Combinations of Retinoic Acid with Either Sodium Butyrate, Dimethyl Sulfoxide, or Hexamethylene Bisacetamide Synergistically Induce Differentiation of the Human Myeloid Leukemia Cell Line HL60

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ABSTRACT

All-trans-retinoic acid (RA), sodium n-butyrate (NaB), hexamethylene bisacetamide (HMBA), and dimethyl sulfoxide (DMSO) induce differentiation of the human acute myeloid leukemia cell line HL60. In the clinic, RA, NaB, or HMBA induce complete or partial remissions. However, the achievement and maintenance of effective plasma concentrations and toxicity have been problems. These difficulties led us to study the interaction of RA with these inducers. We found that combinations of RA with either NaB, HMBA, or DMSO synergistically induced terminal differentiation of HL60. A measure of the effectiveness of these combinations was that the doses of NaB, HMBA, and DMSO required alone to induce half-maximal differentiation were decreased about 4-fold in combination with normal plasma concentrations of about 30 nM RA. RA or NaB alone did not enhance the growth of HL60 cells. In contrast, HMBA or DMSO alone increased growth of HL60 cells even at concentrations that did not induce differentiation. The addition of RA reduced the promotion of growth and increased the extent of terminal differentiation seen with HMBA and DMSO alone. These data suggest that treatment of some malignancies with combinations of RA with HMBA or NaB may maintain differentiation-inducing effects and decrease the problems associated with the achievement and maintenance of effective plasma concentrations as single agents.

INTRODUCTION

Clinical trials of differentiation-inducing agents in patients with leukemia, myelodysplastic syndromes, and solid tumors have included the use of RA2, 13-cis-RA, NaB, and HMBA. These agents induce complete or partial remissions (1–8). However, toxicity and the difficulty of achieving and maintaining effective plasma concentrations complicate this therapy. This led us to investigate the interaction of RA with other inducers in short-term liquid culture of the human myeloid leukemia cell line HL60.

For a wide spectrum of biological phenomena, including treatment of various bacterial infections, cancer chemotherapy, and carcinogenesis, combinations of agents are often more effective than single agents. Our studies show that combinations of RA with either NaB, HMBA, or DMSO are synergistic for inducing differentiation of HL60 cells. These results suggest combinations of agents that may have utility for differentiation-inducing therapy in the clinic.

MATERIALS AND METHODS

Chemicals. RA and NBT were from Sigma Chemical Co. (St. Louis, MO). NaB was from J. T. Baker Chemical Co. (Phillipsburg, NJ).

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1 The abbreviations used are: RA, all-trans-retinoic acid; NaB, sodium n-butyrate; HMBA, hexamethylene bisacetamide; DMSO, dimethyl sulfoxide; 13-cis-RA, 13-cis-retinoic acid; NBT, nitroblue tetrazolium; ED50, the median effect dose; m, the slope of a log dose versus log effect curve; CI, combination index.

2 The abbreviations used are: RA, all- trans-retinoic acid; NaB, sodium n-butyrate; HMBA, hexamethylene bisacetamide; DMSO, dimethyl sulfoxide; 13-cis-RA, 13-cis-retinoic acid; NBT, nitroblue tetrazolium; ED50, the median effect dose; m, the slope of a log dose versus log effect curve; CI, combination index.

HMBA and DMSO were from Aldrich Chemical Co. (Milwaukee, WI). Cells. Maintenance of HL60 cells (passages 18–50) was in suspension culture in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid and 10% (v/v) fetal bovine serum (GIBCO). The cell cultures were grown at 37°C in a humidified atmosphere of 5% CO2 in air and subcultured every 7 days. We determined cell number on an electronic particle counter (Coulter Electronics, Hialeah, FL) and estimated viability by trypan blue dye exclusion.

Induction and Measurement of Differentiation. We induced HL60 cells to differentiate with RA, NaB, DMSO, and HMBA alone and with combinations of RA and each of the other three inducers. We harvested exponentially growing cells by centrifugation and resuspended them in the growth medium at a cell density of 2 × 10⁶/ml. Stock solutions of RA (1 mM) were in ethanol. DMSO and the stock RA solution were serially diluted in ethanol before addition to the culture medium. The final concentration of ethanol was <0.1%. Stock solutions of both NaB and HMBA were in phosphate-buffered saline (1.5 mM KH2PO4-8.1 mM Na2HPO4-136.9 mM NaCl, pH 7.2). For the combination experiments, mixtures of the two compounds were made at a predetermined molar ratio and then serially diluted. The capacity of HL60 to reduce NBT was assessed as the percentage of cells with formazan deposits as described previously (9) and corrected for viability.

Analysis of Combined Drug Effects. The median effect principle described by Chou and Talalay (10) was the basis for analyzing combined drug effects. The principle is described by the median effect equation:

\[
F_r/F_c = (D/ED_{50})^m
\]

where \(F_r\) is the fraction affected and \(F_c\) is the fraction unaffected by the dose \(D\), \(ED_{50}\) is the median effect dose required for 50% differentiation, and \(m\) is the slope of a log dose versus log (fraction affected/fraction unaffected) which signifies the sigmoidicity of an arithmetic dose-effect curve. Dose effects were determined for each agent and for multiple dilutions of a fixed ratio combination. We determined the values of \(ED_{50}\) and \(m\) with the ALLFIT computer program (11). This program uses the four-parameter logistic equation to analyze families of dose-response curves. We set the value for the response when the dose is zero to 5% and the value for the response when the dose is at infinite concentration to 100%. The interaction of two inducers was quantified by determining a CI for each fixed concentration ratio according to the conservative isobologram equation (12):

\[
CI = \frac{(D_1)/(D_0)}{CI_1} \times \frac{(D_2)/(D_0)}{CI_2} = \frac{(D_1)(D_2)}{(D_0)(D_0)}
\]

where \(D_i\) is the dose required to produce \(x\%\) effect alone and \((D_0)\) and \((D_2)\) are the doses of agents 1 and 2 in the mixture that produce the same effect. The relationship between \(D_i\) and \(x\%\) was determined from the parameters, \(m\), \(ED_{50}\), and the median effect equation using a commercially available computer program (13) to calculate the CI values for each fixed concentration ratio of agents. This analysis generates the combination effect as: summation is indicated when CI = 1; synergism is indicated when CI < 1; antagonism is indicated when CI > 1.

Calculation of Dose Reduction Index. The dose reduction index indi-
SYNERGISTIC INDUCTION OF HL60 DIFFERENTIATION

RESULTS

RA in Combination with NaB, DMSO, and HMBA. Fig. 1 shows dose-effect curves for the induction of differentiation of HL60 by RA, NaB, HMBA, or DMSO as single agents and by combinations of NaB, HMBA, or DMSO with RA. These data show the relative potency of the four inducers of differentiation and that, compared to RA, the other inducers alone had much steeper concentration-dependent responses. A measure of this steepness is $m$, the slope of a log dose versus log effect curve. The larger the value of $m$ the greater the steepness of the dose-effect curve and, therefore, the more narrow the range of concentrations over which the agent or combination of agents show a graded effect. An alternative parameter is a ratio of doses required to produce two effects, e.g., $ED_{50}/ED_{20}$. The higher the value of this ratio the greater is the concentration range for the agent alone or in combination to increase differentiation from 20–80%. Table 1 lists values for $ED_{50}$, $m$, and $ED_{50}/ED_{20}$ for each of the inducers alone and in combination with RA, NaB, HMBA, and DMSO. The CI and the dose reduction index are useful for assessing the effect of combinations at various effect levels (12).

The CI and the dose reduction index are useful for assessing the effect of combinations at various effect levels (12). Table 1 shows the CI values for mixtures calculated at the 50% differentiation level. We saw relatively strong synergism (CI < 0.6) with combinations of RA with NaB, HMBA, and DMSO. In addition, we saw marked reductions in the doses of each agent that were needed in combination to achieve the same effect as single agents (Table 2). For example, as single agents, 444 μM NaB and 0.13 μM RA induced half-maximal differentiation. When the two agents were combined at a molar ratio of 3000:1, 74 μM NaB plus 0.025 μM RA induced half-maximal differentiation. The decreases were reflected in dose reduction index values of about 5 for RA and 6 for NaB (Table 2).

Enhancement of Growth by DMSO and HMBA. Two goals of differentiation therapy are that tumor cells gain functional attributes of their normal counterpart and either terminally differentiate or, at least, have a lower proliferative capacity. An increase in proliferative capacity of about 20% is seen at 48 h in HL60 culture grown with either 1.5 mM NaB, 210 mM DMSO, or 4 mM HMBA (14). We also found that HMBA and DMSO enhanced the growth of HL60 cells (Fig. 2). The extent of the enhancement of growth was both dose and time dependent. The increase in cell growth was significant [paired t test, $P < 0.05$ (two tailed)] at HMBA concentrations of 0.5–2.5 mM on Day 1, 0.5–3 mM on Day 2, 0.5–2.5 mM on Day 3, and 0.5–2 mM on Day 4. The maximal enhancement of cell growth was about 30% at 1.5 mM HMBA. We saw similar results with DMSO (Fig. 2) with significant increases in cell growth at DMSO concentrations of 100 mM on Day 1, 50–200 mM on Days 2 and 3, and 50–150 mM on Day 4. With either HMBA or DMSO maximal growth enhancement at Day 4 was at a concentration slightly higher than the threshold concentration for inducing differentiation.

In contrast to HMBA and DMSO we saw essentially no enhancement of growth by either RA or NaB (Fig. 2). With RA there were no growth values significantly greater than the...
Table 1 Parameters for combinations of RA with NaB, HMBA, and DMSO derived from NET assay results

<table>
<thead>
<tr>
<th>Inducer combination (molar ratio)</th>
<th>$ED_{50}$ (µM ± SE)</th>
<th>RA at $ED_{50}$ of combination (µM)</th>
<th>$ED_{50}$ (µM)</th>
<th>CI* ± %SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA (13)</td>
<td>0.13 ± 15</td>
<td>0.85 ± 14</td>
<td>26.55</td>
<td></td>
</tr>
<tr>
<td>NaB (6)</td>
<td>444 ± 5.4</td>
<td>4.50 ± 20</td>
<td>1.85</td>
<td></td>
</tr>
<tr>
<td>HMBA (10)</td>
<td>2,483 ± 3.2</td>
<td>4.34 ± 14</td>
<td>1.89</td>
<td></td>
</tr>
<tr>
<td>DMSO (9)</td>
<td>138,540 ± 2.2</td>
<td>4.72 ± 11</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>NaB:RA</td>
<td>2,000:1 (6)</td>
<td>70 ± 11</td>
<td>1.45 ± 16</td>
<td>6.77 ± 13</td>
</tr>
<tr>
<td></td>
<td>3,000:1 (2)</td>
<td>74 ± 20</td>
<td>1.52 ± 22</td>
<td>6.20 ± 18</td>
</tr>
<tr>
<td></td>
<td>4,000:1 (2)</td>
<td>83 ± 17</td>
<td>1.69 ± 28</td>
<td>5.16 ± 37</td>
</tr>
<tr>
<td>HMBA:RA</td>
<td>500:1 (3)</td>
<td>70 ± 14</td>
<td>0.87 ± 17</td>
<td>24.19 ± 14</td>
</tr>
<tr>
<td></td>
<td>5,000:1 (1)</td>
<td>300 ± 12</td>
<td>1.22 ± 16</td>
<td>9.71 ± 64</td>
</tr>
<tr>
<td></td>
<td>10,000:1 (3)</td>
<td>585 ± 9</td>
<td>2.40 ± 17</td>
<td>3.18 ± 79</td>
</tr>
<tr>
<td></td>
<td>20,000:1 (3)</td>
<td>619 ± 9</td>
<td>2.19 ± 17</td>
<td>3.55 ± 99</td>
</tr>
<tr>
<td></td>
<td>25,000:1 (1)</td>
<td>727 ± 10</td>
<td>2.02 ± 16</td>
<td>3.94 ± 10</td>
</tr>
<tr>
<td>DMSO:RA</td>
<td>100:1 (3)</td>
<td>10.4 ± 17</td>
<td>0.82 ± 24</td>
<td>27.86 ± 12</td>
</tr>
<tr>
<td></td>
<td>1 x 10^3:1 (3)</td>
<td>4,149 ± 10</td>
<td>1.36 ± 16</td>
<td>7.67 ± 22</td>
</tr>
<tr>
<td></td>
<td>2 x 10^3:1 (3)</td>
<td>35,130 ± 9</td>
<td>2.50 ± 18</td>
<td>3.03 ± 12</td>
</tr>
</tbody>
</table>

* Values were calculated from data shown in Fig. 1 and other experiments. The values for $m$, $ED_{50}$, and %SE were obtained from the ALLFIT computer program (11).

The concentration of RA in the mixture inducing half-maximal differentiation calculated by dividing the $ED_{50}$ value of the combination by the molar ratio. In the experiments with combinations, the concentration of RA is so low that the $ED_{50}$ value represents the concentration of the other agent.

Combination index at $ED_{50}$ CI values for each fixed concentration ratio of agents were calculated with a commercially available computer program (13) using the mutually nonexclusive assumption. We used the values for $ED_{50}$ + SE, $ED_{50}$ — SE, $m$ + SE, and $m$ — SE to calculate four values of CI. The mean ± %SD of these four values is listed. When CI = 1, summation is indicated; when CI <1, synergism is indicated; when CI > 1, antagonism is indicated.

Table 2 Dose reductions by combinations of RA with NaB, HMBA, and DMSO

<table>
<thead>
<tr>
<th>Inducer combination (molar ratio)</th>
<th>Dose reduction index* (folds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaB:RA</td>
<td>RA</td>
</tr>
<tr>
<td>2,000:1</td>
<td>3.7</td>
</tr>
<tr>
<td>3,000:1</td>
<td>5.2</td>
</tr>
<tr>
<td>4,000:1</td>
<td>6.2</td>
</tr>
<tr>
<td>HMBA:RA</td>
<td>500:1</td>
</tr>
<tr>
<td>5,000:1</td>
<td>2.1</td>
</tr>
<tr>
<td>10,000:1</td>
<td>2.2</td>
</tr>
<tr>
<td>20,000:1</td>
<td>4.3</td>
</tr>
<tr>
<td>25,000:1</td>
<td>4.3</td>
</tr>
<tr>
<td>DMSO:RA</td>
<td>100:1</td>
</tr>
<tr>
<td>1 x 10^3:1</td>
<td>3.2</td>
</tr>
<tr>
<td>2 x 10^3:1</td>
<td>7.2</td>
</tr>
</tbody>
</table>

* The number of folds of dose reduction needed to achieve half-maximal differentiation in combination compared with each drug alone. The values used for the calculations are the $ED_{50}$ of the combination and the RA concentration in the combination shown in Table 1. Thus, for NaB:RA, 2000:1, drug reduction index for NaB is calculated by 444/70 = 6.3 and the drug reduction index for RA is calculated by 0.13/0.035 = 3.7.

control. With NaB, growth increases of about 5% for 0.6 and 0.8 mM NaB on Day 1 were the only values significantly greater than the control.

The enhancement of growth seen during a 4-day period with relatively low concentrations of either HMBA or DMSO (Fig. 2) persisted for at least 21 days in the presence of 0.5 mM HMBA (Fig. 3) or 25 mM DMSO (data not shown). At Day 21, viabilities were >95%, the percentage of NBT-positive cells was <15%, and the cultures containing either HMBA or DMSO had cell concentrations about 2-fold greater than the control. The population-doubling times were about 33 h in the presence of 0.5 mM HMBA, 34 h in the presence of 25 mM DMSO, and 36 h for the control during these 21 days. Thus, the growth-promoting effects of HMBA or DMSO persisted during the equivalent of a >20,000-fold increase in cell concentration. However, in cultures containing 0.5 mM HMBA we saw a RA of 0.5 mM HMBA, 34 h in the presence of 25 mM DMSO, and 36 h for the control during these 21 days. Thus, the growth-promoting effects of HMBA or DMSO persisted during the equivalent of a >20,000-fold increase in cell concentration. However, in cultures containing 0.5 mM HMBA we saw a RA

Fig. 2. Relative growth as a function of concentration of RA, NaB, HMBA, or DMSO. The cell concentrations are relative to an initial cell number of 2 x 10^5 cells/ml. », Day 1; O, Day 2; •, Day 3; □, Day 4; A, the extent of differentiation (%NBT) at Day 4; bars, SE of 6-13 independent experiments. RA concentrations are plotted on a log scale. Viability was >90% under all conditions.
SYNERGISTIC INDUCTION OF HL60 DIFFERENTIATION

Fig. 3. Cumulative long-term growth of HL60 cells in the presence of 0.5 mM HMBA. HL60 cells (initial density $2 \times 10^3$ cells/ml) were grown in the presence (■) or absence (○) of 0.5 mM HMBA. On Days 4, 8, 12, and 16 a portion of each culture was diluted in fresh medium to a density of $2 \times 10^3$ cells/ml. The results shown were from one experiment. Essentially the same results were seen in two other experiments. Viability was >90%.

Fig. 4. The effect of RA on growth (■, ◇) and differentiation (□, ◆) of HL60 cells in the presence (●, □) or absence (○, ◆) of HMBA. HL60 cells ($2 \times 10^3$ cells/ml) were grown for 4 days in the presence of 0.5 mM HMBA and increasing concentrations of RA. The values for cell number are relative to the cell concentration in the culture without RA and HMBA which was 1.33 ($±0.15$ SE) $\times 10^3$ cells/ml. Each point is the mean of three experiments. The SE of each data point was ≤7% of the mean. Viability was >90% under all conditions.

Fig. 5. Inhibition by RA of the increase in cell growth promoted by HMBA of HL60 cells. HL60 cells ($2 \times 10^3$ cells/ml) were grown in the presence of 1.5 mM HMBA (△), 1.5 mM HMBA plus 10 nM RA (●), or without both HMBA and RA (○). Each point is the mean of three experiments; bars, SE. The SE for data points with no bars is within the symbol. Viability was >90% under all conditions.

dose-dependent decrease in growth that was inversely related to an increase in differentiation (Fig. 4). In the presence of 1.5 mM HMBA, a concentration that gave maximum growth promotion (Fig. 2), the addition of 10 nM RA inhibited growth in a time-dependent manner (Fig. 5). At Day 4 the percentage of differentiated cells of three independent experiments was 7 ± 1 (±SE) in the control, 14 ± 2.1 in the presence of 1.5 mM HMBA, and 52 ± 1.7 in the culture with HMBA and RA.

Fig. 2.

**DISCUSSION**

Our results showing synergism in the induction of differentiation of HL60 by combinations of RA with HMBA or NaB (Fig. 1; Table 1) may have utility in the clinic.

HMBA is under active clinical investigation (4, 15-18) as a differentiation-inducing agent. The dose-limiting toxicity of HMBA is about 1.4 mM and the recommended plasma steady-state concentration for 10-day infusions is about 1 mM (4). At 1 mM HMBA, HL60 growth was enhanced maximally and differentiation was only about 14% (Fig. 2). Thus, if HL60 is a model for treatment with HMBA, there could be clinical difficulties in its use as a sole agent.

The dose-response curves (Fig. 1) and the quantitative information derived from these curves (Tables 1 and 2) showed the effectiveness of combinations of RA with HMBA on differentiation of HL60 cells. The ED$_{50}$ value for HMBA alone was 2.4 mM. However, in the presence of RA the ED$_{50}$ values for HMBA were 0.3–0.7 mM (Table 1). Thus, about 50% of HL60 cells differentiated in 4 days in the presence of a combination of 0.3 mM HMBA and 60 nM RA, a molar ratio of 5000:1. This RA concentration is only about 3-fold greater than the normal plasma concentration (19). Our results raise the possibility that a portion of the clinical response to treatment with HMBA alone is because of synergy with endogenous RA.

Enhancement of growth may complicate treatment with doses of HMBA alone that do not induce high levels of terminal differentiation (Figs. 2 and 3). Growth enhancement may be most troublesome at the 1 mM HMBA plasma concentration recommended for long-term infusion therapy (4). We found that growth enhancement continues at an essentially constant rate for at least 21 days (Fig. 3). However, low concentrations of RA decreased the enhancement of growth by HMBA (Fig. 4). Thus, normal plasma concentrations of RA (10 nM) decreased growth in the presence of 0.5 mM HMBA to levels below those of the control. This decrease of growth was probably a result of the increase in differentiation. We saw similar, but less dramatic, results with the combination of 1.5 mM HMBA and 10 nM RA (Fig. 5).

We included NaB in this study because of its differentiation-inducing activity against a wide variety of malignant cells, including HL60 and cells from patients with acute non-lymphocytic leukemia (20-31). There is also one report of a partial remission after administration of NaB to a child with acute myelogenous leukemia in relapse and refractory to conventional therapy (6).

NaB alone induced differentiation of HL60 with an ED$_{50}$ of about 0.4 mM (Fig. 1 and Table 1). The combinations of RA with NaB shown in Fig. 1 and Table 1 were synergistic with CI$_{50}$ values of <0.5. In the presence of molar ratios of NaB:RA of 2000:1, 3000:1, and 4000:1, 50% of HL60 cells differentiated in 4 days at NaB concentrations of about 70–83 μM and RA
concentrations of about 21–35 nm (Table 1). Thus, the presence of about 28 nm RA reduced the ED₅₀ value for NaB from about 400 to about 75 μM (a dose reduction index value of about 6-fold).

In a clinical study using a continuous i.v. infusion of 4.55 mmol NaB/kg/day for 10 days there was no clinical efficacy and no toxicity in nine patients with acute myeloid leukemias (32). The mean plasma concentration of NaB was about 50 μM. This concentration of NaB is about 6-fold higher than the normal plasma concentration. We do not know of any results on increasing plasma concentrations further.

Based on our results with HL60 (Fig. 2), and many studies with other cell lines, there is minimal induction of differentiation and no growth inhibition at 50 μM NaB. Our data showed that a combination of 60 μM NaB and 30 nm RA induced differentiation of about 40% of HL60 cells (Fig. 1). Higher concentrations of NaB or RA or both increased the extent of differentiation still further. Thus, it is possible that combinations of NaB and RA can be used in the clinic at concentrations that are effective and not toxic.

RA and its isomer 13-cis-RA induce complete remissions in patients with acute promyelocytic leukemia (1–3, 7). RA induces differentiation in primary culture of fresh leukemia cells from patients with acute promyelocytic leukemia (1, 3, 7, 20, 33–36). Based on results with cells from one patient, RA is 10-fold more potent than 13-cis-RA (7). It is of interest that RA is active and 13-cis-RA is inactive in the suppression of growth of transplantable carcinomas in animals (37). In the same study oral administration of RA was more effective than i.p. injection.

RA induces differentiation in primary culture of cells from patients with acute myeloid leukemias classified according to the French-American-British scheme (38) as subtypes acute myeloid leukemia (M₂), acute monoblastic leukemia (M₃), and acute monocytic leukemia (M₄) in addition to acute promyelocytic leukemia, subtype (M₃) (20, 35, 36, 39). Cells from patients with subtype M₂ are resistant (36). Clinical pharmacological studies with 13-cis-RA show that oral dosages increase plasma concentrations to about 1 μM (40, 41). The degree and nature of the toxicity occurring in patients (42) is similar to what is seen for RA at similar oral dosages (3, 7). These results indicate that the plasma concentrations of RA can be increased to levels that we have shown in this study to be synergistic with HMBA and NaB.

Based on the data presented in the current study, we suggest that RA may be useful in combination with other agents in the treatment of some leukemias. Although we saw similar results with HMBA and NaB, NaB may have an advantage clinically because of its greater potency (Fig. 1; Table 1) and its lack of growth-enhancing activity (Fig. 2). The finding that fresh cells because of its greater potency (Fig. 1; Table 1) and its lack of growth-enhancing activity (Fig. 2). The finding that fresh cells entering clinical trials as a differentiating agent. Cancer Treat. Rep., 70:991–995, 1986.


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