ABSTRACT

Butyric acid (BA) induces cytodifferentiation in vitro of a wide variety of neoplastic cells. The potential clinical utility of BA is limited by the apparent difficulty of achieving effective concentrations because of its rapid metabolism and short plasma half-life. In this study we addressed two approaches that may achieve effective concentrations of BA in vivo. One strategy is to use BA derivatives as prodrugs that can be metabolized to yield effective BA concentrations in vivo over a sustained period of time. Another strategy is to define agents that are synergistic with BA so that the desired effect can be achieved at lower concentrations of BA. In this study monobutyrin (MB) and tributyrin (TB) were studied in vitro for their effects on inducing differentiation of human myeloid leukemia HL60 cells and murine erythroleukemia cells. On a molar basis TB was about 4-fold more potent than either BA or MB for inducing differentiation of HL60 cells. BA, MB, or TB induced erythroid differentiation of murine erythroleukemia cells. On a molar basis TB was 3- to 4-fold more potent than BA, whereas MB was much less potent than BA. Combinations of all-trans-retinoic acid with either BA, MB, or TB induced myeloid differentiation of HL60 cells synergistically. We saw marked reductions in the doses of each agent that were needed in combination to achieve the same effect as single agents. For example, 130 μM TB, 110 nM all-trans-retinoic acid, and a combination of 13 μM TB plus 13 nM all-trans-retinoic acid all induced half-maximal differentiation of HL60 cells. Our results suggest that the readily available TB may be an effective prodrug of BA and may be useful either as a sole agent or in combination with other agents for cytodifferentiation therapy of human malignancies.

INTRODUCTION

The successful treatment of human acute promyelocytic leukemia by RA established that cytodifferentiation therapy may have utility in the treatment of human malignancies (1–3). Based primarily on studies in vitro, other cytodifferentiation agents also may have clinical utility. BA is found in some foods and is produced in the mammalian digestive tract by microbial fermentation (4). BA induces cytodifferentiation in vitro of a wide variety of neoplastic cells (5–7). In leukemia cell lines, BA induces differentiation toward either the erythroid, monocytic, or megakaryocytic lineages (5, 8–10).

Clinical trials on human leukemia patients using BA as a sole therapeutic agent have been limited (11, 12). Novogrodsky et al. reported the first clinical application of BA for treatment of leukemia. A child with acute myelogenous leukemia in relapse was administered BA, 5 mg/kg, bid for 12 days. No side effects, none of the patients showed a significant decrease in the level of myeloblasts in the peripheral blood or in the bone marrow. During infusion, the plasma concentration of BA increased about 6-fold over the endogenous level to 39–59 μM. A plasma half-life of 6 min was seen after the infusion was stopped. These results showed that infused BA is rapidly metabolized and that the plasma concentrations were well below the concentrations in the mm range that generally are needed for effects in vitro.

There are at least two approaches that may circumvent the problems associated with the short plasma half-life of BA and the apparent difficulty of achieving effective concentrations in vivo. One strategy is to use BA derivatives that can be metabolized to yield effective BA concentrations in vivo over a sustained period of time (13–16). Another strategy is to find agents that are synergistic with BA so that the desired effect can be achieved at lower concentrations of BA (17).

In the present study we show that MB and TB, two readily available BA derivatives, may function as prodrugs of BA to induce differentiation of human myeloid leukemia HL60 cells and MEL cells. In addition, we show that combinations of BA and TB synergistically induced differentiation of HL60 cells. Taken together, our results suggest that MB and TB are prodrugs of BA and may have utility for cytodifferentiation therapy in human leukemias and other malignancies.

MATERIALS AND METHODS

Chemicals. RA, TB, and nitroblue tetrazolium were from Sigma Chemical Co. (St. Louis, MO). RA was obtained as the sodium salt from J. T. Baker Chemical Co. (Phillipsburg, NJ). MB was purchased from Eastman Chemical Co. (Rochester, NY). Analysis by nuclear magnetic resonance spectroscopy (kindly performed by Dr. Joseph J. Barchi, Jr.) showed that the TB preparation was pure and that the MB preparation contained about 90% MB and several unidentified compounds. No correction was made to the concentrations of MB given in this study based on this analysis.

Cells. HL60 cells (passages 18–50) were from laboratory stocks. The MEL cell line DS195/Sc9, derived from the Friend virus transformed cell line 754A (18), was obtained from Dr. Rifkind. HL60 cells and MEL cells were maintained in suspension culture in Roswell Park Memorial Institute 1640 (GIBCO, Grand Island, NY) supplemented with 10% (v/v) calf serum. The cell cultures were grown at 37°C in a humidified atmosphere of 5% CO2 in air and were 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, BA, MB, and TB alone and with combinations of RA and each of the other three agents. We harvested exponentially growing HL60 cells by centrifugation and resuspended them in the growth medium at a cell density of 2 × 106/ml. Stock solutions of RA (1 mM), MB (1 μM), and TB (1 μM) were in ethanol. These stock solutions were serially diluted in ethanol before addition to the culture medium. The final concentration of ethanol was <0.1%. The stock solution of BA was in phosphate-buffered NaCl solution (1.5 mM KH2PO4, 8.1 mM Na2HPO4, 136.9 mM NaCl, pH 7.2). For the combination experiments, mixtures of the 2 compounds were made at a predetermined molar ratio, usually based on their ED50 values as single agents, and then serially diluted. Differentiation was assessed as the percentage of HL60 cells...
with cell-associated nitroblue diformazan deposits resulting from the reduction of nitroblue tetrazolium (19).

We induced MEL cells to differentiate with BA, MB, and TB following the same procedures used for HL6O cells except that the initial cell concentration was 1 x 10^5/ml and differentiation was expressed as the percentage of MEL cells containing hemoglobin measured by benzidine staining (20).

Analysis of Combined Drug Effects. Isobologram analysis was the basis for analyzing combined drug effects with HL6O cells. Dose effects were determined for each agent and for multiple dilutions of a fixed-ratio combination. The interaction of two inducers was quantified by determining a CI value for each fixed concentration ratio according to the classic isobologram equation (21)

\[
CI = \frac{(D_1)}{(D_{12})} + \frac{(D_2)}{(D_{12})}
\]

where \( D_1 \) is the dose required to produce an effect alone and \( (D) \) and \( (D_2) \) are the doses of agents 1 and 2 in the mixture that produce the same effect. This analysis generates the combination effect as: summation (additivity or zero interaction) 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 indicates how many folds of dose reduction are needed to achieve a given effect in combination compared with each agent alone (21). This was calculated by dividing the ED50 value for a single differentiation agent by the dose of the same agent required in combination with another agent to produce the same effect.

RESULTS

Induction of Differentiation of HL6O cells by BA, MB, and TB alone. Dose-effect curves showed that on a molar basis TB was more potent than either MB or BA in inducing differentiation of HL6O cells (Fig. 1). Based on the ED50 values (Table 1), TB was about 3.7-fold more potent than either BA or MB.

Induction of Differentiation of HL6O Cells by Combinations of RA, TB, and BA. Previous studies showed that combinations of RA and BA synergistically induce differentiation of HL60 cells (17, 22). Therefore, we examined also if TB and MB interacted with BA. Fig. 2 shows the dose-effect curves with various fixed-ratio concentrations of TB with RA. HL6O cells (2 x 10^5/ml) were grown with RA (●) and TB (▲) alone and at TB:RA molar ratios of 500:1 (●) and 2000:1 (▲). The extent of differentiation (percentage of Nitroblue tetrazolium (NBT)) was measured on Day 4. Points, means of 2-7 independent experiments; bars, SE. Viability was >90% when TB was at a concentration >200 µM as a single agent and >125 µM in combination with RA. Under all other conditions viability was >90%.

Table 1 Parameters for combinations of RA, BA, and TB on HL6O cell differentiation

<table>
<thead>
<tr>
<th>Inducer combination, molar ratio</th>
<th>ED50 (µM)</th>
<th>RA at ED50 of combination (µM)</th>
<th>CI50 *</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>0.110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>480</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MB</td>
<td>500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB</td>
<td>130</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA:RA</td>
<td>1000:1</td>
<td>38</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>2000:1</td>
<td>50</td>
<td>0.025</td>
</tr>
<tr>
<td>TB:RA</td>
<td>500:1</td>
<td>10</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>1000:1</td>
<td>13</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>2000:1</td>
<td>24</td>
<td>0.012</td>
</tr>
<tr>
<td>BA:TB</td>
<td>3:1</td>
<td>260</td>
<td>0.91</td>
</tr>
</tbody>
</table>

* Values were calculated from data shown in Figs. 1 and 2 and other experiments.

* The concentration of RA in the mixture inducing half-maximal differentiation calculated by dividing the ED50 value of the combination by the molar ratio. In the experiments with combinations, the concentration of RA is so low that the ED50 value represents the concentration of the other agent.

* Combination index at ED50. CI values for each fixed concentration ratio of agents were calculated as described under "Materials and Methods." When CI = 1, summation (additivity or zero interaction) is indicated; when CI < 1, synergism is indicated; when CI > 1, antagonism is indicated.
agent that were needed in combination to achieve the same effect as single agents (Table 1). For example, as single agents 130 μM TB and 110 nM RA induced half-maximal differentiation. When the two agents were combined at a molar ratio of 1000:1, 13 μM TB plus 13 nM RA induced half-maximal differentiation. These decreases were reflected in dose-reduction index values of 10 for TB and 8.5 for RA.

Analysis of Combined Effects. The interaction between two inducers was visualized by constructing isoboles (23) and quantified by determining CI values (21). As shown in Fig. 3, the 2 isoeffective combinations of RA with BA and the 3 isoeffective combinations of RA with TB were all below and to the left of the summation (additive or zero interaction) isobole displayed by the solid line. The isoeffective combination of BA with TB was close to the summation isobole (Fig. 3, inset). The CI is a sum that measures the divergence between the observed concentrations of agents that in combination produce an effect and the concentrations that produce the same effect as sole agents. The extent to which this value is <1 is a measure of synergism. The extent to which this value is >1 is a measure of antagonism. The CI50 values for combinations of RA either with TB or BA all were ≤0.42, indicating strong synergism (Table 1).

Assessments of the effects of combinations of agents are often made at one effect level (e.g., ED50). However, the CI values for one combination can change from antagonism to synergism (or vice versa) at various effect levels (21, 24, 25). The data in Fig. 4, calculated directly from the data shown in Fig. 2, show that 2 combinations of TB with RA were synergistic at effect levels from 20 to 90% differentiation.

Induction of Differentiation of MEL Cells by BA, MB, and TB. BA, MB, and TB induced erythroid differentiation of MEL cells (Fig. 5). BA and TB induced a maximum differentiation of about 60% that was seen at 4 days with concentrations of BA ≥ 2.5 mM and at 5 days with concentrations of TB ≥ 1.25 mM. In contrast to either BA or TB, maximum differentiation with MB was only about 30% and was seen at 5 days with concentrations ≥ 2.5 mM (Fig. 5).

Assessments of the relative potencies of agents are usually determined from dose-effect curves showing the same maximum effect at

Fig. 4. Changes in the Cl as a function of the extent of differentiation of HL60 cells induced by combinations of TB with RA. The concentrations of TB and RA, either alone or in combination, required to produce the various effects were obtained from the dose-response curves shown in Fig. 2. CI values were calculated as described under "Materials and Methods." The effect levels for differentiation shown on the abscissa were calculated based on a maximum effect of 100% and a minimum (spontaneous) effect of 10%. Thus, e.g., the ED50 values are those concentrations inducing an observed differentiation of 55%.

Fig. 5. Induction of differentiation of MEL cells by BA, MB, and TB. MEL cells (1 X 10⁶/ml) were grown with BA (•, A, ■), MB (○, △, □), or TB (◇, ○, △). The percentage of cells containing hemoglobin was measured by benzidine staining on Days 4 (◇, ○, △), 5 (○, △, ◇), and 6 (□, △, ◇). Point, mean of 1-3 independent experiments. The SE of each data point was <9% of the mean. Viability was >90% under all conditions.
the presence of 1.5 nM BA the differential rate was 0.19 and ap
differentiated cells seen with BA after Day 4 (Fig. 5) were because of
proached a plateau at a concentration of about 4.5 X 10^6 total cells/mi
most constant while the total cell population continued to increase. In
about 0.02 because the concentration of differentiated cells was al
rate was 0.88 to about 4 X 10^6 differentiated cells/mi at Day 4. During
During
30% differentiation, independent of time, were about 0.6 mM for TB,
and 2.5 mM for MB.

3 agents at Days 3, 4, and 5 were about 0.5 mM for TB and 1.5—2 mM
nor the time at which maximum differentiation was attained was
subjected to Day 4 and then a maximum differential rate of
the concentration of MB to 1.5 mM resulted in a longer lag followed by
We found that combinations of RA with either BA, MB, or TB did
not synergistically induce differentiation of MEL cells (data not shown). This result was not surprising because MEL cells are not
induced to differentiate by RA alone (26).

the difficulty of assessing the potencies of BA, MB, and TB for inducing differentiation of MEL cells was complicated because neither the value for maximum differentiation nor the time at which maximum differentiation was attained was
shared by any 2 of the 3 agents (Fig. 5). Thus, a maximum differentiation of about 60% was induced by both BA and TB at different
times and both TB and MB induced a maximum differentiation, with markedly different values, at Day 5. The ED_{50} values for each of
the 3 agents at Days 3, 4, and 5 were about 0.5 mM for TB and 1.5—2 mM
for BA and MB. The concentrations of BA, MB, and TB that induced
30% differentiation, independent of time, were about 0.6 mM for TB,
2 mM for BA, and 2.5 mM for MB.

The difficulty of assessing the potencies of BA, MB, and TB on
differentiation of MEL cells by analysis of dose-response curves (Fig. 5) prompted us to construct differential plots (Fig. 6). These
plots show the changes in the concentrations of differentiated cells in response to an inducer as a function of the total (differentiated plus
undifferentiated) concentrations of cells in the culture. The differential rate

\[
\text{Differential rate} = \frac{\text{Increase in concentration of differentiated cells} \times 10^6}{\text{Increase in concentration of total cells} \times 10^6}
\]

for each curve is influenced both by the rate of differentiation and by
the rate of cell division.

The differential rates for MEL cells were dependent on the concentra-
tion of BA (Fig. 6). In the presence of 3 mM BA the differential rate was 0.88 to about 4 \times 10^6 differentiated cells/ml at Day 4. During
incubation for an additional 2 days the differential rate decreased to
about 0.02 because the concentration of differentiated cells was almost
constant while the total cell population continued to increase. In
the presence of 1.5 mM BA the differential rate was 0.19 and ap-
proached a plateau at a concentration of 4.5 \times 10^6 cells/ml at Day 5. These results show that the decreases in the percentage of
differentiated cells seen with BA after Day 4 (Fig. 5) were because of

the rapid metabolism of BA probably is one reason that the ED_{50}
values for the induction of differentiation by BA are much higher for
MEL cells than for HL60 cells. Thus, the level of BA metabolism in
potential target cells may assist in predicting sensitivity to BA (22).
As shown by Friend et al. (31), a decrease in the percentage of
differentiated MEL cells is seen after BA concentrations drop to levels
that no longer support differentiation. In agreement with Friend et al.
we found that this decrease in the percentage of differentiated cells
after Day 4 (Fig. 5) was because of an increase in the concentration of
non differentiated cells (Fig. 6). The concentration of differentiated
TRIBUTYRIN INDUCTION OF CELL DIFFERENTIATION

TB (Fig. 6) may reflect increasing intracellular concentrations of BA as a function of cell density and time of incubation. It is likely that differentiation of MEL cells is related directly to the intracellular concentration of BA. The intracellular concentration of BA derived from TB probably is dependent on the net effect of several processes, including the rate of uptake of TB; the rate of conversion of TB to BA; the rate of BA catabolism; and the rates of BA efflux from and influx into the cell. Changes in the levels of proteins involved in one or more of these processes may be responsible for the increased rate of differentiation after Day 4 of MEL cells growing with 0.5 mM TB. However, a simpler explanation is based on the hypothesis that a large percentage of BA produced from TB in the cell is excreted into the medium. At low cell densities and at early times the concentration of BA in the medium may be too low for the uptake system to contribute significantly to the total intracellular pool. At higher cell densities and longer incubation times the extracellular BA concentration may reach levels that support a rate of influx that is high enough to elevate the intracellular BA concentration. The elevation in the intracellular BA concentration is reflected in an increase in cell differentiation.

Our evidence indicates that MB also is a prodrug of BA. MB and TB were equipotent in inducing differentiation of HL60 cells (Fig. 1; Table 1). In contrast, BA was more potent than MB in inducing differentiation of MEL cells (Figs. 5 and 6). These results are in agreement with Lea et al. (32) who found that the extent of differentiation of MEL cells over a 4-day incubation period was greater with 1 mM BA than with 2 mM MB.

It is likely that the difference in the relative potency of MB in HL60 cells compared to MEL cells also is related to the net effect of the processes listed above on the intracellular concentration of BA. It will be of interest to measure the reactions involved in the formation of the intracellular BA pool from extracellular BA, MB, and TB. HL60 cells have triacylglycerol acyl hydrolase (lipase) activity (33) which may cleave BA from all three sn positions of TB. To our knowledge, a lipase activity has not been described in MEL cells. However, the differentiation-inducing activities of TB and MB (Fig. 5) suggest that MEL cells have this activity.

Our results showing that TB and MB induce differentiation of HL60 and MEL cells as sole agents (Figs. 1 and 5) and that combinations of TB and RA synergistically induce differentiation of HL60 cells (Figs. 2–4; Table 1) may have clinical applications. Clinical trials on human leukemia patients with the use of BA as a sole therapeutic agent have been limited (11, 12). Novogrodsky et al. (11) found that a child with acute myelogenous leukemia in relapse achieved a partial remission after administration of 4.5 mmol sodium butyrate/kg/day i.v. continuously for 10 days. A second clinical report (12) found no clinical efficacy in nine adult patients with acute myeloid leukemias treated with BA following the same protocol. During infusion, the plasma concentration of BA increased 6-fold over the endogenous level and reached 39–59 μM. A plasma half-life of 6 min was seen after the infusion was stopped. These results showed that the infused BA was rapidly metabolized and that the plasma concentrations reached by a daily dose of 4.5 mmol/kg for 10 days were well below concentrations of BA that are usually needed to elicit antitumor effects in vitro.

It may be noteworthy that a BA plasma concentration of ~50 μM seen with the protocol of Novogrodsky et al. is sufficient to activate fetal hemoglobin production in patients with β-hemoglobinopathies (34, 35). These latter results raise the possibility that some malignancies may respond favorably to relatively low concentrations of BA. A challenge now is to determine whether any of the wide variety of tumor cell types, including leukemia, lymphoma, breast, rectum, colon, liver, cervix, and neural (36–44), that respond to BA in vitro also are responsive to BA in vivo.

For those tumor cell types that are not responsive to low concentrations of BA there is still the problem of how to obtain high plasma concentrations of BA for extended periods of time. Prompted by our initial results on the effectiveness of TB in inducing differentiation of HL60 and MEL cells, a pilot study was performed to measure the BA plasma levels in rats of about 300 g body weight dosed by p.o. gavage with 1 ml (11 mmol/kg) of TB (45). A plasma BA concentration of over 300 μM was seen at 30 min with an estimated plasma half-life of 40 min. A much larger pharmacokinetic study using mice and rats (sponsored by the Pharmacology Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute) essentially has confirmed and extended the pilot study.2 Although more study is needed, it appears that p.o. administration of TB may yield effective levels of BA for extended periods of time.

TB is a well-tolerated agent. No toxicity is seen in mice treated either p.o. or i.p. with a dose of 26.5 mmol/kg (16). In humans, no detectable side effects were seen after 6 premature infants were fed TB for 4 days at doses of about 20 mmol/kg/day (46). While the butyrates are unlikely to produce clinically detectable toxicity, the intensely unpleasant odor of excreted BA may limit its clinical application. Surprisingly, except for one review (6), we are unaware of any published animal or human study that mentions this limitation.

For a wide spectrum of biological phenomena combinations of agents are often more effective than single agents. In this study we found that the ED50 values for induction of differentiation of HL60 cells by BA and TB as sole agents were reduced about 10-fold in the presence of 12–38 nm RA (Table 1). These concentrations of RA are found in normal human blood plasma (47). Thus, in the presence of physiological levels of RA, the 38–50 μM BA needed to induce 50% differentiation of HL60 cells are in the same concentration range that Miller et al. (12) found in patients receiving infusions of BA according to the protocol of Novogrodsky et al. (11). Our results raise the possibility that the positive clinical response to treatment with BA alone reported by Novogrodsky et al. was because of synergy with endogenous RA. This raises the prospect that the spectra of malignancies responding to BA may be greater with higher concentrations of BA or with combinations of BA and other anticancer agents.

Taken together, our results indicate that TB may be a promising candidate as a prodrug of BA, either as a sole agent or in combination with other agents, for cytodifferentiation therapy of human leukemia and other malignancies and possibly for patients with β-hemoglobinopathies.

REFERENCES


Tributyrin: A Prodrug of Butyric Acid for Potential Clinical Application in Differentiation Therapy

Zi-Xing Chen and Theodore R. Breitman