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Structure–activity relationships of methylene or terminal side chain modified retinoids on the differentiation and cell death signaling in NB4 promyelocytic leukemia cells

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Abstract—New structure–activity relationships of a series of methylene or side chain modified retinoids on NB4 acute promyelocytic leukemia cells are investigated. The differentiation- and apoptosis-inducing potential of these compounds is analyzed on the basis of their selective retinoic acid receptor binding profile.

Retinoids are powerful signaling compounds that regulate a plethora of physiological processes during embryogenesis, organogenesis, and in adult organ homeostasis. Due to their differentiation- and apoptosis-inducing activities they are used as therapeutic agents for the treatment of several diseases. Most notably retinoids display a strong cancer therapeutic and cancer preventive activity.[1]

The pleiotropic effects of retinoic acid (RA) analogs are mediated by RAR (retinoic acid receptor) and RXR (retinoid X receptor), which belong to the nuclear receptor superfamily of transcription factors. RAR and RXR bind as heterodimers on the promoter region of retinoic acid target genes to modulate their expression. All three RARs (α, β, γ) are activated by all-*trans* retinoic acid (ATRA) and 9-*cis* RA whereas RXRs (α, β, γ) bind efficiently 9-*cis* RA.[2] There is evidence that RXRs may have alternative ligands[3] and that ATRA may bind to certain 'orphan' nuclear receptors.[4]

Remarkable progress has been made in the past few years on the understanding of the molecular basis of the biological activities, including the cancer therapeutic potential, of retinoids and the cognate receptors. In the case of APL (acute promyelocytic leukemia) the combination of ATRA and chemotherapy induces a complete remission in more than 70% of the treated patients. A *t*(15,17) chromosomal translocation generating a fusion protein between RARα and PML is at the origin of the pathology. The molecular action(s) of the PML–RARα fusion protein and the effect of RA ?tul> are reasonably well understood.[5] Compared to RARα, PML–RARα recruits more efficiently nuclear co-repressors (N-CoRs), which are part of a complex including histone deacetylases (HDACs). Accumulation of these factors at the promoters of retinoic acid-responsive genes leads to transcriptional repression that a physiological concentration of ATRA cannot relieve. This results in a block of differentiation of myeloid progenitor cells at the promyelocytic stage. In addition, the oncogenic potential of the fusion protein can also originate from altered PML functions and distinct interaction profiles with itself and/or other key regulatory factors relative to wild-type PML or RARα. High concentrations of ATRA cause a release of the transcriptional repressor complexes, which restores the 'normal' retinoic acid and PML programs.

ATRA therapy of APL patients has a number of complications; one is that patients can acquire resistance due

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to mutations in the ligand-binding domain of PML–RARα. The other is a relative high toxicity ('retinoic acid syndrome'). For this reason, and as mechanistic tools to unravel and dissect the various signaling capacities of ATRA and RXR ligands ('rexinoids') the synthesis of new retinoic acid analogs is important. Novel retinoids with refined activity characteristics and defined RAR, RXR, and orphan receptor interaction acting as agonists or antagonists have promise of improved therapeutic properties compared to presently available retinoids.[1] In addition synthetic retinoids and rexinoids are also used to explore the structural characteristics of the different ligand-binding domains of the retinoic acid/orphan receptors.[6]

Methylene polyaromatic retinoids have been synthesized in order to create new arotinoids with high binding affinity, isotype selectivity, chemical and metabolic stability. Compound LGD1069 ('targretin' or 'bexarotene') is an RXR-selective agonist with only residual RAR activity that has been approved for the therapy of cutaneous T cell lymphoma and is explored in a number of other therapeutic settings.[7] The 6-substituted-2-naphthoic acid methylene arotinoid, described in Figure 1, shows RARβ,γ selectivity.[8]

Retinoic acid analogs, having two terminal functional groups [carboxyl, carbethoxy and N-(ethylamido) carbonyl], have been investigated in studies of the differentiation of mouse embryonal carcinoma cells.[9]

Here we report a study on the structure–activity relationship of methylene and other side chain modified retinoic acid analogs **1**–**7** (Fig. 2). The biological activity of these retinoids, some of which display significant differentiation and apoptosis inducing potential in the APL model, is discussed in view of their receptor selectivity.

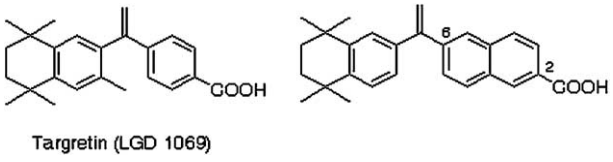

Figure 1. LGD 1069 and 2-naphthoic acid methylene arotinoid.

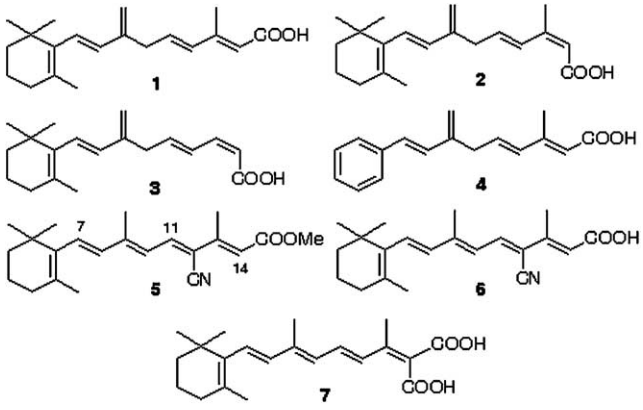

Figure 2. Methylene and other side chain modified retinoids.

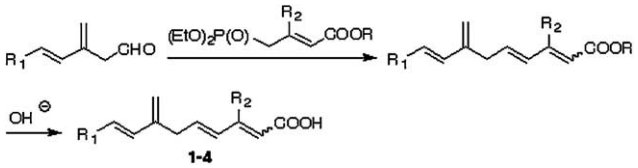

Figure 3. Syntheses of compounds **1**–**4**.

The synthesis of compounds **1**–**4** was achieved by a Wittig–Horner condensation of a suitable alkylidene phosphonate ($R_2 = Me$ or H) with (*E*)-3-methylene-5-(2,6,6-trimethyl-cyclohex-1-en-1-yl)pent-4-enal (compounds **1**–**3**) and (*E*)-3-methylene-5-phenylpent-4-enal (compound **4**) (Fig. 3).[10,11]

Compound **5** was obtained by a condensation (*t*-BuOK, MeOH, rt, 20 h) of β-ionylideneacetaldehyde with methyl (*E*)-4-cyano-3-methylbut-3-enoate. The obtained ester **5** was saponified (KOH, MeOH, H_2O, reflux, 1 h) to provide the acid **6** (Fig. 4).[12]

A vinylogous Stobbe-like reaction of methyl isopropylidenemalonate with the above mentioned methylene aldehyde and further saponification of the obtained alkylidene malonic acid half-ester, provided compound **7**, as shown in Figure 5.[13]

The RAR/RXR selectivity of these compounds was studied using engineered HeLa 'reporter' cell lines. The assay was performed by generation of a chimeric protein, containing DNA-binding domain (DBD) of Gal-4 transcription factor fused to the ligand-binding domain (LBD) of the receptor (RARα,β,γ, or RXRβ). Binding of the retinoid to the LBD leads to activation of a luciferase reporter gene driven by a Gal-4 response element. The generation of luminescence and the fold of

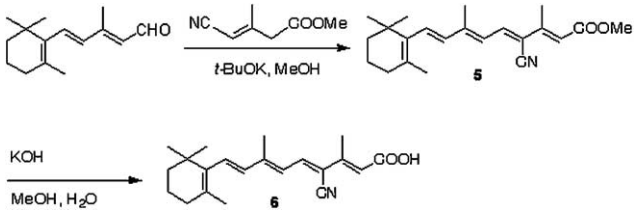

Figure 4. Syntheses of compounds **5** and **6**.

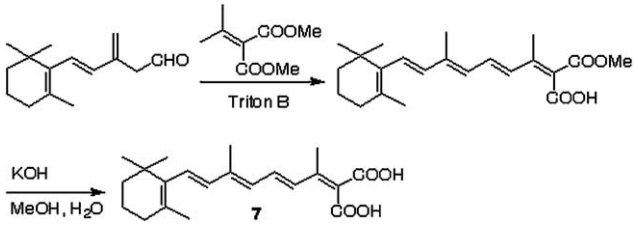

Figure 5. Synthesis of compound **7**.

Table 1. RARα,β,γ, and RXRβ activities for compounds **1**–**7**[a]

Compound	RARα	RARβ	RARγ	RXRβ
ATRA	15.6	9.4	8.9	—
9*Z* RA	—	—	—	14.5
1	7.0	7.2	3.3	5.8
2	11.8	14.5	10.9	2.1
3	7.1	13.1	7.9	2.0
4	4.9	4.0	4.6	13.5
5	1.3	6.7	3.0	1.6
6	23.5	14.6	8.9	2.1
7	22.2	17.9	11.7	3.0

[a] The receptor activation is presented as fold of induction of the basal promoter activity, which is calculated as ratio of the number of photons counted in the presence of the corresponding ligand over the photons counted in the absence of this ligand.

induction were registered by single-photon counting as described in our previous work.[14]

The transcriptional activity of the corresponding receptor was induced at a concentration of 10^{-6} M of each ligand.

Compounds **1**–**7** exert different transcriptional activities through RARα,β,γ, and RXRβ (Table 1; ATRA and 9*Z* RA were used as positive controls for the activation of RARs and RXRs, respectively), which can originate from either a different binding affinity to the respective receptor or a distinct capacity to induce the transcription activation functions of these various receptors. The availability of different RAR/RXR activation profiles in this group of compounds allows correlating the differentiation and apoptosis inducing potential of the tested retinoids with their nuclear retinoic acid receptor activity. The following spectrum of activities was observed:

Compound **1** is agonist for RARα,β; RXRβ and displays a weak RARγ binding activity.

Compounds **2**, **3**, **6**, and **7** are RAR-selective agonists.

Retinoid **4** is a RXRβ agonist and displays some RAR agonistic activity. Interestingly, within its spectrum of activities compound **5** preferentially activates through RARβ, which is generally considered to act as a tumor suppressor.

The above transcriptional profile of retinoids **1**–**7** was then related to their ability to induce maturation and cell death of NB4 APL leukemia cells (Table 2). It has been previously described that RARα agonists induce maturation and apoptosis of these cells.[1] On its own an RXR agonist is devoid of these activities but can synergize with an RARα agonist. Therefore, the NB4 cells constitute a good model to verify the retinoid activity of the compounds.

The differentiation inducing potential of the compounds **1**–**7** was studied by FACScan analysis using specific labeled-antibodies directed against two differentiation markers—CD11c and CD14. Immature NB4 cells can differentiate into the granulocyte lineage and then express the CD11c marker on their surface. They can also follow the monocytic differentiation pathway; in this case the CD14 marker is expressed in addition to CD11c. Treatment of NB4 cells with retinoids **1**, **2**, **3**, **6**, or **7** resulted in expression of only CD11c marker on their surface (Table 2), which was assigned as maturation along the granulocyte lineage. The percentage of positive cells was determined by comparison with the nonspecific binding of isotypic control antibodies. High percentages of CD11c expressing cells are registered after treatment with compounds **1**, **2**, **3**, **6**, or **7**: 93–99% at day 3 and 93–98% at day 6 (not shown in Table 2) with distinct fluorescence intensity reflecting the different expression level of the CD11c marker. Compounds **4** and **5** had considerably lower activity (even during prolonged time of the differentiation- and apoptosis-inducing assays), close to the nontreated cells, and they were not tested in further experiments.

These data confirm RARα activity of the methylene or side chain modified retinoids **1**, **2**, **3**, **6**, and **7**. Compound **4**, which is a predominant RXR-selective ligand, and compound **5**, which displays RARβ selectivity, have weak activities in the differentiation-inducing assays.

Compounds **6** and **7** exhibit the strongest RARα-selective agonistic activity and, consequently, their

Table 2. Differentiation- and apoptosis-inducing potential of compounds **1**–**7** on NB4 promyelocytic leukemia cells

Compound	Mean value of the fluorescence intensity of CD11c–PE expressing cells		% of NB4 cells, labeled with Annexin V–FITC	
	Day 3	Day 6	Day 3	Day 6
Nontreated	11	11	10	8
ATRA	110	197	14	50
TTNPB	106	170	14	35
SR 11237	22	23	11	11
TTNPB/SR	163	215	12	51
1	117	163	18	56
2	38	43	11	14
3	33	35	10	12
4	15	12	9	8
5	10	10	13	8
6	88	126	9	20
7	77	64	11	12

differentiation-inducing potential is stronger than compounds **2** or **3**, which display weaker RARα agonist activity. The strongest differentiation-inducing compound **1** is the only retinoid with both high RARα and RXR agonist activity; the previously reported synergy between these two activities, illustrated in Table 2 by the double treatment with TTNPB (a pan-RAR agonist) and SR 11237 (a pan-RXR agonist), is likely to account for the high differentiation-inducing activity of retinoid **1**.[2]

The ability of the retinoic acid analogs, presented in Table 2, to activate programmed cell death in NB4 cells was detected by FACSsan analysis using Annexin V as marker for apoptotic cells and propidium iodide for discrimination of the necrotic from apoptotic cells. Considerable apoptosis was registered in this group of compounds only for compound **1**, whose nuclear receptor profile contains both RARα and RXR agonist activities.

In conclusion, we report the differentiation- and apoptosis-inducing potential of methylene and side chain modified retinoids. Our data support the following conclusions, (i) RARα ligands are differentiation-competent but RARα and RXR agonist activity is optimal for inducing post-maturation apoptosis; (ii) RARβ-selective agonists on their own are unlikely to have strong differentiation inducing activity in this model and (iii) RXR agonist activity alone does not support efficient induction of either maturation or apoptosis. Clearly, these conclusions are derived from the only existing cellular APL model. It will be interesting to study these compounds, as well as other series of retinoids and rexinoids together with other signaling compounds that have promise for cancer therapy, such as HDAC inhibitors, in other cellular and animal models. Finally, identifying the pathways that are responsible for the induction of differentiation and apoptosis, by using such retinoids as tools, will be a major step forwards to the design of novel types of anti-cancer therapies.

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12. Compound **5**: Yellow crystals, mp 94 °C (ether). IR (film) *v*: 2950, 2929, 2861, 2220, 1715, 1605, 1565, 1361, 1320, 1258, 1224, 1149, 1033, 965, 863 cm^{-1}. ^1H NMR ($CDCl_3$) *δ*: 7.54 (d, 1H, $J = 12.1$ Hz, H_{11}); 6.66 (d, 1H, $J = 12.1$ Hz, H_{10}); 6.58 (d, 1H, $J = 16.1$ Hz, H_7); 6.37 (s, 1H, H_{14}); 6.33 (d, 1H, $J = 16.1$ Hz, H_8); 3.76 (s, 3H, OMe); 2.43 (s, 3H, 13-CH_3); 2.12 (s, 3H, 9-CH_3); 2.10 (m, 2H, 4-CH_2); 1.76 (s, 3H, 5-CH_3); 1.64 (m, 2H, 3-CH_2); 1.50 (m, 2H, 2-CH_2); 1.08 (s, 6H, 1-$(CH_3)_2$) ppm. Anal. Calcd for $C_{22}H_{29}NO_2$: C 77.84, H 8.61, N 4.13; found: C 77.66, H 8.73, N 4.02. Compound **6**: Orange crystals, mp 197 °C (MeCN). IR (film) *v*: 3460, 3425, 2961, 2926, 2864, 2220, 1694, 1611, 1569, 1438, 1382, 1258, 1230, 1195, 1175, 1057, 967, 898, 870 cm^{-1}. ^1H NMR ($CDCl_3$) *δ*: 7.59 (d, 1H, $J = 12.0$ Hz, H_{11}); 6.68 (d, 1H, $J = 12.0$ Hz, H_{10}); 6.62 (d, 1H,

$J = 16.1$ Hz, H_7); 6.40 (s, 1H, H_{14}); 6.35 (d, 1H, $J = 16.1$ Hz, H_8); 2.46 (s, 3H, 13-CH_3); 2.14 (s, 3H, 9-CH_3); 2.08 (m, 2H, 4-CH_2); 1.77 (s, 3H, 5-CH_3); 1.65 (m, 2H, 3-CH_2); 1.50 (m, 2H, 2-CH_2); 1.08 (s, 6H, 1-$(CH_3)_2$) ppm. Anal. Calcd for $C_{21}H_{27}NO_2$: C 77.50, H 8.36, N 4.30; found: C 77.31, H 8.59, N 4.19.

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