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Enhancement of growth and survival and alterations in Bcl-family proteins in β -thalassemic erythroid progenitors by novel short-chain fatty acid derivatives

Serguei Castaneda, Michael S. Boosalis, David Emery, Allison Thies, Douglas V. Faller, and Susan P. Perrine*

Hemoglobinopathy-Thalassemia Research Unit and Cancer Research Center, Boston University School of Medicine, 715 Albany Street, L-908, Boston, MA 02118, USA

Department of Hematopathology, University of Washington, School of Medicine, Seattle, WA 98105–0371, USA Gene Regulation Laboratories, Inc., Newton, MA 02464, USA

Abstract

Accelerated apoptosis of erythroid progenitors is a characteristic of β -thalassemia which presents a significant barrier to definitive therapeutic approaches utilizing induction of endogenous fetal globin gene expression. γ -globin gene expression may not be inducible in, or may not be able to rescue, erythroid cells in which programmed cell death is initiated early in erythroblast development. In this report, short-chain fatty acid derivatives (SCFADs) which induce fetal globin gene expression were tested for their ability to promote proliferation and survival of erythroid progenitors cultured from β -thalassemic subjects, and of cytokine-dependent erythroid cell lines. Certain SCFADs promoted thalassemic Bfu-e growth and cytokine-independent growth and survival of erythroid cell lines. A 40–80% increase in erythroid Bfu-e colony number was observed in cultures established with any of five mitogenic SCFADs, compared to control or butyrate-treated cultures from the same subjects. Immunoblot analysis demonstrated that these same SCFADs also regulated the expression of specific protein inhibitors of apoptosis. Anti-apoptotic ratios of the proteins Bcl- x_L /Bcl- x_S in thalassemic Bfu-e were increased by 30–120% with exposure to the SCFADs, compared to the ratios in the same cells cultured under control conditions. Similar anti-apoptotic increases in Mcl- 1_L /Mcl- 1_S ratios were induced by the SCFADs. These findings suggest that select fetal globin-inducing SCFADs which enhance proliferation of β -thalassemia progenitors may enhance survival of these progenitors by altering levels of Bcl-family protein members. This combination of effects should enhance erythroid cell survival in the β -thalassemia syndromes, allowing fetal globin gene expression to be induced more effectively than currently available, growth-suppressing, fetal globin-inducing agents, such as the butyrates or chemotherapeutic agents.

Keywords

Growth; Survival; Bcl-family protein; β -thalassemia; Erythroid; Fatty acid

Introduction

Pharmacological induction of expression of γ -globin chain synthesis to balance the excess α -globin chains is an established approach to ameliorating the clinical severity of the β -thalassemias. Induction of fetal globin expression has been accomplished in some patients using chemotherapeutic agents such as hydroxyurea, decitabine, and 5-azacytidine, or using the short-chain fatty acids butyrate, phenylbutyrate, and isobutyramide [1–7].

Induction of fetal globin expression alone, however, has resulted in variable and inconsistent hematological responses in total hemoglobin clinically, with significant responses occurring in only one third to two thirds of patients [1,6,8,9]. Possible causes of the lack of correlation between hemoglobin *F* (HbF) induction and amelioration of the anemia include the characteristic spontaneous apoptosis of erythroid progenitors which occurs early in erythroblast development in β -thalassemia, initiated by unbalanced, excess α -globin chains [7,10–12]. This accelerated apoptosis may be aggravated by the cellular growth arrest induced by some of these fetal globin-inducing agents. For example, some histone deacetylase inhibitors, such as the butyrates, are known to inhibit cell proliferation, through arrest in the G₁ phase of the cell cycle, resulting in irreversible apoptosis in certain cell types [13–16]. We previously hypothesized that inhibition of cell proliferation by arginine butyrate and sodium phenylbutyrate may augment the accelerated erythroid apoptosis characteristic of β -thalassemia, and therefore sought to identify alternative HbF-inducing agents which were not growth-inhibitory.

In studies described in this report, novel short-chain fatty acid derivatives (SCFADs) were investigated for their effects on proliferation and survival of β -thalassemia erythroid progenitors, and erythroid cell lines. Certain SCFADs were found to increase thalassemic Bfu-e colony formation and survival, as well as γ -globin expression. These compounds increased ratio of the anti-apoptotic proteins Bcl-x_L and Mcl-1_L to their pro-apoptotic partners Bcl-x_S and Mcl-1_S, respectively. These studies demonstrate that select mitogenic SCFA derivatives provide an anti-apoptotic activity in erythroid cells which should be beneficial for therapeutic application to the β -thalassemias.

Materials and methods

Compounds

Stock solutions of the test compounds were prepared and the pH was adjusted to 7.4 with L-arginine (Ajinomoto, Raleigh, NC). Compounds studied include (AB) butyric acid (Aldrich Chemicals, Milwaukee, WI), sodium α -methylhydrocinnamate (NaST7), sodium 2,2-dimethyl butyrate (NaST20), (Frontage Laboratories, Malvern, PA), sodium 3,4-(methylenedioxy) cinnamate (NaST79), (T.E. Neesby Inc, Fresno, CA), 2-(quinazolin-4-ylamino)butyric acid (RB3), and (4-[(trifluoromethyl)sulfonyl] aniline) acetate (RB4) (SPECS, Rihswijk, Netherlands). All compounds were diluted in Iscove's modified Dulbecco's medium (IMDM), (Mediatech Inc., Herndon, VA).

Cell cultures

Pan-hematologic 32D murine cells (the generous gift of Dr. Daniel Wright) were cultured in RPMI 1640 (Mediatech Inc), 10% heat-inactivated fetal bovine serum (FBS), antibiotics (penicillin-streptomycin) and 2 mM L-glutamine (Gibco, Grand Island, NY), at 37°C in a 5% CO₂ humidified atmosphere. Cultures were supplemented with 0.5 U/ml (50-fold lower than optimal) recombinant mouse interleukin-3 (mIL-3) (Biosource, Camarillo, CA), and the test compounds were added for 36 h. One hour before harvesting, mIL-3 was added to a final concentration of 25 U/ml to some of the cultures, and protein levels were analyzed by immunoblot analysis.

Hematopoietic cultures were established from peripheral blood samples of β -thalassemic patients. CD34⁺ cells were enriched by addition of RosetteSep (Stem Cell Technologies, Vancouver, Canada). The samples were centrifuged over Ficoll-Paque (Amersham Biosciences, Piscataway, NJ) at 700 $\times$ g for 30 min in a Sorvall R/T centrifuge (Sorvall, Osterdone, Germany). CD34⁺ enriched cells were collected and cultured in semi-solid methylcellulose media (H4436), containing an optimal cytokine panel for Bfu-e growth (Stem Cell Technologies), with and without the test compounds, at 37°C in a 5% CO₂ humidified atmosphere. Bfu-e were enumerated after 12 days. After harvesting, cells were incubated overnight in IMDM (Mediatech Inc) with 30% FBS, 2 mM L-glutamine (Gibco), 3 u/ml Erythropoietin (Amgen, Thousand Oaks, CA), with or without the test compounds, and the erythroid cells were collected for analysis of % F-cells, globin mRNA, and Bcl family proteins.

A human cytokine dependent erythroleukemia cell line (TF-1) (American Type Culture Collection, Manassas, VA) was cultured in the presence of RPMI 1640 (Mediatech, Inc.) modified to contain 10 mM HEPES, 4.5 g/L glucose, (American Bioanalytical, Natick, MA), 1 mM sodium pyruvate (Cambrex, Walkerville, MD), 2 mM L-glutamine, 10% FBS, antibiotics (Gibco), supplemented with 4 ng/ml recombinant human GM-CSF (Stem Cell Technologies, Vancouver, Canada). TF-1 cells were incubated at 37°C in a 5% CO₂ humidified atmosphere for 3 days to deplete the cytokine (hGM-CSF), then washed twice with Dulbecco's phosphate-buffered saline (DPBS) (Mediatech, Inc.), and cultured with or without the SCFADs, in the absence of any cytokines, for 36 h and then enumerated, and harvested for globin mRNA determination using the RNase protection assay kit (Ambion, Austin, TX), or treated with 4 ng/ml hGM-CSF for 1 h before harvesting and lysing the cells for protein analysis by immunoblotting or mRNA assay.

Immunoblot analysis

A pan-hematopoietic cell line (32D) or erythroleukemia cell line (TF-1) cultured with or without the test compounds, and Bfu-e colonies from β -thalassemic patients cultured with or without the test compounds, were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA) containing protease inhibitors. The protein extracts were separated on 12% SDS-polyacrylamide gels and transferred to PDVF membranes (Bio-Rad Laboratories, Hercules, CA). Bcl-x was detected with rabbit anti-human Bcl-x_{S/L} (S-18) polyclonal antibody used at 1:300 dilution (Santa Cruz Biotechnology Inc, Santa Cruz, CA) followed by an anti-rabbit IgG horseradish

peroxidase (HRP)-conjugated secondary antibody used at a 1:2500 dilution (Amersham Biosciences). Other antibodies used included anti-Bcl-2, -Mcl-1, -Bax, and - β -actin (Santa Cruz Biotechnology, Inc.). An ECL plus detection system (Amersham Bioscience) and BioMax MS film was used for development (Eastman Kodak Co, Rochester, NY). The image was photographed (Olympus digital camera D-550, Melville, NY) and the density of each band was calculated using the NIH 1.62 software (<http://rsb.info.nih.gov/nih-image>) and normalized to actin as a loading control.

Analysis of F-cells

Immunofluorescent staining and flow cytometry were performed as previously described [17]. Briefly, cells were pelleted by centrifugation, resuspended in 1 ml HBSS with 4% formaldehyde and fixed for 30 min at room temperature. The cells were then permeabilized by serial washes in cold acetone, washed once in cold HBSS/2% FBS, and stained with anti-HbF-PE (BD Biosciences, San Diego, CA) for 30 min on ice. The cells were washed again and analyzed by flow cytometry on a FACScan (Beckton Dickinson, San Jose, CA) using CellQuest software.

Analysis of apoptotic cultures

Cultured cells were washed in DPBS and resuspended in improved MEM media (Mediatech, Inc.) without phenol red, and 10% FBS. Hoechst 33342 dye (2 μ g/ml) (Molecular Probes, Eugene, OR) was added to each cell sample and incubated at 37°C in a 5% CO₂ humidified atmosphere for 10 min. The cells were placed on ice, and the percentage of apoptotic cells was quantitated by FACS analysis using a UV laser (MoFlo, Cytomation, Inc., Fort Collins, CO).

Analysis of globin mRNA by ribonuclease protection assay

For γ -globin mRNA quantification, cultured cells were lysed and total mRNA isolated using a kit (Ambion). Probes for α , β and γ globin were labeled with ³²P, using a glycerolphosphate dehydrogenase (GPDH) probe as an internal control. Hybridized probes and sample RNA are digested, and protected fragments resolved on denaturing polyacrylamide gels, autoradiograms prepared and scanned, and ratios were calculated as previously described and compared to globin mRNA in untreated control cells from the same subject [18].

Results

We reported previously that selected SCFADs could render a growth factor (IL-3)-dependent murine pluripotent cell line independent of growth factor requirements [19]. To determine whether this effect was generalizable to other cell lines normally dependent upon different cytokines which signal through a common pathway, we investigated the effects of certain SCFADs on the human erythroleukemia cell line TF-1, which requires high 4 ng/ml GM-CSF for proliferation [20]. TF-1 cells were cultured without or severely reduced (0.1 ng/ml) GM-CSF, in the presence or absence of selected SCFADs (Table 1). While culture in the presence of arginine butyrate actually inhibited growth, the newer SCFADs all stimulated growth to a statistically-significantly degree (Fig. 1A). The proliferative effects

were most pronounced with RB3 [2-(quinazolin-4-ylamino)butanoic acid] and ST79 [sodium 3,4-(methylenedioxy)cinnamate], but also evident with ST7 [sodium α -methylhydrocinnamate], ST20 [sodium 2,2-dimethylbutyrate] and RB4 [{4-[(trifluoromethyl)sulfanyl]aniline} acetic acid].

A number of studies have suggested that certain hematopoietic cytokines, such as erythropoietin, serve as survival factors by preventing apoptosis, in addition to (or instead of) serving as growth factors. To determine if selected SCFADs could promote survival in hematopoietic cell lines, GM-CSF was depleted and then washed out of TF-1 cell cultures and the effects of SCFADs on survival was analyzed (Fig. 1B). Cell numbers declined in all cultures in the absence of GM-CSF, and declined even more rapidly than control cultures when arginine butyrate was present in the cultures. In contrast, RB3, RB4, ST7, ST20, and ST79 all significantly improved survival of TF-1 cells at days 1, 2, 3, and 4, compared to control conditions. Strikingly, exposure to each of the SCFADs increased total cell numbers at 24 h, in the complete absence of GM-CSF.

A positive effect on cell survival might be expected to be beneficial in other situations where apoptosis is prominent, such as β -thalassemia. Bfu-e were cultured from nine thalassemia patients in the presence of a cytokine mixture which was optimal for Bfu-e growth, in the presence or absence of SCFADs. Each of the SCFADs tested produced a significant increase in Bfu-e number compared to cultures from the same patients grown with optimal cytokine mixture alone (Fig. 1C). In contrast, cultures grown in the presence of arginine butyrate demonstrated a decrease in colony number relative to control conditions.

Hematopoietic cytokines, including erythropoietin, effect their survival (anti-apoptotic) activity by altering the ratios of Bcl-2 family pro-apoptotic and anti-apoptotic proteins in the cell, particularly Bcl-x and Mcl-1 [21–27]. We therefore examined Bcl-2 family protein expression in 32D cells cultured under extremely low IL-3 conditions (Figs. 2A and B). 32D cells cultured with the SCFADs ST20 and ST7 maintained Bcl-x_L/Bcl-x_S ratios at levels comparable to, or higher than, the ratios seen in 32D cultures proliferating in high levels of IL-3. In contrast, cultures grown in the presence of arginine butyrate demonstrate ratios clearly shifted towards apoptosis. Bcl-2 expression in 32D cells did not change among the treatment conditions.

The effect of SCFADs on Bcl-x_L/Bcl-x_S ratios was determined in erythroid cells cultured from subjects with β -thalassemia in the presence of an optimal mixture of cytokines. Each of the SCFADs favorably altered the ratio of Bcl-x_L/Bcl-x_S, ranging from 1.3-fold (ST20) to 2.2-fold (RB3) over control cultures from the same patient (Fig. 3A). In Bfu-e cultured with arginine butyrate, however, the Bcl-x_L/Bcl-x_S ratio fell to less than that of control cultures (<1). Bcl-2 and Bax protein expression in Bfu-e was not altered by any of the treatment conditions (data not shown).

The effect of selected SCFADs on anti-apoptotic protein expression was also studied in TF-1 cells deprived of GM-CSF. All of the SCFADs tested increased the ratio of Bcl-x_L/Bcl-x_S above 1.0 in the cultures, with the largest increase being induced by RB4 (Fig. 3B). Arginine butyrate did not significantly alter the ratio, compared to the GM-CSF-deprived

controls. The anti-apoptotic Mcl-1 protein has been implicated in mediating at least a portion of the pro-survival activity of erythropoietin in erythroid cells, and the anti-apoptotic activity of Mcl-1 may be a function of the ratio of the anti-apoptotic long form (Mcl-1_L) to the pro-apoptotic short form (Mcl-1_S). ST7, ST79, RB3 and RB increased the Mcl-1_L/Mcl-1_S ratio in TF-1 cultures, compared to untreated controls, as did erythropoietin (Fig. 3C). In contrast, arginine butyrate significantly decreased the ratio. Mcl-1 protein levels in Bfu-e cultures from a single thalassemia subject were also assessed. Treatment with the various SCFADs induced Mcl-1 levels up to 1.7 times the control (untreated) levels (Fig. 3D). More striking was the significant depression of Mcl-1 protein levels (8-fold) by exposure to arginine butyrate. In these samples derived from Bfu-e, the Mcl-1_S isoform was not detectable.

The SCFA salt arginine butyrate was initially developed as a therapeutic for the thalassemias because of its activity in increasing expression of the γ -globin gene. The effects of the SCFADs used in the present studies on fetal globin expression was therefore assessed. All of the SCFADs tested stimulated proportions of F cells in β^+ thalassemia cultures above control, ranging from 135% above control for ST7, to 150% above control for RB3 (Fig. 4A) from the same subjects. Arginine butyrate was the most efficient compound at stimulating F cell production, at 170% above control. In TF-1 cells, arginine butyrate, ST20, ST79, and RB3 increased the ratio of γ -globin/ γ -globin + β -globin mRNA expression as demonstrated by a ribonuclease protection assay (RPA) (Figs. 4B and C).

Discussion

Inducing expression of endogenous fetal globin as a therapeutic strategy in β -thalassemia presents a significant challenge, as these erythroid cells undergo programmed cell death early in the developing erythroblast, precipitated by the presence of excess, unbalanced α -globin chains [11,12,28]. Possible causes for the inconsistent hematological responses to fetal globin inducing agents in β -thalassemia include: (1) insufficient induction of fetal globin to sufficiently balance the α -globin chains; (2) induction of fetal globin in only a fraction of erythroid precursor population; and/or (3) irreversible, accelerated apoptosis too early in erythroblast development for the γ -globin gene inducing agents to be effective. Indeed, a major concern as to the feasibility of the strategy of therapeutic induction of endogenous fetal globin is that it may be impossible for fetal globin to salvage a developing erythroblast in which cell death pathway is irreversibly initiated before exposure to a γ -globin gene-inducing agent.

Chemotherapeutic agents induce hemoglobin F expression, but suppress proliferation of bone marrow cells and specifically the proliferation of the erythroid precursor cells in which endogenous fetal globin must be induced. While butyrates increase fetal globin expression in many patients [2,3,29–31], hematological improvement does not consistently follow fetal globin induction in β -thalassemia, suggesting that the factors described above may be blunting the clinical response. Both chemotherapeutic agents and histone deacetylase inhibitors, such as butyrates, inhibit cell proliferation through induction of the cyclin-dependent kinase inhibitors p21^{Cip} and p27^{Kip}, with resulting cell cycle arrest, and are known to induce irreversible arrest or apoptosis in cancer cells [13–16]. Although normal

cells can recover from this butyrate-induced growth arrest, β -thalassemia erythroid cells may be impaired in their ability to recover, due to the additional cellular stresses they endure. The anti-proliferative effects of chemotherapeutic agents or the butyrates may thus explain some of the inconsistency in hematological responses observed. The paradoxical finding of initial responses in fetal globin, which are not always followed by increases in total hemoglobin levels, and which then decline over time (perhaps when the growth arrest predominates), has been observed in some thalassemia patients treated with hydroxyurea or butyrates [3,6,32,33].

Anti-apoptotic proteins which have been established to play a role in erythroid cell survival programs include Bcl-2, Bcl-x_L, and Mcl-1 [27] [21–23]. In particular, the Bcl-x_L protein is essential for survival of erythroid progenitor cells and Bcl-x transcription increases during late erythrocyte differentiation [24]. Erythroid cells from subjects with polycythemia vera survive in vitro without erythropoietin because of constitutive Bcl-x_L expression [34]. Conversely, proteins which have been established to promote apoptosis in erythroid cells include Bax and Bcl-x_S. Ratios of anti-apoptotic to pro-apoptotic proteins pairs of >1.0 are considered to favor overall survival of erythroid cells [35–37]. For example, erythropoietic cytokines have been shown to prolong erythroid survival and retard apoptosis through increasing the ratios of Bcl-x_L/Bcl-x_S, Bcl-2/Bax, and Mcl-1_L/Mcl-1_S. Erythropoietin induces Bcl-x_L expression in human erythroid colony-forming cells, and this induction is mediated by PI3-kinase [38]. The erythropoietin-dependent human leukemia cell line UT-7/EPO undergoes apoptosis upon removal of erythropoietin, accompanied by down regulation of Bcl-x_L. A similar pattern was also observed in normal erythroid progenitor cells [39]. Stem cell factor up-regulates Bcl-2 and Bcl-x_L in erythroid precursors and exogenous expression of these proteins protects erythroblasts from caspase activation and death induced by chemotherapeutic agents [25]. In erythropoietin- or stem cell factor-stimulated human CD34+ cells, there is a marked increase in the levels of Bcl-x_L and Mcl-1 proteins and downregulation of BAX protein as erythroid differentiation progresses [27]. Bcl-2 and Bcl-x_L levels fall coincident with the onset of apoptosis when erythropoietin is withdrawn from the erythropoietin-dependent murine cell line HCD-57, and ectopic expression of Bcl-x_L or Bcl-2 promotes survival and differentiation in the absence of erythropoietin [22].

We have found that the SCFADs tested here favorably alter the Bcl-x_L/Bcl-x_S and Mcl-1_L/Mcl-1_S ratios in the TF-1 cell line and in erythroid precursors from thalassemia subjects. This is in contrast to the effects of arginine butyrate, wherein a marked shift towards pro-apoptotic ratios for Bcl-x and Mcl-1 was induced. Bcl-2/Bax ratios and absolute levels were unchanged by treatment with arginine butyrate or the SCFADs.

The proliferative activity and the anti-apoptotic activity of the novel SCFADs tested here may be mechanistically linked. Cytokines such as IL-3 and erythropoietin induce both types of activities in hematopoietic cells, by signaling through receptors coupled to Jak and Stat5. Erythropoietin stimulates the Bcl-x promoter through its Stat-binding element [23]. Conversely, decreased expression of Bcl-x and increased apoptosis in erythroid progenitors is observed in Stat5 knockout mice [40]. We have previously reported that certain of these novel SCFADs activate Stat5 signaling, through phosphorylation of Stat5, independently of

cytokine receptor activation [19]. It is noteworthy, however, that the proliferative and pro-survival actions of the SCFADs on the human Bfu-e cultures reported here were observed in the presence of optimal concentrations of cytokines during culture, suggesting that these SCFADs have activities beyond those of the cytokines, and perhaps utilize additional pathways.

The combined actions of the novel SCFADs on erythroid cells, an increase in proliferation concomitant with induction of anti-apoptotic ratios of Bcl-2 family proteins, and induction of the fetal globin program, strongly suggests that these compounds will provide three beneficial and potentially synergistic effects for amelioration of the erythroid cell destruction in β -thalassemia. It is possible, however, that the apoptotic program induced by excess α -globin chains may involve a number of effector mechanisms in vivo, not all of which can be prevented by SCFADs through Bcl-2 family proteins. Arginine butyrate induces γ -globin gene expression efficiently, but actually suppresses proliferation because of its histone deacetylase inhibitory activity and induction of the cyclin-dependent kinase inhibitors p21^{Cip} and p27^{Kip} [14–16] and shifts Bcl-2 family protein ratios towards apoptosis. The proliferative and pro-survival actions of the SCFADs, which are independent of fetal hemoglobin induction, may also be of benefit in the treatment of other anemias characterized by decreased cell survival/increased apoptosis such as myelodysplasias, and anemias associated with chemotherapy and radiation exposure [25,41].

A gradual accumulation of F-cells in the circulation, and high-level γ -globin synthesis, is likely to be required for high-level increases in hemoglobin and red blood cell counts in β -thalassemia. These SCFADs reported here, which lack histone deacetylase inhibitory activity, offer an advantage over butyrate, which requires limited administration to avoid growth inhibitory effects on the erythroid compartment. When arginine butyrate administration has been limited to two four-day treatment cycles (or 8 days) per month in β -thalassemia patients, total hemoglobin increases of 1.5–3.9 g/dl above baseline, and mean increases of 2.8 g/dl were achieved. Higher hematologic responses are necessary, however, to render severely anemic β -thalassemia patients independent of transfusion requirements. The SCFADs studied here should not require such restricted titration of dosage, and the ability to administer these fetal globin-inducing agents more frequently than is possible for arginine butyrate should at least double the fraction of erythroid cells in which fetal globin is induced and result in clinical responses of significantly higher magnitude. The fact that these SCFADs are also stimulators of proliferation and survival might be expected to further magnify the beneficial effects of fetal globin induction. Future potential application to human patients will require further investigation of the pharmacokinetic and toxicity profiles in animal models, which is in progress.

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References

1. Steinberg MH, Rodgers GP. Pharmacologic modulation of fetal hemoglobin. *Medicine*. 2001; 80:328–344. [PubMed: 11552087]
2. Collins AF, Pearson HA, Giardina P, McDonagh KT, Brusilow SW, Dover GJ. Oral sodium phenylbutyrate therapy in homozygous beta thalassemia: a clinical trial. *Blood*. 1995; 85:39–43.
3. Cappellini MD, et al. Oral isobutyramide therapy in patients with thalassemia intermedia: results of a phase II open study. *Blood Cells Mol Dis*. 2000; 26:105–111. [PubMed: 10772882]
4. Lowrey CH, Nienhuis AW. Brief report: treatment with azacytidine of patients with end-stage β -thalassemia. *N Engl J Med*. 1993; 329:945.
5. Dunbar C, Travis W, Kan YW, Nienhuis AW. 5-Azacytidine treatment in a beta thalassemia patient unable to be transfused due to multiple allo-antibodies. *Br J Haematol*. 1989; 74:467. [PubMed: 2475157]
6. Singer ST, Sweeters N, Vichinsky E, Wagner EA, Rachmilewitz EA. A dose-finding and safety study of darbopoietin alfa (erythropoiesis stimulating protein) for the treatment of anemia in patients with thalassemia intermedia. *Blood*. 2003; 102:268a.
7. Pootrakul P, et al. A correlation of erythrokinetics, ineffective erythropoiesis, and erythroid precursor apoptosis in thai patients with thalassemia. *Blood*. 2000; 96:2606–2612. [PubMed: 11001918]
8. Rodgers GP, Sauntharajah Y. Advances in experimental treatment of beta-thalassaemia. *Expert Opin Investig Drugs*. 2001; 10:925–934.
9. Perrine SP, et al. Butyrate + EPO in beta thalassemia intermedia: interim findings of a phase II trial. *Blood*. 2002; 100:47a.
10. Schrier SL. Pathobiology of thalassemic erythrocytes. *Curr Opin Hematol*. 1997; 4:75–78. [PubMed: 9107522]
11. Yuan J, et al. Accelerated programmed cell death (apoptosis) in erythroid precursors of patients with severe beta-thalassemia. *Blood*. 1993; 82:374–377. [PubMed: 8329696]
12. Centis F, et al. The importance of erythroid expansion in determining the extent of apoptosis in erythroid precursors in patients with beta-thalassemia major. *Blood*. 2000; 96:3624–3629. [PubMed: 11071663]
13. Wittich S, et al. Structure–activity relationships on phenylalanine-containing inhibitors of histone deacetylase: in vitro enzyme inhibition, induction of differentiation, and inhibition of proliferation in Friend leukemic cells. *J Med Chem*. 2002; 45:3296–3309. [PubMed: 12109913]
14. Vaziri C, Stice L, Faller DV. Butyrate-induced G(1) arrest results from p21-independent disruption of retinoblastoma protein-mediated signals. *Cell Growth Differ*. 1998; 9:465–474. [PubMed: 9663465]
15. Mork CR, Spanjaard RA, Faller DV. A mechanistic approach to anticancer therapy: targeting the cell cycle with histone deacetylase inhibitors. *Curr Pharm Des*. 2005; 11:1091–1104. [PubMed: 15853658]
16. Chen JS, Faller DV. Histone deacetylase inhibition-mediated post-translational elevation of p27Kip1 protein levels is required for G1 arrest in fibroblasts. *J Cell Physiol*. 2005; 202:87–99. [PubMed: 15389542]
17. Emery DW, et al. Selection with a regulated cell growth switch increases the likelihood of expression for a linked gamma-globin gene. *Blood Cells Mol Dis*. 2005; 34:235–247. [PubMed: 15885608]
18. Pace B, Li QL, Peterson K, Stamatoyannopoulos G. alpha-Amino butyric acid cannot reactivate the silenced gamma gene of the beta locus YAC transgenic mouse. *Blood*. 1994; 84:4344–4353. [PubMed: 7527673]
19. Boosalis MS, et al. Short-chain fatty acid derivatives stimulate cell proliferation and induce STAT-5 activation. *Blood*. 2001; 97:3259–3267. [PubMed: 11342457]
20. Kitamura T, et al. Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. *J Cell Physiol*. 1989; 140:323–334. [PubMed: 2663885]

21. Koury MJ, Sawyer ST, Brandt SJ. New insights into erythropoiesis. *Curr Opin Hematol*. 2002; 9:93–100. [PubMed: 11844990]
22. Silva M, Grillot D, Benito A, Richard C, Nunez G, FernandezLuna JL. Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-X(L) and Bcl-2. *Blood*. 1996; 88:1576–1582. [PubMed: 8781412]
23. Silva M, et al. Erythropoietin can induce the expression of bcl-x(L) through Stat5 in erythropoietin-dependent progenitor cell lines. *J Biol Chem*. 1999; 274:22165–22169. [PubMed: 10428780]
24. Tian C, Gregoli P, Bondurant M. The function of the bcl-x promoter in erythroid progenitor cells. *Blood*. 2003; 101:2235–2242. [PubMed: 12411308]
25. Zeuner A, et al. Stem cell factor protects erythroid precursor cells from chemotherapeutic agents via up-regulation of BCL-2 family proteins. *Blood*. 2003; 102:87–93. [PubMed: 12637332]
26. Sawatsri S, Samid D, Malkapuram S, Sidell N. Inhibition of estrogen-dependent breast cell responses with phenylacetate. *Int J Cancer*. 2001; 93:687–692. [PubMed: 11477579]
27. Josefsen D, Myklebust JH, Lomo J, Sioud M, Blomhoff HK, Smeland EB. Differential expression of bcl-2 homologs in human CD34(+) hematopoietic progenitor cells induced to differentiate into erythroid or granulocytic cells. *Stem Cells*. 2000; 18:261–272. [PubMed: 10924092]
28. Bernards R, et al. Tumorigenicity of cells transformed by Adenovirus type 12 by evasion of T-cell immunity. *Nature*. 1983; 305:776–779. [PubMed: 6605479]
29. Ikuta T, Kan YW, Swerdlow PS, Faller DV, Perrine SP. Alterations in protein–DNA interactions in the gamma-globin gene promoter in response to butyrate therapy. *Blood*. 1998; 92:2924–2933. [PubMed: 9763579]
30. Atweh GF, et al. Sustained induction of fetal hemoglobin by pulse butyrate therapy in sickle cell disease. *Blood*. 1999; 93:1790–1797. [PubMed: 10068649]
31. Khachigian LM, Fries JW, Benz MW, Bonthron DT, Collins T. Novel cis-acting elements in the human platelet-derived growth factor B-chain core promoter that mediate gene expression in cultured vascular endothelial cells. *J Biol Chem*. 1994; 269:22647–22656. [PubMed: 8077216]
32. Olivieri NF, et al. Treatment of thalassaemia major with phenylbutyrate and hydroxyurea. *Lancet*. 1997; 350:491–492. [PubMed: 9274590]
33. Hajjar FM, Pearson HA. Pharmacologic treatment of thalassemia intermedia with hydroxyurea. *J Pediatr*. 1994; 125:490–492. [PubMed: 7520935]
34. Silva M, Richard C, Benito A, Sanz C, Olalla I, Fernandez-Luna JL. Expression of Bcl-x in erythroid precursors from patients with polycythemia vera. *N Engl J Med*. 1998; 338:564–571. [PubMed: 9475763]
35. Chao DT, Korsmeyer SJ. BCL-2 Family: regulators of cell death. *Annu Rev Immunol*. 1998; 16:395–419. [PubMed: 9597135]
36. Korsmeyer SJ. BCL-2 gene family and the regulation of cell death. *Int J Radiat Oncol, Biol, Phys*. 1997; 39:98.
37. Opferman JT, Korsmeyer SJ. Apoptosis in the development and maintenance of the immune system. *Nat Immunol*. 2003; 4:410–415. [PubMed: 12719730]
38. Paiboonsukwong K, et al. The signaling pathways of erythropoietin and interferon-gamma differ in preventing the apoptosis of mature erythroid progenitor cells. *Int J Hematol*. 2003; 78:421–428. [PubMed: 14704034]
39. Mori M, et al. Activation of extracellular signal-regulated kinases ERK1 and ERK2 induces Bcl-xL up-regulation via inhibition of caspase activities in erythropoietin signaling. *J Cell Physiol*. 2003; 195:290–297. [PubMed: 12652655]
40. Socolovsky M, Nam H, Fleming MD, Haase VH, Brugnara C, Lodish HF. Ineffective erythropoiesis in Stat5a(–/–)5b(–/–) mice due to decreased survival of early erythroblasts. *Blood*. 2001; 98:3261–3273. [PubMed: 11719363]
41. Parker JE, Mufti GJ, Rasool F, Mijovic A, Devereux S, Pagliuca A. The role of apoptosis, proliferation, and the Bcl-2-related proteins in the myelodysplastic syndromes and acute myeloid leukemia secondary to MDS. *Blood*. 2000; 96:3932–3938. [PubMed: 11090080]

Fig. 1.

(A) Proliferation curves of TF-1 cells treated with SCFADs. TF-1 cell cultures were depleted of GM-CSF as described in Material and methods, and cultured with or without SCFADs, in the absence of any or severely depleted cytokines, for 36 h and then enumerated. AB (Arginine Butyrate), ST7 (sodium α -methylhydrocinnamate), ST20 (sodium 2,2-dimethylbutyrate), ST79 [sodium 3,4-(Methylenedioxy)cinnamate], RB3 [2-(quinazolin-4-ylamino)butanoic acid], and RB4 ({4-[(trifluoromethyl)sulfanyl]aniline} acetic acid). Results from three independent experiments are shown, with bars indicating SE.

ANOVA results for SCFAD-treated cultures compared to controls were as follows: for AB, ST20, ST79, and RB4, $P < 0.05$; for ST7 and RB3, $P < 0.004$. (B) Survival curves of TF-1 cells treated with SCFADs. TF-1 cell cultures were depleted of GM-CSF, and washed to remove any remaining GM-CSF, as described in Material and methods, and cultured with or without SCFADs, in the absence of any cytokines, for 36 h and then enumerated. Results from three independent experiments are shown, with bars indicating SE. ANOVA results for SCFAD-treated cultures compared to controls were as follows: for AB $P < 0.05$; for RB4 and ST 20, $P < 0.015$; for ST7, ST79 and RB3, $P < 0.008$. (C) Effect of selected SCFADs on Bfu-e growth in cultures derived from β -thalassemia patients. CD34+ enriched cells were collected and cultured as described in Materials and methods, in a cytokine mixture designed for optimal Bfu-e growth, with or without SCFADs. Bfu-e were enumerated after 12 days. The number of Bfu-e colonies is expressed as the mean relative to cells from the same patient cultured without SCFADs (control [C]), for nine β -thalassemia patient cultures ($\pm$ SE). Comparison between SCFAD-treated and control values was performed by the Wilcoxon signed-rank test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

Fig. 2.

(A) Bcl-2 family protein expression in 32D cells cultured with SCFADs. 32D cells were cultured under low rIL-3 conditions (0.5 µg/ ml) as described in Materials and methods, in the presence or absence of the indicated SCFADs (B is Arginine Butyrate; 20 is ST20; 7 is ST7; 79 is ST79), or without SCFADs (C) for 36 h, then lysed and analyzed by immunoblotting for Bcl-x or Bcl-2. The Bcl-2 immunoblot was stripped and reprobed for β-actin as a loading control. The sample in lane “IL-3” was derived from a culture treated with high concentrations of rIL-3 (25 U/ml) for 1 h before harvesting. (B) Bcl-x_L/x_S ratios in 32D cells cultured with SCFADs. Ratios are derived from three separate experiments, carried out as described in the legend to A, and quantitated by densitometric scanning.

Fig. 3.

A. Effect of selected SCFADs on Bcl-x_L/Bcl-x_S protein ratios in Bfu-e cultures from β-thalassemia patients. CD34⁺ enriched cells were collected and cultured as described in Materials and methods, in a cytokine mixture designed for optimal Bfu-e growth, with or without the SCFADs. Bfu-e were harvested on day 12, cells were incubated overnight in the presence or absence of the SCFADs, and the cell lysates were analyzed for levels of Bcl-x proteins. Bars represent the mean of Bcl-x_L/Bcl-x_S ratios (±SE) detected by immunoblotting in Bfu-e cultures from four β-thalassemia patients. The Bcl-x_L/Bcl-x_S ratios of the cultures not treated with SCFADs (control [C]) were normalized to 1, and the ratios from cultures treated with specific SCFADs are expressed as fold change of the ratio in the control culture conditions. Comparison between compounds and control values was performed by means of the Wilcoxon signed-rank test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. The insert is a typical immunoblot of Bfu-e-derived Bcl-X protein levels. (B) Effect of selected SCFADs on Bcl-x_L/Bcl-x_S protein ratios in TF-1 cells. TF-1 cell cultures were depleted of GM-CSF as described in Materials and methods, and cultured in the absence of any cytokines, with or without (control [C]) the indicated SCFADs, for 36 h and then harvested and lysed for Bcl-x protein analysis by immunoblotting. Protein ratios were determined by densitometric scanning of the film. (C) Effect of selected SCFADs on Mcl-1_L/Mcl-1_S protein ratios in

TF-1 cells. TF-1 cell cultures were depleted of GM-CSF, cultured in the absence of any cytokines, with or without (control [C]) the indicated SCFADs, as described in the legend to B, and Mcl-1 protein levels were assayed by immunoblotting and quantitated. The lane marked EPO contains lysates from TF-1 cells cultured in the presence of erythropoietin (3 U/ml) for 36 h. The insert depicts an immunoblot of Mcl-1 proteins from a typical experiment, with the position of the Mcl-1_L and Mcl-1_S species indicated. D. Effect of selected SCFADs on Mcl-1 protein levels in Bfu-e cultures from thalassemia subjects. CD34+ enriched cells were collected and treated as described in the legend to B described in Materials and methods, and lysates were immunoblotted for Mcl-1 protein. The level in the untreated control lane (C) was assigned an arbitrary value of 1.0, and the relative levels of Mcl-1 protein in the other lanes are indicated, after normalization to β -actin as a control for loading.

Fig. 4.

(A) Effect of selected SCFADs on percentage of F-cells determined in cultures from β -thalassemia patients. CD34⁺ enriched cells were collected and cultured as described in Materials and methods, in a cytokine mixture designed for optimal Bfu-e growth, with or without the SCFADs. Bfu-e were harvested on day 12, cells were incubated overnight in the presence or absence of the test compounds, and the cells were analyzed for % F-cells. The percentage of F-cells (%F-cells) in cultures exposed to the test compounds is expressed relative to untreated (control) cultures (C) for seven β -thalassemia patient cultures ($\pm$ SE). Comparison between compounds and control values was performed by the Wilcoxon signed-rank test. * $P < 0.05$; ** $P < 0.01$. (B) Effect of selected SCFADs on γ -globin mRNA production in TF-1 cells. TF-1 cells were cultured in the absence of GM-CSF, with or without SCFADs, as described in Materials and methods, for 36 h and then harvested for globin mRNA determination using an RNase protection assay kit. A typical autoradiogram is shown here. 18s RNA serves as a loading control. (C) Effect of selected SCFADs on γ -globin mRNA production in TF-1 cells. γ - and β -globin transcript levels were determined in

TF-1 cells treated as described in the legend to B and the ratios plotted. Bar labeled “GM-CSF” indicates RNA isolated from TF-1 cells cultured in recombinant human GM-CSF at 4 ng/ml.

Table 1

Short chain fatty acid derivatives studied

ID	Compound	Structure
AB	Butyrate
ST7	Sodium α -Methylhydrocinnamate
ST20	Sodium 2,2-Dimethylbutyrate
ST79	Sodium 3,4-(Methylenedioxy)cinnamate
RB3	2-(Quinazolin-4-ylamino)butyric acid
RB4	{4-[(Trifluoromethyl)sulfonyl]anilino} acetic acid