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Design and synthesis of novel derivatives of all-*trans* retinoic acid demonstrate the combined importance of acid moiety and conjugated double bonds in its binding to PML–RAR- α oncogene in acute promyelocytic leukemia

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Abstract

The binding of all-*trans* retinoic acid (ATRA) to retinoid receptor- α (RAR- α) relieves transcriptional repression induced by the promyelocytic leukemia–retinoic acid receptor (PML–RAR) oncoprotein. The ATRA molecule contains a cyclohexenyl ring, a polyene chain containing conjugated double alkene bonds, and a terminal carboxyl group. To determine the contributions of these structural components of ATRA to its clinical efficacy, we synthesized three novel retinoids. These consisted of either a modified conjugated alkene backbone with an intact acid moiety (13a) or a modified conjugated alkene backbone and conversion of the acid group to either an ester (13b) or an aromatic amide (13c). Reporter assays demonstrated that compound 13a successfully relieved transcriptional repression by RAR- α , while 13b and 13c could not, demonstrating the critical role of the acid moiety in this binding. However, only ATRA was able to significantly inhibit the proliferation of APL cells while 13a, 13b, or 13c was not. Furthermore, only 13a led to partial non-significant differentiation of NB4 cells, demonstrating the importance of C9–C10 double bonds in differentiation induced CD11 expression. Our results demonstrate that both the acid moiety and conjugated double bonds present in the ATRA molecule are important for its biological activity in APL and have important implications for the design of future novel retinoids.

Keywords

Acute promyelocytic leukemia (APL); all-*trans* retinoic acid (ATRA); retinoids

Introduction

All-*trans* retinoic acid (ATRA) in combination with chemotherapeutic agents is currently the standard therapeutic approach in newly diagnosed acute promyelocytic leukemia (APL), a subtype of acute myelogenous leukemia (AML) that is characterized by the reciprocal translocation t(15;17) [1, 2]. This translocation results in chimeric fusion of the retinoic acid receptor- α (RAR- α) gene to the promyelocytic leukemia (PML) gene, thereby yielding the PML-RAR- α oncogene [1]. The PML-RAR- α fusion protein has increased binding ability to the transcriptional co-repressors N-CoR and SMRT (nuclear receptor co-repressor and silencing mediator of retinoid and thyroid hormone receptors), resulting in the silencing of RAR target genes, which arrests myelopoiesis at the promyelocytic stage [3]. The efficacy of ATRA in therapeutic doses is thought to be mainly due to the release of co-repressors from PML-RAR- α fusion, thereby stimulating transcription of target genes that restore myeloid differentiation [1, 3]. Though ATRA leads to remission in >90% of patients with APL, its therapeutic course is also characterized by high toxicity and acquired resistance, which has spurred investigators to search for more tolerable and potent compounds. ATRA consists of a cyclohexenyl ring, a polyene chain characterized by conjugated double alkene bonds, and a terminal carboxyl group at position C15 [Figure 1(A)]. The exact contributions of these structural components of ATRA in its binding to RAR- α are not well understood. In an attempt to study the importance of these different components in its binding mechanism, we synthesized three novel retinoic acid analogs (13a, 13b, 13c) with altered structural moieties [Figures 1(B), 1(C), and 1(D)]. Our studies showed that both the acid moiety and conjugated double bonds present in the ATRA molecule are important in its binding to RAR- α and the resulting anti-proliferative and differentiating effects on APL cells.

Methods and materials

Cell lines and cultures

Human NB4 cells (AML type 3 as per French-American-British [FAB] classification, provided by Dr. Gallagher) and ATRA resistant cell lines NB4.007/6 and NB4.306 (provided by Dr. Platanius) were the three APL cell lines used in this study. They were cultured in RPMI medium enriched with 10% fetal bovine serum (FBS). MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) + 10% FBS.

Retinoids

ATRA (Sigma-Aldrich) was dissolved in dimethyl-sulfoxide (DMSO) to a stock solution of 100 mM. Compounds 13a, 13b, and 13c (Figure 1) were synthesized by the procedure detailed in Figure 2.

The synthesis of **13a**, **13b** involved the reaction of methyl magnesium bromide with α -cyclocitral in tetrahydrofuran (THF) to give alcohol **2** as a yellow oil [4]. The alcohol gave satisfactory spectral data and was directly converted to **3** by treatment with triphenylphosphine hydrobromide in methanol. Recrystallization of **3** from methanol/ether (1:6) gave a yellow crystalline solid [5]. Formation of the Wittig reagent from **3** in ether was accomplished with *n*-butyllithium in hexane at room temperature (dark-red color), and

then the Wittig reagent was treated with methyl 4-formybenzoate **4** in ether at -78°C for 10–15 min and next stirred at room temperature under a nitrogen atmosphere for 30 h. After work-up, crude ester **5** was purified by flash column chromatography (hexane/ethyl acetate, 98:2) to give a brown oil in 85% yield [6]. The ester was saponified to generate a white solid, which was filtered, washed with water, and dried. The product was recrystallized from hot ethanol and washed with dry hexane to give acid **6** as white crystals (87% yield) [7]. The structure was confirmed by ^1H , ^{13}C nuclear magnetic resonance (NMR), and nuclear Overhauser effect (NOE) experiment, heteronuclear multiple bond correlation (HMBC), and high-resolution mass spectrometry (HRMS). The compound **13c** was synthesized from **13a** by amide coupling procedure [8].

Cell proliferation assays

NB4, NB4.007/6, and NB4.306 cell lines were treated with 1 μM concentration of ATRA, 13a, 13b, and 13c, and DMSO control. Viable cells were counted at days 1–4 using trypan blue exclusion staining. These experiments were done in triplicate.

Luciferase reporter assays

MCF-7 cells were transfected with a β -galactosidase expression vector and a retinoic acid-responsive elements (RARE)–luciferase plasmid [9] using the superfect transfection reagent as per the manufacturer's recommended procedure (Qiagen). Forty-eight hours after transfection, triplicate cultures were either left untreated or treated with ATRA or retinoids for 16 h. The cells were washed twice with cold phosphate-buffered saline, and after cell lysis, luciferase activities were measured using the protocol of the manufacturer (Promega). The measured luciferase activities were normalized for β -galactosidase activity for each sample.

Flow cytometric analysis for myeloid differentiation

Flow cytometric studies were performed as in previous studies [10]. NB4, NB4.007/6, and NB4.306 cells were treated with ATRA or retinoids 13a, 13b, and 13c for 5 days, and cell differentiation was determined by staining with the anti-CD11b monoclonal antibody. The anti-CD11b monoclonal antibody and a matched isotype control were purchased from Invitrogen (allophycocyanin-conjugated human CD11b antibody) and Becton Dickinson (allophycocyanin-conjugated mouse immunoglobulin G [IgG] antibody), respectively.

Apoptosis

Apoptosis of APL cells with different compounds was studied by flow cytometry using the Vybrant Apoptosis Assay (Invitrogen). Apoptotic cells were evaluated by staining with annexin V–Alex Fluor 488 dye, while necrotic cells were visualized in the same assay by staining with nucleic acid dye, Sytox green (Vybrant Apoptosis Kit; Molecular Probes, Carlsbad, CA). NB4, NB4.007/6, and NB4.306 cell lines were incubated with ATRA and retinoids for 4 days and the assay was done on the fifth day.

Results

The structure of ATRA was modified to synthesize three novel retinoids, consisting of either a modified conjugated alkene backbone with an intact acid moiety (13a) or a modified conjugated alkene backbone and conversion of the acid group to either an ester (13b) or an aromatic amide (13c) (Figures 1 and 2). We first determined the effect of ATRA and the novel retinoids on the retinoic acid receptor. MCF-7 cells were transfected with a plasmid containing a RARE–luciferase construct and treated with these compounds. ATRA led to significant activation of the RAR driven reporter, as expected. Compound 13a showed a significant increase in reporter activity (1.6-fold, p -value 0.04, t -test), though it was less when compared to ATRA. The ester derivative 13b and the amide derivative 13c did not result in any luciferase activity, demonstrating no stimulation of RAR- α mediated gene transcription (Figure 3).

We next determined the effect of ATRA and retinoids on the proliferation of APL cells. We observed that ATRA led to significant inhibition of the proliferation of NB4 cells by day 4 of exposure [Figure 4(A)]. The retinoids 13a, 13b, and 13c resulted in slight reductions in proliferation that did not achieve statistical significance. We further determined for any effects of these retinoids on resistant NB4.007/6 and NB4.306 cell lines. We observed that neither ATRA nor compounds 13a, 13b, and 13c led to any inhibition of proliferation for these cell lines [Figures 4(B) and 4(C)].

To further explore the mechanisms of activity of ATRA and these retinoids on APL cells, we assessed their ability in inducing differentiation by examining for the expression of CD11b, a marker of myeloid differentiation. Under the influence of ATRA, an average of 81% of NB4 cells differentiated into CD11b positive cells as compared to only 5% exposed to DMSO control (p -value of 0.00001, two-tailed t -test). 13a led to differentiation of 24% of NB4 cells, though this difference did not achieve statistical significance. Compounds 13b and 13c did not lead to any significant myeloid differentiation. Neither ATRA nor the retinoids were able to lead to the differentiation of resistant cell lines, NB4.007/6 and NB4.306 (Figure 5).

As ATRA exposure has been shown to lead to decreased proliferation, we also tested the ability of ATRA and retinoids in inducing apoptosis of these cell lines. ATRA did lead to increased apoptosis when compared to DMSO control (p -value of 0.01). 13a showed a minor but significant increase in apoptotic cells (mean 10.77%; p -value 0.047, t -test). 13b and 13c did not lead to any increased apoptosis. None of these retinoids led to any increased apoptosis in the resistant cell lines (Figure 6).

Discussion

ATRA has proven clinical efficacy in acute promyelocytic leukemia, and works by binding to RAR- α and relieving transcriptional repression by the PML–RAR oncoprotein. Efforts to synthesize newer retinoids that improve on the clinical efficacy in leukemia and other cancers are ongoing. In an effort to understand the structure–function relationships of various components of the ATRA molecule to its efficacy in APL, we synthesized novel

retinoids from the ATRA backbone. Our results showed that both the acid moiety and the double alkene bonds of the molecule are important in its binding to RAR- α and differentiation of leukemic blasts. Prior studies with retinoids have equally shown that a C-terminal carboxyl group is essential to RAR binding, independent of whether the compound acts as a RAR agonist or antagonist [11–15]. Our studies expand on this by demonstrating that modification of the ATRA polyene chain with an aromatic backbone as in 13a diminishes ATRA's efficacy. The polyene chain has been shown to cause chemical instability within the ATRA compound, and recent research has focused on substituting the conjugated double bonds with aromatic rings, making the compound more stable with increased bioavailability [16].

Various retinoids with RAR subtype selectivity and aromatic backbones have been synthesized in order to improve selectivity and stability. The concurrent goal is also to find efficient compounds in relapsed cases of APL, as ATRA alone fails to induce a second remission in a majority of patients. A recently synthesized retinoic acid compound called Am80 (tamibaroten) has been shown to be more potent *in vitro*, less toxic (secondary to reduced RAR- γ affinity), and chemically more stable than ATRA [17]. Clinical trials were also able to show that tamibaroten induces remission in up to 58% of patients with relapsed APL [17]. The mechanism of overcoming resistance is thought to be due to a lower affinity to CRABP (cellular retinoic acid binding protein) and subsequent maintenance of higher plasma levels [16]. However, tamibaroten does not seem to be efficient if the acquired resistance is due to molecular alterations of the PML–RAR- α gene, thereby limiting its use [17]. Tamibaroten consists of an aromatic backbone with an interposed amide group. Like ATRA it has a terminal carboxy-group. There are so far no clinical data supporting superiority of this compound over ATRA as a first-line agent.

Binding mechanisms of retinoic acid receptors (RARs) are complex and happen in an allosterically controlled ligand-dependent manner. All RARs consist of an N-terminal activation function (AF-1), a central DNA binding domain (DBD), and a C-terminal ligand binding domain (LDB), which is responsible for retinoid binding [18, 19]. Retinoids can act as agonists or antagonists of RAR function, and their mechanism is determined not only by their chemical structure but also by the ratio of coactivators (Co-A) and co-repressors (Co-R) interacting with RARs [18]. The structural similarity of RARs (α , β , γ) makes it challenging to create receptor subtype-specific agents. ATRA's therapeutic role in APL was mainly thought to be due to its binding to RAR- α and inducing differentiation; however, it was shown recently that retinoids that are RAR unselective—like ATRA—were potent inducers of apoptosis [20, 21]. The exact mechanism of ATRA or retinoid induced apoptosis is not well understood—neither RAR- β or RAR- γ seem to mediate apoptotic activity [22]; however, several other signaling pathways, including mitogen activated protein kinases (MAPKs) [23] and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) [24], are implicated in RAR independent ATRA-induced apoptosis. It can be said that the efficacy of ATRA in APL is most likely the result of multiple not fully understood molecular mechanisms, and this could explain why the search for more potent and less toxic retinoids has so far not yielded a superior therapeutic agent, and ATRA has remained the treatment of choice for APL since it was introduced as a therapeutic agent in the late 1980s.

Future experimental research and clinical trials are necessary to gain a better understanding of ATRA's binding mechanism to RARs, and also its function in other molecular pathways.

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References

1. Melnick A, Licht JD. Deconstructing a disease: RARalpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. *Blood*. 1999; 93:3167–3215. [PubMed: 10233871]
2. Sanz M. Treatment of acute promyelocytic leukemia. *Hematology Am Soc Hematol Educ Program*. 2006;147–155. [PubMed: 17124054]
3. Clarke N, Germain P, Altucci L, Gronemeyer H. Retinoids: potential in cancer prevention and therapy. *Expert Rev Mol Med*. 2004; 6:1–23. [PubMed: 15569396]
4. Fernandez-Mateos A, Mateos Buron L, Martin de la Nava EM, Rubio Gonzalez R. Synthesis of CDE and BCDE molecular fragments of the limonoids havanensin and azadiradione. *J Org Chem*. 2003; 68:3585–3592. [PubMed: 12713364]
5. Rosenberger M, Neukom C, Rosenberger M, Neukom C. Retinoic acid metabolites. 2. Total synthesis of rac-(2E, 4E,6E,8E)-3,7-dimethyl-9-(6-carboxy-2,6-dimethyl-3-oxo-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid and rac-(2E, 4E,6E,8E)-3,7-dimethyl-9-[2,6-dimethyl-6-(hydroxymethyl)-3-oxo-1-cyclohexen-1-yl]-2,4,6,8-nonatetraenoic acid. *J Org Chem*. 1982; 47:1779–1782.
6. Waugh KM, Berlin KD, Ford WT, et al. Synthesis and characterization of selected heteroarotinoids. Pharmacological activity as assessed in vitamin A deficient hamster tracheal organ cultures. Single-crystal X-ray diffraction analysis of 4, 4-dimethylthiochroman-6-ylmethyl ketone 1,1-dioxide and ethyl (E)-p-[2-(4,4-dimethylthiochroman-6-yl)propenyl]benzoate. *J Med Chem*. 1985; 28:116–124. [PubMed: 2578190]
7. Das B, Kabalka G. Design and synthesis of (E)-4-((3-ethyl-2,4,4-trimethylcyclohex-2-enylidene)methyl)benzoic acid. *Tetrahedron Lett*. 2008; 49:4695.
8. Um SJ, Kwon YJ, Han HS, et al. Synthesis and biological activity of novel retinamide and retinoate derivatives. *Chem Pharm Bull*. 2004; 52:501–506. [PubMed: 15133197]
9. Minucci S, Zand DJ, Dey A, et al. Dominant negative retinoid X receptor beta inhibits retinoic acid-responsive gene regulation in embryonal carcinoma cells. *Mol Cell Biol*. 1994; 14:360–372. [PubMed: 8264603]
10. Alsayed Y, Uddin S, Mahmud N, et al. Activation of Rac1 and the p38 mitogen-activated protein kinase pathway in response to all-trans-retinoic acid. *J Biol Chem*. 2001; 276:4012–4019. [PubMed: 11060298]
11. Bourguet W, Ruff M, Chambon P, Gronemeyer H, Moras D. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. *Nature*. 1995; 375:377–382. [PubMed: 7760929]
12. Klaholz BP, Mitschler A, Moras D. Structural basis for isotype selectivity of the human retinoic acid nuclear receptor. *J Mol Biol*. 2000; 302:155–170. [PubMed: 10964567]
13. Pogenberg V, Guichou JF, Vivat-Hannah V, et al. Characterization of the interaction between retinoic acid receptor/ retinoid X receptor (RAR/RXR) heterodimers and transcriptional coactivators through structural and fluorescence anisotropy studies. *J Biol Chem*. 2005; 280:1625–1633. [PubMed: 15528208]
14. Bourguet W, Vivat V, Wurtz JM, Chambon P, Gronemeyer H, Moras D. Crystal structure of a heterodimeric complex of RAR and RXR ligand-binding domains. *Mol Cell*. 2000; 5:289–298. [PubMed: 10882070]

15. Teng M, Duong TT, Klein ES, Pino ME, Chandraratna RA. Identification of a retinoic acid receptor alpha subtype specific agonist. *J Med Chem.* 1996; 39:3035–3038. [PubMed: 8759622]
16. Miwako I, Kagechika H. Tamibarotene. *Drugs Today.* 2007; 43:563–568. [PubMed: 17925887]
17. Ohnishi K. PML-RAR alpha inhibitors (ATRA, tamibaroten, arsenic trioxide) for acute promyelocytic leukemia. *Int J Clin Oncol.* 2007; 12:313–317. [PubMed: 17929112]
18. de Lera AR, Bourguet W, Altucci L, Gronemeyer H. Design of selective nuclear receptor modulators: RAR and RXR as a case study. *Nat Rev Drug Discov.* 2007; 6:811–820. [PubMed: 17906643]
19. Gronemeyer H, Gustafsson JA, Laudet V. Principles for modulation of the nuclear receptor superfamily. *Nat Rev Drug Discov.* 2004; 3:950–964. [PubMed: 15520817]
20. Lu XP, Fanjul A, Picard N, et al. Novel retinoid-related molecules as apoptosis inducers and effective inhibitors of human lung cancer cells in vivo. *Nat Med.* 1997; 3:686–690. [PubMed: 9176499]
21. Cincinelli R, Dallavalle S, Nannei R, et al. Synthesis and structure-activity relationships of a new series of retinoid-related biphenyl-4-ylacrylic acids endowed with antiproliferative and proapoptotic activity. *J Med Chem.* 2005; 48:4931–4946. [PubMed: 16033272]
22. Holmes WF, Soprano DR, Soprano KJ. Synthetic retinoids as inducers of apoptosis in ovarian carcinoma cell lines. *J Cell Physiol.* 2004; 199:317–329. [PubMed: 15095280]
23. Xu QH, Konta T, Furusu A, et al. Transcriptional induction of mitogen-activated protein kinase phosphatase 1 by retinoids - selective roles of nuclear receptors and contribution to the antiapoptotic effect. *J Biol Chem.* 2002; 277:41693–41700. [PubMed: 12186877]
24. Altucci L, Rossin A, Raffelsberger W, Reitmair A, Chomienne C, Gronemeyer H. Retinoic acid-induced apoptosis in leukemia cells is mediated by paracrine action of tumor-selective death ligand TRAIL. *Nat Med.* 2001; 7:680–686. [PubMed: 11385504]

Figure 1.

Molecular structures of ATRA and the synthesized retinoids 13a, 13b, 13c. ATRA consists of a cyclohexenyl ring with a polyene chain with four conjugated double bonds and a carboxyl group at position 15 (A). 13a consists of a modified conjugated alkene backbone while keeping acid moiety intact (B). 13b and 13c are characterized by modified conjugated alkene backbones and conversion of the acid group to either an ester (C) or an aromatic amide (D), respectively.

Figure 2.
Schema of chemical synthesis of retinoids.

Figure 3.

ATRA and 13a increase RARE mediated gene expression. MCF-7 cells transfected with RARE-luciferase construct were incubated with each compound. RARE binding was analyzed by luciferase reporter assay. β -Galactosidase was used as transfection control. Activity is depicted as the ratio of ATRA or retinoid (13a, 13b, 13c) luciferase expression divided by control (DMSO). Compound/control ratio and SEM were calculated through a total number of nine experiments for each compound (* $p < 0.05$, ** $p < 0.01$).

Figure 4.

ATRA inhibits proliferation in the NB4 cell line. The cell lines (NB4, NB4.007/6, and NB4.306) were incubated for the indicated times at 37°C. ATRA and the synthesized retinoids were added daily at a concentration of 1 μ M and viable cells were counted by trypan blue exclusion staining. Standard error (SEM) was calculated from a total number of three experiments (* $p < 0.05$, two-tailed t -test).

Figure 5.

ATRA and 13a enhance differentiation in NB4 cells. All cell lines were incubated for 4 days at 37°C and each retinoid compound was added daily in a concentration of 1 μ M. On the fifth day, the cells were washed and incubated with a CD11 receptor antibody and CD11 expression was measured by flow cytometry. The experiments were repeated four times (***) $p < 0.001$, two-tailed t -test).

Figure 6.

ATRA and 13a increase apoptosis of ATRA sensitive NB4 cells. NB4, NB4.007/6, and NB4.306 were incubated with each retinoid compound respectively for 4 days at 37°C. Each retinoid compound was added daily in a concentration of 1 μ M. On the fifth day, the cells were washed and incubated with annexin V–Alex Fluor 488 dye. The proportion of live, apoptotic, and necrotic cells was measured by flow cytometry. SEM was calculated from a total of four experiments (* $p < 0.05$, ** $p < 0.01$, two-tailed t -test).