Induction of Erythroid Differentiation and Fetal Hemoglobin Production in Human Leukemic Cells Treated With Phenylacetate

By Dvorit Samid, Annie Yeh, and Premakala Prasanna

There is considerable interest in identifying nontoxic differentiation inducers for the treatment of various malignant and nonmalignant blood disorders, including inborn β-chain hemoglobinopathies. Using the human leukemic K562 cell line as a model, we explored the efficacy of phenylacetate, an amino acid derivative with a low toxicity index when administered to humans. Treatment of K562 cultures with pharmacologically attainable concentrations of phenylacetate resulted in erythroid differentiation, evident by the reduced growth rate and increased hemoglobin production. The effect was time- and dose-dependent, further augmented by glutamine starvation (phenylacetate is known to deplete circulating glutamine in vivo), and reversible upon cessation of treatment. Molecular analysis showed that phenylacetate induced γ globin gene expression with subsequent accumulation of the fetal form of hemoglobin (HbF). Interestingly, the addition of phenylacetate to antitumor agents of clinical interest, eg, hydroxyurea and 5-azacytidine, caused superinduction of HbF biosynthesis. The results suggest that phenylacetate, used alone or in combination with other drugs, might offer a safe and effective new approach to treatment of some hematopoietic neoplasms and severe hemoglobinopathies. This is a US government work. There are no restrictions on its use.

It has long been recognized that pharmacologic manipulation of the kinetics of cell growth and differentiation could benefit cancer patients. More recently, it became apparent that similar treatments might also have a beneficial effect in patients with β-chain hemoglobinopathies, due to erythroid maturation with increased fetal hemoglobin (HbF) synthesis. Several antitumor drugs, including hydroxyurea (HU), 5-azacytidine (5AzaC), 5-aza-2'-deoxycytidine (5AzadC), vinblastine, arabinosylcytosine (ara-C), methotrexate, myleran (busulfan), and adriamycin, can increase the production of HbF in experimental models. Moreover, HU and 5AzaC were shown to be active in patients with sickle cell anemia and severe β thalassemia. However, concerns regarding toxic and potential carcinogenic effects of the prevailing antitumor drugs raise the need to identify safe alternatives for long-term treatment of inborn nonmalignant diseases. The accumulation of HbF in adults is thought to be due to changes in the kinetics of erythroid differentiation rather than a direct effect on the fetal globin genes. According to this hypothesis, other agents that can induce differentiation would be expected to affect HbF production. We focus here on the efficacy of a novel nontoxic differentiating agent, phenylacetate.

Phenylacetate is a naturally occurring plasma component capable of conjugating glutamine to yield phenylacetylglutamine (PAG), which is subsequently excreted in the urine. The latter, leading to waste nitrogen excretion, has been the basis for using sodium phenylacetate (NaPA) in the treatment of hyperammonemia associated with inborn errors of urea synthesis or liver failure. Clinical experience indicates that acute or long-term treatment with high doses of NaPA (250 to 550 mg/kg/day, plasma levels 3 to 6 mmol/L) is well tolerated by both infants and adults, essentially free of adverse effects, and effective in reducing plasma glutamine levels. The lack of toxicity of NaPA is not limited to individuals with these metabolic disorders, as documented with normal volunteers. Our laboratory has recently found that NaPA, when used at the pharmacologic concentrations, can affect the maturation of various animal and human cell types in vitro without affecting cell viability. For example, treatment of promyelocytic leukemia HL-60 cells resulted in the rapid decline of myc oncogene expression followed by growth arrest and granulocyte differentiation. Phenylacetate also induced highly efficient adipocyte conversion in mesenchymal C3H 10T1/2 cultures. Unlike the differentiating chemotherapeutic 5AzadC, phenylacetate was neither cytotoxic nor did it cause neoplastic transformation in susceptible cells. The lack of toxicities and the ability to induce cellular differentiation prompted us to examine the effect of NaPA on erythroid maturation and HbF biosynthesis.

The experimental system involved the human leukemic K562 cells, which carry a nonfunctional β globin gene, but produce low levels of the fetal γ globin and of HbF. The K562 cell line was originally established from a patient with chronic myelogenous leukemia in the blast cell transformation, and has since been extensively used as a model in studies of erythroid differentiation and regulation of γ globin gene expression. We show here for the first time that pharmacologically attainable concentrations of NaPA can promote HbF biosynthesis in human cells, and can cause superinduction when combined with other chemotherapeutic agents of interest, ie, HU, 5AzaC, and 5AzadC.

MATERIALS AND METHODS

Cell culture and reagents. Human leukemia K562 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO Laboratories, Grand Island, NY), 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mmol/L L-glutamine, unless otherwise indicated. The suspension cultures were kept in exponential growth phase by diluting every 3 to 5 days.
with fresh medium. Cell viability was determined by trypan blue exclusion. Phenylacetic acid, 4-hydroxyphenyl acetic acid, 3,4-dihydroxyphenyl acetic acid, 2,5-dihydroxyphenyl acetic acid (Sigma, St Louis, MO), and PAG (BRI, Houston, TX) were dissolved in distilled water, and brought to pH 7.4 by the addition of NaOH. 5AzadC, 5AzaC, and HU (Sigma) were also dissolved in distilled water. All drug stock solutions were stored in aliquots at -20°C until used.

**Determination of Hb production.** K562 cells were seeded at 1 X 10^6 cells/mL and treated with the drugs for 4 to 7 days before assay. Qualitative estimation of Hb production was determined by benzidine staining of intact cells in suspension. The Hb concentration resulted in dose-dependent inhibition of cell proliferation, with the IC50 being approximately 8 mmol/L (Fig 1). No toxicity was observed with concentrations as high as 12 mmol/L (cell viability monitored during the first 5 days of treatment was more than 95%). In addition to the cytostatic effect, NaPA induced erythroid differentiation, as evident by the increased number of benzidine-positive cells (Fig 1) and confirmed by quantitative analysis of Hb production (Table 1). The effect of NaPA on cell growth and differentiation was dependent on continuous presence of the drug; upon cessation of treatment, there was a gradual decline in Hb and increased growth rate, reaching control levels after about 2 weeks.

The effect of NaPA on Hb production could be mimicked by the analogue 4-hydroxyphenylacetate (4HPA) (Table 1). Both NaPA and 4HPA are known to conjugate glutamine in humans.10 By contrast, the glutamine-conjugated form, ie, PAG, had no significant effect on either cell proliferation or Hb accumulation. Two other analogues, 3,4-dihydroxyphenylacetate (3,4HPA) and 2,5-dihydroxyphenylacetate (2,5dHPA), were found to be highly cytotoxic (LD50 of 0.35

![Fig 1. The effect of NaPA on cell growth and differentiation. (C) Total cell number and (I) the fraction of benzidine-positive cells were determined after 4 days of continuous treatment. Data represent means ± SD (n = 4). Cell viability was greater than 95%.](image)

<table>
<thead>
<tr>
<th>Treatment (mmol/L)</th>
<th>Benzidine-Positive Cells Fold Increase</th>
<th>Hb Production pg/Cell Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.2 ± 0.8</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>NaPA 2.5</td>
<td>2.7 ± 0.2</td>
<td>0.49 ± 0.02</td>
</tr>
<tr>
<td>5.0</td>
<td>7.0 ± 0.3</td>
<td>3.15 ± 0.20</td>
</tr>
<tr>
<td>10.0</td>
<td>14.6 ± 0.2</td>
<td>2.40 ± 0.16</td>
</tr>
<tr>
<td>4HPA 5.0</td>
<td>7.2 ± 0.1</td>
<td>3.3 ND</td>
</tr>
<tr>
<td>10.0</td>
<td>14.2 ± 0.5</td>
<td>6.46 ND</td>
</tr>
<tr>
<td>3,4dHPA or 2,5dHPA</td>
<td>0.06 &lt;0.5</td>
<td>ND</td>
</tr>
<tr>
<td>PAG 6.0</td>
<td>2.1 ± 0.5</td>
<td>0.37 ± 0.03</td>
</tr>
</tbody>
</table>

Results were determined after 4 days of treatment. Mean ± SD from two or more experiments with duplicates. Cell viability was greater than 95% in all cases except for cultures treated with 0.6 mmol/L 3,4dHPA or 2,5dHPA (< 5.0%, and 47.8% ± 2%, respectively).

Abbreviation: ND, not determined.

**RESULTS**

The effect of NaPA and analogues on cell growth and differentiation. Treatment of K562 cultures with NaPA resulted in dose-dependent inhibition of cell proliferation, with the IC50 being approximately 8 mmol/L (Fig 1).
and 0.6 mmol/L, respectively) and incapable of inducing erythroid differentiation at lower, nontoxic concentrations.

**Regulation of HbF by NaPA.** KS62 cells normally express low but detectable levels of HbF. Protein analysis using anti-HbF antibodies showed that the increase in total Hb in NaPA-treated cells, measured by protein absorbance, was largely due to induction of HbF. Quantitative analysis by densitometry showed an approximately fourfold increase in the amounts of HbF in cells treated with NaPA 10 mmol/L compared with untreated controls (Fig 2A). The latter was associated with similarly elevated steady-state levels of fetal γ globin mRNA (Fig 2B), indicating a pretranslational regulation.

In humans, NaPA causes depletion of circulating glutamine12 due to conjugation of the amino acid to form PAG, an enzymatic reaction known to take place in the liver and kidney. The in vivo reduction in plasma glutamine levels was mimicked in vitro by culturing the KS62 cells in the presence of lowered glutamine concentrations. In agreement with a previous report,21 glutamine starvation alone affected KS62 growth rate (not shown) and Hb production (Fig 3); the effect was further augmented by the addition of NaPA 5 mmol/L. We speculate therefore that, in humans, NaPA may induce erythroid differentiation and HbF production through both depletion of circulating glutamine and a direct effect on erythroid progenitor cells.

**Cooperative induction of HbF by NaPA and other chemotherapeutic drugs.** Despite promising activities of HU, 5AzaC, and 5AzadC in sickle cell anemia, β-thalassemia, and numerous malignancies, their clinical usefulness has been hindered by unacceptable toxicities.3 Combinations with a nontoxic differentiating agent such as NaPA could enhance the efficacy while minimizing the adverse effects of toxic chemotherapies. As shown in Table 2, the addition of NaPA to suboptimal doses of either HU or the cytidine analogues resulted in efficient erythroid differentiation (evident by the increased number of benzidine-positive cells and Hb accumulation) without affecting cell viability. By immunoprecipitation with anti-HbF antibodies (Fig 4) it was shown that the Hb data presented in Table 2 reflect on the accumulation of the fet al form of Hb. While HbF levels increased 2.96-fold by NaPA 5 mmol/L and 4.61-fold by HU 100 μmol/L, cells treated with both drugs had 11.8-fold more HbF than untreated controls. Taken together, results indicate that NaPA and HU may act synergistically to induce HbF production in KS62 cells. The combination of NaPA and 5AzadC had an additive effect. Further studies, summarized in Fig 5, showed that glutamine depletion markedly enhanced the Hb production in cells treated by NaPA in combination with HU, 5AzaC, or 5AzadC. In all cases, cell viability was greater than 95%.

**DISCUSSION**

Differentiating agents selected for their low cytotoxic/carcinogenic potential could be of value for long-term treatment of various blood disorders. Drug toxicity is a particularly important consideration in the case of severe inborn anemias, in view of the overall health condition and the variable life-span of patients. To date, several therapeutic agents have been evaluated for safety and efficacy.

**Table 2. Potentiation of HU, 5AzaC, and 5AzadC Activity by NaPA**

<table>
<thead>
<tr>
<th>Rx</th>
<th>NaPA</th>
<th>Benzidine-Positive Cells (fold increase)</th>
<th>Hb Production (fold increase)</th>
<th>Cell Growth* (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>1.0</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>+</td>
<td>2.95</td>
<td>2.8</td>
<td>68.7 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>HU</td>
<td>–</td>
<td>6.6</td>
<td>5.3</td>
<td>42.9 ± 1.2</td>
</tr>
<tr>
<td>+</td>
<td>14.5</td>
<td>12.6</td>
<td>31.3 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>5AzaC</td>
<td>–</td>
<td>3.0</td>
<td>2.8</td>
<td>64.8 ± 1.5</td>
</tr>
<tr>
<td>+</td>
<td>5.3</td>
<td>4.85</td>
<td>40.7 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>5AzadC</td>
<td>–</td>
<td>3.3</td>
<td>3.1</td>
<td>55.7 ± 0.3</td>
</tr>
<tr>
<td>+</td>
<td>5.9</td>
<td>5.0</td>
<td>45.1 ± 3.0</td>
<td></td>
</tr>
</tbody>
</table>

KS62 cells were treated for 4 days with either NaPA 5 mmol/L, HU 100 μmol/L, 5AzaC 2 μmol/L, 5AzadC 0.3 μmol/L, or the indicated combinations. At the time of assay, untreated control cultures had 1.0 to 1.6 x 10^5 cells, of which 3.4% ± 0.6% were benzidine-positive and the levels of Hb were 0.29 ± 0.02 pg/cell. *Cell viability was greater than 95% in all cases.
Recombinant human erythropoietin, which proved nontoxic and effective in treatment of anemia associated with chronic renal disease, was unfortunately found to be ineffective in sickle cell anemia. The application of 5-AzaC, an antitumor agent with impressive activity in sickle cell disease, has been hindered by concerns regarding its carcinogenic potential. Another active drug, HU, is difficult to use because of the narrow margin between toxicity and the desired effect on HbF production. By contrast, NaPA, shown here to induce erythroid differentiation and HbF production in vitro, is neither cytotoxic nor carcinogenic at effective concentrations (data presented here, in Samid et al., and in Samid et al., manuscript in preparation). The lack of toxicity to humans has long been established. Studies dating back to 1933 documented that administration of phenylacetate 5 to 10 g to humans for 28 days is free of side-effects. More recent experience with patients suffering from urea cycle disorders indicates that the drug is so well tolerated that chronic treatment can be initiated just a few hours after birth.

Using human leukemic K562 cells as a model, it was shown that NaPA can promote the maturation of erythroid progenitor cells that have an active HbF program. The analogue 4HPA was found to be similarly effective, whereas other analogues, including PAG, 3,4dHPA, or 2,5dHPA, were inactive. The accumulation of HbF in NaPA-treated cells was associated with elevated levels of γ-globin mRNA. NaPA, which was shown to cause hypomethylation in plant and murine cells (our unpublished data), could act through demethylation of γ-globin genes. This hypothesis is currently under investigation. Alternatively, the increase in HbF production may be secondary to a reversible block of cell cycle and a change in the kinetics of cell maturation. Indeed, the kinetics of erythroid maturation induced by NaPA resembles that observed with cell-cycle drugs such as 5-AzaC, vinblastine, and ara-C.

In humans, NaPA (as well as 4HPA) may induce erythroid differentiation also through binding to, and depletion of, circulating glutamine. Glutamine is the major nitrogen source for nucleic acid and protein synthesis, and the substrate for energy in rapidly dividing malignant and nonmalignant cells of the erythroid lineage. Glutamine starvation can block cells in the G1 phase of growth and accelerate the kinetics of differentiation. In patients with elevated plasma glutamine levels (1.08 mmol/L ± 0.5 ± 0.17 mmol/L in controls), NaPA treatment caused a reduction to 0.1 to 0.2 mmol/L. The enzymatic reaction leading to glutamine depletion takes place in the mitochondria of liver and kidney cells, and may not occur in leukemic cells treated in vitro. To mimic the in vivo effect, K562 cells were cultured in medium containing lowered amounts of glutamine. In agreement with previous observations, glutamine starvation alone was sufficient to trigger erythroid differentiation with elevated production of Hb, an effect that was further enhanced by the addition of NaPA.

NaPA potentiated the activity of HU, 5-AzaC, and 5AzaDC in K562 cells. With combination treatments, high levels of HbF may be reached using low doses of toxic drugs. Thus far, attempts were made to achieve such a goal by using the combination of HU with the nontoxic agent erythropoietin in the treatment of sickle cell disease. However, while studies confirmed the efficacy of HU, erythropoietin (whether used alone or in combination with HU) showed no activity. The addition of NaPA to HU caused marked induction of HbF synthesis under conditions in which each drug alone produced only a small effect, the results suggesting that the two agents act in a synergistic manner. The concentrations of HU used in our studies are similar to the plasma levels measured in sickle cell anemia patients after an oral administration of 25 mg/kg. This dose, compared with the more effective HU dosage used in humans...
(50 to 100 mg/kg), causes significantly less marrow/hematopoietic toxicity. According to the experimental data, rather high doses of NaPA (millimoles per liter) might be needed to induce significant amounts of HbF. We speculate that such concentrations may be achieved in humans with no significant adverse effects. Pharmacokinetics studies in children with urea cycle disorders\cite{11,12} showed that phenylacetate plasma levels of 3 to 6 mmol/L are essentially toxicity free. Moreover, in one case involving Reye's syndrome, the patient did remarkably well after phenylacetate blood levels reached 10 to 19 mmol/L (Brusilow SW, personal communication, November 1991). However, there is a need for further pharmacokinetics and toxicology studies in patients that do not suffer from such metabolic disorders.

The combination of NaPA with 5AzaC/5AzadC, although not as effective as that with HU, warrants special consideration. The cytosine analogues can benefit patients with leukemia\cite{20,21} as well as those with β-chain hemoglobinopathies.\cite{5} 5AzaC was shown to be active in both sickle cell anemia and β thalassemia, and proved less cytotoxic and more effective than HU in stimulating HbF production. The profound effect of 5AzaC (or 5AzadC) is thought to be due to its ability to perturb erythropoiesis and to inhibit DNA methylation.\cite{32} Hypomethylation can lead to gene activation and cell differentiation; however, it can also promote oncogenesis and the evolution of cells with metastatic capabilities.\cite{20,21} Our experimental data suggest that NaPA could enhance differentiation and prevent neoplastic transformation induced by the cytosine analogues (Prasanna et al, manuscript in preparation).

In conclusion, it appears that NaPA, acting through glutamine depletion and other yet unidentified cellular mechanisms, may induce differentiation in human leukemic cells, promote the production of erythrocytes expressing HbF, and enhance the efficacy of other antitumor drugs while minimizing their adverse effects. NaPA, which has an unpleasant odor, can be substituted by its pro-drug, sodium phenylbutyrate (NaPB), for oral administration.\cite{26} Upon ingestion by humans, phenylbutyrate undergoes β-oxidation to phenylacetate.\cite{33} Both NaPA and NaPB already proved safe for the treatment of infants and adults.\cite{14,26} It seems important therefore to further evaluate the clinical relevance of our experimental data.

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NOTE ADDED IN PROOF

Since the submission of this manuscript, 15 patients with urea cycle disorders treated with phenylbutyrate were examined for the percentage of red blood cells containing fetal hemoglobin (F cells). The mean percentage of F cells in these patients was significantly higher than that in normal subjects.\cite{34} The data lend further support for the proposed use of phenylbutyrate and its active metabolite, phenylacetate, in treatment of β globin hemoglobinopathies.

REFERENCES

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Induction of erythroid differentiation and fetal hemoglobin production in human leukemic cells treated with phenylacetate

D Samid, A Yeh and P Prasanna