Original Research

Identification of fetal hemoglobin-inducing agents using the human leukemia KU812 cell line

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Abstract

Fetal hemoglobin (HbF) ameliorates the clinical severity of sickle cell disease; therefore continued research to identify efficacious HbF-inducing agents is desirable. In this study, we investigated KU812 leukemia cells that express the fetal γ-globin and adult β-globin genes, as a system for screening and discovery of novel HbF inducers. KU812 cells were analyzed in the presence or absence of fetal bovine serum and then expression levels of the globin genes, cell surface markers and transcription factors were quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For comparison, primary erythroid cells were grown in a two-phase liquid culture system. After drug inductions for 48–72 h, globin mRNA and HbF levels were quantified by RT-qPCR and enzyme-linked immunosorbent assay, respectively. Erythroid markers and transcription factors expression levels in KU812 cells were comparable to days 7–14 erythroid cells. We also tested several drugs including butyrate, trichostatin A, scriptaid, suberoylanilide hydroxamic acid and hydroxyurea, which induced γ-globin in KU812 cells; however, some agents also induced β-globin. A novel agent STI-571 was studied in the system, which non-selectively induced the globin genes. Additional studies showed comparable globin gene response patterns in KU812 and primary erythroid cells after treatments with the various drug inducers. Mechanisms of drug-mediated γ-globin induction in KU812 cells require signaling through the p38 mitogen-activated protein kinase pathway similar to that previously demonstrated in primary erythroid cells. These data suggest that KU812 cells serve as a good screening system to identify potential HbF inducers for the treatment of β-hemoglobinopathies.

Keywords: fetal hemoglobin, KU812 cells, primary erythroid cells, γ-globin, β-globin

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Introduction

The human β-hemoglobinopathies are a group of genetic disorders caused by mutations in the β-globin gene. Naturally occurring variants and drug treatments have demonstrated that reactivation of the γ-globin genes to increase fetal hemoglobin (HbF) synthesis is an effective therapeutic option in this group of patients. HbF inhibits hemoglobin S polymerization and ameliorates the clinical manifestations of vaso-occlusion in sickle cell patients. Hydroxyurea (HU) was the first and only drug approved by the Food and Drug Administration for the treatment of sickle cell disease;[1,2] however, HU is not effective in all patients[1] and of minimal benefit in β-thalassemia.[3] Moreover, concerns remain about undesirable side-effects including long-term carcinogenesis.[4] Subsequent clinical trials with other compounds, such as butyrate[5] and decitabine,[6] have shown considerable promise; however, the continued development of potent HbF inducers with low toxicity that can be administered orally is desirable.

Primary erythroid cells remain the best system to confirm HbF-inducing agents *in vitro* and predictor of efficacy *in vivo*. Human burst forming units – erythroid cells in clonogenic assays[7] or erythroid progenitors grown in liquid culture[8] – have been used to evaluate putative HbF inducers. However, these assays are not easily adaptable to large-scale drug screening; thus immortalized cell lines have been investigated for this purpose. Several human leukemia lines have been produced by culturing cells of adult patients with hematopoietic malignancies.[9] The well-known K562 cell line established in 1975[10] is one of the most widely used systems for screening HbF inducers. Subsequently, over 21 'bona fide' cell lines have been derived from acute

myeloid leukemia M6 (erythroid) and M7 (megakaryocytic)[9,11] cancers. Erythroid and/or megakaryocytic cell lines may represent a subpopulation in chronic myeloid leukemia cells in blast crisis since the immunophenotype of erythroleukemia cell lines are not lineage specific. However, a normal bipotent erythroid/megakaryocytic progenitor has been isolated from human bone marrow cells.[12,13]

Six human cell lines including K562 cells carry an embryonic-HbF phenotype (M-TAT, NS-Meg, OCIM1, OCMI2 and AS-E2) and seven cell lines synthesize both γ- and β-globin chains (JK-1, KMOE-2, KU812, LAMA-84, TF-1, TN922 and AP-217).[9] Several of these lines undergo differentiation after drug induction with hemin (Hem), stem cell factor, erythropoietin, trichostatin A (TSA), sodium butyrate (NaB) and dimethyl sulfoxide (DMSO);[9,14,15] however, KU812 cells are unique in that spontaneous differentiation has been observed.[16] The latter characteristic of KU812 cells suggests they might be a good system for screening drugs that stimulate HbF and to determine their specificity for γ-globin gene induction.

In this study, we examined KU812 cells that were initially described as a basophilic precursor line.[17] However, later these cells were demonstrated to be multipotential progenitors that exhibit spontaneous terminal differentiation into erythroid, megakaryocytic or basophilic lineages.[18,19] Terminal erythroid differentiation in the absence of fetal bovine serum is associated with the appearance of erythroblasts containing HbF, hemoglobin A and glycophorin surface markers.[18] KU812 cells also synthesize endogenous interleukin-6 and upon terminal differentiation the receptors for tumor necrosis factor-α and interleuekin-6 are expressed;[20] however, the exact mechanism which sustains terminal differentiation in KU812 cells remains unknown.

To determine the usefulness of KU812 cells as a system for identifying compounds that induce HbF, we first determined the relative stage of KU812 differentiation in comparison with primary erythroid progenitors. Subsequently, we examined the ability of known HbF inducers to activate γ-globin expression and whether novel compounds could be discovered in this system. Erythroid markers and transcription factors expression levels in KU812 cells were comparable to days 7–14 erythroid progenitors. Several drugs induced γ-globin; however, some agents also induced β-globin. HbF was increased in KU812 cells in a pattern comparable to that observed in normal human erythroid progenitors and p38 mitogen-activated protein kinase (MAPK) signaling was required to produce γ-globin activation by NaB and TSA. The usefulness of KU812 cells as a cell culture system to identify novel HbF inducers is discussed.

Material and methods

Tissue culture

K562 and KU812 cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Gibco, Carlsbad, CA, USA) at 37°C, 5% CO_2. KU812 cells were purchased from American Tissue Culture Collection (Manassas, VA, USA) and maintained in culture for two years in the Pace Laboratory before the current studies were completed. KU812 cells were also grown in the absence of fetal bovine serum for five days to determine their ability to undergo spontaneous differentiation. Cell aliquots were removed daily for cell counts, viabilities using 2% trypan blue and cytospin cell preparations for morphology using Geimsa stains; cells were photographed at $\times 40$ using white light on a Nicon Digital Sight DS-L1 camera. RNA was isolated for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, described below. The mRNA levels of γ-globin, β-globin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cell surface markers and transcription factors were measured by RT-qPCR on the days indicated.

Inductions were performed with one million cells treated for 48 h with the following drugs purchased from Sigma (St Louis, MO, USA): 50 μmol/L Hem, 2 mmol/L NaB, 0.5 μmol/L TSA, 10 mmol/L cysteine (Cys), 0.01% DMSO and 100 μmol/L HU. Additional agents tested included 1.0 μmol/L scriptaid (Alexis, Plymouth, PA, USA) and 5 μmol/L suberoylanilide hydroxamic acid (SAHA), a gift from Merck & Company, Inc (Whitehouse Station, NJ, USA). Dose–response studies (0.125–1 μmol/L) were performed with a novel agent STI-571 (Gleevec^{TM}, a gift from Novartis Pharma AG, Basel, Switzerland) to determine if this compound was capable of inducing HbF in erythroid cells.

Two-phase erythroid liquid culture

For studies performed in human erythroid progenitors, peripheral blood buffy coat mononuclear cells were purchased from Carter BloodCare (Fort Worth, TX, USA) in accordance with guidelines of the Institutional Review Board at the University of Texas at Dallas. Erythroid progenitors were generated using the two-phase liquid culture system[8] as follows. During phase 1, cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum and 50 ng/mL each of granulocyte-monocyte colony-stimulating factor, interleukin-3 and stem cell factor. To initiate phase 2 on day 7, the medium was changed and erythropoietin (3 U/mL) and stem cell factor (50 ng/mL) were added. Progenitors were harvested on days 7, 14, 21 and 28 for cell counts and viability using 2% trypan blue. Drug inductions were performed with various agents on day 11 for 72 h; RNA was isolated for RT-qPCR and cytospin preparations for cell morphology using Geimsa and fluorescent antibody stain. The mRNA levels of the genes summarized in Table 1 were measured by RT-qPCR analysis on the days indicated.

RT-qPCR analysis

Total RNA was isolated from samples using RNA Stat-60^{TM} (TEL-TEST 'B' Inc, Friendswood, TX, USA) and used for RT-qPCR analysis as previously published.[21] Briefly, cDNA was prepared using the Improm-II reverse transcriptase system and oligo $(dT)_{15}$ primers (Promega,

Table 1 Characterization of gene expression profile in KU812 and primary erythroid cells

Genes	Primary erythroid cells				KU812 cells†
	Day 7 Mean ± SEM‡	Day 14 Mean ± SEM‡	Day 21 Mean ± SEM‡	Day 28 Mean ± SEM‡	Baseline Mean ± SEM‡
CD34	5.27 ± 0.38	4.23 ± 0.18	1.42 ± 0.04*	1.78 ± 0.03*	3.2 ± 0.236
CD36	22.69 ± 2.4	40.62 ± 0.4	666.66 ± 10.1	311.32 ± 10.7	30.22 ± 1.546
EpoR	7.98 ± 1.5	22.51 ± 0.5*	115.13 ± 2.6*	60.06 ± 1.0*	9.54 ± 2.45
Glycophorin A	0.35 ± 0.30	6.54 ± 1.80*	49.89 ± 17.26*	30.1 ± 2.98*	1.63 ± 0.235
GATA-1	21.19 ± 1.9	76.68 ± 1.0*	278.50 ± 2.9*	91.19 ± 1.1*	24.20 ± 15.684
GATA-2	33.99 ± 3.7	29.01 ± 2.7	11.82 ± 0.3*	20.91 ± 1.2*	16.51 ± 2.94
EKLF	6.44 ± 0.63	33.22 ± 1.8*	127.19 ± 2.5*	85.53 ± 4.6*	34.13 ± 2.239
NF-E2	7.14 ± 1.0	29.82 ± 15.0*	73.90 ± 8.9*	93.23 ± 440.8*	34.82 ± 5.723
γ-Globin	30.82 ± 1.68	39.12 ± 7.25	61.90 ± 17.67	63.22 ± 11.68	41.52 ± 4.278
β-Globin	12.69 ± 1.06	32.96 ± 5.73	134.23 ± 12.68	183.11 ± 17.08	2.23 ± 1.234

NF-E2, nuclear factor-erythroid 2; EpoR, erythropoietin receptor; EKLF, erythroid Krupple-like factor; SEM, standard error of the mean
†KU812 cells were maintained in Iscove's modified Dulbecco medium supplemented with 10% fetal bovine serum. *$P < 0.05$
‡All mRNA values are normalized to GAPDH levels

Madison, WI, USA). qPCR was performed on an iCycler iQ machine (Bio-Rad, Hercules, CA, USA) using a master mix containing Sybergreen iQ Supermix (Bio-Rad) and 100 pmol/L of each gene-specific primer pairs for γ-globin, β-globin and the internal control GAPDH. Standard curves were generated using serial 10-fold dilutions of Topo7 base plasmids carrying a γ-globin cDNA sequence (Topo7-γ-globin), Topo7-β-globin and Topo7-GAPDH. The globin mRNA levels were calculated as a ratio of GAPDH (γ/GAPDH, β/GAPDH) and the γ/β-globin mRNA ratio was calculated by dividing γ/GAPDH by β/GAPDH. The expression levels of CD34, CD36, glycophorin A (GPA), erythropoietin receptor (EpoR), GATA-1, GATA-2, NF-E2 and EKLF were measured by RT-qPCR using SuperArray SYBR Green/Fluorescein PCR master mixes (Frederick, MD, USA) at 0.4 μmol/L concentration. Standard curves were generated using genomic DNA (0.5–500 ng) and mRNA values were normalized by GAPDH.

Enzyme-linked immunosorbent assay

Total hemoglobin was quantified using 20 μL of protein extract from one million KU812 cells mixed with 5 mL of Drabkin's reagent (Sigma), then cyanmethemoglobin was measured at the 540 nm wavelength. HbF levels were measured using the human Hemoglobin F enzyme-linked immunosorbent assay (ELISA) Quantitation Kit (Bethyl Laboratory, Montgomery, TX, USA). Briefly, 96-well plates were coated with sheep anti-human HbF antibody (1 mg/mL); after blocking with 1% bovine serum albumin, horse radish peroxidase-conjugated secondary antibody (1 mg/mL) was added. Raw data were analyzed using GraphPad PRISM (GraphPad Software, Inc, La Jolla, CA, USA) and HbF levels were calculated as a ratio of total hemoglobin corrected for total protein concentrations for each sample (HbF/total Hb/total protein).

Immunohistohemistry

Cytospin cell preparations were fixed with 4% paraformaldehyde in phosphate buffered saline for 15 min, washed and then permeabilized with 0.3% Trition-100 solution for 10 min. Cells were then blocked in a 5% bovine serum albumin solution at room temperature. Immunostaining was performed at 4°C overnight with anti-HbF or anti-hemoglobin A (HbA) fluorescein isothiocyanate (FITC)-conjugated antibody (Bethyl Laboratories, Montgomery, AL, USA) at a 1:200 dilution or anti-HbA phycoerythrin (PE)-conjugated antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) at a 1:100 dilution. Cell nuclei were stained with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Santa Cruz Biotechnology, CA, USA).

KU812 and primary erythroid cells were photographed with an Olympus BX 51 phase-contrast epifluorescent microscope equipped with Hoffman modulation optics. Phase-contrast images were recorded with a CCD camera (1/100 s exposure) and fluorescence images were photographed through 485/20 nm emission and 540/35 nm excitation filters.

p38 MAPK signaling analysis

To determine the time course of p38 MAPK phosphorylation, KU812 cells were treated with NaB (2 mmol/L), TSA (0.2 μmol/L) and anisomycin (ANI) (200 ng/mL) for 1, 6, 24 and 48 h. Western blot was performed as previously published[22] using phosphorylated p38 (p-p38) and total p38 (t-p38) antibodies (1:1000 dilution) purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-rabbit IgG-horseradish peroxidase secondary antibody diluted 1:5000 was used for chemiluminescent detection using the ECL system (Amersham, Piscataway NJ, USA). To demonstrate the role of p38 MAPK in γ-globin induction, KU812 cells were treated for 48 h with NaB and TSA either alone or after a one hour preincubation with the p38 MAPK inhibitor SB203580 (10 μmol/L; Calbiochem, San Diego, CA, USA). ANI (200 ng/mL), a p38 MAPK activator was tested as a positive control. RNA was isolated for RT-qPCR analysis of γ-globin, β-globin and GAPDH expression.

Statistical analysis

The data are reported as the mean ± standard error of the mean from at least five data points generated from independent drug treatments. Data were analyzed by a two-tailed

Student's t-test and values of $P < 0.05$ were considered statistically significant. Statistical analyses were performed using Microsoft Excel (Redmond, WA, USA).

Results

Enhanced globin gene expression occurs during erythroid differentiation in KU812 cells

Previously published studies demonstrated that the γ- and β-globin genes are expressed in multipotential KU812 cells.[18] Therefore, our first set of studies were aimed at establishing the ability of the KU812 cells maintained in our laboratory, to undergo spontaneous erythroid differentiation in the absence of fetal bovine serum and to profile expression levels for a subset of erythroid genes during this process. For comparison, primary erythroid progenitors were grown from peripheral blood mononuclear cells using a two-phase liquid culture system. Cell aliquots were removed either daily from KU812 cells or on days 7, 14, 21 and 28 in primary progenitors for viability and cytospin cell preparations stained with Geimsa to examine cell morphology; in addition RNA was isolated for RT-qPCR gene expression profiling.

Throughout the culture period, KU812 cells maintained viability over 90% whereas decreased cell proliferation was produced in the absence of fetal bovine serum. By day 5, cell morphology consistent with orthochromatophilic erythroblasts along with other immature progenitors was observed (Figure 1a). Over the same period, γ-globin mRNA levels decreased significantly with a concomitant increase in β-globin expression (Figure 1b). However, the total level of γ-globin/GAPDH mRNA remained greater than β-globin/GAPDH (data not shown), suggesting that hemoglobin switching does not occur in KU812 cells. Similar studies in primary progenitors demonstrated erythroid maturation and a marked increase in β-globin expression by day 21 compared with γ-globin (Figure 1c and d). We also determined levels of key trans-factors involved in erythropoiesis for KU812 cells grown in serum-free media (Figure 1e). The significant increase in EpoR, GPA, GATA-1, EKLF and NF-E2 expression levels from day 1 to day 5 was similar to changes in expression levels observed from day 7 to day 14 in primary erythroid cells. These data support the ability of KU812 cells to undergo differentiation with enhanced globin and erythroid-specific gene expression.

KU812 cells express surface markers comparable with days 7–14 erythroid progenitors

Erythropoiesis involves lineage commitment of multipotential stem cells and terminal differentiation mediated by the expression of specific transcription factors and structural proteins required for normal red blood cell physiology. In our system during erythroid commitment, a steady decrease in CD34 expression was observed (5.27 ± 0.38 to 1.78 ± 0.03) with a concomitant 10-fold to 85-fold increase of erythroid-specific markers including CD36, EpoR and GPA (Table 1). The increase in CD36 expression confirms previous reports that CD36-positive cells are usually present in more mature erythroid progenitors.[23] We also measured key transcription factors including GATA-1 which binds in the promoters of several erythroid-specific genes; GATA-1 levels increased 13.1-fold by day 21 while GATA-2 levels decreased 65% consistent with other reports.[24,25] EKLF is an erythroid cell-specific factor that plays a crucial role in β-globin gene expression during adult stage development.[26] NF-E2 is a heterodimeric transcription factor that consists of a hematopoietic-restricted subunit ($p45^{NF-E2}$) and a ubiquitously expressed member of the Maf family of proteins.[27] Expression levels for both factors increased about 13.5-fold during erythroid differentiation and we observed a change in the predominant globin gene expressed from γ-globin (30.82 ± 1.68) to β-globin (183.11 ± 17.08) by day 28, consistent with hemoglobin switching.

Gene profiling was also performed to determine the level of expression of key erythroid genes and hematopoietic factors in KU812 cells maintained in the standard conditions of 10% fetal bovine serum used for induction studies. The expression levels of CD36, EpoR and GPA (Table 1) suggest that KU812 cells have an erythroid phenotype; in addition, levels of the trans-factors including GATA-1, NF-E2 and EKLF were similar to that observed in days 7–14 normal erythroid progenitors as were levels of γ-globin and β-globin gene expression.

Known HbF inducers augment γ-globin gene transcription in KU812 cells

The next set of experiments was aimed at testing the ability of the known HbF inducers including Hem, NaB and HU to modulate γ-globin gene expression in KU812 cells. In addition, three histone deacetylase inhibitors including TSA, scriptaid and SAHA were analyzed. To determine the specificity of drug-mediated gene activation, we monitored γ- and β-globin mRNA levels by RT-qPCR. Hemin treatment induced γ-globin 2.87-fold while β-globin expression was not changed significantly (Table 2). On the other hand, HU activated γ-globin 1.24-fold and decreased β-globin approximately 56%. We also observed γ-globin induction by NaB (1.56-fold; $P = 0.0419$) and SAHA (2.91-fold; $P = 0.0060$) while TSA and scriptaid did not significantly increase γ-globin expression in KU812 cells. Additional studies with the negative control Cys and the drug vehicles ethanol and DMSO demonstrated a lack of significant γ- or β-globin induction by these agents at the concentrations used in our cultures (Table 2).

STI-571 treatment induced γ- and β-globin gene expression in KU812 cells

STI-571 is a compound that inhibits the tyrosine kinase activity of the fusion protein Bcr-Abl, over-expressed in chronic myelogenous leukemia cells.[28] It has been reported that STI-571 stimulates hemoglobin synthesis in K562 cells where an embryonic/fetal pattern of globin gene expression is observed.[28,29] However, it is not known whether STI-571 specifically activates γ-globin expression. Therefore, we first

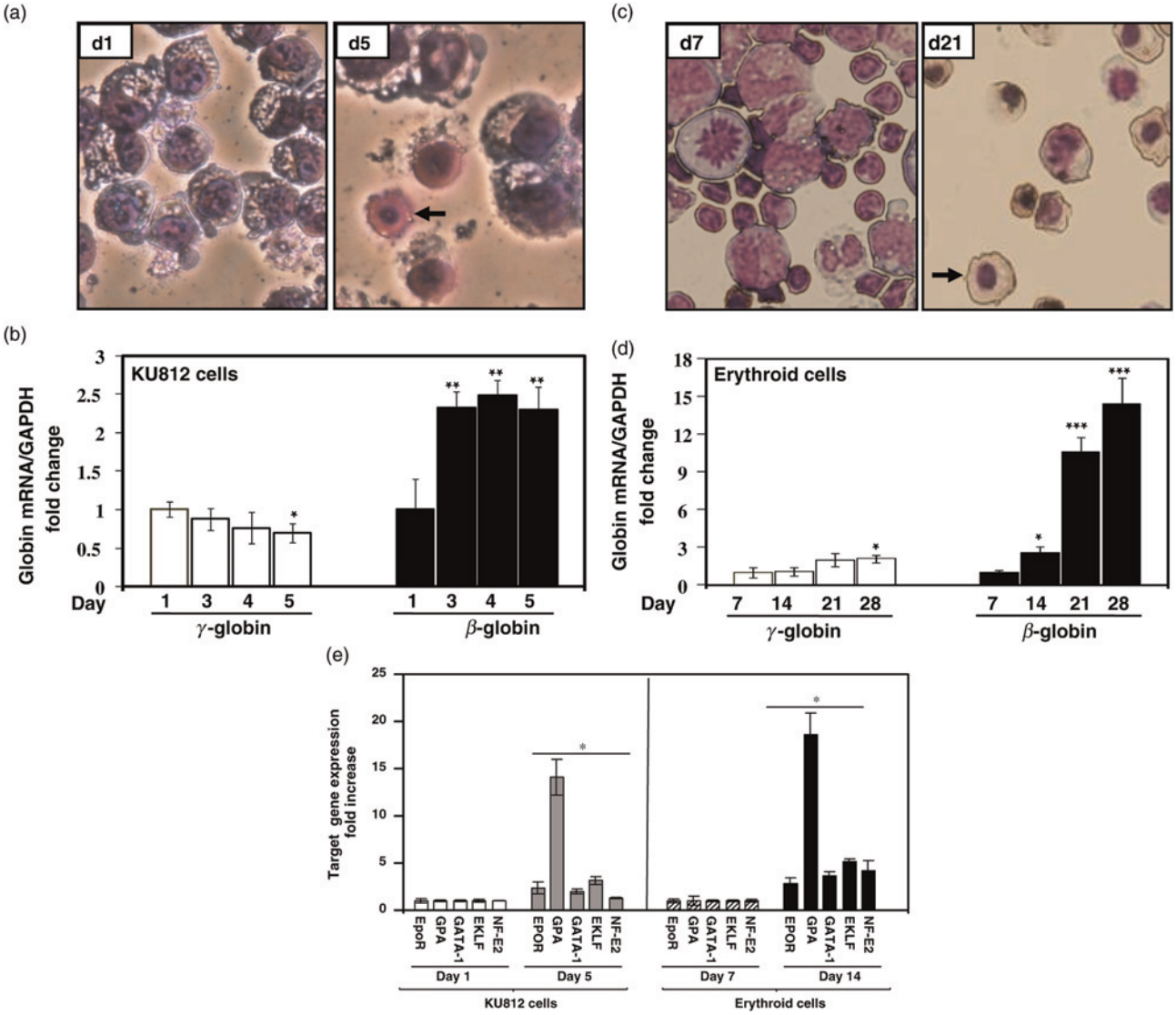

Figure 1 KU812 cells undergo erythroid differentiation in the absence of fetal bovine serum. (a) KU812 cells were grown for five days without fetal bovine serum. Cell aliquots were removed daily for Geimsa stain and RNA isolation for globin gene expression by RT-qPCR analysis. Shown are cytospin preparations of stained KU812 cells on the days indicated. Photographs were taken at $\times 40$ magnification. Note the presence of orthochromatophilic erythroblast by day 5 in culture (black arrow). (b) Bar graphs showing the quantitative RT-qPCR data for γ- and β-globin gene mRNA levels from five independent experiments. Data are presented as the mean $\pm$ SEM, $^{*}P < 0.05$ was considered significant; $^{**}P < 0.01$. (c) Primary erythroid progenitors were grown from peripheral blood mononuclear cells in a two-phase liquid culture system (Material and methods). Cytospin preparations were made at the days shown and stained with Geimsa. Note the appearance of orthochromatophilic erythroblast by day 21 (black arrow). (d) Bar graphs showing the RT-qPCR data for γ- and β-globin gene mRNA levels in erythroid progenitors at the days indicated. $^{***}P < 0.001$ (e) RT-qPCR data for the target genes analyzed in KU812 and primary erythroid cells. NF-E2, nuclear factor-erythroid 2; EpoR, erythroipoietin receptor; EKLF, erythroid Krupple-like factor; SEM, standard error of the mean; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Table 2 Globin gene induction in KU812 cells by pharmacological agents

Compounds	Drugs	γ/GAPD Mean $\pm$ SEM	*P* value	Fold change	β/GAPD Mean $\pm$ SEM	*P* value	Fold change
Untreated cells	None	10.62 $\pm$ 1.124	–	1.00	0.248 $\pm$ 0.019	–	1.00
Hemin	50 μmol/L	30.48 $\pm$ 6.888	0.0423	2.87	0.29 $\pm$ 0.010	0.0758	1.16
Hydroxyurea	100 μmol/L	13.16 $\pm$ 0.390	0.2757	1.24	0.108 $\pm$ 0.002	0.0002	0.437
Butyrate	2 mmol/L	16.52 $\pm$ 0.927	0.0419	1.56	0.077 $\pm$ 0.0017	0.0456	0.31
Trichostatin A	0.2 μmol/L	9.29 $\pm$ 0.4361	0.5349	0.88	0.136 $\pm$ 0.0803	0.1759	0.55
Scriptaid	1.0 μmol/L	10.51 $\pm$ 1.131	0.9763	0.99	0.010 $\pm$ 0.0143	0.0405	0.05
Suberoylanilide hydroxamic acid	2.5 μmol/L	30.95 $\pm$ 3.652	0.0060	2.91	0.241 $\pm$ 0.014	0.8977	0.97
Cysteine	10 mmol/L	9.83 $\pm$ 1.721	0.7201	0.93	0.335 $\pm$ 0.023	0.2281	1.35
Ethanol	1%	15.44 $\pm$ 2.943	0.2005	1.45	0.700 $\pm$ 0.10	0.0912	2.92
Dimethyl sulfoxide	0.01%	8.31 $\pm$ 0.834	0.1737	0.78	0.342 $\pm$ 0.02	0.2700	1.33

SEM, standard error of the mean

tested the effect of this agent in K562 cells treated for 48 h with increasing concentrations of STI-571 (0.125–2 μmol/L). A highly significant 55-fold increase in γ-globin/GAPDH level ($P < 0.001$) was observed at the 2 μmol/L STI-571 concentration (Figure 2a), suggesting this compound might be a potent HbF inducer.

We next asked the question whether KU812 cells could be used to test the specificity of STI-571 for globin gene activation. When KU812 cells were treated with the same STI-571 drug concentrations, both γ- and β-globin mRNA levels were increased from 10- to 18-fold in a dose-dependent manner (Figure 2b). As a result, the γ/β mRNA ratios were not significantly different than levels observed in untreated KU812 cells. To compliment the mRNA data, we also performed immunohistochemistry staining with anti-HbF FITC and anti-HbA PE antibodies and DAPI to visualize cell nuclei. As shown in Figure 2c, we demonstrated a substantial increase in HbF-positive KU812 cells after STI-571 treatment; however, β-globin protein was not detected. Staining was also performed with anti-HbA FITC antibody; however, increased protein was not observed under this condition either (data not shown). These data demonstrated that STI-571 activate both globin genes at the mRNA levels in a non-specific manner.

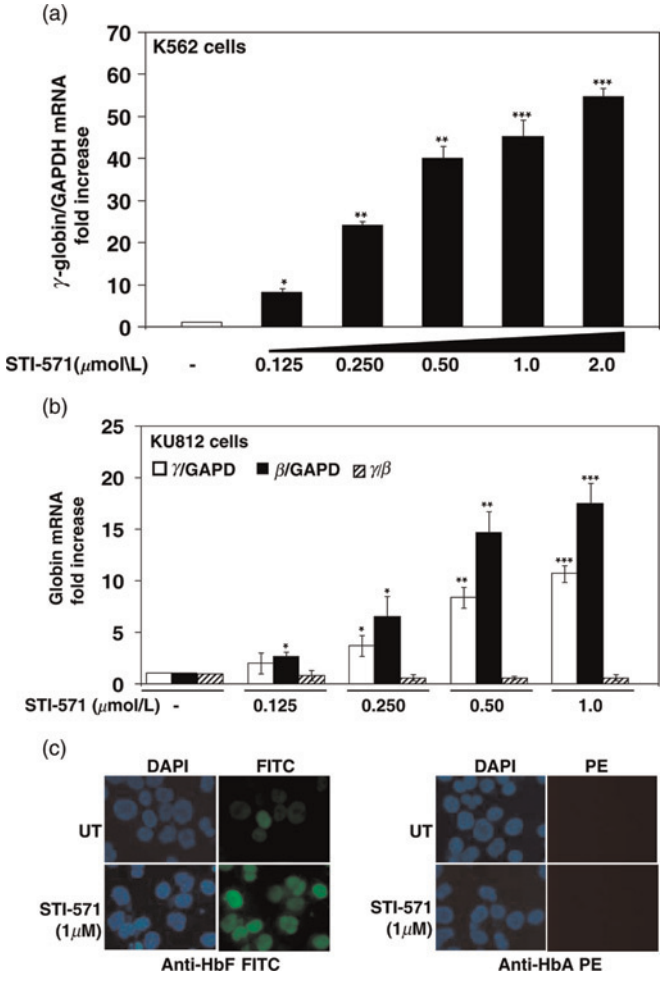

Figure 2 STI-571 induced γ-globin and β-globin gene expression in KU812 cells. (a) K562 cells were treated with STI-571 for 48 h and globin gene expression was quantified by RT-qPCR. The increase in γ-globin/GAPDH mRNA ratio at the different STI-571 concentrations is shown. The mRNA levels in untreated K562 cells (−) were normalized to one. Data are shown as the mean ± SEM. (b) KU812 cells were treated with STI-571 at the concentrations shown and then RT-qPCR analysis was performed for γ-globin, β-globin and GAPDH. The fold increase in γ/GAPDH and β/GAPDH mRNA ratios are shown along by the γ/β-globin ratios (after GAPDH normalization of each globin gene). (c) Immunohistochemistry was performed with DAPI and anti-HbF FITC and anti-HbA PE conjugated antibodies in KU812 cells treated with STI-571 (Material and methods). SEM, standard error of the mean; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole; HbF, fetal hemoglobin; HbA, hemoglobin A; PE, phycoerythrin; FITC, fluorescein isothiocyanate; UT, untreated (A color version of this figure is available in the online journal)

To correlate protein synthesis with mRNA levels, ELISA was performed to quantify HbF after various drug treatments. There was a significant increase in HbF from 1.46 to 6.92-fold for several drug inducers (Table 3). In general, we observed a greater increase in HbF protein than mRNA levels, demonstrating the ability of different pharmacologic agents to stimulate HbF synthesis in KU812 cells. Note the best HbF inducer was HU, the only drug approved for the treatment of sickle cell patients.

Drug-mediated globin gene induction in KU812 and primary erythroid cells show similar expression patterns

Human cell lines that express the adult β-globin gene which can be used to determine the ability of drugs to preferentially induce γ-globin expression and HbF synthesis are desirable. If studies in KU812 cells could predict response in primary cells, this would be a very valuable system since these cells are readily maintained in culture without requirements for specific growth factors. Therefore, data were collected to compare the response of globin genes in KU812 and primary erythroid cells after the various drug treatments. As shown in Figure 3a, the positive control Hem increased γ/β globin mRNA 4.5-fold in primary cells compared with 10-fold induction in KU812 cells; however, scriptaid was a more effective γ-globin inducer in KU812 cells (21-fold versus 9-fold increase). On the other hand, comparable γ/β globin ratios were observed between the two culture systems for NaB, TSA, SAHA and HU; STI-571 stimulated γ- and β-globin similar to our findings in KU812 cells. Fluorescent stain was also completed after STI-571 treatment in primary cells. We observed increased HbF-positive cells (Figure 3b); staining with anti-HbA FITC antibody demonstrated increased HbA-positive cells as well (Figure 3c). From these data, we concluded that the pattern of γ- and β-globin responses observed in KU812 cells was comparable to that obtained in primary erythroid progenitors.

Signaling through the p38 MAPK pathway is required for drug-mediated γ-globin activation in KU812 cells

We performed the last set of experiments to determine whether γ-globin induction in KU812 involves signaling through the p38 MAPK pathway similar to that previously demonstrated for K562 cells[22,30] and primary erythroid

Table 3 Fetal hemoglobin induction in KU812 cells by pharmacological agents

Compounds	Concentration	HbF Mean ± SEM*	*P* value	Fold increase
Untreated cells	None	0.37 ± 0.048	–	1.00
Hemin	50 μmol/L	1.221 ± 0.240	0.02371	3.32
Hydroxyurea	100 μmol/L	2.553 ± 0.056	0.01697	6.92
Butyrate	1 mmol/L	0.543 ± 0.021	0.0332	1.46
Trichostatin A	0.2 μmol/L	1.619 ± 0.174	0.0023	4.41
Scriptaid	1.0 μmol/L	1.225 ± 0.235	0.0234	3.34
Suberoylanilide hydroxamic acid	2.5 μmol/L	1.517 ± 0.831	0.02926	4.10
Cysteine	10 mmol/L	0.4292 ± 0.104	0.4563	1.16
Ethanol	1%	0.368 ± 0.040	0.9743	0.99
Dimethyl sulfoxide	0.01%	0.3998 ± 0.031	0.5782	1.08

*HbF = HbF/total Hb/total protein
SEM, standard error of the mean

cells by our laboratory.[22] To analyze p38 activation after drug treatments, we performed time-course Western blot analysis based on patterns observed in K562 cells.[22] As shown in Figure 4a, treatment with NaB and TSA produced a peak of p-p38 at one hour followed by a drop below baseline by 24 h. By contrast, ANI stimulated p-p38 to maximal levels by 6 h. Interestingly, the levels of t-p38 were decreased as well. We next asked the question whether p38 signaling is involved in γ-globin gene activation. To answer this question, KU812 cells were treated with the various drugs alone or after preincubation for one hour with 10 μmol/L SB203580 to block p-p38 signaling.[31] At steady state, SB203580 treatments produced a 40% drop in γ-globin expression (Figure 4b). Similarly, a 50% drop in gene expression was observed after ANI treatment, suggesting p38 signaling contributes in part to γ-globin activation at steady state. After γ-globin induction by NaB (1.9-fold) and TSA (2.1-fold), we observed 47.4% and 28.5% ($P < 0.05$) gene silencing, respectively, after preincubation with SB203580 (Figure 4b). By contrast, SB203580 had no effect on the ability of Hem to mediate γ-globin induction. Cell viability remained from 80% to 90% for all conditions except ANI where approximately 60% survival was observed; SB203580 treatment had no effect on cell

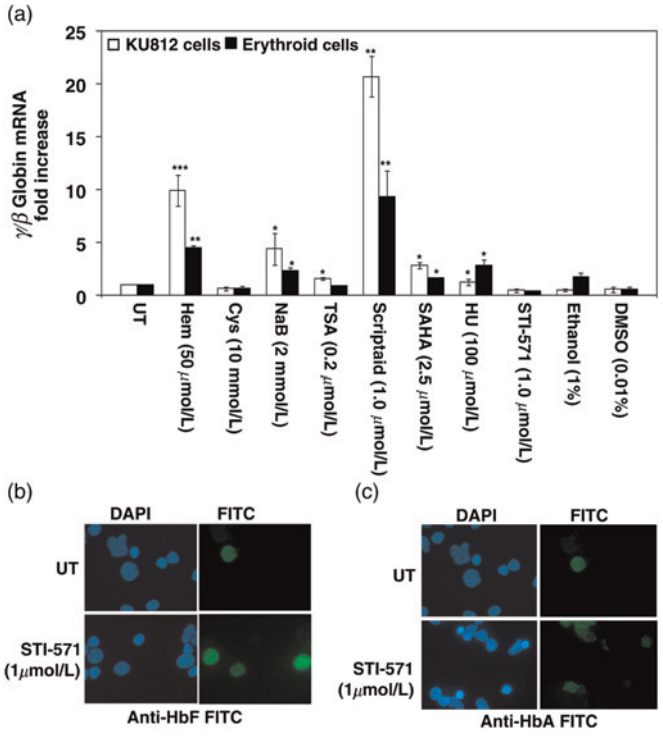

Figure 3 Patterns of γ-globin induction in KU812 cells is similar to that observed in primary erythroid progenitors. (a) The different drugs were tested in KU812 or primary erythroid cells on day 11 in culture (Materials and methods). RNA was isolated for γ-globin, β-globin and GAPDH RT-qPCR. The data are shown as a ratio of γ/β globin mRNA after correcting each gene mRNA level by GAPDH. The globin mRNA levels in untreated cells were normalized to one. (b) Immunohistochemistry was performed with DAPI and anti-HbF FITC-conjugated antibodies in erythroid progenitors treated with STI-571. (c) Immunohistochemistry was performed with anti-HbA FITC-conjugated antibodies in erythroid progenitors treated with STI-571. UT, untreated; Cys, cysteine; Hem, hemin; NaB, sodium butyrate; TSA, trichostatin A; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole; HbF, fetal hemoglobin; HbA, hemoglobin A; PE, phycoerythrin; FITC, fluorescein isothiocyanate (A color version of this figure is available in the online journal)

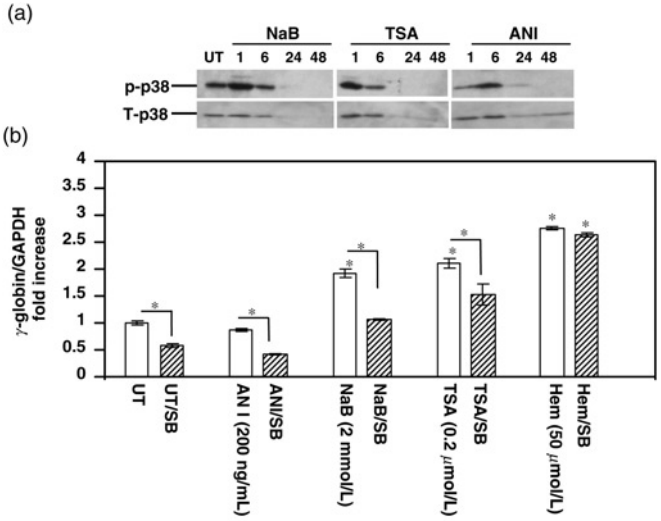

Figure 4 p38 MAPK signaling is required for γ-globin activation in KU812 cells. (a) Western blot analysis was performed to determine phosphorylated p38 (p-p38) and total p38 (t-p38) levels in KU812 cells at the times shown for the different drug inducers (Materials and methods). (b) KU812 cells were treated for 48 h either alone or after one hour preincubation with 10 μmol/L SB203580 (SB). RNA was harvested for RT-qPCR analysis. ANI, anisomycin; UT, untreated; Cys, cysteine; Hem, hemin; NaB, sodium butyrate; TSA, trichostatin A; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MAPK, mitogen-activated protein kinase

viability. These data demonstrate that p38 MAPK signaling is required for HbF induction by NaB and TSA in KU812 cells.

Discussion

Considerable progress has been made toward expanding our understanding of pharmacological induction of HbF using cell lines, animal models and clinical trials in β-hemoglobinopathy patients.[32,33] For the most part, the discovery of HbF inducers has been hypothesis driven; for example, the demethylation of DNA by 5-azacytidine to produce γ-globin reactivation in baboons[34] and humans.[35] Furthermore, cytotoxic agents such as arabinosylcytosine[36] and HU[37] were demonstrated to induce HbF based on perturbations of cell kinetics after drug treatments. Several agents have been tested in immortalized cell lines including K562 and mouse erythroleukemia[38–41] cells, genetically manipulated mice[42,43] and primates.[34,44,45] These systems provided new insights into globin gene regulation and HbF induction in humans.

K562 cells display an embryonic/fetal phenotype, expressing ε-globin and γ-globin, and have been used most commonly to screen potential HbF inducers. It is often not clear whether γ-globin activation is the result of erythroid differentiation or gene-specific induction. A limitation of K562 cells is the lack of β-globin expression despite the presence of a normal β-gene. Therefore, a human erythroid cell line where both γ-globin and β-globin are transcribed to mimic normal erythroid progenitors would be a desirable system for the discovery of novel HbF inducers. Cell lines such as HEL, JK-1, KMOE-2, LAMA-84 and MB-02 have active γ- and β-globin chain synthesis;[9] however, only KU812 cells contain detectable Bart's, fetal and adult hemoglobin protein and can undergo differentiation.[18] Therefore, KU812 cells have the potential to be developed as a system for screening γ-globin gene activators. To determine if this was the case, we analyzed KU812 cell surface markers, some key trans-activators and response to known and novel drug inducers using gene expression profiling in primary erythroid cells for comparison.

KU812 cells have been classified as a multipotential cell line with the ability to differentiate down the basophilic,[17] eosinophilic[19] and erythroid/megakaryocytic lineages.[46] We first confirmed the erythroid phenotype of the KU812 cells maintained in our laboratory using the erythroid markers CD36, and the EpoR. These markers were expressed at levels comparable to days 7–14 erythroid progenitors grown *in vitro*. In general, erythroid differentiation proceeds in response to cell signaling stimuli and transcriptional programs regulated by lineage-restricted DNA-binding proteins.[47,48] In KU812 cells, levels of the transcription factors GATA-1, NF-E2 and ELKF were increased during normal erythropoiesis and spontaneous differentiation of KU812 cells in serum-free medium supporting the utility of the latter as a model system.

Pharmacological agents such as DMSO, NaB, phorbol esters or Hem have been used to stimulate numerous cell lines to differentiate along the erythroid, megakaryocytic or monocyte/macrophage lineages. We tested several pharmacological agents in KU812 cells to determine the level of γ-globin induction relative to that of β-globin. Two drugs activated γ-globin but had no effect on β-globin (Hem and SAHA) or activated γ-globin with a concomitant decrease in β-globin (HU, NaB, TSA and scriptaid). To compliment the mRNA data, we observed HbF induction by ELISA for six of the agents tested. These data demonstrate the ability to distinguish which chemical compounds activate γ-globin and/or β-globin transcription and HbF synthesis using KU812 cells.

We also tested a new compound STI-571 to determine its specificity for γ-globin activation and compared the response produced in primary erythroid cells. STI-571 is an anticancer compound that selectively inhibits the Bcr-Abl tyrosine kinase.[28] This agent induced γ-globin 55-fold in K562 cells, whereas the induction was much less dramatic in KU812 cells. However, we gained the new knowledge that STI-571 also activated β-globin, ultimately producing little change in the γ/β ratio in KU812 cells, suggesting that STI-571 would not be an effective HbF inducer; this conclusion was supported by data obtained in primary erythroid cells. Using γ-globin gene activation and β-globin-gene silencing as the optimal responses desired for an effective HbF inducer, then scriptaid, SAHA and HU would have been predicted to be the best HbF inducers. Our published studies in β-YAC transgenic mice treated with scriptaid[49] demonstrated *in vivo* HbF induction while the success of HU therapy in adults[1,2] and children[50] with sickle cell disease support our conclusions in KU812 cells.

Studies aimed at comparing drug response in KU812 cells with those produced in primary erythroid progenitors were also performed. Although the magnitude of the response to Hem and scriptaid was greater in KU812 cells, the general pattern of γ-globin inducibility was the same in both cell culture systems. Our final set of experiments confirmed a role for p38 MAPK signaling in mechanisms of γ-globin induction in KU812 cells. We observed p38 activation which was required for maximal γ-globin gene induction by NaB and TSA lending support of KU812 cells as a screening system to identify HbF inducers.

For many years K562 cells have been used as the main model for drug screening. However, increased γ-globin gene transcription in these cells may be a consequence of increased globin gene expression during terminal differentiation, rather than direct γ-globin trans-activation. A second system, mouse erythroleukemia cells, display an adult pattern of globin synthesis; however, mice lack a fetal developmental stage of globin gene expression. The generation of stably transfected mouse erythroleukemia cells with incorporated DNA fragments of the human γ-globin gene failed to demonstrate proper regulation of the introduced gene.[51] Similarly, mouse erythroleukemia cells containing a human β-YAC transgene were not correctly regulated.[52] Skarpidi *et al.*[51] developed a rapid method for detecting HbF inducers using a DNA construct, in which the coding sequences of firefly and renilla luciferase genes were substituted for human γ- and β-globin, respectively, in stably transfected murine GM979 cells. This system was

used to screen short-chain fatty acid derivatives as HbF inducers. Testing the dual reporter in human KU812 cells might prove to be a suitable alternative; however, a recognized limitation of using cells lines is that the trans-factor environment will reflect the stage of developmental maturation of those cells. In KU812 cells, the level of γ-globin is many folds greater than β-globin supporting a fetal-type erythroid environment; therefore, the final test of the ability of a drug to act as an HbF inducer in adult-type cells will ultimately require primary cultures and/or *in vivo* testing.

Normal erythroid progenitors grown from $CD34^+$ cells in liquid culture remain the standard for validating the efficacy of HbF-inducing agents. While liquid culture systems have overcome limitations of cell numbers, there remain challenges related to the cost of CD34+ cells, a longer growth period to generate erythroid progenitors and a requirement for multiple growth factors. Therefore, an immortalized human cell line that is readily maintained in culture with active γ-globin and β-globin gene expression such as KU812 cells would be useful as a screening tool. This would allow the establishment of stable cell lines with reporter constructs that can be used for high throughput drug screening such as that established in GM979 cells.[51] Newer cell lines such as JAS-R that secrete erythropoietin[53] might be another candidate to be investigated in the future.

Author contributions: All authors participated in the design and interpretation of the studies, data analysis and review of the manuscript. SZ and SS carried out drug induction studies in KU812 and K562 cells. T-FL and AM completed protein quantification and fluorescent microscopy. WL conducted primary culture experiments. VR completed the p38 MAPK signaling studies. SZ and BP wrote the manuscript. All individuals who made contributions to this study are included as authors.

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