# A Retinoid/Butyric Acid Prodrug Overcomes Retinoic Acid Resistance in Leukemias by Induction of Apoptosis

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## Abstract

**Some success in overcoming retinoic acid (RA)-resistance has been reported for acute promyelocytic leukemia in cell lines and the clinic by combining histone deacetylase inhibitors, like sodium butyrate (NaB), with RA. This epigenetic therapy counteracts the effects of nuclear corepressors, causing a DNA conformation that facilitates RA-induced gene transcription and cell differentiation. In an effort to improve delivery of each drug, we have synthesized retinoyloxymethyl butyrate (RN1), a mutual prodrug of both RA and butyric acid. RN1 targets both drugs to the same cells or cellular compartments to achieve differentiation at lower concentrations than using RA and NaB alone. In an RA-resistant cell line, which is not responsive to RA and NaB given together at the same concentration, RN1 inhibited growth substantially. This growth inhibition is caused by an increase in apoptosis and a minimal induction of differentiation, rather than the more complete granulocytic differentiation as seen in the RA-sensitive cell line. The different phenotypes induced by RN1 in RA-sensitive *versus* RA-resistant cells are reflected by altered patterns of gene expression. In addition to acute promyelocytic leukemia cells, RN1 induces apoptosis of other RA-resistant leukemic cell lines with blocked transcriptional pathways, but not normal human peripheral blood mononuclear cells. RN1, therefore, is a novel retinoid that may be more widely active in hematologic malignancies than RA alone.**

## Introduction

Abnormal transcriptional regulation resulting in the repression of differentiation pathways may mediate the phenotype of numerous malignancies, especially leukemia. "Epigenetic" or "transcription" therapy is emerging as a possible treatment for diseases associated with aberrant transcription factors and transcriptional repressors. Transcription therapy may be defined as "the repression/derepression of target genes of transcription factors involved in disease pathogenesis" (1). Acute promyelocytic leukemia (APL) has provided a model both for understanding how increased transcriptional repression can lead to blocked differentiation and uncontrolled growth, as well as for the development of transcription therapy. The promyelocytic leukemia protein (PML)/retinoic acid receptor α (RARα) fusion oncoprotein characteristic of APL binds transcriptional corepressor complexes, which contain histone deacetylase (HDAC) activity (2). The HDAC activity is responsible for keeping the chromatin in a "closed" conformation that is inaccessible to the transcriptional machinery (3). Even in the presence of physiologic levels of retinoic acid (RA), PML/RARα maintains its association with HDACs and blocks transcription. Pharmacologic levels of RA overcome this transcriptional repression, providing the basis for differentiation therapy using RA in APL patients (4–6). However, RA-resistant APL and other leukemic cells characterized by aberrant transcriptional repressors do not respond to RA alone (7). APL cells containing the PLZF/RARα, non-APL cells with the AML1/ETO translocation, and lymphoblastic leukemia cells with the TEL/AML1 are resistant to RA; all have increased binding of corepressor/HDAC complexes and inappropriate transcriptional repression of target genes (8–10). *In vitro*, RA in combination with HDAC inhibitors, such as trichostatin A or NaB, can induce a variable increase in growth arrest and differentiation of these cells (2). *In vitro* results have been supported by a report that treatment of an RA-refractory APL patient with RA combined with phenylbutyrate, also an HDAC inhibitor, led to increased histone acetylation in blood and bone marrow mononuclear cells, differentiation of the malignant clone, and complete clinical remission (11). However, other similar patients did not respond to the same combination therapy (12). These data suggest that the use of HDAC inhibitors in combination with RA may be an effective treatment for RA-refractory APL, but improvements in therapy are needed to increase the percentage of patients who respond.

This synergistic action between RA and NaB provided the rationale for the development of retinoyloxymethyl butyrate (RN1). RN1 is a mutual prodrug of RA and butyric acid, which has been shown to function at lower concentrations than RA or butyric acid alone in the RA-sensitive leukemic

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cell line, HL-60 (7). We hypothesized that targeted delivery of a non-specific inhibitor of HDAC and RA would broaden the spectrum of activity of RA to induce specific pathways of differentiation and cell cycle arrest that are blocked in many cancer cells.

To test this hypothesis, we employed a well-defined model of RA-resistant APL, as well as other RA-resistant leukemic cell lines. We found that RN1 induced differentiation in RA-sensitive APL cells as well or better than RA alone. RN1 also significantly inhibited the growth of an APL subclone that was resistant to treatment with RA or RA given together with NaB. We examined the effects of RN1 on the expression of differentiation markers and known RA target genes and promoters. In contrast to its effects on RA-sensitive APL cells, in the RA-resistant, NB4-MR4 clone, RN1 not only partially induced differentiation and known transcriptional targets of RA, but caused substantial caspase activation and apoptosis. We then examined the effects of RN1 on RA-resistant non-APL cell lines and found significant cell death on RN1 treatment. In contrast, RN1 treatment was not toxic to normal human peripheral blood mononuclear cells (PBMCs). These data suggest that RN1 may be a useful therapeutic not only in RA-responsive and non-responsive APL, but also in other malignancies characterized by transcriptional repression.

## Results

### *RN1 Induces Growth Arrest and Differentiation in RA-Sensitive APL Cells*

First, we compared the ability of RA and RN1 to induce growth arrest and differentiation in NB4 cells. NB4 cells were treated for 5 days with media alone, RA, sodium butyrate (NaB), RA and NaB together, or RN1. NaB had no effect on the growth of NB4 cells, while treatment with RA, RA plus NaB, or RN1 completely inhibited their growth (Fig. 1A). In addition, we found that RN1, like RA, inhibited NB4 cells by differentiation. The induction of differentiation by these treatments was confirmed by the nitroblue tetrazolium (NBT) assay, CD11b expression, and morphology. Indeed, when assayed after 5 days of treatment with RA, RA plus butyrate, or RN1, nearly 100% of cells stained positive for NBT and CD11b (data not shown). However, when assayed after only 2 days of treatment (Fig. 1B), RN1 induced differentiation significantly better than RA. Surprisingly, the addition of only 10 μM butyrate to RA significantly enhanced the induction of NBT, in a manner similar to the superinduction seen with hexamethylene bisacetamide (HMBA; 13). These data are consistent with results seen in HL-60, a retinoid-sensitive AML cell line in which RN1 was shown to be more effective than RA (7).

In addition, we tested the ability of RN1 to degrade the oncogenic PML/RARα protein that is characteristic of these APL cells. Like RA and RA combined with butyrate, RN1 induced degradation of the PML/RARα fusion protein (data not shown). Taken together, these data suggest RN1 functions in a manner similar to RA in RA-sensitive NB4 cells, but either RN1 or the addition of small amounts of butyrate to RA may facilitate a faster response.

### *RN1 Inhibits the Growth of RA-Resistant APL Cells*

To begin to study RN1 in RA-resistant models, we used the retinoid-resistant subclone of NB4, R4. The R4 cell line has a point mutation in the RARα portion of the PML/RARα oncoprotein that results in a dominant negative inhibition of the co-expressed wild-type RARα that cannot be reversed by RA (14). RA resistance in the R4 subclone can be reversed to a limited degree by combination of RA with the HDAC inhibitor, TSA (2). R4 cells were treated for 5 days with media alone, RA, NaB, RA and NaB together, or RN1 (Fig. 2A). As expected, the low dose of 10 μM NaB alone had no effect on the growth of R4 cells. As previously published, RA does not inhibit growth of R4 cells. In contrast, RN1 significantly inhibited R4 cell growth while concomitant treatment of the same concentrations of RA and butyrate did not. R4 cells were inhibited by the combination of RA and NaB, but only when millimolar concentrations of NaB were used (data not shown).

To determine whether RN1 induced differentiation of R4 cells as in NB4 cells, we looked at two markers of granulocyte

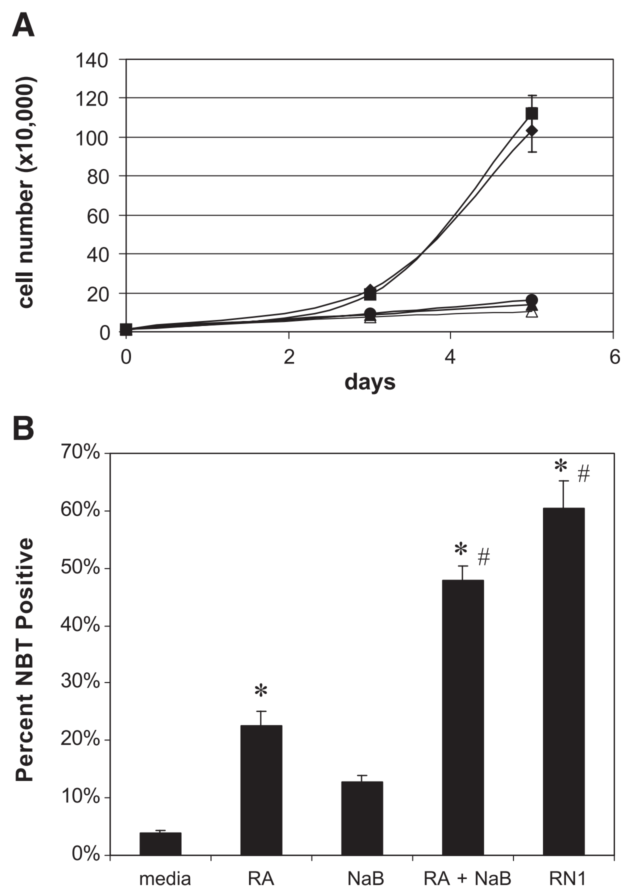


**FIGURE 1.** RN1 induces growth arrest and differentiation in the NB4 cell line. **A.** NB4 cells were treated with media (♦), NaB (■), RA (▲), NaB and RA (●), or RN1 (△). In NB4 cells, RA, RA plus NaB, and RN1 significantly inhibited growth ($P < 0.001$). *Points,* average of three separate wells; *bars,* SE. These data are representative of seven different growth curves. **B.** The percentage of NBT-positive cells was determined after 2 days of treatment with media, RA, NaB, RA and NaB, or RN1. *, indicates a significant difference from media-treated cells ($P < 0.04$). #, indicates a significant increase from RA treatments ($P < 0.02$).

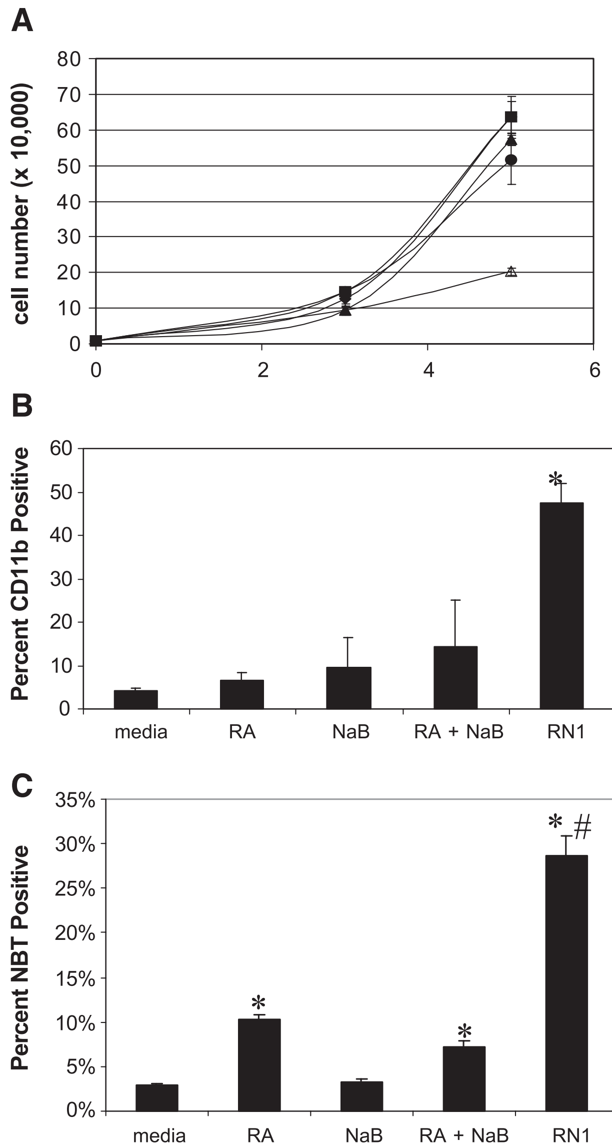


**FIGURE 2.** RN1 inhibits growth and induced differentiation marker expression in R4 cells. **A.** R4 cells were treated with media (♦), NaB (■), RA (▲), NaB and RA (●), or RN1 (△). Only RN1 significantly inhibited growth ($P < 0.02$). *Points,* average of three separate wells; *bars,* SE. These data are representative of seven different growth curves. **B.** Cells were treated with media, RA, NaB, RA and NaB, or RN1 for 5 days. Cells were then stained with antibodies against human CD11b and analyzed by flow cytometry for surface expression. *, indicates a significant difference from media-treated cells ($P < 0.04$). **C.** The percentage of NBT-positive cells was determined after 5 days of treatment with media, RA, NaB, RA and NaB, or RN1. *, indicates a significant difference from media-treated cells ($P < 0.04$). #, indicates a significant difference from all treatments ($P < 0.001$).

differentiation: CD11b surface antigen expression and NBT assay. CD11b antigen expression was analyzed on viable cells only. Only RN1 increased the percentage of R4 cells that stained positive for CD11b after 5 days (Fig. 2B). In R4 cells after 5 days, RN1 treatment resulted in approximately 30% NBT-positive cells as compared to under 10% NBT-positive cells seen after the same concentration of RA plus NaB was added separately (Fig. 2C). No increase in NBT expression was seen earlier in the treatment period in R4 cells, which contrasts to that seen in response to RN1 in NB4 cells. Interestingly, RN1-induced differentiation as assessed by NBT assay is significantly higher than previously reported for TSA and RA in R4 cells, although still lower than that induced in NB4 cells (Fig. 1 and data not shown; 2).

### *RN1 Induces Apoptosis in RA-Resistant R4 Cells*

Although several markers of granulocytic differentiation were moderately up-regulated by RN1 treatment, these cells did not have a differentiated phenotype by morphology nor did RN1 induce degradation of the PML/RARα fusion protein (data not shown). Therefore, we tested whether RN1 caused apoptosis of R4 cells. NB4 and R4 cells were treated for 5 days, and the percentage of cells with sub-$G_0$-$G_1$ propidium iodide (PI) staining was determined by flow cytometry. Both RA and RN1 caused only a minimal increase in apoptosis of NB4 cells (Fig. 3A). In R4 cells, however, RN1 induced significantly more apoptosis than any other treatment group, indicating that although RN1 induced a partially differentiated phenotype, the decrease in cell number was due also to increased cell death. In addition, we tested for the presence of activated caspase-3 using a caspase-3 inhibitor, DEVD-FMK,

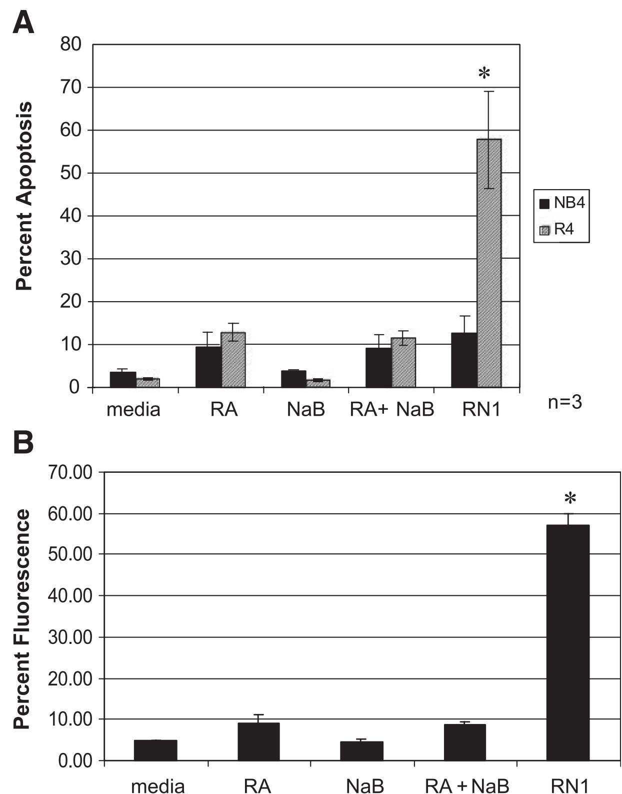


**FIGURE 3.** RN1 induces apoptosis in R4, but not NB4 cells. **A.** DNA fragmentation. NB4 (■) and R4 (▨) were treated for 5 days with media, RA, NaB, RA plus NaB, and RN1. Cells were stained with PI and the percentage of sub-$G_0$-$G_1$ staining was quantified by flow cytometry. **B.** Activation of caspase-3. R4 cells were treated for 3 days with media, RA, NaB, RA plus NaB, and RN1. Cells were stained with RED-DEVD-FMK, and the percentage of fluorescent cells was determined by flow cytometry.

conjugated to a fluorescent tag. Only activated caspase-3 binds this compound and this binding can be detected and quantified using flow cytometry. R4 cells were treated with media, RA, NaB, RA plus NaB, and RN1 for 3 days, a time point before the large increase in DNA fragmentation detected by PI staining. Only RN1-treated R4 cells exhibited a significant increase in the percentage of caspase-3 activation (Fig. 3B). These data confirm that RN1, but not RA or RA combined with an equal concentration of butyrate, induces apoptosis in R4 cells.

*Expression of Putative RA Target Genes by RN1 in R4 Cells*

RN1's ability to induce differentiation markers and apoptosis in the RA-resistant R4 cells led us to hypothesize that RN1 might not activate the same set of genes in R4 cells that have been associated with response to RA in NB4 cells. The signaling pathways leading to granulocytic differentiation are incompletely understood and the mechanisms by which retinoids induce apoptosis even less so. Because RN1, but not RA, is able to induce partial differentiation in R4 cells, we compared the genes/activities induced by RN1 and RA to identify those required for differentiation. Consistent with previous reports that RARβ may be a marker for RA-induced granulocytic differentiation, we found that RARβ mRNA expression increased in NB4 cells after treatment with any retinoid (Fig. 4A). The lane corresponding to RN1-treated NB4 cells is underloaded as shown by the decrease in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and does not correspond to less RARβ induced by RN1 (RARβ:GAPDH densitometry ratio = 0.77) as compared to RA-treated cells (RARβ:GAPDH densitometry ratio = 0.63). However, no treatment induced RARβ mRNA expression in R4 cells. We then asked whether RN1 induced histone acetylation, as might be expected from its butyrate moiety. We examined the acetylation of histone H4 in the area of RARβ promoter containing the RA response element by the chromatin immunoprecipitation assay. All retinoid-containing treatments induced acetylation of the RARβ promoter in NB4 cells, while none of the treatments induced acetylation of the RARβ promoter in the RA-resistant R4 cell line (Fig. 4B). RN1 was unable to induce acetylation of the portion of the RARβ promoter containing the RARE at any time point or concentration tested in R4 cells. Thus, activation of RARβ is not required for the partial differentiation or apoptosis in R4 cells by RN1.

Other gene families have been proposed to be critically involved in myeloid differentiation pathways. Members of the C/EBP family of transcription factors have been implicated in the regulation of myeloid differentiation, and RA treatment of APL cells induces C/EBP expression coinciding with granulocytic differentiation in APL cells (15). Initially we compared the C/EBPβ mRNA expression following 24 h treatment with media, RA, NaB, RA plus NaB, or RN1 in NB4 and R4 cells. We found that both RA and RN1 could induce C/EBPβ mRNA levels to the same extent in either NB4 or R4 cells (Fig. 4C), suggesting that the transcriptional block in differentiation of R4 cells is independent or subsequent to the induction of C/EBPβ. Next, we tested C/EBPε mRNA levels and as expected, we found that a 24-h treatment with RN1 as well as RA ± butyrate induced C/EBPε mRNA expression by Northern blotting in NB4 cells (Fig. 4C). In R4 cells, RA plus butyrate and RN1 induced C/EBPε mRNA while RA alone was ineffective, suggesting a role for C/EBPε in the partial differentiation induced by RN1 in these RA-resistant cells.

In addition to RARβ and C/EBPβ/ε, we tested the ability of RN1 to restore transcription of several other genes induced by

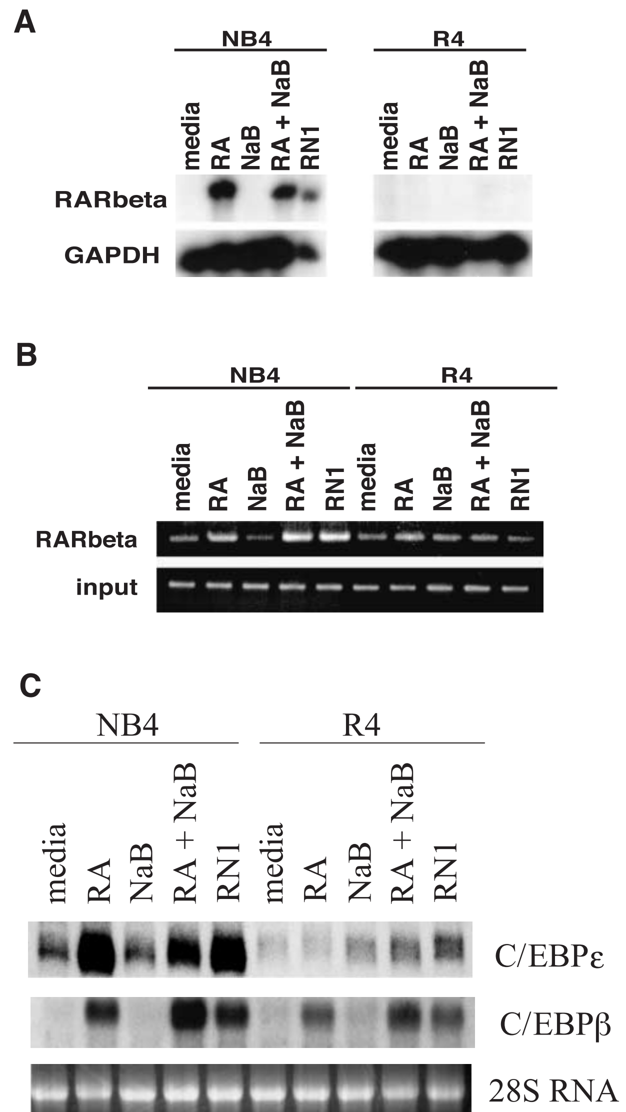


**FIGURE 4.** RN1 induces RARβ expression in NB4, but not R4 cells. **A.** RARβ RNase protection assay comparing RARβ mRNA expression in NB4 and R4 cells treated for 24 h with media, RA, NaB, RA plus NaB, and RN1. **B.** Chromosomal immunoprecipitation (ChIP) assay of NB4 and R4 cells treated for 1 h with media, RA, NaB, RA and NaB, or RN1. Immunoprecipitated DNA was detected by sequence-specific PCR for RARβ promoter sequences and electrophoresed in agarose gels. *Input,* amplification of 1% of the total material used for immunoprecipitation. **C.** Northern blots of total RNA from NB4 and R4 cells treated with media, RA, NaB, RA plus NaB, or RN1. Blots were probed for CCAAT enhancer binding protein ε (*C/EBPε*; *top*) and C/EBPβ (*middle*). Loading was controlled by visualization of the 28s ribosomal band via ethidium bromide staining (*bottom*).

RA in NB4 cells, including RARα2 (16), RIG-E (17), transglutaminase 2 (18), and BFL-1 (19). None of these genes were induced in R4 cells by RA or RN1 (data not shown). We also tested the ability of RN1 to induce hyperacetylation of promoters other than RARβ. The p21 promoter was acetylated in response to RN1 only in NB4 cells, but not R4 cells (data not shown). These data show that RN1 restores transcription of only a limited number of RA-target genes and suggest that critical genes for overcoming RA resistance remain to be described.

### *RN1 Arrests the Growth and Induced Apoptosis in Non-APL Malignancies*

Recently, the fusion proteins from other malignancies with core binding factor translocations have been shown to form complexes with HDACs. AML cells with the t{8;21} translocation express the AML1/ETO fusion oncoprotein, which complexes with HDACs and represses wild-type AML1 target gene transcription. Treatment of cell lines and patient cells expressing the AML1/ETO fusion protein with a combination of RA and the HDAC inhibitor, TSA, partially restores RA signaling and induces differentiation (9). The TEL/AML1 fusion protein is expressed in common acute lymphoblastic leukemia cells and forms a stable complex with the corepressor, N-CoR, which in turn, recruits HDACs (10). Therefore, it is reasonable to test whether RN1 might have activity in these malignancies, as well.

Kasumi cells, which express the AML1/ETO fusion protein, and REH cells, which express the TEL/AML1 fusion protein, were treated for 5 days with media, RA, NaB, RA plus NaB, or RN1 (Fig. 5). REH cells were treated with all compounds at 10 μM as in all previous experiments. Kasumi cells, however, exhibited significant growth inhibition to 10 μM RA; therefore, the growth curves were done with all treatments at 1 μM. In both cell lines, RN1 inhibited growth significantly better than RA alone or RA plus NaB, confirming that other malignant cells with aberrant transcriptional repression could be inhibited by RN1. No differentiation was seen in Kasumi cells, which are known to express granulocyte differentiation markers and RARβ mRNA after treatment with RA and the HDAC inhibitor, TSA (9). We could find no evidence of increased NBT activity or RARβ expression after treatment with RN1 (data not shown).

In contrast, we found that RN1 decreased growth of these malignancies by inducing apoptosis. Cells were treated for 5 days and the percentage of cells with sub-$G_0$-$G_1$ PI was determined by flow cytometry. As seen with R4 cells, RN1 induced significantly more apoptosis than RA alone or in combination with NaB in both REH (Fig. 5C) and Kasumi (Fig. 5D) cell lines. These data extend the data in APL cells and suggest that RN1 may be useful clinically for the growth arrest of other leukemic cells.

### *RN1 Does Not Inhibit Normal PBMCs More Than RA Alone*

To determine if the actions of RN1 were specific to the malignant cell lines tested above, we isolated normal human PBMCs and after stimulation with phytohemagglutinin (PHA) and Interleukin 2 (IL-2), treated them with 10 or 1 μM RN1 or RA and butyrate, alone or in combination. After 5 days, the number of viable cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay was used in lieu of counting viable cell numbers with trypan blue because of the large amount of cell death and debris inherent to cultures of normal cells. When compared to media-treated cultures, RA, RA plus butyrate, and RN1 all reduced the number of viable PBMCs, but no treatment's reduction was statistically significant (Fig. 6). As a control for the assay, NB4 cells were treated for 5 days with the same combination of compounds and the cell viability assessed. The results from NB4 cells confirm the results from the growth curves in Fig. 1. The number of viable NB4 cells was reduced significantly with any treatment containing RA including RN1 (Fig. 6). In addition, at 10 μM, RN1 reduces the number of viable NB4 cells better than RA alone or RA in combination with butyrate. These results indicate that while RN1 inhibits growth of NB4 cells, it does not significantly alter the number of viable normal PBMCs in culture after 5 days.

## Discussion

Aberrant transcriptional repression has been increasingly implicated in the etiology of acute leukemias, leading to the development of "transcription therapy." Such a therapy would combine elements to facilitate transcriptional initiation of blocked differentiation pathways and to inhibit HDAC-mediated repression. To this end, RN1 was created as a mutual prodrug of RA and butyric acid. In fact, RN1 induces growth arrest and differentiation in retinoid-sensitive leukemias as well or better than RA or simultaneous treatment of RA and butyrate.

Even though RN1 induced some markers of differentiation in the RA-resistant R4 cells, no morphologic changes consistent with granulocytic maturation were seen. RN1 did not restore transcription of other putative RA-target genes in R4 cells other than C/EBPε, suggesting that C/EBPε may be more important to granulocytic differentiation than RARβ. Rather, RN1 induces significant apoptosis in R4 cells, in contrast with NB4 cells where both RA and RN1 induce differentiation with minimal apoptosis. At day 5, RN1-treated R4 cells do not appear morphologically differentiated, but only 50% of the cells are remaining. Of these cells, a significant number exhibit increased differentiation marker expression. Although caspase-3 is cleaved and activated in R4 cells following RN1 treatment, we did not see a decrease in bcl-2 levels in R4 cells that could account for an increase in cell death, although in NB4 cells, both RA and RN1 decrease bcl-2 protein levels (data not shown). Thus, RN1 appears to initiate both the differentiation process and an apoptotic mechanism within R4 cells.

RN1 inhibits growth of other hematologic malignancies that express fusion proteins responsible for the aberrant recruitment of HDACs. Importantly, RN1 was not only effective in another subtype of AML, but also induced growth arrest and apoptosis in a lymphoblastic leukemia line, suggesting the therapeutic potential of RN1 may not be limited to AML. In contrast to R4 cells, where partial differentiation occurs post-RN1 treatment, the apoptotic pathway is exclusively induced in Kasumi cells by RN1. We have additional data from other cell lines, including

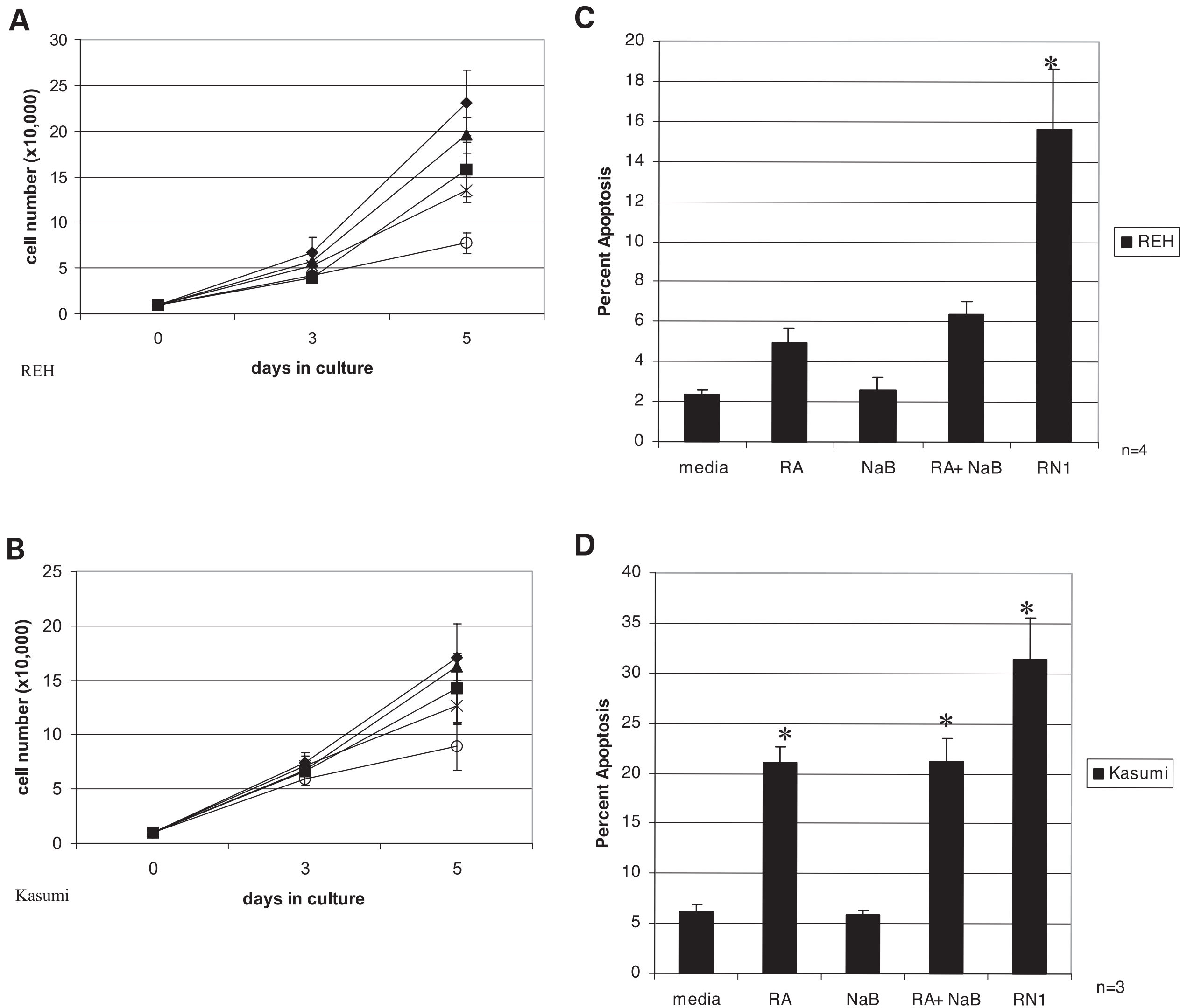


**FIGURE 5.** RN1 induces growth arrest in other non-APL leukemic cell lines. REH (**A**) or Kasumi (**B**) cells were treated with media (♦), NaB (■), RA (▲), NaB and RA (×), or RN1 (○). In REH cells, all treatments were dosed at 10 μM, while Kasumi cells were dosed at 1 μM. RN1 significantly inhibits growth of REH ($P < 0.001$) and Kasumi ($P = 0.001$) cells. *Points,* average of three separate wells; *bars,* SE. These data are representative of three growth curves. **C** and **D**. REH (**C**) and Kasumi (**D**) cells were treated with media, NaB, RA, NaB and RA, or RN1 for 5 days. Cells were stained with PI and the percentage of sub-$G_0$-$G_1$ staining was quantified by flow cytometry.

two diffuse large B-cell lymphoma lines, that RN1 induces apoptosis without expression of differentiation markers (data not shown). The response in non-APL cells further suggests a different mechanism for RN1.

RN1 is not, however, a differentiating or cytotoxic agent in all cells. As shown in Fig. 6, the number of viable normal human PBMCs is not significantly altered by treatment with RN1. Although a slight reduction was seen with RN1 treatment, this reduction was no different than that seen with RA and butyrate in combination. In addition, RN1 did not inhibit the growth of two additional RA-resistant APL cell lines more than RA and butyrate in combination (data not shown). This suggests that, while RN1 may be a useful therapeutic in some tumors, it may not be universally effective, but that it is not likely to be more detrimental to normal tissues than RA itself.

Retinoids are well known for their use in differentiation therapy of APL, but considerable evidence exists that they cause cancer cell death. Retinoids have been shown to induce apoptosis in solid tumor models including pancreatic cancer (20), prostate cancer (21), lung cancer (22), and melanoma (23). Retinoids can also induce apoptosis in lymphoma cell lines and exhibit antitumor activity in patients with relapsed lymphoma (24, 25). RA induces apoptosis of B-cell chronic lymphocytic leukemia cells via caspase-3 activation (26). In fact, RA may induce a post-maturation apoptosis in APL cells via the tumor-specific death ligand, TRAIL (27). Specifically, *N*-(4-hydroxyphenyl)-*all-trans*-retinamide (4-HPR) induces apoptosis in the RA-resistant NB306 cell line, where RA alone has no effect (28). 4-HPR also induces apoptosis via activation of caspase-3 in acute lymphoblastic leukemia cells (29). However, 4-HPR-induced apoptosis is also associated with

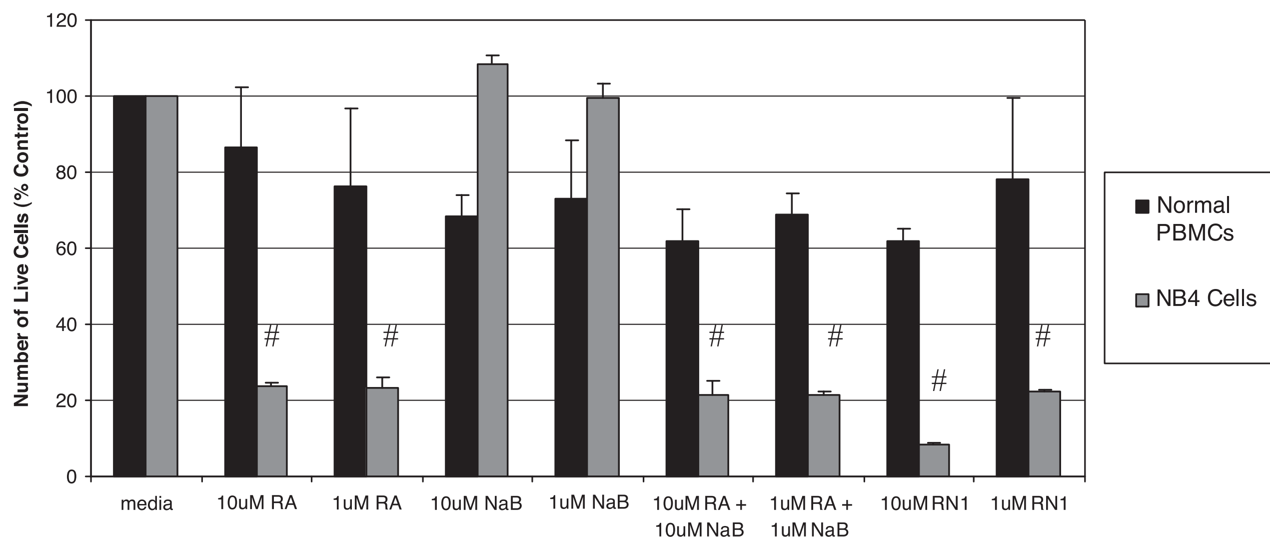


**FIGURE 6.** RN1 does not alter the number of viable normal PBMCs. Normal human PBMCs were isolated using a Ficoll gradient. Cells were stimulated with PHA and IL-2 and treated with media, NaB, RA, NaB and RA, or RN1 (1 and 10 μM) for 5 days. An MTT assay was performed as an indication of the number of viable cells and the results were calculated as percentage of media-treated cells. ■, PBMCs; ■, NB4 cells. Data are representative of an *n* of 3 for PBMCs and an *n* of 2 for NB4 cells. #, indicates a significant difference from media-treated cells ($P < 0.001$).

decreased bcl-2 expression, while RN1 is not. Thus, other retinoid compounds do induce apoptosis and some mechanisms may be similar to those involved in RN1-induced apoptosis of R4 cells.

In addition, HDAC inhibitors are known to induce apoptosis in a variety of tumor types. Valproic acid induces apoptosis in MT-450 breast carcinoma cells and HT-29 colonic cancer cells (30). Chlamydocin, a naturally occurring HDAC inhibitor, induces apoptosis in A2780 ovarian cancer cells in a caspase-3-dependent manner (31). Also, suberoylanilide hydroxamic acid (SAHA) induces apoptosis in a number of B-cell malignancies, including multiple myeloma (32).

Clearly, RN1 has increased antitumor activity as compared to RA and a micromolar dose of butyrate given concomitantly. The mechanism by which RN1 works more effectively is unclear. The higher potency of RN1 may result from the RA fragment imparting lipophilicity and facilitating the butyric acid's entry into the cell or the nucleus. In addition, delivery of RA and butyrate within a single molecule may effectively cause an increase in local concentrations of each drug at the RARE of critical target genes.

RN1 has significant growth inhibitory activity in both RA-sensitive and -resistant APL, although potentially through different mechanisms. Furthermore, RN1 appears to have significant activity in several non-APL, and even non-AML cell lines. The mechanisms by which RN1 induces apoptosis must be dissected, and we have begun cDNA microarray analysis to help answer this question. It also will be important to test the efficacy of RN1 *in vivo* against malignant cells in which RA resistance can be overcome *in vitro* by RN1. We plan to focus further investigations on the mechanisms of action of RN1 *in vitro* and *in vivo* to develop better therapy for leukemias, especially those resistant to many current treatment protocols.

## Materials and Methods

### *RN1 Synthesis*

RN1 synthesis, performed in the dark and under $N_2$, was based on the procedure previously described (7). The procedure was improved by using an excess of base ($K_2CO_3$), and the final purification step was conducted with flash chromatography on a silica gel column (Merck, Germany). The changes resulted in higher yield (97%) and greater purity (>95%). The chemical structure is shown in Fig. 7.

### *Cell Lines and Chemicals*

All cell lines were grown in RPMI 1640 (Invitrogen Inc., Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; Wisent Inc., St-Bruno, Quebec, Canada) and incubated in a humidified chamber at 37°C with 5% $CO_2$. NB4 cells are derived from an APL patient (33). The NB4-MR4 (R4) cell line is a subclone selected for resistance to RA and characterized to have a mutation in the ligand-binding domain of the PML-RARα protein (14). Kasumi cells are a human AML cell line that express the AML1-ETO fusion oncoprotein (a kind gift from Dr. Daniel Tenen, Harvard Medical School). REH cells are a human lymphocytic leukemia cell line purchased from ATCC that express the TEL-AML1 oncoprotein.

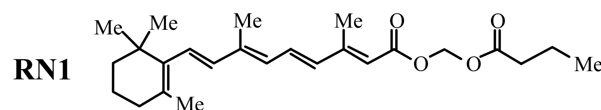


**FIGURE 7.** The structure of RN1.

In growth curves, $1 \times 10^4$ cells were plated and treated on day 0 and the cultures replenished with new media and treatment on day 3. Cells were enumerated on days 3 and 5. RA, butyrate, and RN1 were used at 10 μM in all experiments within this manuscript unless otherwise noted.

### PI Staining

Quantitation of apoptotic cells was performed as previously described (34). Cells were washed in 4°C PBS/5% FBS/0.01 M $NaN_3$, pelleted, and resuspended in 0.5 ml of hypotonic fluorochrome solution containing 50 μg/ml PI (Sigma), 0.1% sodium citrate, and 0.1% Triton X-100. Cells were analyzed by flow cytometry. Cells undergoing DNA fragmentation and apoptosis were shown to be those in which PI fluorescence was weaker than the typical $G_0$-$G_1$ cell cycle peak.

### Surface Antigen Phenotyping

Surface antigens were detected by flow cytometry. Cells were washed with PBS supplemented with 5% FBS and 0.01 M sodium azide, resuspended with the phycoerythrin (PE)-labeled CD11b (Beckman Coulter, Mississauga, Ontario, Canada) or an IgH isotype control antibody, and incubated for 30 min on ice in the dark. Cells were then washed with PBS/5% FBS/0.01 M $NaN_3$. Cells were fixed in 1% paraformaldehyde and placed on ice in the dark before analysis. The gates for positive-staining cells were determined by comparison with cells stained with the isotype-matched control antibodies.

### Caspase-3 Activity Assay

Cells were treated for 3 days and harvested into microcentrifuge tubes. The cells were incubated with Red-DEVD-FMK (Oncogene Research Products, San Diego, CA) for 1 h at 37°C in 5% $CO_2$. Subsequently, cells were washed twice and the sample stored on ice until analysis by flow cytometry.

### NBT Assay

NBT assays were performed as described previously with slight modifications (35). Briefly, cells were harvested after treatment and the cells enumerated. Subsequently, $5 \times 10^5$ cells were resuspended in 500 μl of media. NBT solution (500 μl of 1 mg/ml NBT) was added and activated with 0.25 μg/ml phorbol 12-myristate 13-acetate (PMA). Cells were incubated for 30 min at 37°C. After incubation, the percentage of NBT-positive cells was determined by visual inspection and enumeration of the blue-staining cells.

### MTT Assay

PBMCs were obtained from a healthy normal donor after obtaining informed consent and were collected into tubes containing 7.2 mg $K_2$EDTA. The blood was diluted 1:3 in PBS, layered onto an equal volume of Ficoll-Plaque PLUS (Amersham Biosciences, Piscataway, NJ), and centrifuged at 1500 rpm for 30 min. The medium layer, containing lymphocytes, platelets, and hematopoietic colony forming units, was collected and washed twice in PBS. The number of nucleated cells was measured using 3% acetic acid and trypan blue to allow lysis of RBC and enumeration of viable cells, respectively. PBMCs were seeded at $2 \times 10^6$/ml and stimulated with PHA (10 μg/ml; Becton Dickinson and Co., Sparks, MD) for 2 days. The cells were diluted 1:2 in RPMI containing IL-2 (20 units/ml; Peprotech, Rocky Hill, NJ) and when indicated, treated with test compounds. At the end of the 5-day treatment period, 0.5 mg/ml of MTT (Sigma, Oakville, Ontario, Canada) was added to each well and the plates were incubated at 37°C for 4 h. The medium was then removed and formazan crystals were dissolved in DMSO. Optical density was measured at 570 nm using an ELX 800 Universal Microplate Reader (Bio-Tek Instruments, Inc, Winooski, Vermont).

### Transient Transfection Assay

APL ($5 \times 10^6$ cells/transfection) cells were rinsed in serum-free OPTI-MEM (Invitrogen) and transfected by electroporation with 10 μg/transfection of the reporter plasmid βRE-tk-chloramphenicol acetyltransferase (CAT; 36) and 5 μg/transfection of pCMV-β-galactosidase as an internal control for transfection efficiency. Cells were electroporated and replenished with media and grown for 48 h in the absence or presence of drugs. The CAT activity was measured using a modified protocol of the organic diffusion method as described previously (37). The CAT counts were normalized with β-galactosidase activity to obtain the relative CAT activity.

### Chromosomal Immunoprecipitation Assay

ChIP assays were performed after a 1-h treatment as previously described (38). Anti-acetylated histone H4 antibodies (Upstate Biotechnology Inc., Lake Placid, NY) were used to immunoprecipitate DNA/protein complexes. Immunoprecipitated and input DNA was recovered by phenol/chloroform extraction and ethanol precipitation and analyzed by PCR. Rabbit immunoglobulin was used in the immunoprecipitation as a negative control for non-specific precipitation of DNA. No band could be amplified from rabbit immunoglobulin-precipitated products (data not shown). Input DNA is amplified as a control for the amount of product added to initial immunoprecipitation. RARβ PCR reaction parameters were an initial hot start at 95°C for 4 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation for 2 min at 72°C. Primer pair to amplify RARβ promoter was 5′-TCC TGG GAG TTG GTG ATG TCA G-3′ and 5′-AAA CCC TGC TCG GAT CGC TC-3′. PCR products were analyzed by 3% agarose gel electrophoresis and visualized by ethidium bromide staining.

### Northern Blotting

Total RNA was isolated using guanidinium thiocyanate extraction as described (39). Ten micrograms of total RNA were electrophoresed and transferred to Zeta-probe membrane (Bio-Rad, Mississauga, Ontario, Canada). Blots were probed with $^{32}$P-labeled probes derived from restriction digests of plasmid DNA. C/EBε plasmid was a kind gift from Dr. Daniel Tenen (Harvard Medical School).

### RNase Protection Assay

Fifty micrograms of total RNA were used for RNase protection assays as previously described (40). Hybridization of cRNA probes was performed at 50°C overnight, followed by the addition of 350 μl of RNase digestion buffer [10 mM Tris-Cl (pH 7.5), 300 mM NaCl, 5 mM EDTA] containing RNase T1 (Roche Diagnostics, Laval, Quebec, Canada). RNase digestion was performed at 30°C for 1 h. The RNase-resistant fragments were resolved by electrophoresis on 6% urea-polyacrylamide sequencing gels and visualized by autoradiography.

### Statistical Analysis

The significance of data was determined using SPSS version 8.0. Analysis of variance followed by Tukey's post hoc tests was used to determine if cell treatments produced significant changes.

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