Evaluation of Differentiation-Inducing Activity of Retinoids on Human Leukemia Cell Lines HL-60 and NB4

Yuichi HASHIMOTO, Hiroyuki KAGECHIKA, Emiko KAWACHI, Hiroshi FUKASAWA, Go SAITO and Koichi SHUDO

Institute of Molecular and Cellular Biosciences, and Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan. Received May 15, 1996; accepted July 11, 1996

Retinoids, including all-trans-retinoic acid (ATRA), its isomers, and fifty synthetic retinoids (retinoobenzoic acids), were tested for differentiation-inducing activity on human leukemia cell lines HL-60 and NB4. Binding activity of typical retinoids to nuclear retinoic acid receptors (RARs) was also investigated. A good linear correlation between the ED50 values of differentiation-inducing activity towards HL-60 cells and those towards NB4 cells was found. Binding activities of retinoids to RARx and RARβ also correlated well to the differentiation-inducing activities.

Key words: retinoid; differentiation; induction; HL-60 cell; NB4 cell; retinoic acid receptor

Human leukemia cell line HL-60 was the first human cell line found to be induced to differentiate to mature granulocytes by all-trans-retinoic acid (ATRA).13 This differentiation-inducing activity of ATRA on HL-60 cells initiated the development of the differentiation-therapy concept. Since then, many synthetic retinoids have been prepared and evaluated on the basis of their HL-60 cell differentiation-inducing activity. Though HL-60 was first reported to be established from a patient with acute promyelocytic leukemia (APL), FAB-M3 according to the French-American-British classification, it is now recognized as being derived from acute myeloblastic leukemia (AML) on maturation (FAB-M2)3 because the cells deviate from the genetic definition of APL (vide infra).4,5

The effects of retinoids are believed to be elicited by binding of retinoids to retinoic acid receptors (RARs).6–8 RARs are members of the steroid/thyroid nuclear receptor superfamily, and three subtypes of RAR, i.e., RARx, RARβ and RARγ, are so far known.6–9 RAR(s) in HL-60 cells activated by a retinoid acts as a specific transcription factor and changes the pattern of gene expression, which in turn causes the induction of cell differentiation. We have shown that HL-60 cells express RARs α and β.10,11 Expression of RARγ has not been detected in HL-60 cells.12 RAR is known to act as a homodimer or as a heterodimer of RAR and another type of nuclear receptor, retinoid X receptor (RXR).13,14 Three subtypes of RXR have been found. RXR is believed to be a receptor of 9-cis-retinoic acid (9CRA).15 ATRA does not bind to RXR, while 9CRA binds to both RXR and RAR.15,16

Clinical trials have revealed that ATRA is a potent drug which can induce complete remission of a specific type of leukemia, i.e., APL.17,18 The complete remission of APL caused by ATRA was reported to correlate with ATRA-induced differentiation of APL cells to mature granulocytes.19 Though ATRA-induced differentiation of APL cells is a similar phenomenon to that of the non-APL cell line HL-60 at the cell biological level, the effectiveness of ATRA as a differentiation-therapy agent for treatment of human leukemia seems to be restricted to APL at present.

* To whom correspondence should be addressed.
constructed as previously described using plasmids containing human RARx cDNA (pSG5-RARx0) and human RARβ cDNA (pSG-RARβ0), which were supplied by Prof. Chambon (Inserm, Strasbourg, France).

Expression and extraction of the products were performed as described. Extracted recombinant RARs were purified by the usual method using an amylose-resin column. The retinoid-binding activity of recombinant RARs was estimated by the nitrocellulose filter binding assay method. Briefly, purified recombinant RARs were incubated with 8 nM [3H]Am80 in the absence or presence of various concentrations of a competitor at 4°C for 16 h. The incubation mixture was absorbed by suction onto a nitrocellulose membrane. Radioactivity that remained on the membrane was measured using a liquid scintillation counter. The difference of the radioactivity thus measured between the mixture incubated with [3H]Am80 alone and that with [3H]Am80 in the presence of an excess amount (0.8 μM) of Am80 was defined as the specific binding activity of 100%. The IC50 value of a test compound defined as the concentration of the competitor necessary to inhibit the specific binding of 8 nM [3H]Am80 with the efficiency of 50%. The binding activity of a test compound is presented as the inhibition constant (Ki value), which was calculated according to the equation 

\[ K_i = \frac{IC_{50}(1 + [L])/K_d} \]

where [L] is the concentration of [3H]Am80 (8 nM) and Kd is the dissociation constant of Am80 determined by Scatchard analysis (5.45 × 10⁻⁹ M and 3.27 × 10⁻⁸ M for the recombinant RARs x and β, respectively).

RESULTS

Differentiation-Inducing Activity of Typical Retinobenzoic Acids on NB4 Cells  First, we assayed the differentiation-inducing activity of typical retinobenzoic acids, i.e., Am80, Am580, Ch55, Re80 and Am555S (Fig. 2), on NB4 cells. These retinobenzoic acids are all more potent than ATRA in differentiation-inducing assay using HL-60 cells and were chosen because they possess unique characteristics: (i) Am80 and Am580 bind and activate both RARx and RARβ, but not RARγ or RXRα; (ii) Ch55 binds and activates all three RAR subtypes but it does not bind cellular retinoic acid binding protein (CRABP); (iii) Re80 possesses the most potent differentiation-inducing activity on HL-60 cells among the compounds we have so far assayed; and (iv) Am555S is a unique silicon-containing compound. ATRA and its isomers were also assayed.

The results are shown in Fig. 3 and Table 1. All the compounds tested showed potent differentiation-inducing activity on NB4 cells. The ED50 values of these compounds and the order of potency towards NB4 cells were similar to those towards HL-60 cells (Table 1). In addition, the shape of the dose–response curves, morphology of the differentiated cells, and the time course of the induced differentiation of NB4 cells were similar to those of HL-60 cells.

Re80 is a very potent differentiation-inducer for both HL-60 and NB4 cells. The potency of Ch55 indicates that retinoid-binding to CRABP is not necessary for the
retinoid-induced cell differentiation of either NB4 or HL-60 cells. Potent activity of Am80 and Am580 indicates that activation of RARα and/or RARβ is enough to induce both HL-60 and NB4 cell differentiation, though we do not know the effects of these compounds on PML/RARα. It is at least clear that retinoid-binding to RXRs or RARγ is not necessary for NB4 or HL-60 cell differentiation. 9CRA, which binds and activates both RARs and RXRs, showed slightly weaker activity than ATRA towards NB4 cells.

Structure–Activity Relationship of Retinobenoic Acids in Differentiation-Inducing Assay Using NB4 Cells and Its Correlation with That Using HL-60 Cells Next, we analyzed the structure–activity relationship of retinobenoic acids for induction of NB4 cell differentiation. Our previous results indicate that the bulkiness of the substituents (R's in Fig. 1) is critical for HL-60 cell differentiation. The results obtained are shown in Table 1. Fig. 2 shows the structures of retinobenoic acids assayed.

Table 1. Differentiation-Inducing Activities of Typical Retinoids on NB4 and HL-60 Cells

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>ED₅₀ value (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NB4</td>
</tr>
<tr>
<td>ATRA</td>
<td>3.1 × 10⁻⁹</td>
</tr>
<tr>
<td>9CRA</td>
<td>1.0 × 10⁻⁹</td>
</tr>
<tr>
<td>Am80</td>
<td>2.2 × 10⁻⁹</td>
</tr>
<tr>
<td>Am580</td>
<td>3.2 × 10⁻¹⁰</td>
</tr>
<tr>
<td>Am585</td>
<td>2.0 × 10⁻¹⁰</td>
</tr>
<tr>
<td>Ch55</td>
<td>2.0 × 10⁻⁹</td>
</tr>
<tr>
<td>Re80</td>
<td>7.1 × 10⁻¹¹</td>
</tr>
</tbody>
</table>

Numbers indicate the structures (Fig. 2) of the compounds.
Table 2. Differentiation-Inducing Activities of Am-Series Retinobenzoic Acids on NB4 and HL-60 Cells

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>ED₅₀ value (m) NB4</th>
<th>ED₅₀ value (m) HL-60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Am00 (9)</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Inactive</td>
</tr>
<tr>
<td>Am20 (10)</td>
<td>CH₃CH₂</td>
<td>CH₃CH₂</td>
<td>H</td>
<td>3.9 x 10⁻⁸</td>
</tr>
<tr>
<td>Am30 (11)</td>
<td>H</td>
<td>(CH₃)₂CH</td>
<td>H</td>
<td>8.9 x 10⁻⁷</td>
</tr>
<tr>
<td>Am32 (12)</td>
<td>(CH₃)₂CH</td>
<td>H</td>
<td>H</td>
<td>3.9 x 10⁻⁷</td>
</tr>
<tr>
<td>Am55 (13)</td>
<td>(CH₃)₂C</td>
<td>H</td>
<td>(CH₃)₂C</td>
<td>8.9 x 10⁻⁹</td>
</tr>
<tr>
<td>Am66 (14)</td>
<td>(CH₃)₂CH</td>
<td>H</td>
<td>(CH₃)₂CH</td>
<td>2.6 x 10⁻⁸</td>
</tr>
<tr>
<td>Am68 (15)</td>
<td>(CH₃)₂CH</td>
<td>(CH₃)₂CH</td>
<td>H</td>
<td>1.0 x 10⁻⁸</td>
</tr>
<tr>
<td>Am80 (4)</td>
<td>(CH₃)₂C(CH₃)₂C(CH₃)₂</td>
<td>H</td>
<td>3.2 x 10⁻¹⁰</td>
<td>7.9 x 10⁻¹⁰</td>
</tr>
</tbody>
</table>

The r value for the correlation between ED₅₀ values for NB4 and HL-60 cells was 0.976. Numbers indicate the structures (Fig. 2) of the compounds.

Table 3. Differentiation-Inducing Activities of Ch-Series Retinobenzoic Acids on NB4 and HL-60 Cells

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>ED₅₀ value (m) NB4</th>
<th>ED₅₀ value (m) HL-60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch00 (21)</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>Inactive</td>
</tr>
<tr>
<td>Ch20 (22)</td>
<td>CH₃CH₂</td>
<td>CH₃CH₂</td>
<td>H</td>
<td>1.3 x 10⁻⁷</td>
</tr>
<tr>
<td>Ch30 (23)</td>
<td>(CH₃)₂CH</td>
<td>(CH₃)₂CH</td>
<td>H</td>
<td>2.1 x 10⁻⁹</td>
</tr>
<tr>
<td>Ch40 (24)</td>
<td>H</td>
<td>(CH₃)₂C</td>
<td>H</td>
<td>3.1 x 10⁻⁸</td>
</tr>
<tr>
<td>Ch55 (7)</td>
<td>(CH₃)₂C</td>
<td>H</td>
<td>(CH₃)₂C</td>
<td>1.1 x 10⁻¹⁰</td>
</tr>
<tr>
<td>Ch60 (25)</td>
<td>(CH₃)₂CH</td>
<td>H</td>
<td>(CH₃)₂CH</td>
<td>8.8 x 10⁻⁹</td>
</tr>
<tr>
<td>Ch80 (26)</td>
<td>(CH₃)₂C(CH₃)₂C(CH₃)₂</td>
<td>H</td>
<td>2.4 x 10⁻¹⁰</td>
<td>6.4 x 10⁻¹⁰</td>
</tr>
<tr>
<td>Ch40S (28)</td>
<td>H</td>
<td>(CH₃)₂Si</td>
<td>H</td>
<td>Inactive</td>
</tr>
<tr>
<td>Ch60S (29)</td>
<td>(CH₃)₂Si</td>
<td>H</td>
<td>(CH₃)₂Si</td>
<td>3.2 x 10⁻¹⁰</td>
</tr>
<tr>
<td>Ch55G (31)</td>
<td>(CH₃)₂Ge</td>
<td>H</td>
<td>(CH₃)₂Ge</td>
<td>1.6 x 10⁻⁸</td>
</tr>
<tr>
<td>Ch60G (29)</td>
<td>(CH₃)₂Ge</td>
<td>H</td>
<td>H</td>
<td>1.5 x 10⁻⁸</td>
</tr>
</tbody>
</table>

The r value for the correlation between ED₅₀ values for NB4 and HL-60 cells was 0.927.

differentiation-inducing and RAR-binding activities of retinobenzoic acids, 7,8,25–27) The present results, shown in Tables 2 and 3, indicate that the structure-activity relationship observed for NB4 cell differentiation-induction is the same as that for HL-60 cell differentiation-induction. Correlation factors between the ED₅₀ values for NB4 and HL-60 cells are r = 0.976 for the Am-series of retinobenzoic acids (Table 2) and r = 0.927 for the Ch-series of retinobenzoic acids (Table 3). 31) These results suggest that the target molecule(s) of retinoids in NB4 cells is the same, or at least possesses the same ligand-selectivity, as that in HL-60 cells.

The correlation between the ED₅₀ values of forty-three retinoids, including ATRA, its isomers, Am- and Ch-series retinobenzoic acids, and other types of retinobenzoic acids, in the assay using NB4 cells and those in the assay with HL-60 cells was analyzed (Fig. 4). A good correlation with an r value of 0.911 was observed. 31) Rather large deviations from the linear correlation were found for the compounds with lower activities (ED₅₀ values higher than 10⁻⁶ M). This might reflect a generally inferior specificity of less potent compounds.

RAR-Binding Activity of Retinobenzoic Acids and Its Correlation with Differentiation-Inducing Activity Binding activities of various retinobenzoic acids to RARα and RARβ were measured by means of binding competition with [³H]Am80 using recombinant RARs. As already reported, 29,33,34) the recombinant RARs used possess ligand-affinity and ligand-selectivity which are very similar to those of the corresponding human RARs. Therefore, the recombinant RARs can be regarded to have essentially identical (or at least very similar) ligand-binding characteristics to those of human RARs, and it should be possible to discuss the correlation between differentiation-inducing activity and RAR-binding activity of the compounds on the basis of the ligand-binding activity.
determined using recombinant RARs.

A high correlation was found between RARα-binding and RARβ-binding activities, with the $r$ value of 0.84 (Fig. 5). However, we did find potent retinobenzoic acids which greatly deviate from the correlation curve (Fig. 5), i.e., Am555S (dot No. 6 in Fig. 5) and Am68P (dot No. 19 in Fig. 5) (structures are shown in Fig. 6).28) Am555S possesses more than 200-fold higher binding affinity for RARα than for RARβ (Fig. 5). Comparison of the $K_i$ values of Am555S with those of Am68P indicates that the former possesses more than 30-fold higher binding affinity than the latter to RARα. Conversely, Am68P has more than 30-fold higher binding affinity than Am555S for RARβ. In spite of this large difference in RAR-subtype-selectivity of these two retinobenzoic acids, it is noteworthy that Am555S and Am68P showed comparably potent cell differentiation-inducing activities, with $ED_{50}$ values of the order of $10^{-8}$ M on both NB4 and HL-60 cells.

Correlations between RARs-binding activities and cell differentiation-inducing activities towards NB4 and HL-60 cells were analyzed (Fig. 7), and a linear correlation with $r$ values of 0.81—0.96 between RARs-binding activities and differentiation-inducing activities was observed for both cell types.

RARα-selective Am555S and Am68P, which possesses only weak binding affinity to RARα (1/30 of that of

![Graph](image1)

**Fig. 4. Comparison of $ED_{50}$ Values of Various Retinoids on NB4 and HL-60 Cells**

Vertical and horizontal scales: $Log ED_{50}$ values for HL-60 cells and NB4 cells, respectively. ○, ATRA and its isomer,51-52, Am-series of retinobenzoic acids including Am80, Am550, Am555S and Am68P,24,25, Ch-series of retinobenzoic acids including Res8,27, other retinobenzoic acids.29-30 The line was drawn by the least-squares method ($r = 0.91$). Numbers indicate the compounds whose structures are shown in Fig. 2.

![Graph](image2)

**Fig. 5. Correlation between Binding Activities of Retinoids to RARα and RARβ**

Vertical and horizontal scales: $Log K_i$ values in ligand-binding competition experiments for RARβ and RARα, respectively. The line was drawn by the least-squares method ($r = 0.84$). Numbers indicate the compounds whose structures are shown in Fig. 2.

![Graph](image3)

**Fig. 6. Structures of Am555S and Am68P**

![Graph](image4)

**Fig. 7. Correlations between Differentiation-Inducing Activities and RAR-Binding Activities**

Correlations between $K_i$ values in ligand-binding competition experiments for RARα (○) or RARβ (●) and $ED_{50}$ values in cell differentiation-inducing assay for NB4 cells (a) or HL-60 cells (b). The lines were drawn by the least-squares method and the $r$ values are shown in the figures. Numbers indicate the compounds whose structures are shown in Fig. 2.
Am555S) but 30-fold higher activity to RARβ than Am555S, showed cell differentiation activities on both NB4 and HL-60 cells with similar potency. In addition, ED_{50} values for HL-60/NB4 cell differentiation-induction activity of Am555S and Am68P coincide well with K_{i} values for RARx-binding affinity and RARβ-binding affinity, respectively. These results suggest that activation of either RARx or RARβ is sufficient to induce cell differentiation.

**DISCUSSION**

Various retinoids, including ATRA, its isomers, and retinobenzoic acids, were assayed for cell differentiation-inducing activities towards NB4 and HL-60 cells, and we concluded that this activity of the compounds towards NB4 cells is highly correlated with that towards HL-60 cells. The finding suggests that the results of cell differentiation-inducing assay using HL-60 cells can predict the activity towards APL cells. Retinoid-induced morphological changes of HL-60 cells are more distinct than those of NB4 cells. Therefore, we believe that an assay system using HL-60 cells would be effective for guiding the development of superior differentiation-therapy agents for the treatment of APL.

The binding affinities of various retinoids to RARx and RARβ were measured. Analysis of the correlations of the results suggested that RARx/RARβ-binding affinity correlates with the cell differentiation-inducing activities, and, in comparing Am555S and Am68P, that activation of either RARx or RARβ is sufficient to induce cell differentiation of both NB4 and HL-60 cells. Though confirmation of these suggestions should wait for creation of RARx-specific and RARβ-specific retinoids, the suggestions have implications for the molecular mechanism of retinoid-induced cell differentiation of NB4 and HL-60 cells, i.e., RARx and RARβ appear to have mutually compensatory roles in cell differentiation-induction. Therefore, the potency of cell differentiation-inducing activity of retinoids towards HL-60 cells would depend on the higher of the binding affinities of the compound to RARx or RARβ. In fact, the potencies of the HL-60 cell differentiation-inducing activities (ED_{50} values) of Am555S and Am68P seem to coincide with the binding affinities (K_{i} values) to RARx and RARβ, respectively. If this were the case, it would be a plausible hypothesis that the target molecule of retinoids in NB4 cells for cell differentiation-induction is also either RARx or RARβ, because both Am555S and Am68P are similarly active in NB4 cell differentiation-induction. Though we do not know the ligand selectivity of PML/RARx, it might resemble that of RARx, rather than that of RARβ. The potent cell differentiation-inducing activity of RARβ-selective Am68P, though the selectivity is not high, would then suggest that activation of RARβ plays an important role in induction of NB4 cell differentiation. We cannot exclude the possibility that binding of retinoids to PML/RARx also plays a critical role in inducing differentiation NB4 cells. However, reconstruction of the PML-containing nuclear body in NB4 cells, which has been proposed to be the mechanism of retinoid-induced NB4 cell differentiation, must be a secondary phenomenon, because the characteristics, including the kinetics, of the retinoid-induced differentiation of NB4 cells and of HL-60 cells which contain a normal nuclear body are similar.

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