

Abrogation of IL-3 Requirements and Stimulation of Hematopoietic Cell Proliferation *In Vitro* and *In Vivo* by Carboxylic Acids

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ABSTRACT: Short-chain fatty acids, such as butyrate and propionate, induce fetal globin gene expression and are under clinical investigation in the β -hemoglobinopathies. Limitations of the short-chain fatty acids as therapeutics include their rapid metabolism and a tendency to induce cell growth arrest if administered for prolonged periods. In studies described here, the cellular effects of other inducers of fetal globin, phenoxyacetic acid and derivatives of short-chain fatty acids and cinnamic acids, were investigated in the human erythroid cell line K562, the IL-3 dependent multi-lineage cell line (32D), and in mice and primates. Several test compounds supported 32D cell proliferation despite a 50-fold depletion of IL-3, which resulted in growth arrest and apoptotic death in control cells. The degree of proliferation induced by certain test compounds was similar to the degree of proliferation induced by Erythropoietin and G-CSF in the cells. Eight of ten compounds induced γ globin mRNA in K562 cells. A 2.5 to 6-fold increase in reticulocytosis was observed *in vivo* in mice treated with two prototype compounds. Pharmacokinetic studies of three prototype compounds demonstrated millimolar plasma concentrations after single oral doses for many hours in primates. These findings identify orally bioavailable compounds which induce γ globin gene expression and hematopoietic cell proliferation through an activity which partially abrogates requirements for IL-3. Such compounds provide potential for oral therapeutics which stimulate proliferation of hematopoietic cells of multiple lineages, as well as inducing fetal globin.

Keywords: globin genes, cell proliferation, hematopoiesis, growth factors, mice, primates

INTRODUCTION

The β globin disorders are characterized by mutant or decreased β globin production, and also by accelerated apoptosis in the β -thalassemias (1-5). Reactivation of expression of

the developmentally silenced γ globin gene can compensate for decreased or mutant β globin, and has become one accepted approach to therapy (1-4, 6-7). Butyrate can stimulate γ globin production when given on an intermittent basis (8-20). However, butyrate tends to accelerate

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apoptosis by inducing growth arrest in the G1 phase of the cell cycle, which may compromise its beneficial effects on γ globin gene expression if given continuously (9,21-26). Furthermore, butyrate must be infused intravenously due to its rapid metabolism through beta oxidation. Phenylbutyrate has similarly been effective in inducing fetal globin in some patients, but also requires administration of large doses due to its relatively short half-life (27-28). Orally bio-available γ globin stimulants which do not inhibit hematopoietic cell growth and are resistant to beta oxidation would be useful for therapeutic application in these conditions.

In the studies described here, compounds which have been synthesized or selected for resistance to beta oxidative metabolism and glucuronidation were investigated for their effects in stimulating γ globin gene expression in a human erythroid-like cell line and for their effects on cell growth utilizing a multi-lineage murine hematopoietic cell line, 32D. This cell line is dependent on IL-3 for growth and terminally differentiates in the presence of G-CSF or erythropoietin (29-31). 32D cells undergo apoptotic cell death in the absence of IL-3 and do not proliferate when IL-3 concentrations are reduced by 50-fold below the levels required for maximal proliferation (29,30). No condition or growth factor has previously been found to abrogate the IL-3 dependency of this cell line (32). In the presence of IL-3 depletion, 32D cells also terminally differentiate along the erythroid lineage in the presence of erythropoietin or terminally differentiate into mature granulocytes in the presence of G-CSF (29-30, 33). Some test compounds which stimulated γ globin expression also supported proliferation of this multi-lineage cell line and prevented apoptotic cell death when IL-3 was withdrawn. *In vivo* activity was also found with a prototype test compound administered to mice. Finally, half-lives of several hours were found when three prototype compounds were orally administered to baboons, demonstrating their resistance to beta oxidation and potential therapeutic utility.

MATERIALS AND METHODS

Treatment of K562 Cells and Analysis of Globin mRNA

K562 cells which were kindly provided by Dr. George Atweh were cultured with 10% fetal bovine serum (Sigma, St. Louis, MO) and RPMI media (Grand Island Biological Company, Grand Island, New York) in a humidified atmosphere with 5% CO₂/95% air. Compounds were tested at a final concentration of 1 mM at neutral pH and included butyric acid, phenoxyacetic acid, dimethylbutyric acid, alpha-methylhydrocinnamic acid, 4-methoxyocinnamic acid, cis and trans 2-methoxycinnamic acid, phenylpropionic acid, DL β -amino-n-butyric acid, 2 methylhydrocinnamic acid, 3-(3,4 dimethoxyphenyl) propionic acid and 2,5- and 3,4-(dimethoxyphenyl) acetic acid (all from Aldrich Chemical Company, St. Louis, MO). After 3 days of culture with these agents, mRNA was purified and α , β , and γ globin mRNA was analyzed by primer extension using oligonucleotide primers and quantitation on a PhosphoImager as previously described (34-36). A representative autoradiogram and a summary of the globin expression induced by the effective compounds is shown in Figure 1 and Table 1.

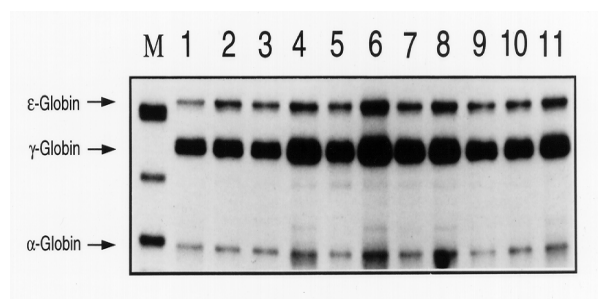


Figure 1. Primer extension analysis of globin mRNA demonstrates increases in γ globin mRNA was induced over constitutive levels in untreated control K562 cells (Lane 1), by treatment with butyric acid (Lane 2), phenoxyacetic acid (Lane 3), α methylhydrocinnamic acid (Lane 4), 2,2 dimethylbutyric acid (Lane 5), trans 2-methoxyhydrocinnamic acid (Lane 6), 2-methylhydrocinnamic acid (Lane 7), cis 2-methoxyhydrocinnamic acid (Lane 8), 3,4-(dimethoxy phenyl) acetic acid (Lane 9), 3-(3,4 dimethoxyphenyl) propionic acid (Lane 10) and 2, 5-(dimethoxyphenyl) acetic acid (Lane 11).

Table 1. Effect of Compounds on Fetal and Alpha Globin mRNAs in K562 Cells

Compound ^b	Radioactivity ^a		
	Fetal Globin (γ)	Alpha Globin (α)	γ/α
Control	915479	118789	7.7
Arginine butyrate	2176523	296132	7.3
Phenoxyacetic acid	2755891	507148	5.4
α -Methylhydrocinnamic acid	1648056	92979	17.7
2,2-Dimethylbutyric acid	1697936	178751	9.5
trans- 2-Methoxycinnamic acid	957146	36751	26.0
2-Methylhydrocinnamic acid	1388899	89473	15.5
cis-2-Methoxycinnamic acid	2255627	105452	21.4
(3,4-Dimethoxyphenyl)acetic acid	1206529	106875	11.3
3-(3,4-Dimethoxyphenyl)propionic acid	1858358	191985	9.7
(2,5-Dimethoxyphenyl)acetic acid	1240100	85941	14.4

^a Radioactivity was determined by phosphorimager.

^b Compounds were tested at a final concentration of 1 mM.

Proliferation Studies Using 32D Cells

32D cells were cultured in RPMI media with 10% fetal bovine serum (Sigma, St. Louis, MO), 100 mM glutamine (GIBCO), and murine IL-3 (20 U/ml) (Biosource International, Camarillo, CA). Growth factor controls used included the standard concentration of IL-3 required for proliferation of 32D cells (25 U/ml) and a 50-fold lower concentration (0.5 U/ml), alone and with erythropoietin (3U/ml) or G-CSF (100 U/ml), (Amgen, Thousand Oaks, CA). Additional compounds studied, besides those listed above, included dimethyl hydroxy acetic acid, dimethyl propionic acid, dimethyl phenoxyacetic acid, dimethyl methoxyacetic acid (all from T.E. Nesby Corporation, Fresno, CA), dihydrocinnamic acid, and β aminohydrocinnamic acid (Sigma Chemical Company, St. Louis, MO). The test compounds were added separately at final concentrations of 1 mM. As a cell density of $2.5 \cdot 10^5$ is necessary for growth of this cell line, this density was maintained by passing the cells at three day intervals into fresh media with supplements and test compounds or by

concentrating the cells if apoptosis occurred. Proportions of cells which were viable or apoptotic, and the fraction of cells in each part of the cell cycle was assessed by incubating the cells with Trypan blue and enumeration, and with propidium iodide staining and FACScan analysis on days 1, 3, 5, and 9 of culture using established methods (37, 38).

In Vivo Administration in Mice

To determine if two prototype compounds have *in vivo* activity in stimulating erythropoiesis, α methylhydrocinnamic acid and dimethylbutyric acid were individually administered to C57 black mice according to regulations of the Committee on Animal Care at the University of Southern Alabama, as previously described (16,39). Test compounds were administered by intraperitoneal injection three times per day for seven days at a total daily dose of 300 mg/kg. Blood (50 ml) was sampled from the retro-orbital space and reticulocytes were quantitated by staining with 1% brilliant cresyl blue and counting the percentage of

reticulum positive cells in 1000 cells. Reticulocytes were compared to control mice which were injected with the same volume of normal saline and which received a 50 ml daily phlebotomy for 21 days without a significant change in hematocrit or a significant increase in reticulocyte counts (B. Pace, unpublished observations).

Pharmacokinetic Studies in Baboons

Baboons were cared for according to regulations of the Committee on Animal Care at the University of Oklahoma Health Sciences Center. The baboons were maintained with chronic indwelling venous and arterial catheters which were maintained with sterile technique for blood sampling as previously described (15). Compounds were administered by nasogastric tube and blood was collected to determine drug plasma levels at regular intervals following single oral doses. The test compounds were analyzed after ether extraction of the plasma, separation by HPLC, and quantitated by comparison to a spiked internal standard of heptanoic acid according to established methods (40).

RESULTS

Effects of the test compounds on globin gene expression were assessed by comparing the ratios of γ globin: α globin mRNA. The ratio of γ globin mRNA in treated cells were compared to γ globin mRNA in control cells, and was adjusted for quantity of mRNA using α globin. γ : α globin mRNA ratios increased up to 26-fold greater than that in untreated (control) K562 cells in the presence of 7/10 test compounds, as shown in Table 1. The most active compounds in stimulating γ globin compared to control cells were phenoxyacetic acid and 2-methylhydrocinnamic acid. Curiously, phenoxyacetic acid also increased the amount of α globin by more than 3-fold over untreated K562 cells, which typically have an α thalassemic phenotype. Two cinnamic acid derivatives decreased the amount

of α globin compared to control cells (Table 1). The compounds which produced the highest γ : α globin mRNA ratios (corrected for amount of mRNA) are α methylhydrocinnamic acid, 2-methoxycinnamic acid, and 2-methylhydrocinnamic acid. Phenoxyacetic acid increased γ globin approximately 3-fold above control levels and α globin by 4-fold. These results are consistent with previous findings that phenoxyacetic, dimethyl fatty acids, and cinnamic acid compounds stimulate γ globin expression in erythroid progenitors cultured from human subjects and from CD34+ cells isolated from fetal liver (25).

Under culture conditions containing recombinant murine IL-3 at 25 U/ml, the optimal concentration for proliferation of 32D cells, apoptosis was detected in less than 10% of the cell population and 32D cells doubled after 3 days. However, 80% of the 32D cells underwent apoptosis (assessed by propidium iodide staining) when IL-3 levels were decreased by 50-fold, from 25 U/ml to 0.5 U/ml, and all of the 32D cells underwent apoptosis in the absence of any IL-3 (Figure 2). In the presence of 0.5 U/ml IL-3 and addition of erythropoietin or G-CSF, cell proliferation occurred along the erythroid and myeloid pathways respectively with cell numbers increasing by 2-3 fold over 5 days, shown in figure 2, consistent with previous reports. Addition of certain of the test compounds at 1 mM with the same low level of IL-3 produced a 2.5 - 3-fold increase in cell proliferation above that observed with the marginal IL-3 concentration alone. In the presence of phenoxyacetic acid, α methylhydrocinnamic acid, dimethylbutyric acid, DL- β amino-n-butyric acid and dimethyl hydroxyacetic acid, cell proliferation increased 2 to 3-fold despite IL-3 depletion (0.5 U/ml), a similar degree of proliferation as was induced by Erythropoietin or G-CSF under the same conditions. In contrast, addition of 1 mM Butyrate with the low concentration of IL-3 resulted in cell death. These findings are shown in figure 2.

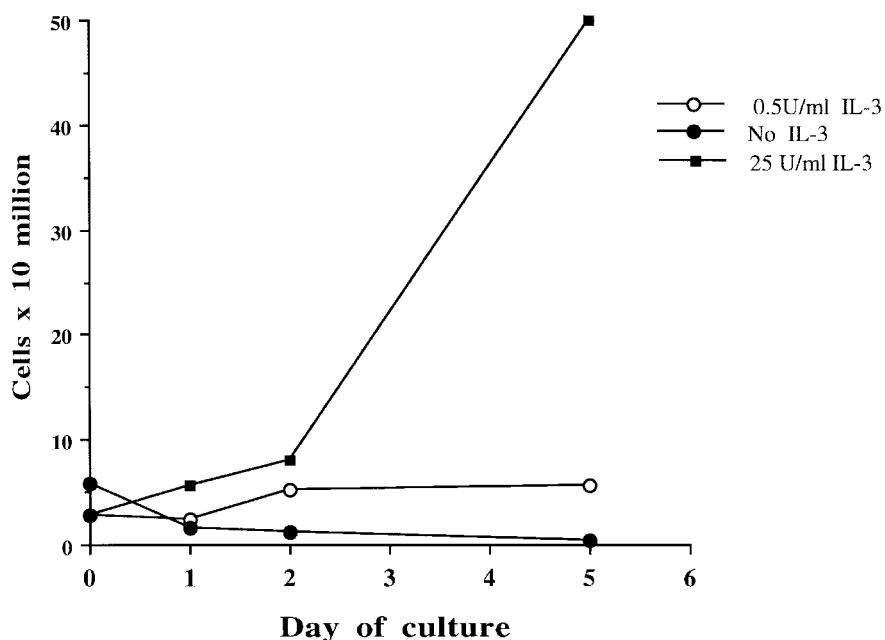


Figure 2. A. Comparison of proliferation of 32D cells in the presence of optimal IL-3 (50 U/ml), IL-3 depletion (0.5 U/ml) and in the absence of IL-3, which results in uniform cell death by apoptosis.

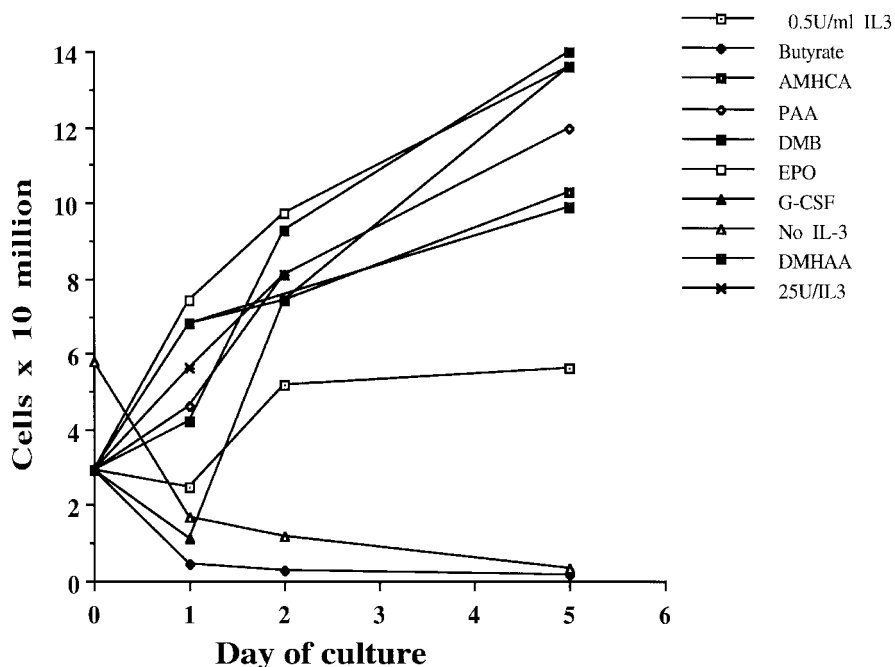


Figure 2. B. Comparison of proliferative rates of IL-3 dependent 32D cells in the presence of low (0.5 U/ml) IL-3 alone and with erythropoietin (3 U/ml), G-CSF (100 U/ml), and 1.0 mM phenoxycetic acid (PAA), α -methylhydrocinnamic acid (AMHCA), dimethylbutyric acid (DMB), butyrate, dimethylhy-droxyacetic acid (DMHAA). Withdrawal of IL-3 and addition of butyrate to 32D cells in (0.5 U/ml) IL-3 condition resulted in cell death. Addition of test compounds resulted in continued cell proliferation at rates similar to those induced by erythropoietin or G-CSF.

To determine oral bioavailability, pharmacokinetic studies of certain prototype test compounds were performed in juvenile baboons. Millimolar plasma levels were detected following single oral doses of phenoxyacetic acid, dimethylbutyric acid, and α -methylhydrocinnamic acid and these levels persisted for 7 hours or longer. Calculated half-lives ($t_{1/2}$) were 6.5, 6.8, and 7 hours respectively, following doses of 100-500 mg/kg (Figure 3). These peak plasma levels are higher than the concentration of compound which was required for γ globin stimulation in primary hematopoietic cells *in vitro* (25).

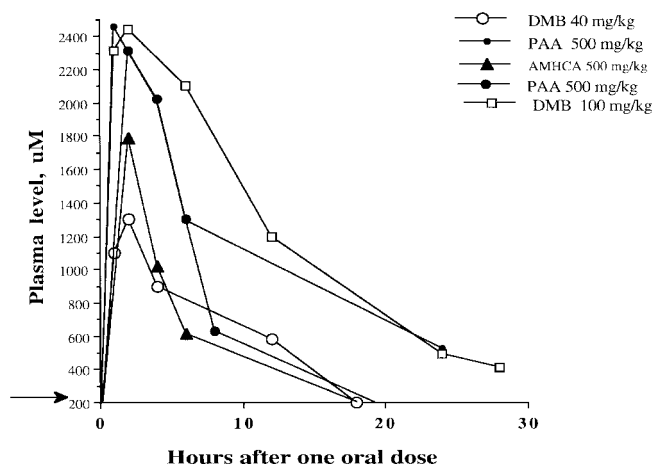


Figure 3. Pharmacokinetics in primates after oral administration of single doses of phenoxyacetic acid (PAA), α -methylhydrocinnamic acid (AMHCA), and dimethylbutyric acid (DMB). Plasma levels persisted in a range well above the concentration (0.2mM) which is necessary for hematologic activity *in vitro*, (shown by the arrow), for at least six hours.

To determine how general the effects of these compounds may be, two prototype compounds, α -methylhydrocinnamic acid and dimethylbutyric acid, were also administered to mice. Administration of the compound resulted in a 2.5- to 6-fold (250-600%) increase in reticulocytes over baseline (Figure 4). Reticulocytosis was observed in a step-wise manner and in a time-frame consistent with the time required for development and maturation of late and early murine erythroid progenitors (3 and

6 days, respectively). Reticulocytes increased by only 6-8% after 21 days of saline-injections in control mice phlebotomized to the same (50 ml/day) degree, and hematocrits did not change in controls over this time frame (B. Pace, unpublished observations).

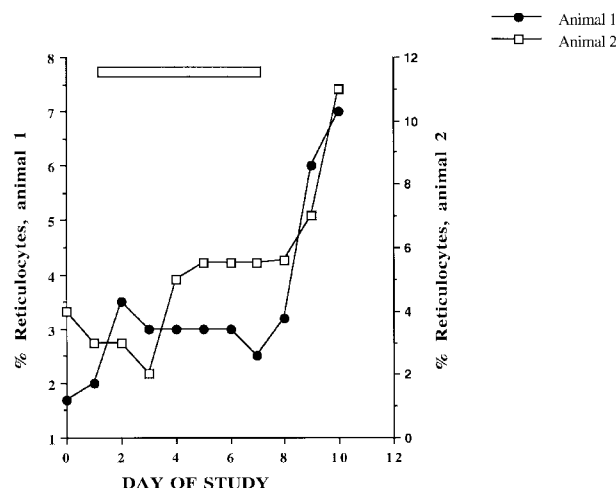


Figure 4. Induction of reticulocytosis in C57 mice treated from days 1-7 with α -methylhydrocinnamic acid (300 mg/kg/day), designated by the horizontal bar. Increases of 2.5-fold and 6-fold over baseline reticulocytes was observed. A similar increase was not observed in controls which were similarly handled and treated with saline injections.

DISCUSSION

Suppression or inhibition of erythropoiesis and general hematopoiesis in a dose-dependent fashion are limitations of the butyrates and hydroxyurea, respectively, in the treatment of the β -hemoglobinopathies. Further disadvantages of the butyrates as optimal therapeutics include their lack of oral bioavailability and their extremely rapid metabolism *in vivo*. The current studies arose from our search to identify novel orally-bioavailable compounds with long plasma half-lives, which induce γ globin gene expression without simultaneously inducing cell growth arrest. Extensive investigation of agents which affect hematopoiesis during the past decade has focused on multipotential hematopoietic growth factors which stimulate proliferation of multiple

lineages, the lineage-specific growth factors erythropoietin and G-CSF, the differentiating agents DMSO, butyric acid, retinoic acid, and HMBA, and inhibitory factors, such as TGF- β and IFN- γ . Previous comparison of the effects of butyric acid, which inhibits erythroid proliferation and α amino-n-butyric acid, which slightly stimulates erythroid progenitor growth (13), suggested that simple compounds with slight modifications may also modulate erythroid cell growth. The findings herein now demonstrate that several classes of simple short-chain fatty acid derivatives, with specific modifications in structure, stimulate the proliferation of hematopoietic cells and can decrease the requirements for the multipotential growth factor IL-3. Abrogation of the IL-3 requirements of 32D cells by substances other than IL-3 has not been previously found (32). As these simple compounds diffuse into cells freely without requiring receptors and diffuse into mitochondria, the compounds may exert their growth stimulating activities through metabolic pathways, through traditional signaling pathways, and/ or through transcriptional regulation of growth-related genes. Further studies are required to determine the exact mechanisms of action, whether certain compounds may induce primarily differentiation or proliferation or both, and which compounds, if any, act solely on specific hematopoietic lineages.

The pattern of globin gene stimulation induced in K562 cells by some of these compounds is complex, in that certain compounds (butyric and phenoxyacetic acid) stimulated expression of both α and γ globin mRNA, which may represent an effect of inducing differentiation of these cells. Other compounds (2-methoxyhydrocinnamic acid) curiously decreased expression of α globin, which accentuates the K562 α thalassemic phenotype. Such an effect would not be deleterious in human β -thalassemia, and might even serve to improve globin chain balance more than simply stimulating γ globin expression

would. Phenoxyacetic acid induced both γ globin mRNA as well as cellular proliferation. Such compounds particularly merit further investigation for future consideration as therapeutics of the β -thalassemias, as the accelerated erythroid apoptosis characteristic of these diseases severely limits the time-frame during which any γ globin stimulant can act to improve globin chain balance before cell death occurs.

Several of the derivatized compounds studied here would not undergo rapid metabolism *in vivo*, as do the simple fatty acids. The phenoxyacetic and phenylakyl acids and the dimethylated carboxylic acid derivatives were synthesized or selected for their structural resistance to usual routes of metabolism *in vivo*. Two of these prototype compounds, α methylhydrocinnamic acid and dimethylbutyric acid, did indeed have erythropoietic activity when administered to mice, and three compounds have prolonged half-lives in the baboon after oral administration. This result is significant because mice have higher rates of metabolism than do primates, including humans, and because even higher doses of butyrate were previously not active Hb F-inducers in mice, although they were active in primates (13, 44). These and similar compounds, particularly with modifications at the fourth position of a phenyl group and the 2,2 dimethyl substituted carboxylic acids, are attractive as hematopoietic stimulants and as fetal hemoglobin-inducing agents. Further investigation of their safety and efficacy *in vivo* appear warranted.

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