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A Comparative Analysis of the Effects of Sodium Butyrate and Hydroxyurea on Erythroid Differentiation, Cell Proliferation, and Fetal Hemoglobin Production in K562 Cells

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ABSTRACT
This study examines the dose- and time-dependent effects of sodium butyrate (NaB) on cell proliferation, erythroid differentiation, and fetal hemoglobin (HbF) production in human erythroleukemia K562 cells. Building on previous evidence that sodium butyrate influences K562 cell growth and hemoglobin production, the study focuses on determining how different concentrations and durations of NaB exposure affect cellular responses. K562 cells will be exposed to sodium butyrate at concentrations of 0.01, 0.1, and 1.0 mM under single and continuous exposure conditions. Cell proliferation and viability will be evaluated at predetermined time points, and changes in total hemoglobin production will be assessed using spectrophotometric analysis. Fetal hemoglobin production will be evaluated using high-performance liquid chromatography (HPLC). Morphological changes associated with erythroid differentiation will also be examined using scanning and transmission electron microscopy. The study will compare responses across NaB concentrations and exposure durations to identify concentration- and time-dependent patterns in K562 cell proliferation and hemoglobin production. Particular attention will be given to the previously observed response to 0.1 mM NaB, which produced the greatest total hemoglobin production, and to the earlier detection of HbF following exposure to higher concentrations of NaB. The findings are expected to clarify the relationship between sodium butyrate exposure, K562 cell proliferation, erythroid differentiation, and fetal hemoglobin induction. The study may provide additional insight into the potential of sodium butyrate as an inducer of fetal hemoglobin production and establish a basis for further investigation of pharmacological approaches to erythroid differentiation and hemoglobin induction.
Keywords: Sodium butyrate, K562 cells, Erythroid differentiation, Fetal hemoglobin, Hemoglobin production, Cell proliferation, Dose-dependent response, Time-dependent exposure, Erythroleukemia, and Pharmacological induction.
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INTRODUCTION

Sickle cell disease (SCD) is an inherited hemoglobin disorder characterized by the production of abnormal sickle hemoglobin (HbS), which can polymerize under deoxygenated conditions and contribute to red blood cell deformation, hemolysis, vaso-occlusion, recurrent pain, and progressive organ damage **(Ware et al., 2017)**. One of the most important naturally occurring modifiers of SCD severity is fetal hemoglobin (HbF). Unlike HbS, HbF contains γ-globin rather than β-globin chains and does not participate in HbS polymerization, thereby reducing the intracellular concentration of HbS available for polymerization and sickling **(Ware et al., 2017; Estepp et al., 2017)**. Consequently, increased HbF can dilute intracellular HbS and reduce the tendency of red blood cells to sickle. Clinical and experimental evidence has therefore established HbF induction as an important therapeutic strategy for β-hemoglobin disorders (Rodgers et al., 1993; Ware et al., 2017). Higher HbF levels have also been associated with fewer complications and hospitalizations among individuals with sickle cell anemia (Ware et al., 2017; Estepp et al., 2017). According to Ware et al. (2017), hydroxyurea is one of the best-established pharmacological agents for increasing HbF production in individuals with SCD. By increasing HbF and through additional effects on erythroid cells, leukocytes, reticulocytes, and vascular biology, hydroxyurea can reduce vaso-occlusive complications and other manifestations of SCD (Ware et al., 2017). Nevertheless, the search for additional HbF-inducing agents remains important because responses to hydroxyurea vary among individuals and because long-term pharmacological treatment requires continued monitoring. Earlier approaches to HbF induction included agents such as 5-azacytidine and cytarabine, but their cytotoxic properties and clinical limitations stimulated interest in alternative agents capable of activating fetal-globin expression with potentially different toxicity profiles (Constantoulakis et al., 1989).

Sodium butyrate (NaB), a short-chain fatty acid and histone deacetylase inhibitor, emerged as a particularly important candidate because experimental studies demonstrated that butyrate can reactivate fetal-globin gene expression (Constantoulakis et al., 1989). Constantoulakis et al. (1989) demonstrated that sodium butyrate increased the frequency of F-positive erythroid progenitor colonies and stimulated γ-globin expression in erythroid progenitors from individuals with sickle cell anemia and β-thalassemia. The investigators also reported evidence that sodium butyrate acted at the level of globin gene expression rather than simply increasing the overall number of progenitor cells. Earlier experimental observations had established that butyrate could promote erythroid differentiation in leukemic erythroid models, providing an important basis for subsequent investigations of its effects on fetal-globin expression (Andersson et al., 1979; Villeval et al., 1983). Clinical evidence subsequently provided additional support for the potential of butyrate as an HbF-inducing agent. Perrine et al. (1993) administered continuous intravenous arginine butyrate to patients with sickle cell anemia and β-thalassemia for two or three weeks, beginning at 500 mg/kg/day. Fetal-globin synthesis increased in all six treated patients, with increases ranging from 6% to 45% above pretreatment levels; the proportion of F-reticulocytes approximately doubled, while γ-globin mRNA increased approximately two- to six-fold (Perrine et al., 1993). These findings demonstrated that butyrate could stimulate fetal-globin expression in humans and provided an important foundation for subsequent investigation of butyrate-based approaches to hemoglobin induction (Perrine et al., 1993). The clinical relevance of butyrate exposure was further supported by pharmacokinetic investigations showing that sodium and arginine butyrate could be administered in vivo, although their pharmacokinetic properties and metabolic handling were important considerations for therapeutic application (Daniel et al., 1989). Subsequent clinical studies of related butyrate compounds, including sodium phenylbutyrate, also demonstrated increases in fetal hemoglobin in patients with sickle cell disease, further supporting the broader concept that butyrate-related compounds can stimulate HbF production (Dover et al., 1992, 1994).

The cellular mechanisms underlying the response to sodium butyrate are particularly relevant to studies using human erythroleukemia K562 cells. K562 cells are a well-established experimental model for investigating erythroid differentiation because they can be induced to acquire erythroid characteristics and synthesize hemoglobin. Early studies of erythroleukemic cells and their derivatives demonstrated that these cells could undergo differentiation-related changes under appropriate experimental conditions (Orkin et al., 1975). Subsequent work specifically established the erythroid properties of K562 cells and demonstrated that agents such as hemin, butyrate, and 12-O-tetradecanoylphorbol-13-acetate could alter their erythroid phenotype (Villeval et al., 1983). Previous research has demonstrated that sodium butyrate can induce erythroblastic differentiation and increase hemoglobin accumulation in K562 cells, with evidence implicating Giα2 signaling in the differentiation response (Davis et al., 2000). Additional research has shown that sodium butyrate can induce cell-cycle arrest and erythroid differentiation in K562 cells, including changes associated with p18INK4C expression (Li et al., 2008). Sodium butyrate has also been shown to influence signaling pathways involved in erythroid differentiation, including inhibition of ERK and activation of p38 MAP kinase pathways (Witt et al., 2000). Collectively, these findings indicate that the biological effects of NaB extend beyond direct modulation of globin gene expression and may involve coordinated alterations in cell-cycle regulation, intracellular signaling, chromatin structure, and erythroid differentiation.

The therapeutic rationale for increasing HbF is particularly important because clinical studies have demonstrated a relationship between HbF concentration and disease severity in SCD. Hydroxyurea therapy can increase HbF and improve clinical outcomes, while erythropoietin has also been investigated as an adjunct capable of augmenting the HbF response to hydroxyurea (Rodgers et al., 1993). Evidence from pediatric populations suggests that achieving clinically meaningful HbF thresholds during hydroxyurea therapy is associated with important clinical benefits (Estepp et al., 2017). These findings reinforce the importance of identifying cellular mechanisms and experimental agents capable of enhancing fetal-globin production. At the same time, the historical development of HbF-inducing therapies illustrates the need to balance globin induction with cellular viability, treatment tolerability, and sustained biological activity. Thus, investigation of NaB in an experimental erythroid model remains relevant for understanding how concentration and duration of exposure influence the relationship between cellular proliferation and hemoglobin-associated differentiation.

The present study extends this body of research by examining the concentration- and time-dependent effects of sodium butyrate on K562 cell proliferation and hemoglobin-associated differentiation. Specifically, K562 cells were exposed to 0.01, 0.1, and 1.0 mM NaB under single- and continuous-exposure conditions. Cell growth was monitored over time, while total hemoglobin was assessed spectrophotometrically and HbF was examined using cation-exchange high-performance liquid chromatography (HPLC). A separate experiment

compared hydroxyurea alone with a combination of hydroxyurea and 0.1 mM NaB. The experimental design therefore permitted assessment of whether increasing NaB concentrations and longer exposure periods were associated with changes in cellular proliferation, total hemoglobin production, and the timing of detectable HbF production. The central purpose of the study was to determine whether sodium butyrate could promote hemoglobin-associated erythroid responses while maintaining viable K562 cell growth. Particular attention was given to whether an intermediate concentration of NaB could produce a more favorable balance between proliferation and hemoglobin production than a higher concentration. This question is important because an agent that increases HbF or total hemoglobin but simultaneously causes substantial cellular loss may have limited biological usefulness. Accordingly, the study considered cell growth, total hemoglobin, and HbF as complementary outcomes rather than interpreting hemoglobin induction independently of cellular proliferation.

LITERATURE REVIEW

Fetal Hemoglobin and Sickle Cell Disease

Fetal hemoglobin is an important biological modifier of sickle cell disease because HbF interferes with the polymerization of HbS. HbF contains α2γ2 rather than α2βS2, and the γ-globin chains do not participate in the polymer formation responsible for red blood cell sickling (Rodgers et al., 1993; Ware et al., 2017). Consequently, individuals with higher HbF levels generally experience less severe disease manifestations. Experimental and clinical evidence has demonstrated that pharmacological stimulation of HbF production can therefore represent an important approach to reducing the consequences of SCD (Rodgers et al., 1993; Ware et al., 2017). The importance of HbF is also demonstrated by observations among children receiving hydroxyurea. In the HUSTLE cohort, children who achieved HbF levels greater than 20% experienced fewer hospitalizations and lower odds of vaso-occlusive pain and acute chest syndrome than children with lower HbF levels (Estepp et al., 2017). These findings reinforce the biological rationale for investigating compounds capable of increasing HbF production.

Pharmacological Induction of HbF

Hydroxyurea is currently a major pharmacological strategy for inducing HbF in SCD. Its ability to increase HbF contributes to reduced HbS polymerization and fewer vaso-occlusive complications. However, the response to hydroxyurea is not uniform, and its effects extend beyond HbF induction. Consequently, researchers have continued to investigate alternative compounds that may stimulate fetal-globin expression through different cellular mechanisms (Ware et al., 2017). Earlier investigations examined DNA-demethylating and cytotoxic agents such as 5-azacytidine and cytarabine. Constantoulakis et al. (1989), for example, compared butyrate with these approaches and reported that sodium butyrate induced HbF in erythroid progenitors and acted synergistically with 5-azacytidine, although the same synergistic effect was not observed with cytarabine. The findings suggested that sodium butyrate could influence γ-globin expression through effects on globin genes or chromatin structure.

Sodium Butyrate and Fetal-Globin Expression

Sodium butyrate has received considerable attention because of its capacity to stimulate fetal-globin expression in experimental systems and humans. In erythroid progenitors obtained from patients with HbSS and β-thalassemia, sodium butyrate increased HbF expression and increased the proportion of cultured erythroblasts producing HbF (Constantoulakis et al., 1989). Perrine et al. (1993) subsequently provided clinical evidence that butyrate could stimulate fetal-globin production in patients with β-hemoglobin disorders. Continuous intravenous arginine butyrate administration produced measurable increases in fetal-globin synthesis, F-reticulocytes, and γ-globin mRNA. These findings were particularly important because they demonstrated that the fetal-globin response observed in experimental systems could also occur in patients.

Sodium Butyrate and K562 Erythroid Differentiation

The K562 human erythroleukemia cell line provides a useful experimental model for studying erythroid differentiation and hemoglobin synthesis. Earlier research demonstrated that K562 cells possess erythroid characteristics and can produce fetal and embryonic hemoglobins. Butyrate treatment increased hemoglobin-associated characteristics in these cells, although the response did not represent complete terminal differentiation

(Villeval et al., 1983). Subsequent studies provided stronger evidence that sodium butyrate can promote erythroid differentiation in K562 cells. Davis et al. (2000) reported that sodium butyrate induced K562 cells to differentiate toward the erythroid lineage and acquire the capacity to synthesize hemoglobin. Their findings also implicated Gαi2 signaling in the differentiation process. Similarly, Li et al. (2008) demonstrated that sodium butyrate induced G0/G1 cell-cycle arrest and erythroid differentiation and identified increased p18INK4C expression as part of the response. Other mechanistic investigations have suggested that sodium butyrate influences erythroid differentiation through intracellular signaling pathways. Witt et al. (2000) reported decreased ERK and JNK phosphorylation and increased p38 phosphorylation following butyrate treatment of K562 cells. Inhibition of p38 activity abolished butyrate-induced hemoglobin expression, suggesting that p38 signaling contributes to the erythroid response. Together, these studies indicate that sodium butyrate does not simply increase hemoglobin through a single pathway but may influence cell-cycle progression, chromatin regulation, intracellular signaling, and erythroid gene expression.

Research Gap

Although previous studies **(such as Constantoulakis et al., 1989; Perrine et al., 1993; Davis et al., 2000; Witt et al., 2000; Li et al., 2008)** have established that butyrate can stimulate fetal-globin expression and erythroid differentiation, important questions remain concerning the relationship between NaB concentration, duration of exposure, cellular proliferation, total hemoglobin accumulation, and the timing of HbF detection. A higher concentration may produce a more rapid differentiation-associated response but may also reduce cellular proliferation or survival. Conversely, a lower or intermediate concentration may maintain cellular growth while producing a delayed but potentially stronger hemoglobin response. This study addresses this issue by examining three NaB concentrations—0.01, 0.1, and 1.0 mM—under both single and continuous exposure conditions. The study also compares NaB with hydroxyurea and evaluates their combination. This design provides an opportunity to distinguish between the timing of HbF induction and the overall biological effectiveness of treatment. The study's methodology specifically incorporated cell-growth measurements, total hemoglobin determination, and HPLC-based HbF detection to provide complementary measures of the cellular response.

METHODOLOGY

This study employed an experimental laboratory design to investigate the effects of sodium butyrate (NaB) on the growth and hemoglobin production of human erythroleukemia K562 cells. The experimental design was structured to evaluate cellular responses across three concentrations of NaB (0.01, 0.1, and 1.0 mM) under both single-exposure and continuous-exposure conditions. Hemin at a concentration of 25 μM was incorporated as a positive control for cellular induction, while untreated K562 cells served as the control group. A separate experiment evaluated hydroxyurea (HU) at 1 μM alone and in combination with 0.1 mM NaB. The principal outcomes were changes in K562 cell growth and total hemoglobin production, while fetal hemoglobin (HbF) production was subsequently assessed using cation-exchange high-performance liquid chromatography (HPLC). The experimental procedures were designed to permit comparisons of cellular responses according to NaB concentration, treatment condition, and duration of exposure.

Cell Culture

Human erythroleukemia K562 cells were maintained as suspension cultures in RPMI 1640 culture medium supplemented with 10% fetal calf serum and antibiotics. Cultures were maintained under standard controlled cell-culture conditions and were periodically diluted with fresh culture medium to maintain the cells in the exponential phase of growth. Before initiation of the experimental treatments, K562 cells were collected and prepared for exposure by centrifugation, washing with phosphate-buffered saline (PBS), and resuspension in appropriate culture medium. Cell numbers were determined using a hemacytometer, and cell viability was assessed using the Trypan Blue Exclusion Test. This procedure allowed viable cells to be distinguished from nonviable cells before the cells were assigned to the respective experimental treatment groups.

Sodium Butyrate Treatment

K562 cells were exposed to NaB at concentrations of 0.01, 0.1, and 1.0 mM. These concentrations were selected to examine the effects of increasing NaB exposure on K562 cell growth and hemoglobin-associated responses. Two experimental exposure conditions were used: single exposure and continuous exposure. In the

single-exposure experiment, cells received NaB once and were subsequently monitored for changes in cell growth over a seven-day period. Cell growth was assessed on Days 1, 2, 3, 4, 5, 6, and 7 to characterize the temporal response following a single exposure. The untreated K562 cells served as the control, while cells treated with 25 μM hemin served as the positive-control group. For the continuous-exposure experiment, K562 cells were maintained under repeated exposure to the designated concentrations of NaB and monitored for 21 days. Cell growth was evaluated on Days 1, 3, 7, 10, 14, 18, and 21. This extended exposure period was used to determine whether repeated exposure to NaB produced a different pattern of cellular growth from that observed following a single treatment. The use of both exposure conditions allowed the study to distinguish short-term responses from responses associated with prolonged or repeated NaB exposure.

Hydroxyurea and Combination Treatment

A separate experiment was conducted to examine the effects of hydroxyurea and its combination with NaB on K562 cell growth and hemoglobin production. Cells were exposed to 1 μM HU alone or to a combination of 1 μM HU and 0.1 mM NaB. The treatment groups included untreated K562 cells, 1 μM HU, 0.1 mM NaB, and the combined treatment of 1 μM HU with 0.1 mM NaB. Cell growth was evaluated after 1, 3, and 7 days of exposure. The purpose of this experiment was to determine whether simultaneous exposure to HU and NaB produced a greater cellular response than either treatment alone. The 0.1 mM NaB concentration was selected for the combination experiment because the preceding NaB experiments demonstrated a comparatively strong cellular response at this concentration.

Preparation of Treatment Groups

K562 cells were initially placed in culture flasks at approximately 0.3×10^5 cells per flask. Treatment solutions were prepared at the designated concentrations and added to the appropriate experimental cultures. Control cultures received an equivalent volume of culture medium without the test compound. For continuous-exposure experiments, treatment-containing medium was supplied to the appropriate cultures according to the designated exposure schedule. All cultures were maintained under standard cell-culture conditions throughout the experimental period. At the end of each designated exposure period, cells were collected and prepared for the appropriate analysis. Depending on the experimental endpoint, collected cells were used for cell counting, total hemoglobin determination, or HPLC analysis of hemoglobin fractions. The treatment structure allowed cellular growth and hemoglobin-related outcomes to be evaluated at corresponding exposure periods.

Determination of Cell Growth

Cell growth was determined by measuring the number of viable K562 cells following each designated exposure period. After incubation, cells were collected and washed with PBS before being resuspended for cell counting. Cell numbers were determined using a hemacytometer, while cell viability was evaluated using the Trypan Blue Exclusion Test. Cells capable of excluding the dye were considered viable, whereas cells that incorporated the dye were considered nonviable. For the single-exposure experiment, cell growth was evaluated over Days 1–7 among the control, hemin, 0.01 mM NaB, 0.1 mM NaB, and 1.0 mM NaB groups. For the continuous-exposure experiment, cell growth was assessed over Days 1–21 using the same treatment conditions. The analysis focused on the direction, timing, and relative magnitude of changes in viable cell numbers following exposure. Particular attention was given to periods of increasing and decreasing cell growth because the observed K562 growth pattern did not follow a consistently linear trajectory.

Determination of Total Hemoglobin

Total hemoglobin production was determined in K562 cells following exposure to NaB, HU, and the combined NaB-HU treatment. Cells were collected at the designated exposure periods, washed with PBS, and pelleted by centrifugation. The resulting cell pellets were lysed by resuspension in double-distilled water. The cell lysates were subsequently prepared for spectrophotometric analysis. For ultraviolet-visible spectrophotometric analysis, the cell lysates were centrifuged and the resulting supernatants were analyzed over a wavelength range of 350–650 nm. Hemoglobin-associated absorbance was evaluated at approximately 414 nm. Total hemoglobin production was examined at Days 1, 3, and 7, allowing comparisons among the control and NaB treatment groups, as well as among the HU and combination-treatment groups. The spectrophotometric measurements provided an estimate of total hemoglobin associated with the treated K562 cell populations.

Fetal Hemoglobin Analysis

Fetal hemoglobin production was evaluated using cation-exchange HPLC. Cell lysates obtained from control and NaB-treated K562 cells were analyzed against established hemoglobin standards to identify individual hemoglobin fractions. The standards included hemoglobins A, F, S, and C. The approximate retention times used for identification were 4.6 minutes for HbF, 7.05 minutes for HbA, 8.1 minutes for HbS, and 9.7 minutes for HbC. K562 samples were evaluated at selected exposure periods to determine the presence and timing of detectable HbF production. Control samples were analyzed after 1, 3, and 7 days, while the 0.01 mM NaB group was examined after 3 and 7 days. The 0.1 mM NaB group was analyzed after 1, 3, and 7 days, and the 1.0 mM NaB group was examined after 1 and 3 days. Samples in which the HbF chromatographic peak was weak were concentrated and subsequently reinjected to improve the detection and visualization of the HbF peak. The HPLC analysis was primarily intended to determine whether HbF was detectable following NaB exposure and to establish the approximate time at which HbF became detectable at each treatment concentration. Comparisons were therefore made according to NaB concentration and duration of exposure.

Analysis of Hemoglobin Chromatograms

The HPLC chromatograms were evaluated by comparing the retention times of peaks observed in K562 cell lysates with those obtained from the hemoglobin standards. A chromatographic peak occurring at approximately 4.6 minutes was interpreted as corresponding to the HbF fraction based on the established standard profile. Peaks occurring at approximately 7.05, 8.1, and 9.7 minutes were compared with the expected retention times for HbA, HbS, and HbC, respectively. Control and NaB-treated samples were compared to determine whether HbF was detectable at each exposure period. The analysis also considered changes in the relative visibility of the HbF peak following concentration of samples. This approach was particularly important for samples in which HbF was present at a level that produced a weak chromatographic signal before concentration. Because the available HPLC results primarily established the presence, absence, and relative prominence of HbF peaks, the chromatographic analysis was interpreted descriptively. Absolute HbF concentrations were not calculated because a quantitative HbF standard curve was not available for the reported analysis. Consequently, the HPLC findings were used primarily to establish the timing and relative detection of HbF rather than to provide an absolute measurement of HbF production.

Data Analysis

The experimental findings were analyzed descriptively according to treatment group and duration of exposure. For the single-exposure experiment, patterns of K562 cell growth were compared among untreated control, hemin, 0.01 mM NaB, 0.1 mM NaB, and 1.0 mM NaB groups over seven days. The analysis considered changes in cell growth across successive days and the relative response associated with each NaB concentration. For the continuous-exposure experiment, K562 cell growth was compared among the treatment groups over the 21-day observation period. Particular attention was given to the timing and direction of increases and decreases in cell growth and to whether repeated NaB exposure was associated with recurring patterns of cellular growth. This approach was used to characterize the temporal behavior of K562 cells under prolonged NaB exposure. For the hydroxyurea experiment, cell growth was compared among the control, 1 μM HU, 0.1 mM NaB, and combined 1 μM HU plus 0.1 mM NaB groups at Days 1, 3, and 7. The combination treatment was evaluated to determine whether exposure to both compounds resulted in a greater cellular response than exposure to either compound individually. Total hemoglobin production was compared among treatment groups at Days 1, 3, and 7 using the spectrophotometric absorbance measurements. Particular attention was given to the treatment concentration and exposure duration associated with the greatest observed hemoglobin response. For HbF, HPLC chromatographic peaks were compared with the established hemoglobin standards, with the primary outcome being the timing of detectable HbF production following exposure to the different NaB concentrations.

Methodological Alignment with the Study Outcomes

The methodology was structured to correspond directly with the experimental outcomes presented in Figures 1–9 and Tables 1–2. The single-exposure experiment and repeated cell counting generated the cell-growth data presented in Figure 1, whereas continuous NaB exposure and repeated cell counting generated the 21-day growth patterns presented in Figure 2. The hydroxyurea and NaB combination experiment provided the comparative cell-growth findings presented in Figure 3. Spectrophotometric analysis of cell lysates provided the

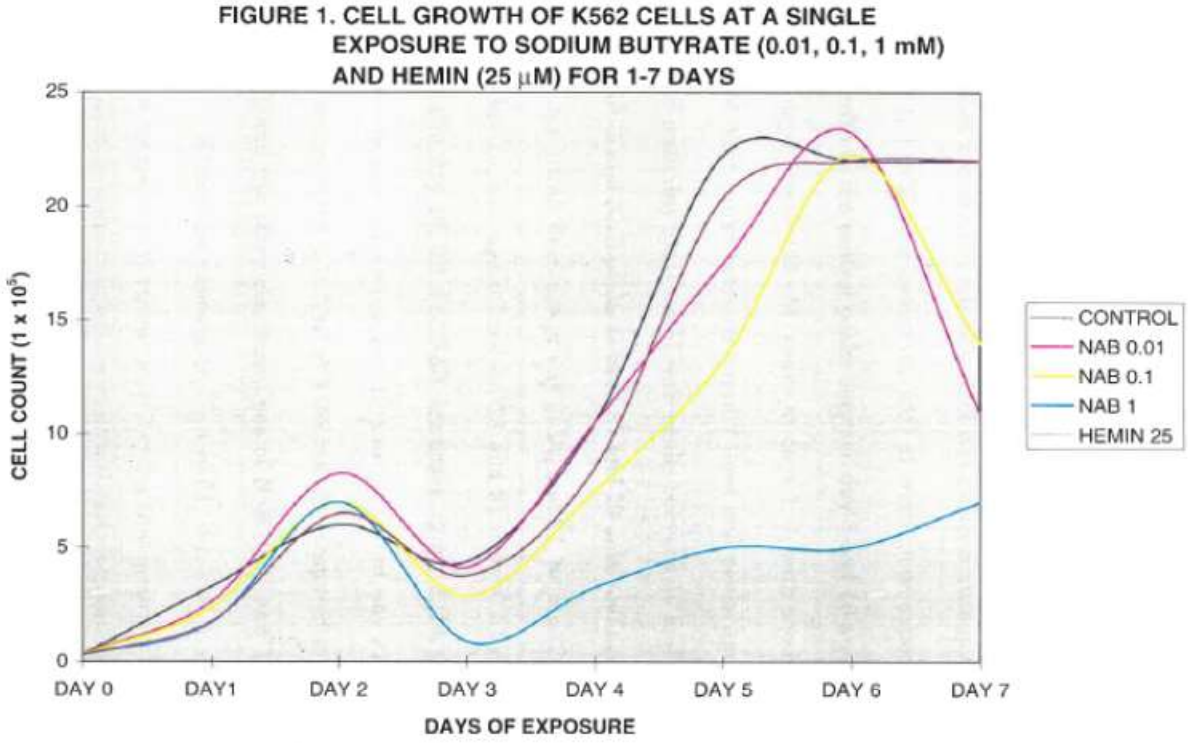
total hemoglobin measurements reported in Tables 1 and 2 and summarized in Figure 4. HPLC analysis of the hemoglobin standards established the reference retention times used to identify hemoglobin fractions in Figure 5. Subsequent HPLC analysis of untreated K562 cells provided the control chromatographic profiles shown in Figure 6, while analysis of cells exposed to 0.01, 0.1, and 1.0 mM NaB provided the HbF findings presented in Figures 7, 8, and 9, respectively. The methodology was designed to determine whether sodium butyrate altered K562 cell growth and whether changes in cellular growth were accompanied by increased total hemoglobin and HbF production. The use of multiple NaB concentrations and different exposure durations permitted assessment of concentration- and time-dependent cellular responses. The comparison of single and continuous exposure provided additional information concerning the effects of treatment duration, while the hydroxyurea experiment provided an opportunity to examine whether combining two pharmacological treatments produced an enhanced cellular response. The integration of cell-growth measurements, spectrophotometric hemoglobin determination, and HPLC analysis provided complementary approaches for evaluating the effects of NaB on K562 cellular proliferation and erythroid-associated hemoglobin production.

RESULTS AND ANALYSIS

Cell Growth of K562 Cells

Figure 1

Cell Growth of K562 Cells at a Single Exposure to Sodium Butyrate (0.01, 0.1, and 1mM) and Hemin (25μM) for 1-7 Days



The effects of sublethal or sub-treatment concentrations of sodium butyrate (NaB) on the growth of K562 cells were examined over a seven-day period. K562 cells were exposed to 0.01, 0.1, and 1.0 mM NaB, with untreated control and 25 μM hemin groups included for comparison. The pattern of cell growth demonstrated that the response of K562 cells to NaB was both concentration- and time-dependent. Rather than producing a continuous increase or decrease in cell growth, NaB exposure was associated with alternating periods of increased and decreased cellular proliferation. During the first day of exposure, cell growth increased in all treatment groups, although the untreated control showed slightly greater growth than the NaB-treated

groups. By Day 2, cell growth reached an early peak across the treatment groups, with cells exposed to 0.1 mM NaB showing slightly greater growth than the other NaB concentrations. This observation suggests that the intermediate concentration of NaB may have provided a more favorable cellular response during the early phase of exposure. In contrast, the higher concentration of 1.0 mM NaB appeared to exert a greater inhibitory influence on cell proliferation.

A reduction in cell growth was observed on Day 3 in the control and treatment groups. The reduction was most apparent in cells exposed to 1.0 mM NaB. Because a similar decrease was observed in the untreated control, the Day 3 reduction may reflect a normal fluctuation in the growth characteristics of the K562 cell culture rather than an effect exclusively attributable to NaB. The subsequent increase in cell growth on Day 4 supports the possibility that the cells were undergoing a temporary growth transition or lag phase before entering another period of active proliferation. Following the Day 3 reduction, cell growth increased again on Day 4 in the control, hemin, and all NaB treatment groups. The control and 25 µM hemin groups reached their highest levels of growth around Day 5 and subsequently showed a tendency to level off by Day 6. This pattern is consistent with a transition from active proliferation toward a more stable growth phase. The response to NaB differed according to concentration. Cells treated with 0.01 and 0.1 mM NaB continued to increase through approximately Day 6, followed by a decline through Day 7. Interestingly, the 0.01 mM NaB group showed slightly greater cell growth than the 0.1 mM group during this later period. The 1.0 mM NaB treatment demonstrated a different pattern, with reduced cell growth followed by a slight recovery toward Day 7. The lower growth observed at 1.0 mM NaB suggests that increasing NaB concentration may place greater stress on the cells and consequently limit sustained proliferation.

The single-exposure experiment demonstrates that NaB does not produce a simple linear effect on K562 cell growth. Instead, the response appears to involve alternating periods of proliferation and reduced growth. The 0.01 and 0.1 mM concentrations generally supported greater cell growth than the 1.0 mM concentration, whereas the 1.0 mM treatment appeared to have a stronger growth-inhibitory effect. The relatively favorable response observed with 0.1 mM NaB during the early period of exposure is particularly important because this concentration subsequently demonstrated a strong association with hemoglobin production. The observed fluctuations can therefore be characterized as a cyclic or oscillatory growth response following a single NaB exposure. However, because measurements were collected at discrete time points and no formal longitudinal statistical analysis was reported, the term "cyclic" should be interpreted as a descriptive pattern rather than evidence of a statistically established biological cycle.

Figure 2

Cell Growth of K562 Cells Continuously Exposed to Sodium Butyrate (0.01, 0.1, and 1mM) and Hemin (25µM) for 1-21 Days

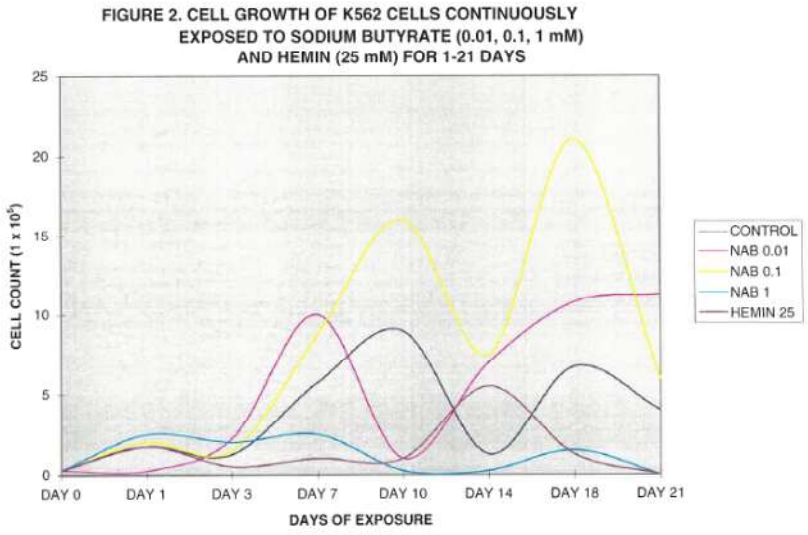


Figure 2 extends the analysis by examining K562 cell growth following continuous exposure to NaB for 21 days. The longer observation period provides evidence that K562 cells exhibit substantial temporal variation in their growth response to continuous exposure. Unlike the single-exposure experiment, which primarily demonstrated fluctuations over seven days, continuous exposure revealed repeated periods of increased and decreased cell growth over an extended period. The untreated control demonstrated periods of increased cell growth, with prominent peaks occurring between Days 7 and 10 and again between Days 14 and 21. These fluctuations indicate that the K562 cells themselves have a dynamic growth pattern even in the absence of treatment. Therefore, changes observed in the NaB-treated groups should be interpreted in relation to the natural growth behavior of the control cells. The 25 μM hemin-treated cells also demonstrated a variable growth pattern. Growth was observed at Days 1 and 7, with the highest peak occurring around Day 14. This was followed by reductions in growth around Day 3, Day 10, and the period between Days 18 and 21. These results indicate that hemin exposure was associated with repeated changes in cellular growth rather than a sustained increase in proliferation.

Cells continuously exposed to 0.01 mM NaB demonstrated an early period of increased growth between approximately Days 3 and 7. Growth subsequently declined around Day 10 before increasing again around Day 18 and remaining relatively stable through Day 21. This pattern suggests that the lower NaB concentration was associated with relatively prolonged cellular adaptation to continuous exposure. The 0.1 mM NaB treatment produced one of the most prominent growth responses. Cell growth increased substantially between approximately Days 10 and 14 and increased again around Day 18. Compared with the other treatment groups, the 0.1 mM NaB concentration produced the greatest overall cell growth. This finding is important because 0.1 mM NaB also produced the greatest amount of total hemoglobin after seven days in the hemoglobin analysis. Thus, among the concentrations examined, 0.1 mM NaB appears to provide a particularly favorable experimental condition for examining the relationship between K562 cell proliferation and erythroid-associated differentiation. The 1.0 mM NaB group showed an increase in cell growth between approximately Days 7 and 10, followed by a decrease between Days 10 and 14. A subsequent increase occurred around Day 18, followed by another decrease around Day 21. Although the 1.0 mM concentration was capable of supporting periods of increased cell growth, its repeated reductions in growth suggest that the higher NaB concentration may have produced greater cellular stress or reduced the ability of cells to maintain sustained proliferation.

Taken together, the continuous-exposure data demonstrate that NaB-induced changes in K562 cell growth are dependent on both concentration and duration of exposure. The growth patterns appear to contain approximately two major periods of increased growth during the 1–21-day observation period. The recurrence of these fluctuations in both treated and untreated cells indicates that intrinsic characteristics of K562 cell growth contribute to the observed pattern. Nevertheless, differences among the NaB concentrations indicate that treatment modifies the magnitude and timing of these growth responses. The particularly strong growth response observed with 0.1 mM NaB supports its selection for subsequent experiments involving hydroxyurea and hemoglobin production. Importantly, the data suggest that an intermediate NaB concentration may provide a balance between maintaining viable cell growth and promoting erythroid-associated cellular responses, whereas the higher 1.0 mM concentration may impose greater limitations on sustained cell proliferation.

Figure 3

Cell Growth of K562 Cells Exposed Continuously to Hydroxyurea (1μM), and Combination of Hydroxyurea (1μM) and Sodium Butyrate (0.1 mM) for 1, 3 and 7 Days

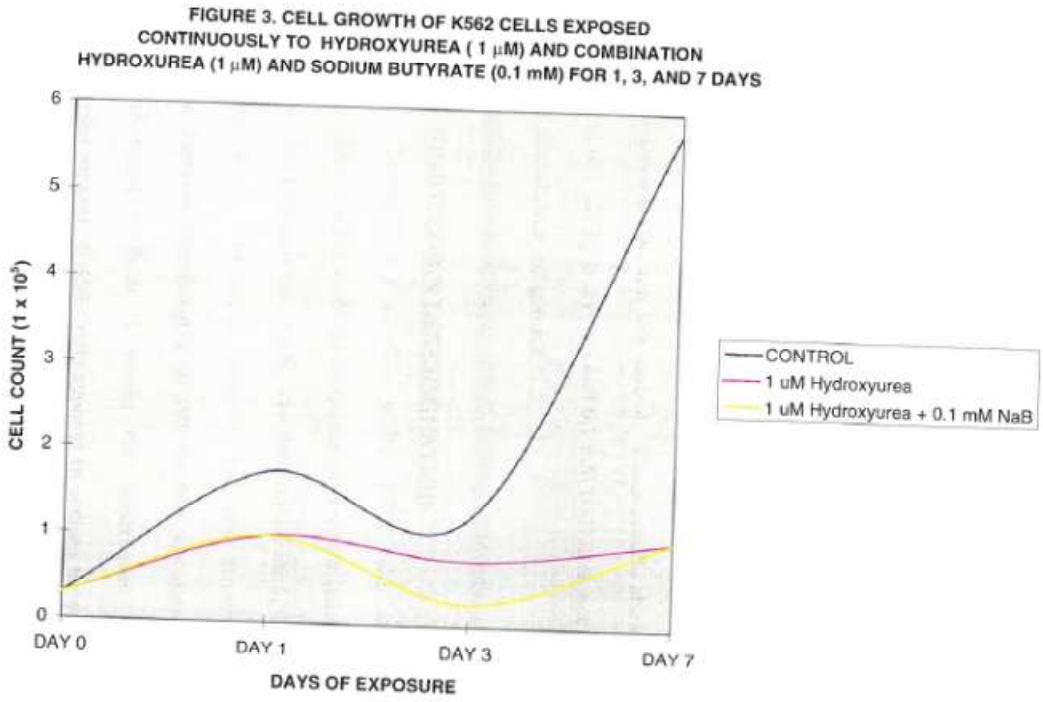


Figure 3 presents the effects of hydroxyurea (HU) alone and in combination with 0.1 mM NaB on K562 cell growth over Days 1, 3, and 7. An untreated control was included to determine whether the treatments altered the natural growth pattern of the cells. On Day 1, the control and treatment groups showed relatively similar levels of cell growth. The 1 μM HU group demonstrated slightly greater growth than the combination treatment consisting of 1 μM HU and 0.1 mM NaB. However, both treatment groups remained below the control. This observation indicates that neither treatment produced a strong stimulatory effect on K562 proliferation during the initial exposure period. By Day 3, cell growth decreased in both the HU-only and HU-plus-NaB groups. The decline was more pronounced in the combination treatment than in either the control or HU-only group. This finding suggests that the simultaneous exposure to HU and NaB did not enhance short-term cell proliferation. Instead, the combined treatment appeared to produce a greater reduction in cell growth during this period. By Day 7, cell growth increased in the control and treatment groups. The recovery suggests that the reduction observed at Day 3 was not necessarily associated with irreversible loss of cellular growth capacity. However, the results do not demonstrate that combining HU with 0.1 mM NaB produces an additive or synergistic stimulatory effect on K562 cell growth. Overall, the growth data suggest that the combination of HU and NaB may temporarily suppress cell proliferation more strongly than HU alone. Therefore, based solely on cell growth, the combination does not demonstrate evidence of synergy in stimulating K562 proliferation. Rather, the results suggest that NaB and HU may influence cellular growth through mechanisms that do not simply reinforce one another.

Total Hemoglobin Determination

Total hemoglobin production was evaluated in K562 cells exposed to different concentrations of NaB, HU, and the combination of NaB and HU. The cells were examined after 1, 3, and 7 days of exposure. Following treatment, the cells were lysed using double-distilled water, and absorbance measurements were obtained across

wavelengths ranging from 350 to 650 nm using a UV-visible spectrophotometer. Measurement of total hemoglobin provides an additional indicator of the erythroid-associated response of K562 cells. Importantly, changes in hemoglobin production should be interpreted separately from changes in cell number because increased total hemoglobin may result from increased hemoglobin production per cell, increased cell number, or a combination of both. Therefore, the hemoglobin results provide complementary information to the cell-growth findings.

Table 1

Total Hemoglobin Content of K562 Cells Exposed to Different Concentrations of Sodium Butyrate (0.01, 0.1 & 1.0 mM) for 1, 3, and 7 Days

TABLE 1

TOTAL HEMOGLOBIN CONTENT OF K562 CELLS
EXPOSED TO DIFFERENT CONCENTRATIONS OF SODIUM BUTYRATE
(0.01, 0.1 & 1.0 mM) FOR 1, 3, AND 7 DAYS

TIME OF EXPOSURE & TREATMENT	ABSORBANCE	TOTAL Hb (mg/ml)
DAY 1 - Control	0	0
DAY 1 - N = 0.01	0	0
DAY 1 - N = .1	0	0
DAY 1 - N = 1	0	0
DAY 3 - Control	0	0
DAY 3 - N = 0.01	0	0
DAY 3 - N = .1	0	0
DAY 3 - N = 1	0.11540	0.0143
DAY 7 - Control	0.48838	0.052
DAY 7 - N = 0.01	0.27570	0.0358
DAY 7 - N = .1	0.6	0.078
DAY 7 - N = 1	0.24885	0.032

Table 1 presents the absorbance measurements and total hemoglobin content of K562 cells exposed to 0.01, 0.1, and 1.0 mM NaB. The findings demonstrate a time-dependent accumulation of detectable hemoglobin following NaB exposure. Hemoglobin was detected in K562 cells following seven days of exposure to all three NaB concentrations. In contrast, hemoglobin was detected as early as Day 3 in cells exposed to 1.0 mM NaB. This earlier detection suggests that the higher NaB concentration may accelerate the appearance of detectable hemoglobin-associated products. However, the earlier detection should not be interpreted as evidence that 1.0 mM NaB produced the greatest overall differentiation response, because the 0.1 mM treatment produced the greatest amount of total hemoglobin by Day 7. The 0.1 mM NaB treatment produced the highest total hemoglobin content at Day 7. This finding is particularly noteworthy because 0.1 mM NaB also produced the greatest cell growth response in the continuous-exposure experiment. Together, these observations suggest that 0.1 mM NaB represents a favorable concentration for maintaining K562 cell growth while promoting hemoglobin production. The response at 1.0 mM NaB is also important. Although hemoglobin became detectable earlier at this concentration, the higher concentration was associated with reduced cell growth and increased cell loss during prolonged exposure. Thus, an earlier appearance of hemoglobin does not necessarily

indicate a superior overall treatment effect. A treatment may stimulate hemoglobin production while simultaneously compromising cell proliferation or viability. These findings indicate that the relationship between NaB concentration and hemoglobin production is not strictly linear. Instead, the intermediate concentration of 0.1 mM appears to provide the most favorable balance between cellular growth and hemoglobin production under the experimental conditions examined.

Table 2

Total Hemoglobin Content of K562 Cells Exposed to 1 µM Hydroxyurea and Combination of Sodium Butyrate (0.1 mM) and Hydroxyurea (1 µM) for 1, 3, and 7 Days

TABLE 2

TOTAL HEMOGLOBIN CONTENT OF K562 CELLS EXPOSED TO 1.0 µM HYDROXYUREA AND COMBINATION OF 0.1 mM SODIUM BUTYRATE AND 1.0 µM HYDROXYUREA FOR 1, 3 AND 7 DAYS

TIME OF EXPOSURE & TREATMENT	ABSORBANCE	TOTAL Hb (mg/ml)
DAY 1 - Control	0	0
DAY 1 - 1.0 µM hydroxyurea	0	0
DAY 1 - 0.1 mM sodium butyrate & 1.0 µM hydroxyurea	0	0
DAY 3 - Control	0	0
DAY 3 - 1.0 µM hydroxyurea	0	0
DAY 3 - 0.1 mM sodium butyrate & 1.0 µM hydroxyurea	0	0
DAY 7 - Control	0.48838	0.052
DAY 7 - 1.0 µM hydroxyurea	0.06	0.0078
DAY 7 - 0.1 mM sodium butyrate & 1.0 µM hydroxyurea	0.03771	0.005

Table 2 summarizes total hemoglobin production in K562 cells exposed to 1 µM HU, 0.1 mM NaB combined with 1 µM HU, and the untreated control. The results indicate that the combination treatment produced less total hemoglobin than the HU-only treatment and the control. The lower hemoglobin content observed in the combination treatment indicates that adding 0.1 mM NaB to HU did not enhance hemoglobin production under the conditions examined. Instead, the combination appeared to reduce the hemoglobin response relative to HU alone. Consequently, there is no descriptive evidence from these results that the combination treatment produced a synergistic effect on hemoglobin production. The finding is consistent with the cell-growth data presented in Figure 3. The combination treatment produced a greater reduction in cell growth at Day 3 than HU alone. Therefore, the reduced hemoglobin content associated with the combination treatment may reflect reduced cellular proliferation and/or altered erythroid-associated activity. Because total hemoglobin was measured rather than hemoglobin normalized to cell number or total cellular protein, additional experiments

would be required to determine whether the lower hemoglobin concentration resulted primarily from fewer cells, reduced hemoglobin production per cell, or both.

Figure 4

Total Hemoglobin Content of K562 Cells Exposed to Sodium Butyrate (0.01-1 mM), 1 μM Hydroxyurea and Combination of Sodium Butyrate (0.1 mM) and Hydroxyurea (1 μM) for 1, 3, and 7 Days

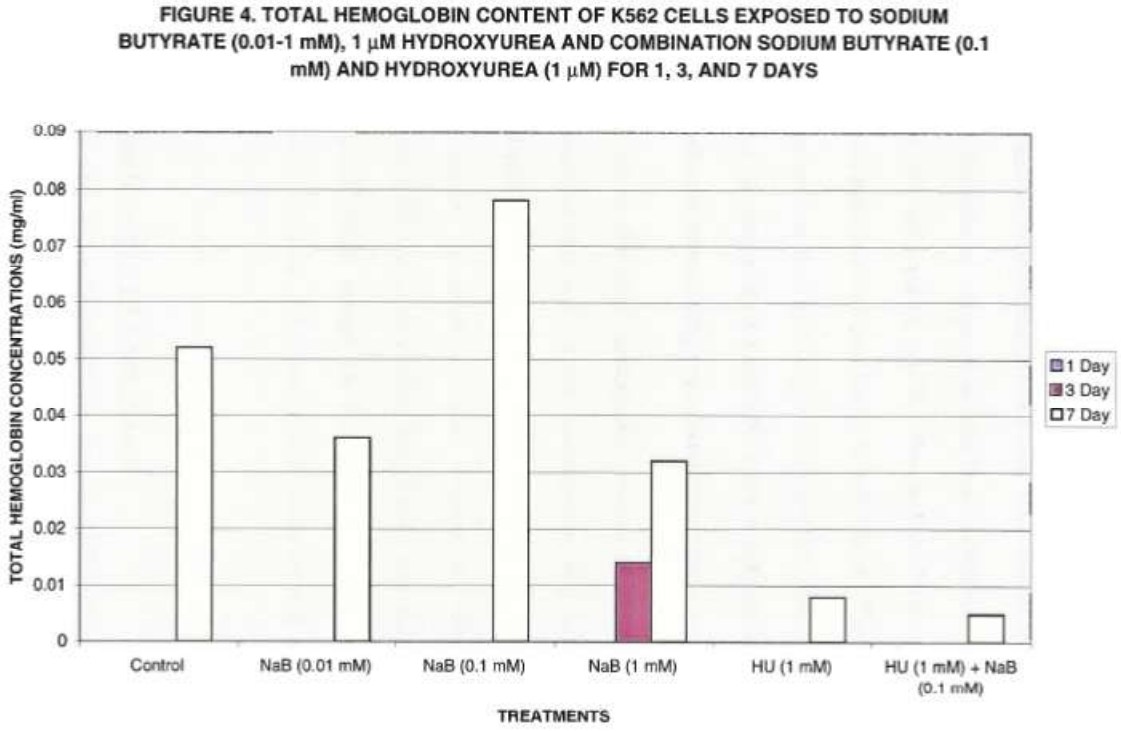


Figure 4 provides a comparative representation of total hemoglobin production following exposure to NaB, HU, and the NaB-HU combination. The seven-day control produced more hemoglobin than the 0.01 mM NaB, 1.0 mM NaB, HU, and combination-treatment groups. However, the 0.1 mM NaB group produced the highest total hemoglobin level among the treatments examined. The particularly strong response observed with 0.1 mM NaB indicates that this concentration may be more effective than the other tested concentrations in promoting hemoglobin accumulation in K562 cells. This finding corresponds with the results shown in Table 1 and provides additional support for identifying 0.1 mM NaB as an important concentration for subsequent investigations. The results also demonstrate that greater drug concentration does not necessarily produce greater hemoglobin production. Although 1.0 mM NaB produced detectable hemoglobin earlier than the lower concentrations, it did not produce the greatest total hemoglobin at Day 7. This distinction is important because it demonstrates that the timing of hemoglobin appearance and the final amount of hemoglobin produced are separate outcomes. Overall, the data suggest that NaB has a concentration-dependent effect on K562 cellular responses, with 0.1 mM producing the most favorable response under the experimental conditions. The results also suggest that prolonged exposure to a higher NaB concentration may adversely affect cell growth, thereby limiting the total cellular capacity for hemoglobin production.

Fetal Hemoglobin in K562 Cells

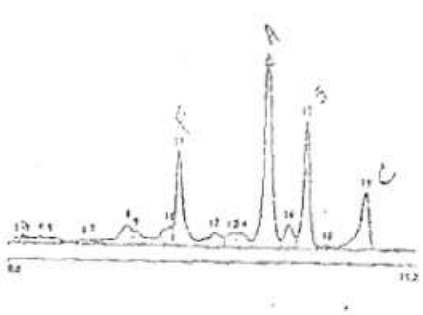
The ability of K562 cells to produce fetal hemoglobin (HbF) following exposure to NaB was investigated using high-performance liquid chromatography (HPLC). Hemoglobin analysis can be performed

using several chromatographic approaches, including anion-exchange, cation-exchange, and reversed-phase HPLC. Cation-exchange HPLC provides a method for separating and identifying different hemoglobin fractions based on their chromatographic characteristics. In the present analysis, HPLC was used to determine whether HbF could be detected in K562 cells following exposure to NaB. Samples collected from control and NaB-treated cells were compared with hemoglobin standards representing HbA, HbF, HbS, and HbC. Identification of hemoglobin fractions was based on their characteristic retention times. The HPLC standards established the approximate retention times for the hemoglobin fractions used in the analysis. HbF eluted at approximately 4.6 minutes, HbA at approximately 7.05 minutes, HbS at approximately 8.1 minutes, and HbC at approximately 9.7 minutes. These retention times provided the reference points for evaluating chromatographic peaks obtained from the K562 cell samples.

Figure 5

High Performance Liquid Chromatography (HPLC) of Standards AFSC Utilizing Cation-Exchange Chromatography

FIGURE 5. HIGH PERFORMANCE LIQUID CHROMATOGRAPH OF STANDARDS AFSC UTILIZING CATION-EXCHANGE CHROMATOGRAPHY.

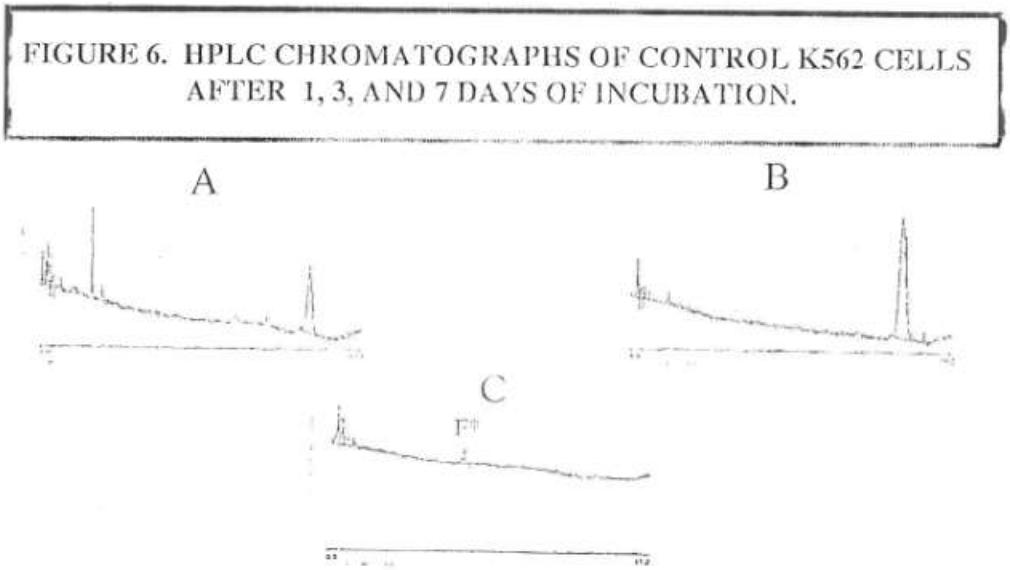


F=Fetal hemoglobin; A=Normal adult hemoglobin;
S=Sickle hemoglobin; C=Hemoglobin C

Figure 5 establishes the chromatographic profiles of the hemoglobin standards. The retention time of approximately 4.6 minutes was used to identify HbF, while retention times of approximately 7.05, 8.1, and 9.7 minutes corresponded to HbA, HbS, and HbC, respectively. The establishment of these standards was essential for interpreting the K562 samples because identification of HbF was based on comparison of the sample chromatograms with the known retention time of the HbF standard. The chromatographic approach therefore provided qualitative evidence for the presence or absence of HbF in the experimental samples.

Figure 6

HPLC Chromatographs of Control K562 Cells After 1, 3, and 7 Days of Incubation

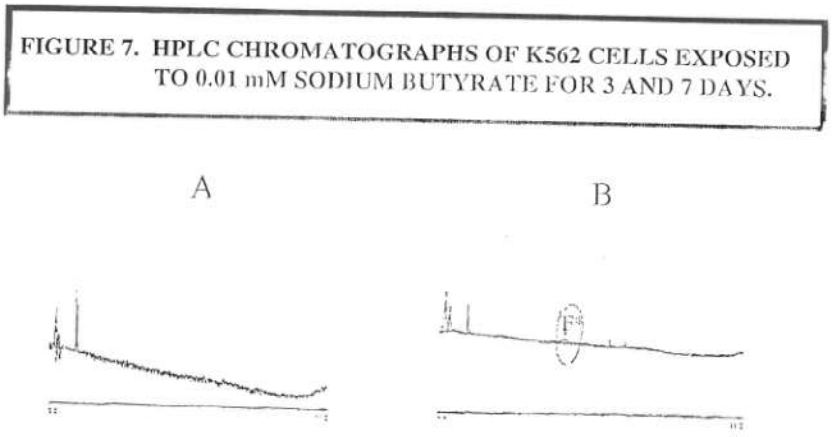


(A) Control K562 cells after 1 day incubation. (B) Control cells after 3 days incubation. (C) Control cells after 7 days of incubation. F*=Fetal hemoglobin (HbF) peak.

Figure 6 demonstrates the HPLC profiles of untreated K562 cells after 1, 3, and 7 days of incubation. No clear HbF peak was observed in the control samples after one or three days of cultivation. However, some HbF production was observed at Day 7. This finding indicates that prolonged cultivation alone may be associated with a low level of HbF production in K562 cells. A relatively large peak was observed at approximately 9.7 minutes in the Day 1 and Day 3 control samples. Because this retention time was close to that associated with HbC, the peak initially appeared consistent with HbC. However, after concentration of the sample, the peak disappeared, and the same peak was not observed in the Day 7 sample. Therefore, the identity and biological significance of this peak could not be conclusively established. The disappearance of the peak following concentration suggests that the observed chromatographic signal may have resulted from a non-specific component, sample-related artifact, or another substance with chromatographic behavior similar to the HbC standard. Consequently, this peak should not be interpreted as definitive evidence of HbC production by K562 cells. The appearance of HbF at Day 7 in the untreated control is noteworthy because it establishes that HbF may be detectable under the culture conditions even without NaB exposure. Thus, the interpretation of HbF induction by NaB should account for the possibility of a baseline or spontaneous HbF response associated with prolonged K562 cell cultivation.

Figure 7

HPLC Chromatographs of K562 Cells Exposed to 0.01 mM Sodium Butyrate for 3 and 7 days

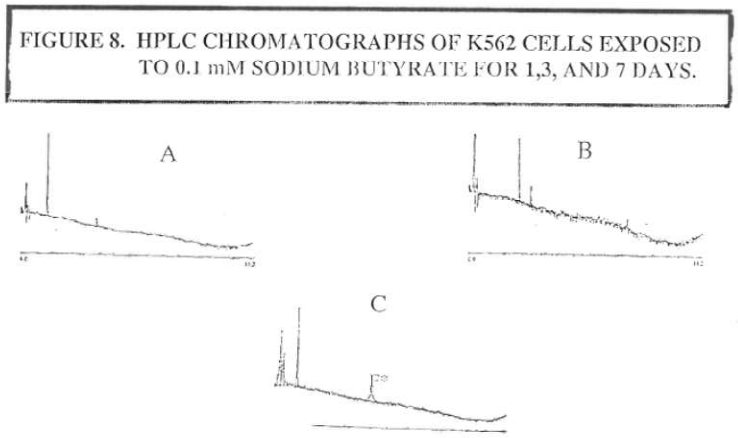


(A) Shows K562 cells after 3 days of exposure to 0.01 mM sodium butyrate. (B)Shows K562 cells after 7 days of exposure to 0.01 mM sodium butyrate. F*=Fetal hemoglobin (HbF) peak.

Figure 7 shows the HPLC chromatographs of K562 cells exposed to 0.01 mM NaB for three and seven days. No detectable HbF peak was observed after three days of exposure. However, an HbF peak was observed after seven days. Following concentration of the Day 7 sample, the HbF peak became more prominent. This increase in peak visibility supports the interpretation that HbF was present in the sample, although the concentration was initially low. The results indicate that 0.01 mM NaB was associated with detectable HbF production after prolonged exposure, whereas HbF was not detectable at the earlier three-day time point. These findings suggest that the effect of low-dose NaB on HbF production may require several days to become detectable. The delayed appearance of HbF also corresponds with the total hemoglobin results, in which hemoglobin production became more apparent following longer exposure.

Figure 8

HPLC Chromatographs of K562 Cells Exposed to 0.1 mM Sodium Butyrate for 1, 3, and 7 days



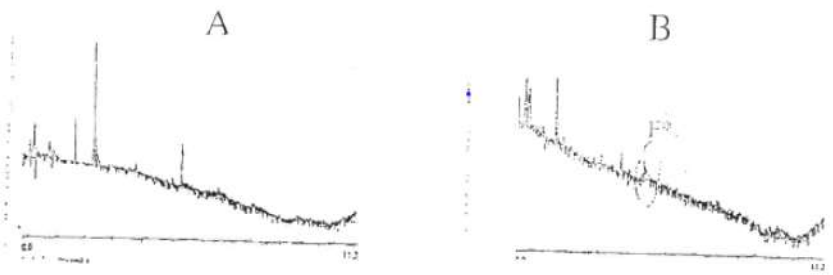
(A) Shows K562 cells after 1 day of exposure to 0.1 mM sodium butyrate. (B)Shows K562 cells after 3 days of exposure to 0.1 mM sodium butyrate. (C) Shows K562 cells after 7 days of exposure to 0.1 mM sodium butyrate. F*=Fetal hemoglobin peak.

Figure 8 presents the HPLC chromatographs of K562 cells exposed to 0.1 mM NaB for one, three, and seven days. HbF was identified at Day 7, whereas no clearly detectable HbF signal was observed at the earlier time points. The HbF peak at Day 7 was larger than the corresponding peak observed following exposure to 0.01 mM NaB. This observation suggests that 0.1 mM NaB may promote a stronger HbF response than the lower NaB concentration after prolonged exposure. However, the peak decreased in size following concentration of the sample. This observation indicates that the chromatographic response was sensitive to sample preparation and concentration. Although the presence of HbF is supported by the retention time, the qualitative nature of the analysis prevents determination of the precise amount of HbF produced. The 0.1 mM NaB results are particularly important when considered together with the total hemoglobin data. The same concentration produced the greatest total hemoglobin content at Day 7 and demonstrated a relatively prominent HbF chromatographic peak. Thus, 0.1 mM NaB appears to be an effective concentration for inducing hemoglobin-associated responses in K562 cells under the conditions of this experiment.

Figure 9

HPLC Chromatographs of K562 Cells Exposed to 1 mM Sodium Butyrate for 1 and 3 days

FIGURE 9. HPLC CHROMATOGRAPHS OF K562 CELLS EXPOSED TO 1 mM SODIUM BUTYRATE FOR 1 AND 3 DAYS.



(A) Shows K562 cells after 1 day of exposure to 1 mM sodium butyrate. (B)Shows K562 cells after 3 days of exposure to 1 mM sodium butyrate. F*=Fetal hemoglobin (HbF) peak.

Figure 9 shows the HPLC chromatographs obtained from K562 cells exposed to 1.0 mM NaB. No HbF was identified after one day of exposure. However, an HbF peak was observed after three days. Concentration of the sample increased the size and visibility of the HbF peak, providing additional qualitative evidence for the presence of fetal hemoglobin. The earlier appearance of HbF in the 1.0 mM NaB group compared with the 0.01 and 0.1 mM groups suggests that the higher concentration may accelerate the onset of detectable HbF production. However, this apparent acceleration must be considered together with the cell-growth findings. The 1.0 mM NaB treatment was associated with lower cell growth and substantial cell loss during prolonged exposure. Consequently, although HbF became detectable earlier at the higher concentration, prolonged exposure was not favorable for maintaining the K562 cell population. Samples from Day 7 of the 1.0 mM NaB treatment were not analyzed because of substantial cell death and a severe reduction in the number of recoverable cells. This limitation is important when comparing the three NaB concentrations because the absence of a Day 7 HPLC measurement for the 1.0 mM group prevents direct comparison of late-stage HbF production across all treatment concentrations.

Overall Analysis of Cell Growth and Hemoglobin Production

Collectively, the results demonstrate that NaB produces concentration- and time-dependent effects on K562 cells. The response was not characterized by a simple relationship in which increasing NaB concentration continuously increased either cell growth or hemoglobin production. Instead, the findings indicate that different concentrations produce distinct temporal patterns. The 0.01 mM concentration generally supported continued cell growth and produced detectable HbF after seven days. The 0.1 mM concentration produced the strongest overall combination of cell growth and hemoglobin production. In particular, 0.1 mM NaB generated the greatest total hemoglobin content at Day 7 and produced a relatively prominent HbF signal. These findings identify 0.1 mM as an experimentally important concentration for further investigation of NaB-induced erythroid-associated responses in K562 cells. The 1.0 mM concentration produced a different biological profile. Although HbF was detectable as early as Day 3, cell growth was comparatively lower and prolonged exposure resulted in substantial cell loss. Therefore, the higher concentration may promote an earlier differentiation-associated response while simultaneously compromising cellular expansion or survival. This finding emphasizes the importance of distinguishing between induction of hemoglobin production and maintenance of viable cellular proliferation.

The results from the HU experiment further demonstrate that combining treatments does not necessarily enhance the response. The combination of 1 μM HU and 0.1 mM NaB produced less cell growth at Day 3 and less total hemoglobin than HU alone. Thus, the combination did not demonstrate a synergistic effect on either cell proliferation or hemoglobin production under the conditions examined. The HPLC findings provide additional evidence that NaB exposure is associated with HbF production in K562 cells. HbF was detected after seven days with 0.01 and 0.1 mM NaB, while HbF was detected earlier, at Day 3, with 1.0 mM NaB. The delayed appearance of HbF at the lower concentrations compared with the higher concentration suggests that NaB concentration may influence the timing of the HbF response. Nevertheless, the 1.0 mM concentration was associated with substantial cell loss, indicating that earlier HbF detection was accompanied by reduced cellular sustainability. An important limitation of the present HPLC analysis is that the results were primarily qualitative. Although the chromatographic retention time allowed HbF to be identified, a standard curve was not available to determine the precise quantity of HbF produced. Consequently, the magnitude of HbF induction cannot be accurately compared numerically among treatment groups. Future experiments should incorporate a validated HbF standard curve, appropriate internal standards, replicate samples, and normalization of HbF to cell number, total protein, or another appropriate measure of cellular content.

The findings support the conclusion that NaB influences both K562 cell growth and hemoglobin-associated differentiation in a manner dependent on concentration and duration of exposure. Among the concentrations examined, 0.1 mM NaB produced the most favorable overall response, characterized by comparatively strong cell growth and the highest total hemoglobin production at Day 7, together with detectable HbF. In contrast, 1.0 mM NaB appeared to produce an earlier HbF response but was associated with reduced cellular growth and increased cell loss during prolonged exposure. These findings suggest that the biological effects of NaB involve a balance between maintaining cellular proliferation and promoting erythroid-associated differentiation. The results therefore provide a basis for further investigation of NaB as an inducer of erythroid differentiation in K562 cells. Future studies should quantify HbF using an HPLC calibration curve, normalize hemoglobin production to viable cell number, assess cell viability, and employ appropriate statistical analyses to determine whether the observed differences among treatment groups and time points are statistically significant.

DISCUSSION OF FINDINGS

Sodium Butyrate and K562 Cell Growth

The findings of this study demonstrate that sodium butyrate produced concentration- and time-dependent effects on K562 cell growth. Following a single exposure, K562 cells did not demonstrate a simple linear increase or decrease in proliferation. Instead, the cells exhibited alternating periods of increased and decreased growth over the seven-day observation period. The 0.01 and 0.1 mM NaB groups generally maintained greater cellular growth than the 1.0 mM group, while the 1.0 mM treatment demonstrated a stronger inhibitory pattern. The lower growth observed at 1.0 mM is consistent with the possibility that increasing NaB concentration produces greater cellular stress and limits sustained proliferation. This finding is consistent with previous research showing that sodium butyrate can induce erythroid differentiation while simultaneously affecting cell-cycle progression in K562 cells. Li et al. (2008) reported that sodium butyrate induced G0/G1 cell-cycle arrest as part of the erythroid differentiation process. Therefore, reduced proliferation following NaB exposure should not necessarily be interpreted as an absence of biological activity. Rather, it may reflect a shift

in cellular behavior from active proliferation toward differentiation-associated processes. The present results are particularly relevant because the 1.0 mM concentration produced the strongest evidence of reduced sustained proliferation while also producing earlier HbF detection.

The continuous-exposure experiment reinforced the concentration- and time-dependent nature of the cellular response. During the 21-day observation period, untreated and treated K562 cells demonstrated repeated increases and decreases in growth. Importantly, similar fluctuations were present in the untreated controls, indicating that part of the observed variation may represent the intrinsic growth behavior of K562 cultures rather than a direct effect of NaB. Nevertheless, treatment modified the magnitude and timing of these changes. The 0.1 mM NaB group produced the greatest overall growth response, particularly during the later exposure period. The strong response observed at 0.1 mM is important because it occurred simultaneously with the strongest hemoglobin response. This suggests that the intermediate NaB concentration may provide a favorable experimental balance between maintaining viable cell proliferation and promoting erythroid-associated hemoglobin production. Such a balance is biologically important because differentiation-inducing treatments may become less useful if the concentration required to stimulate differentiation substantially compromises cellular survival.

Sodium Butyrate and Total Hemoglobin Production

The total hemoglobin findings provide additional evidence that sodium butyrate stimulated erythroid-associated responses in K562 cells. Hemoglobin was detected after seven days in all three NaB treatment groups, whereas hemoglobin became detectable as early as Day 3 in cells exposed to 1.0 mM NaB. However, the 0.1 mM treatment produced the greatest total hemoglobin content at Day 7. The finding that 1.0 mM NaB produced earlier detectable hemoglobin but did not produce the greatest final hemoglobin content is particularly important. It indicates that the speed of hemoglobin induction and the magnitude of the final hemoglobin response are not necessarily equivalent outcomes. A higher concentration may accelerate the initiation of an erythroid-associated response, but excessive exposure may simultaneously reduce proliferation and cellular sustainability. In contrast, 0.1 mM NaB appeared to support continued cell growth while producing the highest total hemoglobin level at Day 7.

This result agrees with previous experimental studies demonstrating that sodium butyrate can enhance hemoglobin synthesis in K562 cells. Villeval et al. (1983) found that K562 cells possess erythroid properties and that butyrate can enhance hemoglobin-associated responses. More specifically, Davis et al. (2000) demonstrated that sodium butyrate induced K562 erythroblastic differentiation and increased hemoglobin accumulation. These earlier findings provide biological support for the present observation that NaB exposure was associated with increased hemoglobin production. However, total hemoglobin should be interpreted cautiously because the measurement was not normalized to viable cell number or total cellular protein. As the study results appropriately recognize, an increase in total hemoglobin may reflect greater hemoglobin production per cell, a greater number of cells, or both. Thus, the particularly strong total hemoglobin response observed with 0.1 mM NaB should be interpreted as evidence of an overall increase in hemoglobin-associated cellular content rather than definitive evidence that each individual cell produced more hemoglobin.

Sodium Butyrate and Fetal Hemoglobin Production

The HPLC findings provide direct qualitative evidence that NaB exposure was associated with detectable HbF production in K562 cells. The HbF standard eluted at approximately 4.6 minutes, and this retention time was subsequently used to identify HbF in the K562 samples. The untreated control samples did not show a clear HbF peak at Days 1 and 3, although HbF became detectable by Day 7. This finding is important because it indicates that prolonged K562 cultivation itself may permit a low level of HbF production. Consequently, the NaB-associated HbF response should be interpreted relative to the baseline response of untreated cells. At 0.01 mM NaB, HbF was not detected at Day 3 but became detectable at Day 7. The HbF peak became more prominent after concentration of the sample, supporting the interpretation that HbF was present at a relatively low level initially. At 0.1 mM NaB, HbF was similarly detected at Day 7, but the HbF signal was relatively more prominent than that observed at 0.01 mM. This result is especially meaningful because 0.1 mM NaB also produced the highest total hemoglobin content at Day 7. The 1.0 mM NaB treatment produced a different pattern. HbF was detected as early as Day 3, demonstrating that the highest concentration appeared to accelerate the onset of detectable HbF production. However, this treatment was also associated with lower cell

growth and substantial cell loss during prolonged exposure. Day 7 HPLC analysis was not possible because of the substantial loss of recoverable cells.

These findings are broadly consistent with the earlier literature demonstrating that butyrate stimulates fetal-globin expression. Constantoulakis et al. (1989) reported increased HbF expression in erythroid progenitors following sodium butyrate exposure and suggested that the compound acts directly or indirectly at the level of globin gene expression and chromatin regulation. Perrine et al. (1993) subsequently demonstrated increased fetal-globin synthesis in patients with sickle cell anemia and β-thalassemia following continuous arginine butyrate administration. The present K562 findings therefore extend the broader experimental evidence that butyrate can stimulate fetal-globin-associated responses. The results also support an important distinction between earlier HbF induction and overall treatment effectiveness. Although 1.0 mM NaB produced the earliest detectable HbF signal, it was not the most favorable treatment when cell growth and total hemoglobin production were considered together. In contrast, 0.1 mM NaB produced the strongest combination of cell growth, total hemoglobin production, and detectable HbF. Thus, the results suggest that the optimal experimental concentration may not be the concentration that produces the earliest molecular response.

Comparison of Sodium Butyrate and Hydroxyurea

The hydroxyurea experiment provided an additional comparison between two pharmacological approaches associated with HbF induction. Hydroxyurea is well established as an HbF-inducing therapy for SCD and has demonstrated clinical benefits through reduction of HbS polymerization and vaso-occlusive complications. In the present experiment, however, the combination of 1 μM hydroxyurea and 0.1 mM NaB did not produce an enhanced cellular response. On Day 3, the combination treatment produced a greater reduction in cell growth than hydroxyurea alone. By Day 7, cell growth recovered in the treatment groups, but the results did not demonstrate an additive or synergistic stimulatory effect of combining the two agents. The total hemoglobin findings produced a similar pattern. The combination of HU and NaB produced less total hemoglobin than HU alone and the untreated control. Therefore, under the experimental conditions used in this study, the addition of NaB to HU did not enhance total hemoglobin production. These findings should not be interpreted as evidence that NaB and HU cannot interact positively under any circumstances. Rather, they indicate that the particular concentrations, exposure schedule, and K562 experimental conditions used in this study did not produce an apparent synergistic effect. This observation is interesting in light of earlier work demonstrating that butyrate can interact differently with other pharmacological HbF-inducing agents. Constantoulakis et al. (1989) reported synergistic HbF induction when NaB was combined with 5-azacytidine but not when it was combined with cytarabine. Thus, the biological effects of combination therapy may depend strongly on the specific agents, concentrations, exposure periods, and cellular context involved.

Above all, the findings demonstrate that sodium butyrate influenced K562 cell proliferation and hemoglobin-associated responses in a concentration- and time-dependent manner. The most important finding was that 0.1 mM NaB produced the most favorable overall response. This concentration supported comparatively strong cell growth, produced the greatest total hemoglobin content at Day 7, and generated a detectable HbF response. The 1.0 mM concentration produced an apparently faster HbF response, with HbF detectable by Day 3, but this response was accompanied by reduced cellular growth and substantial cell loss during prolonged exposure. Therefore, the findings suggest a potential trade-off between rapid induction of erythroid-associated responses and maintenance of viable cellular proliferation. The 0.01 mM concentration, meanwhile, supported cell growth but required a longer exposure period before HbF became detectable. These findings are consistent with the broader literature showing that sodium butyrate can induce erythroid differentiation and hemoglobin synthesis in K562 cells (Villeval et al., 1983; Davis et al., 2000; Li et al., 2008). They are also consistent with experimental and clinical evidence demonstrating that butyrate can stimulate fetal-globin expression in erythroid progenitors and patients with β-hemoglobin disorders (Dover et al., 1992; Dover et al., 1994; Constantoulakis et al., 1989; Perrine et al., 1993). Nevertheless, the HbF findings in the present study should be regarded as qualitative rather than quantitative evidence. The HPLC analysis established the presence and approximate timing of HbF based on chromatographic retention time, but a validated calibration curve was not available to determine the precise amount of HbF produced. Consequently, the study cannot establish statistically or quantitatively that 0.1 mM produced a specific percentage increase in HbF relative to 0.01 or 1.0 mM NaB. Similarly, the spontaneous appearance of HbF in the Day 7 untreated control indicates that prolonged culture may itself contribute to HbF detection.

The findings provide preliminary evidence supporting sodium butyrate as an experimental inducer of erythroid-associated hemoglobin production in K562 cells. The particularly favorable response at 0.1 mM suggests that intermediate NaB concentrations deserve additional investigation. Future studies should incorporate biological and technical replicates, formal statistical testing, quantitative HbF calibration using validated standards, and normalization of HbF and total hemoglobin to viable cell number or total cellular protein. Future research should also examine whether the apparent earlier HbF induction at 1.0 mM reflects genuine acceleration of erythroid differentiation or is partly associated with reduced cellular proliferation. Additional markers of erythroid differentiation, such as glycophorin A, γ-globin mRNA, and relevant erythroid transcription factors, could help distinguish between increased hemoglobin accumulation and broader differentiation. This would be particularly useful because previous research indicates that sodium butyrate can alter cell-cycle regulation and intracellular signaling during K562 erythroid differentiation (Li et al., 2008; Witt et al., 2000). The study supports the conclusion that sodium butyrate has biologically meaningful effects on K562 cells and that these effects depend strongly on both concentration and duration of exposure. Among the concentrations examined, 0.1 mM NaB appears to provide the most favorable balance between cellular proliferation and hemoglobin-associated differentiation, whereas 1.0 mM NaB appears to accelerate HbF detection at the expense of sustained cellular growth and survival. These findings provide a useful experimental basis for further investigation of sodium butyrate as a potential HbF-inducing compound and for understanding the cellular mechanisms linking butyrate exposure, erythroid differentiation, and fetal hemoglobin production.

Limitations

Several limitations should be considered when interpreting the findings of this study. The investigation was conducted using the human erythroleukemia K562 cell line, which provides a useful experimental model for studying erythroid differentiation and hemoglobin production but does not fully reproduce the physiological environment of human erythropoiesis or sickle cell disease. Consequently, responses observed in K562 cells cannot be assumed to occur at the same magnitude or through the same mechanisms in patients with sickle cell disease. In addition, the study evaluated only three concentrations of sodium butyrate—0.01, 0.1, and 1.0 mM. Although these concentrations allowed comparison of low, intermediate, and high exposure levels, they do not establish the complete dose-response relationship for NaB. Additional concentrations between and outside the levels examined may produce different cellular responses. The finding that 0.1 mM NaB produced the most favorable overall response therefore applies specifically to the concentrations and experimental conditions examined. Furthermore, the HPLC analysis of fetal hemoglobin was primarily qualitative. HbF was identified according to its chromatographic retention time, but a validated calibration curve was not available to determine the precise quantity of HbF produced by each treatment group. Consequently, the study establishes the presence and timing of detectable HbF but does not provide a precise quantitative estimate of HbF induction. The results also showed that HbF could be detected in untreated K562 cells after seven days of culture, indicating that prolonged cultivation may contribute to baseline HbF production.

Another important limitation concerns the measurement of total hemoglobin, which was not normalized to viable cell number, total cellular protein, or another measure of cellular content. Therefore, increased total hemoglobin could reflect increased cell numbers, increased hemoglobin production per cell, or a combination of these factors. Similarly, the lower hemoglobin observed following the hydroxyurea-NaB combination cannot be attributed definitively to reduced hemoglobin synthesis per cell because the combination treatment also produced lower cell growth. Moreover, the study relied primarily on descriptive analysis. Formal statistical tests were not reported to determine whether differences among treatment groups and exposure periods were statistically significant. The observed fluctuations in cell growth were therefore interpreted as descriptive temporal patterns rather than statistically established biological cycles. An additional limitation is that the 1.0 mM NaB group experienced substantial cell loss during prolonged exposure, preventing HPLC evaluation at Day 7. This limits direct comparison of late-stage HbF production across all three NaB concentrations. Finally, the study examined only a limited set of cellular outcomes and did not directly measure molecular markers of erythroid differentiation, γ-globin gene expression, or the intracellular signaling pathways responsible for the observed response.

Future Studies

Future studies should address these limitations through a more comprehensive experimental design. First, subsequent research should include biological and technical replicates and apply appropriate statistical methods to determine whether observed differences in cell growth, total hemoglobin, and HbF production are

statistically significant. Longitudinal statistical approaches would be particularly useful for evaluating the time-dependent growth patterns observed following single and continuous NaB exposure. Second, future investigations should evaluate a broader range of NaB concentrations and exposure durations. Concentrations surrounding the 0.1 mM level could help determine whether the observed favorable response represents a true optimal concentration or simply the strongest response among the three concentrations examined. Additional exposure periods could also clarify whether the delayed HbF response observed at lower NaB concentrations eventually exceeds the response produced by higher concentrations. Third, future studies should incorporate quantitative HPLC analysis using validated HbF standards and calibration curves. HbF should ideally be normalized to viable cell number, total protein, or another appropriate cellular denominator. This would allow researchers to distinguish increased HbF production per cell from increases resulting primarily from differences in cell proliferation.

Fourth, future research should examine molecular indicators of erythroid differentiation. Measurements of γ-globin mRNA, γ-globin protein, erythroid transcription factors, and erythroid surface markers could provide stronger evidence concerning the biological mechanisms underlying NaB-induced differentiation. These measurements would also help determine whether the earlier HbF detection observed with 1.0 mM NaB represents accelerated erythroid differentiation or a response associated with cellular stress. Fifth, additional studies should investigate the mechanisms through which NaB influences K562 cells. Sodium butyrate is known to influence cellular processes associated with chromatin regulation and erythroid differentiation, and previous research has implicated cell-cycle regulation and intracellular signaling pathways in the response to NaB. Mechanistic studies could therefore help explain why 0.1 mM NaB yielded the most favorable combination of cell growth and hemoglobin production, whereas 1.0 mM resulted in earlier HbF detection but poorer sustained cell growth.

Finally, future research should examine NaB in more clinically relevant experimental models, including primary erythroid progenitor cells obtained from individuals with sickle cell disease or β-thalassemia. Studies using primary human cells would provide stronger evidence concerning whether the findings observed in K562 cells translate to human erythropoiesis. Additional studies could also evaluate different treatment schedules and combinations with established HbF-inducing therapies to determine whether specific concentrations or sequential exposure strategies can enhance HbF production without compromising cellular viability. Therefore, future studies should move from qualitative observation toward quantitative, mechanistic, and clinically relevant investigation. Such research would help determine whether sodium butyrate can sustain HbF induction while preserving erythroid cell viability, and whether its biological effects can translate into safer, more effective approaches for disorders involving abnormal hemoglobin production.

CONCLUSION AND POLICY IMPLICATIONS

This study examined the effects of sodium butyrate on K562 cell growth and hemoglobin-associated erythroid responses under different concentrations and exposure conditions. The findings demonstrate that NaB produced concentration- and time-dependent effects on K562 cells rather than a simple linear response. The effects of NaB differed according to both concentration and duration of exposure, demonstrating the importance of considering dose and treatment schedule when evaluating pharmacological induction of erythroid differentiation. Among the three concentrations examined, 0.1 mM NaB produced the most favorable overall response. This concentration was associated with comparatively strong cell growth, the greatest total hemoglobin production at Day 7, and detectable HbF. These findings suggest that 0.1 mM may provide a useful experimental concentration for further investigation of NaB-induced erythroid responses in K562 cells. The 1.0 mM concentration produced a different response. Although HbF was detected earlier, at Day 3, the higher concentration was associated with reduced cellular growth and substantial cell loss during prolonged exposure. This finding demonstrates that earlier detection of HbF does not necessarily indicate a superior overall biological response. Rather, an effective erythroid-inducing treatment must balance hemoglobin induction with maintenance of viable cellular populations.

The study also found that combining 1 μM hydroxyurea with 0.1 mM NaB did not produce an apparent synergistic response. The combination produced lower cell growth at Day 3 and lower total hemoglobin than hydroxyurea alone. Therefore, under the experimental conditions examined, combining the two agents did not improve the measured cellular outcomes. The HPLC findings further support an association between NaB exposure and HbF production. HbF became detectable after seven days of exposure to 0.01 and 0.1 mM NaB, whereas the 1.0 mM treatment produced detectable HbF by Day 3. However, because the HPLC analysis was

qualitative and the untreated control also demonstrated some HbF after prolonged culture, these findings should be interpreted as preliminary evidence of HbF induction rather than definitive quantitative proof of increased HbF synthesis. In the nutshell, the findings provide experimental evidence that sodium butyrate can influence K562 cell proliferation and hemoglobin-associated erythroid responses. The results particularly identify 0.1 mM NaB as the most favorable concentration among those tested, because it produced the strongest overall balance between cellular growth and hemoglobin production. The study therefore provides a foundation for further investigation of sodium butyrate as an experimental HbF-inducing compound.

Policy Implications

Although this was an in vitro laboratory study and therefore cannot directly establish clinical effectiveness, the findings have several potential implications for sickle cell disease and other β-hemoglobin disorders. Importantly, the results support continued research into pharmacological strategies that increase HbF production. Because HbF can reduce the tendency of HbS to polymerize, approaches that safely increase HbF may contribute to reducing disease severity and improving health outcomes among individuals with sickle cell disease. At the same time**,** the findings highlight the importance of dose optimization in the development of HbF-inducing therapies. The observation that 1.0 mM NaB produced earlier HbF detection but also greater cellular loss demonstrates that maximizing drug concentration may not maximize therapeutic benefit. Policy and research priorities should therefore emphasize treatments that achieve meaningful HbF induction while preserving cellular viability and minimizing toxicity. Moreover, the findings support continued investment in translational research on butyrate-based and other HbF-inducing therapies. Previous clinical research has demonstrated that butyrate can stimulate fetal-globin expression in patients with β-hemoglobin disorders, providing a basis for continued investigation. However, laboratory evidence should be followed by appropriately designed preclinical and clinical studies before changes to clinical treatment guidelines are considered.

More broadly**,** the findings reinforce the importance of supporting research into multiple HbF-inducing strategies rather than relying exclusively on a single pharmacological approach. Hydroxyurea remains an important HbF-inducing therapy, but variation in treatment response creates a continuing need for alternative or complementary approaches. In this context**,** the lack of an enhanced response from the HU-NaB combination in this study suggests that combination therapy should be evaluated empirically rather than assumed to be beneficial. From a public health policy perspective**,** investment in sickle cell disease research should include support for translational studies that connect laboratory discoveries with clinical outcomes. Research funding could prioritize studies that evaluate HbF induction, treatment safety, long-term effectiveness, treatment adherence, and disparities in access to disease-modifying therapies. Such research is particularly important because the potential value of a new HbF-inducing treatment depends not only on its biological effectiveness but also on whether it can be safely, affordably, and equitably delivered to patients. Equally important, the findings emphasize that laboratory discoveries should be interpreted cautiously when developing health policy. The present study was conducted in K562 cells, and its HPLC findings were qualitative. Therefore, the results do not provide sufficient evidence to recommend sodium butyrate as a clinical treatment for sickle cell disease. Instead, they provide a scientific rationale for additional research. Accordingly, future policy-oriented research should connect experimental evidence with patient-centered outcomes, including HbF levels, frequency of vaso-occlusive crises, acute chest syndrome, hospitalization, quality of life, treatment toxicity, and long-term organ complications. Overall, the study contributes to the growing evidence that pharmacological manipulation of erythroid differentiation and fetal hemoglobin production may represent an important avenue for addressing β-hemoglobin disorders. In particular, sodium butyrate, particularly at the 0.1 mM concentration examined in this study, demonstrated a favorable experimental profile by supporting K562 cell growth while producing the highest total hemoglobin response and detectable HbF. However, these findings should be regarded as preliminary and hypothesis-generating. Therefore, further quantitative, mechanistic, preclinical, and clinical research is necessary before sodium butyrate can be considered for incorporation into clinical treatment or health policy.

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