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 (5AzA-C). 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.

© 1993 by The American Society of Hematology.

Enhanced Fetal Hemoglobin Production by Phenylacetate and 4-Phenylbutyrate in Erythroid Precursors Derived From Normal Donors and Patients With Sickle Cell Anemia and β-Thalassemia

By Eitan Fibach, Premakala Prasanna, Griffin P. Rodgers, and Dvorit Samid

Expression of Globin Genes is tightly regulated during ontogeny. In humans, globin production is characterized by two major "switches": production of embryonic hemoglobins (Hbs) switches after the first 2 months of gestation to the production of two types of fetal Hb (HbF) and then again, at birth, to the adult Hbs (HbA and HbA). Various genetic abnormalities, including deletion of a portion of the globin gene cluster, or point mutations within or outside of the cluster, have been described that increase HbF synthesis in postnatal life. An increase in HbF is frequently observed in the inherited hemoglobinopathies, sickle cell (SS) anemia and β-thalassemia (β-thal) syndromes, and can be acquired, as observed in juvenile chronic myelogenous leukemia or acute erythroidic stress.

Increased HbF levels ameliorate the clinical symptoms associated with SS disease and would be expected to be beneficial in β-thal by reducing the imbalance in α-to non-α-globin chain. In SS anemia, HbF-containing red blood cells (F cells) have lower concentrations of sickle Hb (HbS), and HbF directly inhibits polymerization of HbS, accounting for the lower propensity of such cells to form intracellular polymers and cause pathophysiologic consequences.

Several pharmacologic agents have been used to stimulate HbF synthesis in vivo. Initial studies involving 5-azacytidine (5AzA-C) or hydroxyurea (HU) treatment of baboons and of patients with β-thal or SS anemia showed considerable stimulation of HbF production. However, concerns regarding potential carcinogenesis by 5AzA-C and dose-limiting myelotoxities of HU have led to intensive search for less toxic drugs that would stimulate HbF production in the adult. Along these lines, promising effects have been observed with butyrate and derivatives, as well as erythropoietin (Epo) in combination with agents such as HU.

Recent studies have focused on sodium phenylacetate (NaPA) and its precursor, sodium 4-phenylbutyrate (NaPB), as potential therapeutic agents for treatment of β-chain hemoglobinopathies with low toxicity. Our laboratory first demonstrated that NaPA, used at pharmacologically attainable, well-tolerated concentrations (3 to 6 mmol/L), can promote HbF biosynthesis in human K562 leukemic cells that carry two copies of a nonfunctional β-globin gene. Subsequently, patients with urea-cycle disorders treated with NaPB were shown to have significantly higher percentage of F cells compared with normal subjects. On ingestion by humans, NaPB is converted by β-oxidation to NaPA; NaPA, in turn, conjugates glutamine to form phenylacetylglutamine (PAG). It was of interest therefore, to explore further the activity of these compounds in erythroid progenitors derived from normal donors and from patients with severe inborn hemoglobinopathies.

Our present studies exploited a novel, two-phase liquid culture procedure yielding large (up to 5 × 10⁸) and pure (95% to 98%) populations of erythroid cells, in which cell biology and Hb production closely mimic that occurring under physiologic conditions, and which responds to agents known to affect HbF levels. The results indicate that both NaPB and NaPA, but not PAG, stimulate HbF production in primary cultures derived from normal donors, as well as from SS anemia and β-thal patients.

From the Department of Hematology, Hadassah University Hospital, Jerusalem, Israel; Laboratory of Chemical Biology, National Institute of Diabetes and Digestive and Kidney Diseases, and Clinical Pharmacology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD.

Submitted March 23, 1993; accepted June 11, 1993.

Supported in part by Elan Pharmaceutical Corp Grant No. G174ED, and by the Israel Science Foundation administered by The Israel Academy of Sciences and Humanities.

Address reprint requests to Dvorit Samid, PhD, Clinical Pharmacology Branch, National Cancer Institute, Bldg 10, Room 12C103, Bethesda, MD 20892.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.

Blood, Vol 82, No 7 (October 1), 1993: pp 2203-2209

2203
MATERIALS AND METHODS

Patients. The study group included patients with homozygous SS disease or β-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 β-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 α-minimal essential medium supplemented with 10% fetal calf serum (FCS) (both from Gibco, Grand Island, NY), 1 µg/mL cytosporine A (Sandoz, Basel, Switzerland), and 10% conditioned medium collected from bladder carcinoma 5637 cultures.27 In phase II, the nonadherent cells were recultured in α-medium supplemented with 30% FCS, 1% deionized bovine serum albumin, 2 mmol/L 2-mercaptoethanol, 1.5 mmol/L glutamine (unless otherwise indicated), 1 × 10^{-6} 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. 5AzA 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.25 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 procedure25 (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.30 The human globin cDNA probes included JW101 (α), JW102 (β), and a 0.6-kb EcoRl/HindIII fragment of the 3' end of human G-γ-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. Cytosis 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 6 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 β-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...

![Fig 1. Dose-response of cultured erythroid precursors to NaPB and NaPA. Cells derived from peripheral blood of normal donors were cultured in the two-phase liquid culture system. Drugs were added on day 6, and the number of viable cells per milliliter (×10^4) was determined on day 13. The cultures were then harvested and lysed. Hb proteins chromatographed, and %HbF calculated (σ). Data points represent the mean ± SE of six cultures derived from different donors. No changes in cell viability were observed with NaPA doses as high as 10 mmol/L, or with NaPB at 3 mmol/L.](www.bloodjournal.org)
PHENYL-FATTY ACIDS STIMULATE FETAL HEMOGLOBIN

Fig 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 (× 10⁴/ml), and the amounts of Hb (picograms) per cell (MCH) (B) Total Hb (micrograms) per milliliter culture (a), and %HbF (b). 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%.

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, NaPA (5 mmol/L) increased HbF production by NaPA or NaPB in primary cultures of normal of 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 phenyl-

acetate conjugation to glutamine with subsequent excretion in the urine. 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. 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, 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 chlorine 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 5AzA. 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 5AzA (2 μmol/L) (from 2.5- to 6.6-fold). The results, in agreement with previous observations, indicate that addition of NaPA to suboptimal, nontoxic doses of the other drugs can potentiate HbF production. The various treatment regimens 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 as its precursor, NaPB, as potentially nontoxic novel therapies for cancer and inborn β-chain hemoglobinopathies. 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 maturation with increased fetal γ-globin expression, and potentiated the activity of other agents of clinical interest, including HU, 5AzA, and butyrate.
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 (i.e., 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 (e.g., macrophages, lymphocytes, or stromal cells) or specific growth factors (e.g., 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

![Fig 3. Stimulation of HbF production by erythroid cells derived from normal individuals and patients with SS anemia or β-thal. NaPA was added to 7-day phase II cultures and the proportion of HbF determined on day 13. %HbF was determined by HPLC analysis. Data points represent mean ± SE of four determinations.](image-url)
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.

to fivefold increased steady-state mRNA levels in cells treated with NaPA or NaPB. The latter was accompanied by a decline in the amount of β-globin transcripts (less than twofold), while α-globin expression was unchanged. Activation of the fetal globin gene could be secondary to removal of negative trans-acting proteins from the γ-globin promoter region, or to exposure of regulatory elements to positive transcriptional factors. NaPA, which was shown to cause hypomethylation in plant36 and murine cells (Prasanna et al, unpublished data), could affect DNA-protein interactions subsequent to demethylation of γ-globin genes.

It is well documented that the γ-globin genes are methylation-dependent and can be readily activated by hypomethylating drugs (e.g., 5AzaC).8-11 The mechanisms of γ-globin activation by the phenyl-fatty acids are currently under investigation.

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.37 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.3 Drug-induced cytostasis 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.18 In a short-term trial with arginine butyrate administered by continuous intravenous infusions to patients with SS disease or major β-thal, Perrine et al18 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,

Fig 5. Increased levels of γ-globin mRNA in cell treated with NaPA or NaPB. Drugs were added to 7-day phase II cultures derived from a normal donor (lanes 1 to 3) and from a SS patient (lanes 4 and 5). Cytoplasmic RNA (20 μg/mL) was extracted 4 days later and subjected to Northern blot analysis. Blots were probed consecutively with 32P-labeled cDNA probes for γ-, β-, and α-globins, and exposed to x-ray film for 3 days, 6 hours, and 1.5 hours, respectively. Untreated controls (lanes 1 and 4): NaPA 5 and 10 mmol/L (lanes 2 and 3, respectively); NaPB 2.5 mmol/L (lane 5). Relative changes in globin expression induced by NaPA in cells derived from a SS patient (not shown) were comparable to that in lanes 1 through 3.
NaPB and NaPA, in patients with $\beta$-globin disorders is yet to be determined. Clinical experience with NaPA, involving patients with either urea-cycle disorders or lysinuric protein intolerance, 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 and capable of inducing HbF in the urea-cycle patients. 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 $\beta$-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.

ACKNOWLEDGMENT

We thank E.A. Rachmilewitz, A.N. Schechter, and C.E. Myers for their interest and support of these studies, A. Treves for culturing cells from thalassemic patients, and A. Agarwal for excellent technical assistance.

REFERENCES

10. Charache S, Dover G, Smith K, Talbot CC, Moyer S: Treatment of sickle cell anemia with 5-azacytidine results in increased fetal hemoglobin production and is associated with nonrandom hypomethylation of DNA around the $\gamma$-delta globin complex. Proc Natl Acad Sci USA 80:4842, 1983
26. Fibach E, Burke LP, Schechter AN, Rodgers GP: Hydroxyurea increases fetal hemoglobin in cultured erythroid cells derived from normal and patients with sickle cell anemia or $\beta$-thalassemia. Blood 81:1630, 1993
34. Loo YH, Miller KA, Nowlin J, Horning MG: Identification


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

E Fibach, P Prasanna, GP Rodgers and D Samid