I270 and I263 in RARα and RARβ, respectively), the left part of the ligands is pushed away from helix H5 in the direction of helix H11, loop 11-12 and H12. This shift generates most of the differences in the ligand-binding characteristics of RAR isotypes reported in the table.

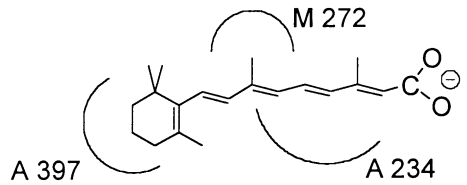

Figure 3. Amino acid environment of the retinoic acid in RARγ LBP. RARγ-specific residues that diverge in RARα and RARβ are shown together with the regions of RA contacting these residues.

Table 3. Cell differentiation potential of compounds **1**–**7**

Retinoid-induced CD11c expression in NB4 leukemia cells						
Compound	None	DMSO	ATRA	TTNPB	**1**	**2**
CD11c[a]	18	17	96	97	14	89
Compound	**3**	**4**	**5**	**6**	**7**	
CD11c[a]	91	37	35	93	84	

[a]% of CD11c-expressing cells.

As expected, due to the very weak RARα agonist activity, compound **1** is inactive in the differentiation assay, while the weak activity of compound **5** which has strong RXR and moderate RARα agonist activity is not immediately apparent.

In summary, new selective retinoic acid analogues, having coumarin or planar polycyclic ring systems in the hydrophobic moieties of their molecules, were synthesized, their differential RAR/RXR activity was established and the factors affecting the structure–activity relationships were analyzed. The results from the receptor selectivity study are useful in the design of new selective ligands for treatment of particular types of cancers that display different tissue distribution of the retinoic acid receptors. The search of new effective retinoids is directed to biologically active compounds with reduced toxicity (teratogenicity, retinoic acid syndrome) and reduced tendency to generate resistance (which can, at least in part, be correlated with a high metabolic rate of the compound) compared to ATRA. In addition to isotype-selectivity, the possibility to generate (i) retinoids and rexinoids that induce a defined pattern of interaction of the receptor heterodimer with co-activator (HAT, SMCC), and co-repressor (HDAC) complexes,[16] (ii) function-selective[17] (transactivation or transrepression) retinoids and rexinoids, and (iii) rexinoids[18] which affect heterodimeric signaling with partners other than RAR will lead to compounds with significant promise for cancer therapy and prevention.

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