

Erythroid Progenitor Proliferation is Stimulated by Phenoxyacetic and Phenylalkyl Acids

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ABSTRACT: Short-chain fatty acids, such as butyrate and propionate, are under investigation as therapeutic stimulants of fetal hemoglobin production in the β -hemoglobin disorders. Significant limitations to these fatty acids and derivatives as optimal therapeutics are their rapid metabolism *in vivo* and their induction of cell growth arrest in the G₁ phase of the cell cycle. This antiproliferative activity is related to their inhibition of metabolic transport pumps which are essential for cell proliferation. Other small carbon compounds, the phenylalkyl acids, phenoxyacetic acids, and phenylacetic acids, which are structurally resistant to oxidative metabolism, are shown here to induce fetal globin production in human erythroid cultures at concentrations of 0.2 mM, lower than those required for most other fatty acids. Certain of these compounds were found not to inhibit cellular neutral amino acid transport function in erythroid cells, nor to inhibit erythroid colony (Bfu-e) growth. Certain of these compounds even stimulated human Bfu-e proliferation *in vitro* beyond that induced by optimal concentrations of hematopoietic growth factors. The combination of increased fetal globin chain production by these compounds and their stimulatory effects on erythropoiesis result in an increase in Hb F-expressing erythroid cells in culture several-fold greater than that achieved by the butyrates. These new compounds thus have the potential to provide superior therapy for the β -hemoglobinopathies and other anemias.

Keywords: Fetal hemoglobin, hemoglobinopathies, phenylalkylacids, phenoxyacetic acids, erythropoiesis, metabolic transport

INTRODUCTION

Short-chain fatty acids have been shown to regulate specific gene expression at the transcriptional level in a variety of tissues (1). In erythroid cells of several species, acetate, butyrate, propionate, phenylbutyrate, and longer chain fatty acids stimulate embryonic or fetal globin gene expression, through an action on the γ -globin gene

promoter (2-11). Accordingly, butyrate, phenylbutyrate, isobutyramide, and valproic acid are currently being evaluated clinically as fetal globin stimulants in the β -hemoglobin disorders (12-16). One factor which limits the efficacy of many of these agents as optimal therapeutics is their typically rapid oxidative metabolism (17). Furthermore, high concentrations of these butyrates and phenylbutyrates inhibit cell

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proliferation by inducing growth arrest in the late G₁ phase of the cell cycle (4,5, 16,18-21). This antiproliferative effect may be mediated in part through their inhibition of the function of a metabolic transport pump which is essential for cell growth, the System A transport system for neutral amino acids (22-23). These inhibitory effects of the butyrates and resulting G₁ growth arrest are particularly undesirable in the β -thalassemias -- hyperproliferative conditions in which excess, unbalanced α -globin chains accelerate apoptosis of erythroid precursor cells (24). We and others have evaluated several generations of other small carbon compounds for activity in stimulating fetal globin expression in erythroid progenitor cell cultures, promoter-reporter gene systems, and animal models (2-11). While many short-chain carbon compounds of similar sizes but diverse structures were shown to induce fetal hemoglobin production, they were often not useful *in vivo* (8,16). Millimolar levels of butyrate, valerate, and propionate were required to stimulate the fetal globin gene promoter, while the longer chain fatty acids were more toxic or less active than butyrate in the baboon (6,8). Liakopoulou identified some substituted fatty acids which preserved the fetal globin-stimulating actions of butyrate; however, most required 5-15 mM concentrations for activity *in vitro*, levels which are difficult to maintain *in vivo* (8). Thus, additional, more practical, longer-lived and potent stimulants of fetal hemoglobin are desirable for use as therapeutics.

Phenoxyacetic and phenylalkylacids possess steric resistance to oxidative metabolism and renal filtration. These compounds, therefore, have long physical and biological half-lives *in vivo*. Accordingly, representative agents from these classes and some synthetic derivatives were examined for their activities on proliferation, amino acid transport, and γ -globin gene stimulation in human erythroid cells. A number of compounds were identified which did not inhibit metabolic transport or erythroid cell proliferation, and were potent stimulants of fetal globin expression.

MATERIALS AND METHODS

Erythroid Progenitor Cultures

Mononuclear cells were separated from heparinized samples of peripheral blood from normal subjects, from patients with sickle cell anemia, or from fetal liver, by centrifugation over Ficoll-Paque (Pharmacia, Piscataway, New Jersey), and cultured in semi-solid media with 0.9% methylcellulose and maximal concentrations of the hematopoietic growth factors IL-3, GM-CSF, IL-6, and Stem Cell Factor (SCF) without serum, (Stem Cell Technologies, Vancouver, B.C.) and 2 U/ml erythropoietin, as previously described (4,10-11). In some experiments, CD34⁺ cells were isolated by labeling the mononuclear cells with an anti-CD34 antibody, followed by panning on goat anti-murine IgG-coated flasks (Applied Immune Sciences, Santa Clara, CA), according to the manufacturer's directions. Cultures were incubated in a humidified CO₂ incubator at 37°C, and erythroid colonies were enumerated at days 12-14. Erythroblasts were harvested and subjected to globin chain electrophoresis, and quantitation of the globin chains was performed by laser densitometry (2,10-11).

Metabolic Transport Studies

Growth arrest in late G₁ of the cell cycle can occur through amino acid (nutrient) deprivation (23), a previously reported action of butyrate in erythroblasts (22). Erythroblasts isolated from fetal liver samples (Advanced Biological Resources, Alameda, California), a source which provides a cell population of greater than 90% pure erythroblasts, were used for these studies. The cells were incubated in RPMI media with 5% fetal calf serum alone (controls) or in 0.5 - 2 mM concentrations of the compounds being tested, in a humidified incubator at 37° C in 5% CO₂/95% air for 3 days. Amino acid uptake into cells by System A transport was examined using established methods (22), with ³H-methyl aminoisobutyric acid used as the substrate.

Assays were performed in duplicate. The function of the Pgp-170 (mdr-1) efflux pump was assayed in K562 cells using the rhodamine 123 uptake method (25). Cells were cultured alone, or with 1.0 - 2.0 mM concentrations of the compounds under investigation, as described above. Treated cells were incubated with rhodamine 123 and intracellular rhodamine levels were analyzed by FACScan analysis.

RESULTS

Analysis of Fetal Globin Production

Addition of 3-phenylbutyrate, α -methylhydrocinnamic acid, 2,2-dimethylbutyric acid,

phenoxyacetic acid, or thiophenoxyacetic acid to erythroid cultures of peripheral blood from sickle cell and normal subjects resulted in significantly increased proportions of fetal globin chain production (2-6 fold), with absolute increases of γ -globin ranging from 12-38% of non- α -globin above the levels found in untreated cultures from the same subject ($p \leq 0.01$, chi-square). Results from a representative sickle cell subject's cultures with tested compounds added at a concentration of 0.2 mM are shown in Figure 1. Stimulation with these compounds was found in cultures from at least four different samples. Concentrations of compounds above 0.5 mM did not produce greater elevations of fetal globin production (not shown).

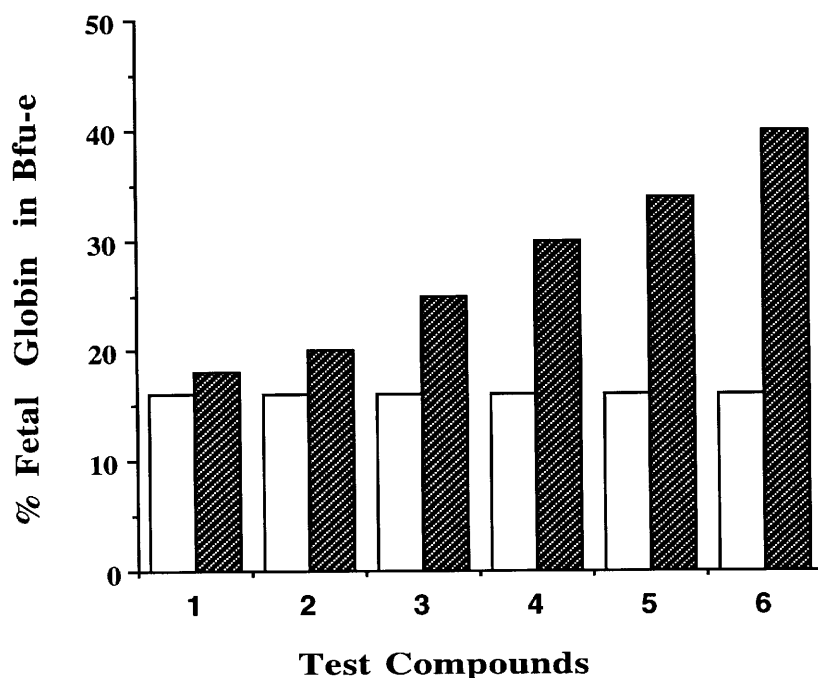


Figure 1. Comparison of fetal globin produced in erythroid progenitor colonies developing in the presence or absence of test compounds. Fetal globin production in erythroid colonies cultured from a normal individual with maximal concentrations of IL-3, erythropoietin, GM-CSF, SCF and IL-6 are shown in white; production from erythroid colonies cultured from the same sample with the test agents in addition to the growth factors are shown in the darker shaded bars. Bars 1 - 6 represent fetal globin produced in Bfu-e treated respectively with α -methylcinnamic acid, 3-phenylbutyrate, α -methylhydrocinnamic acid, 2,2-dimethylbutyric acid, phenoxyacetic acid, and thiophenoxyacetic acid, respectively, at 0.2 mM concentrations. Significant increases in fetal globin were induced by the 5 test compounds in bars 2-6 ($p < .05$, paired t-test), from absolute levels of 16% of non- α -globin (control) to 20-40% of non- α globin synthesized in Bfu-e cultured with the test compounds.

Analysis of Bfu-e Colony Production

Erythroid colony formation (Bfu-e) from peripheral blood of a normal subject (Figure 2), or subjects with sickle cell anemia (Figure 3), and from CD34⁺ cells isolated from fetal liver (Figure 4), in the presence or absence of some of the test compounds was quantitated. Addition of certain of the new compounds at the time the cultures were established resulted in significant increases in Bfu-e production compared to control cultures from the same samples cultured with optimal concentrations of the hematopoietic cytokines alone ($p < 0.02$, paired t-test and chi-square). Phenoxyacetic acid, 2,2-dimethylbutyric acid, and α -methylhydrocinnamic acid had the most

consistent and potent stimulatory effects (beyond the activity of the included hematopoietic growth factors) in normal, sickle cell, and fetal stem cell cultures ($p < 0.05$, paired t-test). In experiments in which arginine butyrate alone was added (Figure 2), Bfu-e production was significantly inhibited, producing only 16% of the number of Bfu-e developing in (untreated) control cultures. The combination of phenoxyacetic acid and arginine butyrate, however, did not show the same inhibitory activity. In contrast, the numbers of Bfu-e produced were greater than for either phenoxyacetic acid or arginine butyrate alone, suggesting a synergistic effect on cell proliferation (Figure 3).

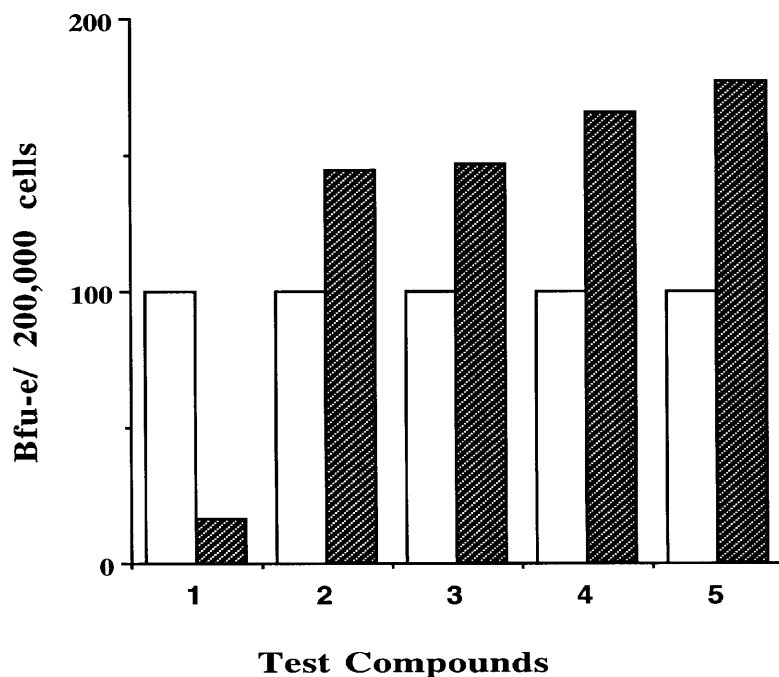


Figure 2. Effects of the tested compounds on erythroid colony (Bfu-e) proliferation in culture. Erythroid colony numbers developing from a normal subject in the presence of growth factors alone are shown in the light bars; Bfu-e colony number relative to control from treated cultures are shown in the darker bars. Added compounds are: [1] 0.1 mM Arginine Butyrate, (16% of control); [2] 0.5 mM 2-(4'-methoxyphenoxy)propionic acid; [3] α -methyl-hydrocinnamic acid; [4] 0.2 mM phenoxyacetic acid; and [5] 0.2 mM 4-chlorophenoxy-2-propionic acid. All changes from control were significant, ($p < 0.05$, chi-square).

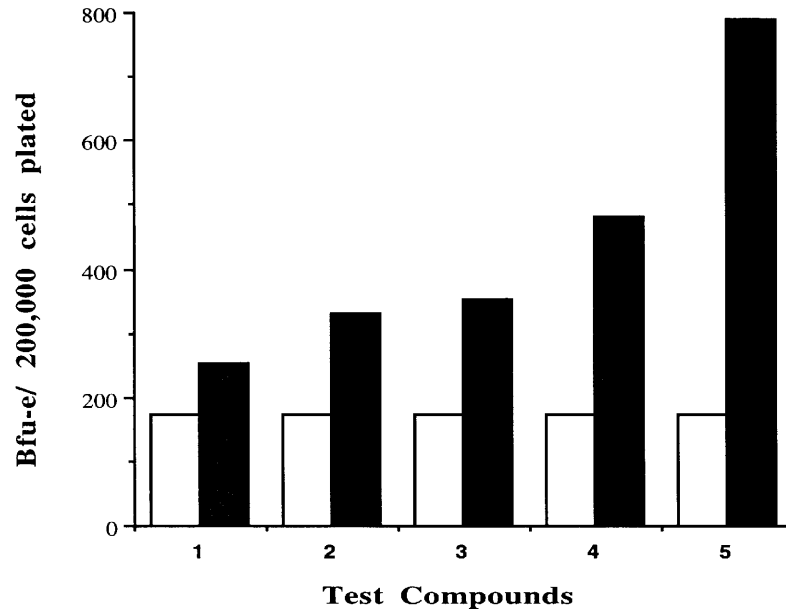


Figure 3. Effects of tested compounds on erythroid colony (Bfu-e) proliferation in sickle cell anemia. Erythroid colony numbers developing from a subject with sickle cell disease in the presence of optimal growth factors alone are shown in the light bars; colony numbers from treated cultures are shown in the darker shaded bars. Added compounds are: [1] α -methyl hydrocinnamic acid; [2] 2-(4'-methoxyphenoxy) propionic acid; [3] 2,2-dimethylbutyric acid; [4] 0.2 mM phenoxyacetic acid; and [5] phenoxyacetic acid plus 0.1 mM arginine butyrate. Test agents are at concentrations of 0.1-0.2 mM. Changes over control were significant ($p < 0.05$, paired t-test and chi-square).

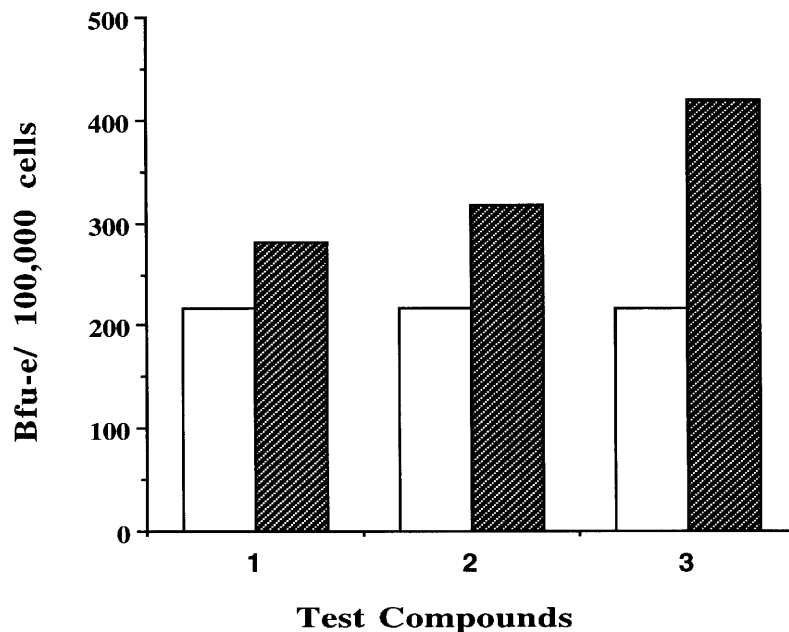
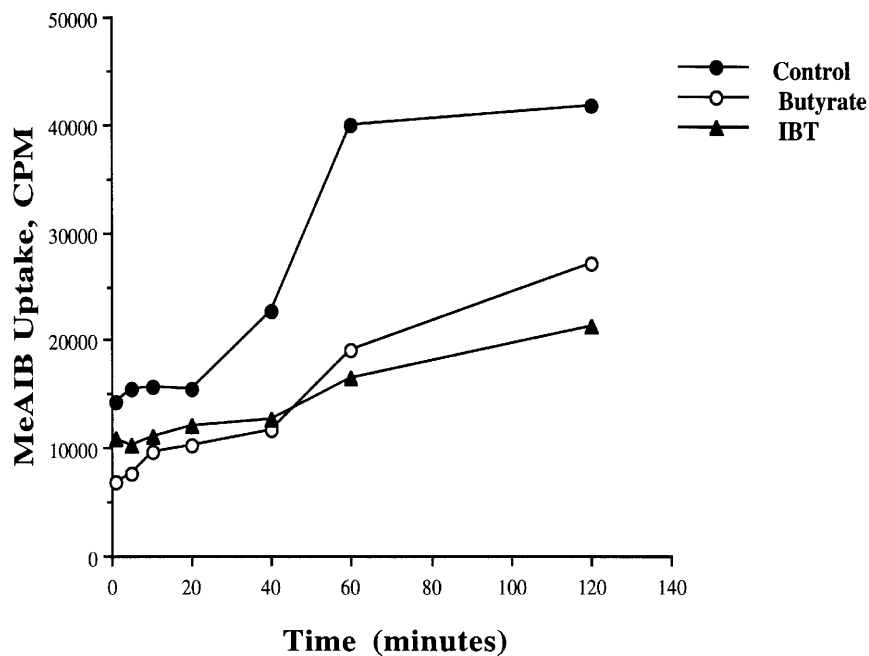


Figure 4. Effects of tested compounds on fetal erythroid (Bfu-e) proliferation. Erythroid colony numbers developing from CD34⁺ cells isolated from fetal liver in the presence of a panel of hematopoietic growth factors alone are shown in the light bars; colony numbers from treated cultures are shown in the darker shading. Added compounds were: [1] 2-(4'-methoxyphenoxy)propionic acid; [2] phenoxyacetic acid; [3] α -methyl-hydrocinnamic acid, all at 0.2 mM. All changes above control were significant ($p < 0.025$).

(A)



(B)

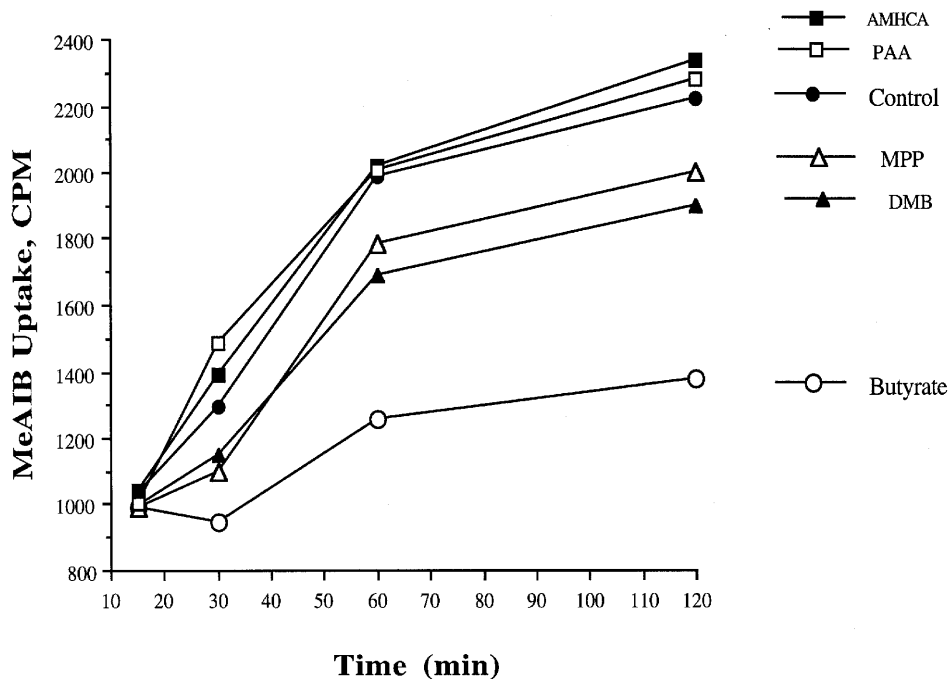


Figure 5. Uptake of a radio-labeled neutral amino acid (3H-methyl-aminoisobutyric acid) into erythroblasts incubated in the presence or absence of the test compounds. (A) Arginine butyrate (AB) and isobutyramide (IBT) (2.0 mM) treatment produced a marked reduction in the transport of this synthetic amino acid, compared to uptake into untreated (Control) fetal liver erythroid cells. (B) Arginine butyrate inhibited amino acid uptake relative to untreated (Control) cells or cells incubated with phenoxyacetic acid (PAA) or α -methylhydrocinnamic acid (AMHCA), which did not inhibit amino acid transport. 2-(4'-methoxyphenoxy) propionic acid (MPP) and 2,2-dimethylbutyric acid (22DMB) treatment produced a moderate inhibition of transport. Compounds were tested at 1 mM.

These results indicate that some of the phenylalkyl and phenoxyacetic acids can stimulate fetal hemoglobin production without toxic effects on erythroid cell growth, and some agents even stimulate hematopoietic colony formation.

Cellular Transport Analyses

Metabolic transport studies revealed striking differences between the effects of arginine butyrate and certain of the test compounds on uptake of neutral amino acids into erythroid cells. While arginine butyrate and isobutyramide inhibited System A pump function by 50-80% compared to untreated cells, α -methylhydrocinnamic acid and phenoxyacetic acid, at the same concentration as arginine butyrate or isobutyramide, did not inhibit this essential transport mechanism (Figures 5A and 5B). Hydrocinnamic acid and 2,2-dimethylbutyric acid produced intermediate levels of inhibition, resulting in 10-20% less uptake of the labeled amino acid substrate than in untreated (control) cells.

Butyrates have also been reported to inhibit the P-glycoprotein (mdr-1) export system (26), leading to increased intracellular accumulation of some drugs and drug metabolites. Evaluation of the P-glycoprotein transport system in K562 erythroid cells revealed only small (2.5-8%) increases in rhodamine accumulation in cells treated with both butyrate and the test compounds relative to untreated K562 cells (data not shown).

The results of these two metabolic transport assays suggest that nutrient deprivation is more likely to explain the erythroid cell growth inhibition resulting from exposure to butyrate than is excessive intracellular accumulation of substrates or drugs due to decreased efflux secondary to inhibition of P-glycoprotein-mediated transport systems.

DISCUSSION

Studies in our laboratory and others have demonstrated that fetal globin gene expression is induced by several fatty acids (1-10, 16). Many fatty acids like butyrate, acetate, and derivatives

such as phenylbutyrate, stimulate fetal globin expression *in vitro*, although requiring concentrations as high as 5-7.5 mM (6, 8). The newly-developed compounds tested here, at concentrations of 0.1-0.2 mM, induced fetal globin synthesis *in vitro* to levels comparable to, or superior to, the levels achieved by other fatty acids at 1 - 5 mM concentrations.

Continuous infusions of high doses of certain simple fatty acids, however, may adversely affect erythroid cell proliferation *in vivo*, and, if continued for prolonged periods, may inhibit or counter-act their beneficial activity in stimulating fetal globin (16). Studies of the molecular actions of butyrate in other cell lineages have provided some insight into how this antiproliferative activity may occur. Butyrate is well-known to cause growth arrest in the late G₁ phase of the cell cycle in a variety of cell types (18-22). One mechanism by which this may occur is through nutrient deprivation, *via* inhibition of the metabolic amino acid transport system which is most closely associated with proliferation, the System A transport system (22-23). Alternatively, butyrate has also been shown to inhibit the P-glycoprotein transport system (26), which could in theory result in the accumulation of toxic levels of drugs or metabolites within erythroid cells, and thereby adversely affect growth. These adverse activities of the butyrates on cellular transport mechanisms could result either in deprivation of essential nutrients or in the accumulation of high intracellular concentrations of drugs, which may all contribute to cell growth arrest. While growth arrest induced by butyrate may initiate apoptosis in tumor cells, the butyrate G₁ arrest is usually reversible in normal cells. It is not known, however, whether fragile thalassemic erythroblasts, in which the normal program of apoptosis is already accelerated due to accumulation of excess toxic unmatched alpha chains, can similarly recover (24).

The two metabolic transport systems which are most important for cellular proliferation and function, one for influx and one for efflux, were therefore examined to determine the effects of the newly-developed fetal-hemoglobin-inducing

compounds in human erythroid cells. In marked contrast to butyrate and isobutyramide, phenoxyacetic acid and α -methylhydrocinnamic acid did not inhibit the transport of neutral amino acids into erythroblasts. Other related compounds, hydrocinnamic acid and 2-(4'-methoxyphenoxy) propionic acid, produced intermediate degrees of System A transport inhibition. Competition experiments demonstrated that this inhibition is not due to competition between these compounds and the amino acid transport substrates (SPP, unpublished). Rhodamine 123 uptake studies demonstrated that the MDR pump function was only slightly decreased (by 6-8%) in K562 cells treated with butyrate or isobutyramide, and the test compounds had similar minimal effects. Thus, the inhibitory effects of butyrate on neutral amino acid transport systems in erythroid cells do not extend to all cellular transport mechanisms. Furthermore, these results suggest that exposure of developing erythroid cells to high levels of butyrate for prolonged periods are likely to result in a relative deprivation of amino acids essential for the synthesis of proteins such as hemoglobin. One prediction from these findings is that intermittent butyrate therapy may be more effective than continuous therapy for raising hemoglobin levels in medical conditions with underlying destruction of erythroid precursors, and this has been observed *in vivo* (15-16).

The utility of fatty acids in stimulating fetal globin expression must thus be balanced against their well-characterized action in inducing cell growth arrest. An orally bioavailable agent which acts at low doses, is metabolically stable and non-toxic, and which stimulates fetal hemoglobin production and erythroid cell proliferation would be particularly beneficial in disorders characterized by apoptosis of developing erythroblasts. The new compounds examined here were effective in stimulating fetal globin expression at 0.1 to 0.2 mM concentrations. In addition, rather than being growth-inhibitory, certain of the new compounds also produced a substantial increase in the number of Bfu-e per culture, as well as an increase in the size of the

individual Bfu-e colonies (not shown). Because this stimulatory effect of the new compounds on γ -globin synthesis and on Bfu-e number occurred in the presence of optimal concentrations of a panel of potent hematopoietic growth factors, and in isolated stem cell cultures from fetal sources which are known to be unresponsive to cytokines (27), this erythropoietic effect is not likely to be mediated through accessory cells or stimulation of growth factor elaboration. In early studies, these new compounds appear to be orally bioavailable and well-tolerated in baboons, and demonstrate the predicted resistance to metabolism (SPP, in preparation).

These results indicate that certain phenylalkyl, phenylacetic, phenoxyacetic acids and derivatives stimulate both progenitor cell proliferation and fetal globin expression. Thus, in contrast to the first and second generations of fatty acid drugs which inhibit erythroid cell metabolism and proliferation at therapeutic concentrations, this combination of cellular growth- and Hb F-stimulatory properties demonstrated by these newly identified compounds should produce a synergistic increase in Hb F-expressing erythroblasts *in vivo*.

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