In both sickle cell (SS) anemia and β-thalassemia (β-thal), an increase in fetal hemoglobin (HbF) ameliorates the clinical symptoms of the underlying disease. Several pharmacologic agents have been used to elevate HbF levels in adults; however, concerns regarding adverse effects of the prevailing drugs raise an urgent need for other agents capable of stimulating HbF production. We show here that sodium phenylacetate (NaPA) and its precursor, sodium 4-phenylbutyrate (NaPB), can enhance HbF production in cultured erythroid progenitor derived from normal donors and patients with SS anemia or β-thal, when used at pharmacologic concentrations. Treatment resulted in (1) reduced cell proliferation, (2) elevated hemoglobin (Hb) content per cell (mean cellular Hb [MCH]), and (3) an increased proportion of HbF produced, associated with elevated levels of γ-globin mRNA. Moreover, the active phenyl-fatty acids, with NaPA as a prototype, potentiated HbF induction by other drugs of clinical interest, including hydroxyurea (HU), sodium butyrate, and 5-azacytidine (5AzAC). Efficacy could be further enhanced by introducing chlorine substituents at the phenyl ring to increase drug lipophilicity. Our findings indicate that NaPA and NaPB, both already proven safe and effective in treatment of children with urea cycle disorders, might benefit also patients with severe hemoglobinopathies. The two-phase liquid culture procedure used in this study should prove valuable in further studies exploring the mechanisms of HbF induction by these agents, and might provide an assay to predict patient response in the clinical setting.

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MATERIALS AND METHODS

Patients. The study group included patients with homozygous SS disease or \( \beta \)-thal who had been admitted to the Clinical Center of the National Institutes of Health (NIH) for routine evaluation, or normal blood donors from the Department of Transfusion Medicine (NIH). After informed consent was obtained, approximately 20 to 25 mL of blood was obtained for erythroid cell culture studies (see below). The diagnosis of SS anemia or \( \beta \)-thal was made on the basis of (1) hemoglobin electrophoresis on alkaline cellulose acetate and on acid citrate dextrose; (2) peripheral blood examination; and, occasionally, (3) DNA and RNA analysis of bone marrow aspirates. When possible, diagnosis was confirmed by family studies. Routine hematologic profiles were performed on a Coulter Model S (Hialeah, FL).

Erythroid cell cultures and reagents. The two-phase liquid culture procedure has been previously described in detail.24,25 Briefly, peripheral blood mononuclear cells were isolated by centrifugation on a gradient of Ficoll-Hypaque and cultured for 7 days (phase I) in \( \alpha \)-minimal essential medium supplemented with 10% fetal calf serum (FCS) (both from Gibco, Grand Island, NY), 1 \( \mu \)g/mL cyclosporine A (Sandoz, Basel, Switzerland), and 10% conditioned medium collected from bladder carcinoma 5637 cultures.27 In phase II, the nonadherent cells were recultured in \( \alpha \)-medium supplemented with 30% FCS, 1% deionized bovine serum albumin, \( 1 \times 10^{-5} \) mol/L 2-mercaptoethanol, 1.5 mmol/L glutamine (unless otherwise indicated), \( 1 \times 10^{-4} \) mol/L dexamethasone, and 1 U/mL human recombinant Epo (Ortho Pharmaceutical, Raritan, NJ). These cultures yielded up to \( 10^9 \) erythroid cells per milliliter blood. Cell viability was determined by Trypan Blue exclusion. Phenylacetic acid, 4-phenylbutyric acid, p-chlorophenylacetic acid, and butyric acid (Sigma, St Louis, MO) were dissolved in distilled water and brought to pH 7.0 by the addition of NaOH. 5-AzaC and HU were purchased from Sigma, and PAG was a gift from S. Brusilow (Johns Hopkins, Baltimore, MD).

Determination of erythroid maturation and Hb synthesis. Differentiation was assessed morphologically by preparing cytocentrifuge slides (Shandon, Cheshire, UK) stained with alkaline benzidine and Giemsa. The number of Hb-containing cells was determined using the benzidine-HCl procedure.28 Hbs were characterized and quantitated by cation exchange high-performance liquid chromatography (HPLC) of cell lysates as previously described.29 Total Hb in lysates prepared from a known number of Hb-containing (benzidine-positive) cells was measured using either the tetramethylbenzidine procedure30 (Sigma kit, catalog no. 527) or by cation exchange HPLC (measuring the total area under the chromatogram). Standard Hb solutions (Isolab, Akron, OH) were used for reference. Mean cellular Hb (MCH) was calculated by dividing the total Hb content of the lysate by the number of benzidine-positive cells.

Northern blot analysis and DNA probes. Cytoplasmic RNA was separated on 1% agarose-formaldehyde gels. RNA isolation, gel electrophoresis, transfer onto Nytran membranes (Schleicher & Schuell, Keene, NH), hybridization with radiolabeled DNA probes, and autoradiography (Kodak x-ray film XAR, Rochester, NY) have been previously described.31 The human globin cDNA probes included JW101 (\( \alpha \)), JW102 (\( \beta \)), and a 0.6-kb EcoRI/HindIII fragment of the 3′ end of human G-\( \gamma \)-globin gene. Probes were labeled with \( [32P]dCTP \) (New England Nuclear, Boston, MA) using a random primed DNA labeling kit (Boehringer, Mannheim, Germany).

RESULTS

Effect of NaPA and NaPB on cell growth and Hb production. Addition of NaPA or NaPB to phase II erythroid cultures resulted in reduced cell proliferation (Fig 1 and 2), with no apparent change in cell viability. Cytoplasmasis was associated with a decline in total Hb produced per culture; however, both Hb content per cell (MCH) and the proportion of HbF (%HbF) increased after treatment (Fig 2). The extent of changes observed was dose- and time-dependent: the earlier the drugs were added during the second phase of growth, the higher was the increase in %HbF; however, cell yields were proportionally decreased. For example, addition of 5 mmol/L NaPA to normal precursors on day 2 caused an approximately 90% decrease in cell number along with a 12-fold increase in %HbF, as determined on day 13. When treatment was initiated on day 6, cell number decreased only by 60% compared with controls, and %HbF increased 3.3-fold. To obtain sufficient cells for further analysis, subsequent experiments involved the addition of drugs on days 6 and 7, and cells were harvested on day 13. Under these conditions, results were reproduced in cultures derived from six normal donors, as well as four patients with SS anemia and four patients with \( \beta \)-thal (Fig 3). NaPA (5 mmol/L) and NaPB (2.5 mmol/L) caused a significant increase in both MCH (38% to 100%) and the proportion of
PHENYL-FATTY ACIDS STIMULATE FETAL HEMOGLOBIN

HbF produced. In the case of homozygous SS patients, %HbF was elevated 2.0- to 4.1-fold (mean, 3.0-fold) by 5 mmol/L NaPA, and 3.2- to 5.6-fold (mean, 4.0-fold) by 2.5 mmol/L NaPB. The latter was associated with a 12% ± 3% decrease in HbS levels, with no change in HbA2 (Fig 4).

Induction of fetal globin gene expression. As in K562 cells,19 increased HbF production by NaPA or NaPB in primary cultures of normal SS cells appears to be due to pretranslational regulation of γ-globin expression. Northern blot analysis (Fig 5) showed a dose-dependent increase (up to fivefold) in the steady-state levels of γ-globin mRNA, accompanied by a slight decrease (less than twofold) in the amount of β-globin transcripts. There was no change in α-globin expression.

Effect of PAG and glutamine depletion. PAG, the end-metabolite of both NaPB and NaPA, is formed by phenylacetate conjugation to glutamine with subsequent excretion in the urine.22,23 The latter leads to depletion of circulating glutamine. It was of interest, therefore, to examine the effects of PAG and of glutamine starvation on erythroid proliferation and HbF accumulation. PAG was found to be inactive, as previously observed in other experimental models.18,31 Since glutamine conjugation takes place in the kidney and liver, and may not occur in the cultured erythroid precursors, the in vivo conditions were mimicked by growing phase II cultures in medium without or with varying concentrations of glutamine. In contrast to that observed with K562 cells,18 glutamine starvation of the nonmalignant erythroid cells had no significant effect on either cell growth or HbF production, nor did it enhance the efficacy of NaPA (data not shown). The differences in responses of the malignant versus nonmalignant cells are likely to be due to differences in glutamine metabolism (see Discussion).

Design of active analogs and combination treatment protocols. Focusing on NaPA as a prototype drug, we found that chloride substituents at the phenyl ring, which enhance drug lipophilicity and antitumor activities (Samid et al, unpublished data), also increase the efficacy of HbF stimulation in the erythroid precursors. For example, we found that treatment of cultures derived from a normal donor with 3 mmol/L p-chlorophenylacetate caused 58.9% inhibition of cell proliferation, and a 3.9-fold increase in %HbF (from 0.92 ± 0.1 to 3.6 ± 0.25% HbF). In this case, up to 6 mmol/L NaPA was needed to produce a comparable effect.

We next examined the effect of NaPA in combination with other drugs of clinical interest, ie, HU, sodium butyrate, and 5AzaC. When used alone in cultures derived from normal donors (HbF base levels of 0.8% to 2.0%), NaPA (5 mmol/L) and HU (0.05 mmol/L) increased %HbF by 3.5- and 2.0-fold, respectively; the combination of the two resulted in a 4.7-fold increase in HbF. NaPA also augmented HbF stimulation by butyrate (0.5 mmol/L) (from 3.1- to 7.15-fold), and of 5AzaC (2 μmol/L) (from 2.5- to 6.6-fold).

The results, in agreement with previous observations,19 indicate that addition of NaPA to suboptimal, nontoxic doses of the other drugs can potentiate HbF production. The various treatment regiments produced significant cytostasis (data not shown); however, there was no significant change in cell viability, as determined by Trypan Blue exclusion.

DISCUSSION

There is a considerable interest in NaPA and its precursor, NaPB, as potentially nontoxic novel therapies for cancer19,31,32 and inborn β-chain hemoglobinopathies.19,20,33 Our data demonstrate the enhancement of HbF production by these phenyl-fatty acids in cultured erythroid cells derived from normal individuals and patients with SS disease and β-thal. Both NaPA and NaPB, used at pharmacologically attainable concentrations, promoted erythroid matura-

![Graph A](image)

**Figure 2.** Time-dependent changes in cell proliferation and Hb production. NaPA (5 mmol/L) was added on days 2, 4, 6, and 8 of phase II cultures derived from normal donors, and the cells were analyzed on day 13. (A) Number of Hb-containing cells per milliliter (X 10^4) (●), and the amounts of Hb (picograms) per cell (MCH) (□). (B) Total Hb (micrograms) per milliliter culture (a), and %HbF (●). Data points represent the means of four determinations. The deviation of results of each determination from the mean did not exceed 10%. NaPB at 2.5 mmol/L produced comparable effects (not shown). In all cases, cell viability was greater than 95%.
NaPA and NaPB are closely related compounds. NaPB is metabolized in the mitochondria to NaPA by β-oxidation. NaPA, in turn, can be converted back to NaPB through an enzymatic reaction catalyzed by medium-chain fatty acid elongase. The end-metabolite of both NaPB and NaPA (ie, PAG), is formed in the liver and kidney by conjugation of NaPA to glutamine with subsequent excretion in the urine. Both NaPA and NaPB were found to increase total Hb accumulation per cell (MCH) and the proportion of HbF (%HbF) in a dose- and time-dependent manner. NaPB, the more lipophilic of the two, was more active on a molar basis. By contrast, neither PAG nor glutamine depletion affected Hb synthesis. Consistent with induction of terminal differentiation, treatment with NaPB/NaPA led to reduced cell proliferation, yet no change in cell viability was observed at the concentrations used. Preliminary data, focusing on NaPA as a prototype drug, indicate that drug potency can be further enhanced by introducing chlorine substituents to the phenyl ring. Analogs with halogen substitutions have increased lipophilicity, improved uptake by cells, and enhanced potency also as antitumor agents (Samid et al, manuscript in preparation).

Increased HbF production in treated cells did not require the mediation of accessory cells (eg, macrophages, lymphocytes, or stromal cells) or specific growth factors (eg, granulocyte-macrophage colony-stimulating factor) that enhance HbF production in the progeny of early erythroid progenitors. Therefore, it appears that responses to NaPA/NaPB depend on drug interaction with relatively late erythroid precursors that are already engaged in Hb production.

The effects of NaPA/NaPB on HbF were reproduced in cultures of cells derived from normal individuals who exhibit low HbF levels, as well as from patients with hemoglobinopathies and elevated HbF amounts. However, there was considerable heterogeneity in responses. While two thalassemic patients had more than a threefold increase in %HbF after treatment with 5 mmol/L NaPA, cells from two other patients showed only 1.3- and 1.5-fold increases. These findings are reminiscent of the clinical experience with HU; significantly increased HbF production was found in approximately 75% of the HU-treated SS anemia patients. No molecular, biochemical, cellular, or clinical parameter was found to predict the outcome of treatment. In ongoing studies, we are now comparing the response to HU of cultured cells derived from patients with hemoglobinopathies to their clinical responses to determine the predictive value of the two-phase liquid culture system. Interestingly, our preliminary findings (unpublished) indicate that cells derived from SS patients with poor response to HU in vivo and in vitro do respond to NaPB with a significant induction of HbF, thus offering a possible alternative to those who fail to respond to HU therapy.

The mechanisms of HbF stimulation by NaPB and NaPA are not known. Accumulation of HbF in adults could result from a direct effect on γ-globin genes. Molecular analysis showed marked upregulation of γ-globin, as indicated by up
PHENYL-FATTY ACIDS STIMULATE FETAL HEMOGLOBIN

Fig 4. Effect of NaPA on the proportions of Hb species in cultured erythroid precursors derived from a patient with SS anemia. NaPA was added to 7-day phase II cultures. The cells were harvested and lysed on day 13, and the proportions of HbF (●), HbA2 (□), and HbS (■) were determined following separation on cation exchange HPLC.

Drug-mediated changes in Hb could be secondary to a reversible block of cell cycle and/or a change in the kinetics of cell maturation. The effect of NaPA and NaPB on erythroid proliferation and HbF production resembles that observed with cell-cycle drugs such as 5AzaC, vinblastine, and cytarabine. In inducing cytostasis, the drugs could select out a minor, preexisting subpopulation of F cells with growth advantage. This mechanism may be particularly effective for cells derived from patients with β-thal, where F cells may be resistant to ineffective erythropoiesis, i.e., premature death of the erythroid precursors due to imbalance in globin chain synthesis. Drug-induced cytosis in vivo might potentially ameliorate the erythroid hyperproliferative effects associated with major thalassemia syndromes (i.e., massive marrow expansion, hyperuricemia, hypermetabolic state).

The promise of nontoxic inducers of HbF production for treatment of β-chain hemoglobinopathies was recently demonstrated in clinical studies with the fatty acid butyrate. In a short-term trial with arginine butyrate administered by continuous intravenous infusions to patients with SS disease or major β-thal, Perrine et al. documented a significant and rapid increase in γ-globin synthesis, with no significant adverse effects. The levels of HbF achieved should ameliorate the clinical symptoms of the underlying diseases.

The tolerance and efficacy of the phenyl-fatty acids,
NaPB and NaPA, in patients with β-globin disorders is yet to be determined. Clinical experience with NaPA, involving patients with either urea-cycle disorders or lysinuric protein intolerance,\textsuperscript{38,39} indicates that long-term treatment with high drug doses (250 to 550 mg/kg/d; phenylacetate plasma levels, 3 to 6 mmol/L) is well tolerated by both infants and adults. Oral NaPB, substituting for NaPA, which has an offensive odor, was shown to be safe at similarly high doses,\textsuperscript{23} and capable of inducing HbF in the urea-cycle patients.\textsuperscript{20} The demonstrated stimulation of HbF production, lack of adverse clinical effects, and easy oral administration make NaPB an attractive candidate for use (alone or in combination with other agents) in treatment of severe inborn β-chain hemoglobinopathies. Preliminary clinical studies focusing on SS disease are encouraging, as they confirm that oral NaPB can increase F-cell numbers in these patients without obvious significant adverse effects.\textsuperscript{40}

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Enhanced fetal hemoglobin production by phenylacetate and 4-phenylbutyrate in erythroid precursors derived from normal donors and patients with sickle cell anemia and beta-thalassemia

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