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Novel therapeutic candidates, identified by molecular modeling, induce γ -globin gene expression in vivo[☆]

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Abstract

The β -hemoglobinopathies and thalassemias are serious genetic blood disorders affecting the β -globin chain of hemoglobin A ($\alpha_2\beta^A_2$). Their clinical severity can be reduced by enhancing expression of fetal hemoglobin (γ -globin), producing HbF ($\alpha_2\gamma_2$). In studies reported here, γ -globin induction by 23 novel, structurally-unrelated compounds, which had been predicted through molecular modeling and *in silico* screening of a 13,000 chemical library, was evaluated *in vitro* in erythroid progenitors cultured from normal subjects and β -thalassemia patients, and *in vivo* in transgenic mice or anemic baboons. Four predicted candidates were found to have high potency, with 4- to 8-fold induction of HbF. Two of these compounds have pharmacokinetic profiles favorable for clinical application. These studies thus effectively identified high potency γ -globin inducing candidate therapeutics and validated the utility of *in silico* molecular modeling.

[☆]*Contributors:* SPP, DVF, MT, GLW, and CHL designed the studies and evaluated the results. SAC, MSB, RM, DWE, MSM, and LS performed experiments. RB performed molecular modeling. SPP, DVF, CL, and SAC wrote the manuscript. All authors approved the final manuscript.

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Conflict of interest: SPP and RB are inventors on a patent application relating to this work. The authors declare that they have no other competing interests.

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Keywords

Butyrate; fetal hemoglobin; hemoglobinopathy; small molecules

Introduction

The β -globin disorders are serious genetic blood diseases and are designated as a global health burden [1]. These conditions become clinically apparent only after fetal hemoglobin (HbF, $\alpha_2\gamma_2$) is suppressed in infancy. HbF induction, even to small degrees, is established to ameliorate β -globin disorders, as γ -globin chains can functionally replace deficient β -globin, reducing anemia in β -thalassemia, and inhibit polymerization of sickle hemoglobin (HbS) in sickle cell disease [2–18]. Only one therapeutic, hydroxyurea (HU), is approved for treatment of sickle cell disease and is beneficial in approximately 50% of patients [19]. Additional therapeutic agents, particularly non-cytotoxic agents which can be used in combination with hydroxyurea, are needed [3,20]. Prior generation HbF-inducers, arginine butyrate (AB) and sodium phenylbutyrate (SPB), have demonstrated proof-of-concept, but require high doses which are difficult for long-term use and broad patient application [9–12,15,21]. Orally-active HbF-inducers which have adequate efficacy at doses which are more tolerable for long-term use would provide a significant advance for treating these globally-prevalent conditions [3–5,22]. Prolongation of erythroid survival by stimulation of HbF production would be particularly beneficial in β -thalassemias, a condition in which apoptosis of erythroid precursors occurs early in developing erythroblasts [5,7,16,23–29].

Several short-chain fatty acids and derivatives (SCFAs; SCFADs), including butyrate, sodium phenylbutyrate, valproate, and isobutyramide, have induced HbF and produced clinical effects in small clinical trials, and demonstrated proof-of-principle for this pharmacological approach [4,5,9–15,30,31]. One limitation of these therapeutic agents is that several are histone deacetylase inhibitors, and although potent γ -globin inducers, also suppress erythroid cell growth [32,33]. Arginine butyrate was particularly active, but requires intravenous administration, due to its short plasma half-life, intermittent administration due to its anti-proliferative effects. The large oral dose requirements for sodium phenylbutyrate limited its application. Orally-active γ -globin inducers with higher potency and without large dose regimens, genotoxicity, or cytotoxicity, are desirable for long-term definitive therapy of patients with β -globin diseases.

In a program to identify additional therapeutic candidates, we employed iterative pharmacophores, pseudo-binding site modeling, and *in silico* high-throughput screening. Compounds predicted by the modeling to be HbF-inducers were screened for fetal globin induction in promoter assays, including a dual-luciferase promoter gene assay which detects only strong and specific inducers of the γ -globin promoter, rather than general globin gene induction [34] [35].

We have now evaluated 14 candidates for γ -globin-inducing activity in human erythroid progenitors and confirmed their activity. Of these, two compounds (RB7 and RB16) were found to have γ -globin inducing activity *in vivo* without toxicity and with favorable pharmacokinetic profiles for therapeutic application.

Material and Methods

Molecular modeling and test compounds

Molecular modeling was carried out as detailed previously [35]. The TFIT program within the FLO/QXP Molecular Modeling software (cmcma@ix.netcom.com; ThistleSoft,

Colebrook, CT) was used to construct pharmacophores. The “pseudo” receptor was constructed with the PSEUD module of FLO and docking was carried out using the S-DOCK+ program of FLO. Figures were prepared using PyMol [35].

The candidate compounds identified through this molecular modeling and *in silico* screening (designated RB compounds) were obtained from Specs (Wakefield, RI) [35]. Arginine butyrate (AB; The University of Iowa, Iowa City, IA) and sodium 2,2-dimethyl butyrate (ST20; Frontage Laboratories, Malvern, PA) were utilized as positive control compounds as established inducers of γ -globin gene activity [2,4,5,7,22]. A schema illustrating the testing is shown in Fig. 1A. A schematic of two of the active compounds, RB7 and RB16, within the “pseudo” receptor is shown in Fig. 1B, i and ii, respectively.

Erythroid progenitor cultures and F-cells by FACs

Progenitor cultures were performed to evaluate potential cytotoxicity and γ -globin gene expression. Peripheral blood samples from 9 different patients with β -thalassemia were collected in heparin and studied with approval of the Institutional Review Board of the Boston University School of Medicine, and all participants gave written informed consent. Erythroid progenitors were cultured from peripheral blood samples, as previously described [28]. Briefly, CD34+ cells were enriched using the RosetteSep reagent and were cultured in serum-free methylcellulose media (H4436) containing EPO (3 U/ml) and IL-3 (20 ng/ml) to support Bfu-e growth (STEMCELL Technologies, Vancouver, BC, Canada). Cultures were established with or without the test compounds, added at the time the cultures were initiated and on the last day of culture. Each compound was tested over a concentration range, and then at the optimal concentrations an average of 4–6 times. Erythroid colonies were enumerated on day 14. Cultured erythroid cells were analyzed for the fraction of cells expressing HbF (% F-cells) by immunofluorescent staining and flow cytometry, as previously described [28,36]. In brief, pelleted cells were first fixed with 1 ml of 4% formaldehyde in Hank's Buffered Saline Solution (HBSS) for 30 minutes. The cells were then permeabilized with serial incubations in 50:50 acetone:water, acetone, and 50:50 acetone::water at 4°C for 3 minutes each, separated by centrifugation at 0°C for 3 minutes each. Following a wash in cold HBSS containing 2% fetal bovine serum (FBS) and pelleting by centrifugation, the cells were stained for 30 minutes on ice with a PE-conjugated mouse anti-human fetal hemoglobin antibody (BD Biosciences, San Jose, CA). The cells were again washed with HBSS/FBS and then analyzed on a FACScan flow cytometer using CellQuest software (BD Biosciences). The fraction of cells expressing HbF was determined by setting gates based on unstained and isotype controls.

Two-phase erythroid suspension cultures

CD34+ cells were obtained from the NHLBI Program of Excellence in Gene Therapy, National Hematopoietic Cell Processing Core, Fred Hutchinson Cancer Center (Seattle, WA) using a protocol approved by the University of Washington Institutional Review Board. Mobilized CD34+ cells from healthy adults were cultured in a two-phase liquid system, containing fetal bovine serum and growth factors including rHuIL-3 (50 ng/ml), rHuSCF (100 ng/ml), and Flt-3 ligand (100 ng/ml) for 7 days, followed by rHuEPO at 4 U/ml (R&D Systems, Minneapolis, MN) for 14 days to enrich for erythroid progenitors, as previously described [30,37]. Test compounds were added to second phase cultures 2 days after initiation. Every 2 days thereafter, cells were enumerated by hemocytometer counting and mRNA was harvested. Cell lysates were also collected from the second phase erythroid cultures on day 14 for hemoglobin analysis by high-performance liquid chromatography (HPLC). Erythroid differentiation was assessed by flow cytometry, using anti-CD34-PE, anti-CD45-PerCP-Cy5.5, anti-CD71-APC, and anti-CD235a-FITC antibodies (BD Biosciences). Matching isotype control antibodies were used as negative controls.

mRNA analysis by real-time PCR and analysis of HbF by HPLC

Serial RNA samples were isolated from purified erythroid cells in suspension cultures, and quantitative PCR was performed, as previously described [30]. Briefly, total RNA was isolated using the RNeasy Plus Mini Kit (QIAGEN, Valencia, CA), reverse transcription was performed using the GeneAmp RNA PCR kit (Applied Biosystems), and PCR was performed using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) on a Opticon Monitor instrument (MJ Research, Watertown, MA). Primers sequences were as follows: β -actin, GTGGGGCGCCCCAGGCACCA and CTCCTTAATGTACGCACGATTTC; G3PD, TCCCATCACCATCTTCCA and CATCACGCCACAGTTTCC; α -globin, TGGGGTAAGGTCGGCGCGCA and TGCACCGCAGGGGTGAATC; β -globin, GGTGGTCTACCCTTGGACCC and GATACTTGTGGGCCAGGGCA; γ -globin, AGACGCCATGGGTCATTTCACA and GCCTATCCTTGAAAGCTCTGCAT. β -Actin and G3PD mRNA levels were employed for standardization.

HbF was measured by cation exchange high-performance liquid chromatography, using a 35 $\times$ 4.6 mm; 3 mm PolyCAT A Column, following the directions of the manufacturer (The Nest Group, Southborough, MA), as previously described [30,38].

Studies in transgenic mice

Murine experiments were conducted in accordance with the Canadian Council on Animal Care guidelines and approved by the Clinical Research Institute of Montreal Animal Care Committee. The γ 8653 transgenic mice with the human μ LCR -201 A γ -globin promoter (initially provided by George Stamatoyannopoulos) were bred in our laboratory (MT) for more than 40 generations onto C57Bl6/J mice to obtain a pure genetic background for this analysis. Two to four animals per group were administered either normal saline, ST20 (sodium 2,2-dimethyl butyrate) at 500 mg/kg, or RB16 at 50 mg/kg, in the same volumes, once daily for 5 days per week for 2 weeks. Test compounds were prepared as sterile, neutral pH, aqueous solutions. Peripheral blood was collected to determine γ -globin mRNA levels at baseline and on day 14 and/or 21 of the study.

Analysis of γ -globin mRNA in transgenic mice by real-time PCR—Isolated total RNA was used as a template for cDNA synthesis and a real-time PCR was performed using primer sets specific for the S16 and human γ -globin genes, under conditions previously described [39]. S16 RNA levels were used for standardization.

Studies in non-human primates

Pharmacokinetic profiles and assays of γ -globin expression were performed with the approval of the Institutional Animal Care and Use Committees of the University of Oklahoma Health Science Center and of Boston University School of Medicine. Test agents were administered in juvenile baboons (*Papio hamadryas anubis*) rendered anemic, as previously described [22,40]. Briefly, animals were chronically phlebotomized on a daily basis to achieve stable anemia, maintaining a total hemoglobin level of 7.0 to 7.5 g/dl. Candidate compounds were administered intravenously or orally once daily in single doses to determine pharmacokinetic profiles, or once daily in single doses, 4–5 days per week for 4–5 weeks, to assess γ -globin inducing activity. When oral bioavailability was established and intravenous and oral pharmacokinetic profiles were determined to be similar, intravenous dosing was used to avoid the repeated anesthesia required for oral administration. The compound RB7 was tested in a range from 25 to 200 mg/kg and then administered intravenously at doses of 50 or 200 mg/kg once daily, 5 days per week for 4 weeks, to evaluate γ -globin gene expression in two baboons, 6202 and 01505. The compound RB16 was administered intravenously at a dose of 10 mg/kg once daily, 4–5 days

per week for four weeks, to assess γ -globin gene expression in baboon 5002. Levels of γ -globin mRNA and globin chain synthesis were assayed in baboons before and during treatment with test compounds. A washout period was provided between administration of different compounds in the same baboon.

Analysis of γ -globin mRNA in baboons by RPA—Levels of γ -globin mRNA expression were assayed by RNase protection (RPA) in peripheral blood, as previously described [28,40,41]. Relative mRNA expression levels were determined by the relative change (fold-increase) of γ -globin above 18S gene expression, compared to baseline levels (day 0 of treatment). The band densities were measured using ImageJ software version 1.42 (National Institutes of Health, Bethesda, MD).

Globin chain synthesis by chromatography—Globin protein chain synthesis was assayed in ^3H -leucine-labeled reticulocytes by column chromatography, as previously described [22,40]. The amount of globin synthesis was calculated from the radioactivity that co-chromatographed with an OD of 280 nm. Globin ratios were calculated as $\gamma/(\gamma + \beta) \times 100$ and $\beta/(\gamma + \beta) \times 100$.

Pharmacokinetic profiles—Plasma concentrations of the test compounds were determined by liquid chromatography/mass spectrometry (Central Valley Toxicology, Clovis, CA) after oral or intravenous administration to assess half-lives and oral bioavailability of selected compound, as previously described [22].

Statistical analyses

All statistical analyses were performed using GraphPad Prism software version 5.02 (GraphPad Software, La Jolla, CA). Values obtained from cultures performed with each test compound were compared to values from the untreated or vehicle-treated controls cultured from the same subject or cell source. Comparison of values between each single test compound and the controls was performed by means of a one-sample *t*-test. Statistical differences between the values obtained in mice treated with normal saline and each test compound, and between the values of both test compounds, were determined using a paired *t*-test; *p* values < 0.05 were considered significant.

Results

Molecular modeling was carried out as detailed previously [35]. A 13,000 compound library was screened by docking into a “pseudo” receptor. Out of 630 compounds selected, 26 compounds with the most favorable binding energy from the 13,000 library were evaluated in a reporter gene assay, which detects preferentially strong inducers of the γ -globin gene promoter, rather than general globin gene inducers [34], as previously reported [35]. A schema illustrating our evaluation *in vitro* and *in vivo* is shown in Fig 1A. A schematic of two of the active compounds, RB7 and RB16, within the “pseudo” receptor is shown in Fig. 1B, i and ii, respectively.

Pharmacodynamic studies in vitro

The structures and active concentrations of 14 novel compounds and 2 positive control compounds tested in erythroid progenitors cultured from thalassemia patients are shown in Table 1. Induction of γ -globin (fetal globin) by test compounds was observed in erythroid progenitors cultured from individuals expressing both elevated basal HbF levels (β -thalassemia patients) (Fig. 2), and low basal levels of γ -globin (normal adults) (Fig. 3). Treatment with most of test compounds resulted in a mean increase in F-cell proportions by 40% to 100% compared to respective vehicle-treated control cultures from the same subjects

with β -thalassemia (Fig. 2). The most consistent increases in γ -globin expression relative to baseline were observed in 6/6 subjects' cultures with the compound RB13. Exposure to RB3, RB7, RB13, RB14, and RB17 also consistently enhanced F-cell production in cultured progenitors, in at least 3 of 4 β -thalassemia patient cultures.

To further evaluate the effects of the most potent test compounds on γ -globin induction and cell proliferation, CD34+ cells from normal adults expressing low γ -globin baseline levels were cultured over a concentration range of the compound RB7, the most potent agent in enhancing the γ -globin promoter in the dual-luciferase assay [35], as shown in Fig. 3. Relative γ - and β -globin gene expression levels were maximal at day 13 of culture, with higher γ/β globin ratios showing a dose-dependency on the RB7 concentration. Fetal globin protein increased from nearly undetectable levels in untreated control cultures (<1%) to 19% at 100 μ M RB7 (Fig. 3A-B). HbF production increased in parallel with RB7 exposure, in a dose-dependent manner (up to an 18-fold increase at 100 μ M) (Fig. 3C-D). There was no suppression of erythroid progenitor proliferation in the presence of even the highest concentrations of compound RB7 (Fig. 3E).

We reported previously that select SCFADs enhance erythroid growth in cultures established from patients with β -thalassemia [28]. Neutral or stimulatory effects, rather than inhibitory effects, on erythroid proliferation are desirable in therapeutics for anemic disorders. Stimulatory activity of a number of the test compounds on the growth of erythroid progenitors cultured from subjects with β -thalassemia was observed, particularly with RB14 and RB16 (Fig. 4). Addition of these compounds resulted in the production of 2-fold more erythroid colonies than in vehicle-treated controls from the same subject. In contrast, exposure to the HDAC inhibitor arginine butyrate reduced colony numbers by 10–25%. Test compounds RB3 and RB17 also augmented erythroid proliferation *in vitro* consistently, increasing Bfu-e colony growth from samples obtained from 3 of 4 patients with β -thalassemia.

Pharmacokinetic studies in vivo

Although the 14 novel compounds evaluated enhanced γ -globin expression in human primary erythroid cultures, most of the test compounds had relatively brief half-lives of less than one hour when administered to baboons, or produced undetectable detectable plasma levels. However, plasma levels of two candidate compounds, RB7 and RB16, persisted at the concentrations targeted for *in vitro* activity in erythroid progenitors for at least 8 hours after single intravenous or oral doses (Fig. 5). Both compounds were rapidly absorbed after oral administration, detected in plasma within 15 minutes after an administered dose, with peak levels (C_{max}) detected at 15–60 minutes (T_{max}) after oral dosing (Table 2). The area under the curve after intravenous administration was similar to that observed after oral administration of the test compounds, shown in Fig. 5A,C and Table 2. Plasma concentrations after intravenous and oral dose administration demonstrated oral bioavailability of 53% for RB7 at a 50 mg/kg dose and an oral bioavailability of 81% for RB16 at a 10 mg/kg dose (Table 2). These doses produced plasma levels equivalent to concentrations required for induction of γ -globin mRNA expression in baboons (Fig. 5A,C). Targeted plasma levels were maintained consistently in 3 different baboons with single doses of RB16 at 10 mg/kg per dose (Fig. 5E). The targeted concentration levels were maintained for at least 8 hours in plasma after single oral doses as low as 25 mg/kg for RB7 and 10 mg/kg for RB16 (Fig. 5). The projected human equivalent doses are 1–5 mg/kg, as humans had required 10–20% of the doses necessary in juvenile baboons to induce γ -globin using SCFADs with a half-life of 11 hours in previous studies [18]. This is 1/10 the doses required for other SCFAD therapeutics.

In vivo studies in transgenic mice or baboons

The two most active candidate compounds in inducing γ -globin expression *in vitro* that also had favorable pharmacokinetic profiles, RB7 and RB 16, were further evaluated in γ 8653 transgenic mice or in anemic baboons. The compound RB16 was administered in transgenic mice for two weeks at 50 mg/kg. Treatment with this compound resulted in an average 7-fold increase in γ -globin gene expression levels above the same animal's baseline, and above the levels in saline-treated control animals. The increase was higher than in the positive control (sodium 2,2-dimethyl butyrate, ST20) at 500 mg/kg, which produced a ~3-fold increase above control (Fig. 6). To evaluate the effect of RB7 and RB16 treatment on endogenous γ -globin gene induction in primates, these compounds were next tested in phlebotomized baboons. Administration of RB7 (50 mg/kg doses) resulted in a peak increase in γ -globin mRNA expression levels an average of 8-fold over baseline levels (Fig. 7A). Treatment with RB16 (10 mg/kg doses) induced a 4-fold peak increase in relative γ -globin mRNA expression above baseline (Fig. 7B). These doses required for RB7 and RB16 induction of γ -globin mRNA *in vivo* were significantly less than doses required for γ -globin induction by sodium 2,2 dimethylbutyrate (150 mg/kg) [22]. Fetal globin gene expression increased within 4- 5 days of administration of RB7 and RB16 (Fig. 7A and B). Corresponding synthesis of γ -globin protein by treatment with compound RB7 is illustrated in Fig. 7C, rising from undetectable γ -globin protein synthesis at baseline to 7% γ -globin synthesis during treatment. Monitoring of behavior, appetite, growth, and laboratory tests, including hematology and chemistry panels showed no adverse side effects in the baboons treated for up to 4 weeks with the 2 lead candidate compounds.

Discussion

A large body of biochemical, molecular, and clinical evidence has established that persistence of, or pharmacologic induction of fetal globin (γ -globin) is beneficial in β -thalassemia and sickle cell disease [2,4,5,7]. Thus, inducing γ -globin expression has become one accepted approach to the treatment of β -hemoglobinopathies [2,4,5,7]. Proof-of-principle has been established in patients treated with prior generation short-chain fatty acids (SCFAs) and derivatives (SCFADs) including arginine butyrate, phenylbutyrate, and isobutyramide, which were first evaluated for γ -globin-inducing activity in the predictive cellular and animal models utilized here [15,22,31,40,42–46]. Treatment with AB and SPB have reduced anemia, decreasing or eliminating transfusion requirements, in β -thalassemia patients [9–16]. Subjects with prolonged high levels of the SCFAs propionic acid and α -amino-n-butyric acid secondary to metabolic abnormalities also have elevated HbF [47,48]. Yet, high dose requirements and complex treatment regimens have limited the development of the first generations of SCFAD therapeutics for long-term therapy. While SCFADs have not been found to be mutagenic, those SCFADs which are also HDAC inhibitors have additional limitations with respect to long term use, including rapid metabolism and suppression of erythropoiesis due to cell cycle arrest, which make these SCFADs difficult for patients to tolerate long-term, or require intermittent dosing [9–11,31]. An unmet medical need therefore still exists for treatment of the β -thalassemias and hemoglobinopathies, particularly for agents that could be used in combination with other potential therapeutics.

We have identified previously a certain orally-active SCFADs which induce γ -globin expression *in vivo* and which also do not inhibit erythroid cell growth [22,28,29]. The proliferative SCFADs sodium 2,2-dimethylbutyrate (ST20) and sodium α -methylhydrocinnamate (ST7) stimulated erythroid colony formation in culture beyond that produced by optimal hematopoietic growth factors alone, and increased red cell counts in anemic phlebotomized baboons, and raised hematocrits in transgenic and normal mice [22,28]. Yet, because of their relatively low potency, these second generation agents may

require daily doses of 2–3 grams in adult humans. As multiple factors contribute to the anemia of β -globin disorders [4,16], an optimal therapeutic agent for long-term treatment of β -hemoglobinopathies should: 1) induce high-level γ -globin gene expression at low micromolar or nanomolar concentrations; 2) enhance survival, or inhibit apoptosis, of erythroid progenitors to allow a temporal window for the HbF induction to occur; and 3) provide therapeutic levels for six to ten hours after oral doses, a time-frame that was effective for treatment using arginine butyrate in β -thalassemia and sickle cell patients [16,21].

One of the compounds studied here for *in vivo* activity, 3-(2-oxo-2H-chromen-3-yl) benzoic acid (RB7), has been documented to have novel targeted mechanisms of action, inducing γ -globin expression through alterations in transcriptional complexes with displacement of a repressor complex consisting of HDAC3 and NCoR, and recruitment of a specific erythroid transcription factor (EKLF) to the γ -globin gene promoter [41,49]. The demonstration of potent *in vivo* activity by RB7 in this report indicates this candidate compound may provide a targeted “smart drug” approach with intriguing specificity [41,49].

The *in vitro* culture studies demonstrated that 13 of 14 novel predicted candidate compounds stimulate production of erythroid cells expressing HbF (F-cells) by 40–70% above untreated control cultures from normal individuals (who have low basal levels of HbF). The compounds increased F-cell proportions by 100% above untreated controls in progenitors cultured from patients with β -thalassemia intermedia (who have high basal levels of HbF). Nine compounds produced these responses at concentrations which are one log lower (5–20 μ M) than those required for AB or ST20 (active at 100–500 μ M). The lead compound RB7 produced an absolute increase in HbF protein of ~20% (20-fold above baseline). Additionally, RB7 treatment produced a marked 7-fold induction in the $\gamma/(\gamma + \beta)$ globin chain synthesis in a baboon with no detectable baseline γ -globin expression during a brief (2 week) treatment. This degree of induction would be expected to produce highly significant HbF levels in patients with sickle cell disease, due to the typical 2- to 3-fold selective survival advantage of cells containing HbF [2]. Because HbF expression, and consequent hematologic responses, were generated in human patients treated continuously for 6–12 hours daily with butyrate (which has a plasma half-life of only 5–15 minutes), we sought a similar 6–12 hour duration of drug levels in selecting the candidate compounds to develop for clinical use [9,22]. The present pharmacokinetic studies identified several compounds in which low oral doses produced plasma levels persisting above the predicted active concentrations for at least 8 hours in baboons. As other SCFAD therapeutics have required doses in humans that were 12 to 20% of doses required in juvenile baboons, human equivalent doses of RB7 and [(4,6-dimethoxypyrimidin-2-yl)sulfanyl]-3-methylbutyric acid (RB16) are predicted to be in the range of 1–5 mg/kg, [3,50]. These projected dose requirements offer a significant advance in potency, compared to the 20 to 35 gm required for sodium phenylbutyrate or butyrate, and even compared to ST20, which requires 150 to 200 mg/kg in baboons and 20 to 30 mg/kg in humans [9–12,15,50]. Slow release formulation may allow the more rapidly metabolized candidates, RB3, 13, 14, and 19, to also be clinically useful. However, acetic acid {4-[(trifluoromethyl)sulfanyl]anilino} (RB4), which has potent activity in inducing γ -globin gene expression and erythropoiesis *in vitro* [28], produced in methemoglobinemia in the baboons, and therefore is not attractive as a clinical candidate.

As expected, the activity of the two agents tested *in vivo* did not directly correlate with the magnitude of induction they produced in the γ -globin gene promoter reporter-based assays or the *in vitro* erythroid cultures, likely related to factors involving cellular uptake, oral bioavailability, and metabolism.

Our ‘pseudo’ receptor binding model predicted these novel candidate compounds through *in silico* screening of chemical libraries [35]. The findings described here confirm that novel candidate small molecules identified by this approach indeed induce γ -globin expression in primary erythroid cells and in relevant animal models, and two of these compounds have pharmacokinetic properties well-suited for clinical use.

Conclusion

These *in vivo* studies confirm that *in silico* molecular modeling has significant predictive power for identifying inducers of γ -globin gene expression with *in vivo* activity in relevant animal models. These candidate molecules identified are structurally dissimilar to previous SCFAD inducers of γ -globin gene expression, demonstrating the power of the model. Compounds with 3- to 8-fold higher potency in inducing γ -globin gene expression, at one log lower concentrations compared to prior generation clinically-active agents, were identified. These studies confirm the utility of *in silico* molecular modeling using a pseudo-receptor model to predict pharmacologic candidates.

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Fig. 1.
(A) Schema use for evaluation of compounds identified by *in silico* pharmacophore screening. **(B)** The most active compounds, RB7 (i) and RB16 (ii), docked into the “pseudo” receptor. Receptor carbons are shown in light blue, compound carbons are colored magenta (i) or white (ii). Oxygen atoms are colored red and nitrogen atoms are colored dark blue, and sulphur atoms are colored yellow. Some of the propane molecules were deleted for clarity.

Fig. 2. Effects of test compounds on γ -globin expression in β -thalassemia erythroid progenitors. Percentage of fetal hemoglobin expressing cells (% F-cells) in Bfu-e cultured from patients with β -thalassemia. The values shown are the means of the peak values $\pm$ SEM and expressed relative to control cultures from the same patients with β -thalassemia. Control values are shown by the dotted lines. Statistical significance is denoted by * $p < 0.05$ or ** $p < 0.01$.

Fig. 3.

Effects of the compound RB7 on erythroid progenitors cultured from normal adult CD34+ cells. (A) γ -globin and (B) β -globin expression profiles by day of culture: vehicle-treated (control) (0 μ M; $\circ$) or treated with RB7 at concentrations of 10 μ M ($\bullet$), 20 μ M ($\square$), or 100 μ M ($\blacksquare$). Values shown are the mean mRNA levels of γ -globin $\pm$ SEM relative to β -actin and G3PD. (C) Hemoglobin A (adult) or F (fetal) assayed by HPLC in control cells or treated with RB7 at 10, 20, or 100 μ M. Arrows indicate fetal hemoglobin (HbF, F) and adult hemoglobin (HbA, A) peaks. (D) Dose-response effect of increasing concentrations of RB7 on HbF. (E) Effect of treatment with RB7 at different concentrations on proliferation of erythroid progenitors through the culture period. Fetal hemoglobin induction by RB7 was not associated with inhibition of erythroid growth, in contrast to the effects of HDAC inhibitors.

Fig. 4.

Effects of candidate compounds on proliferation of erythroid progenitors. Bfu-e were cultured from patients with β -thalassemia and exposed to the test compounds as indicated. The values shown are the means of the peak values $\pm$ SEM and expressed relative to vehicle-treated control cultures from the same source. Statistical significance is denoted by * $p < 0.05$.

Fig. 5.

Pharmacokinetic profiles of test compounds RB7 and RB16 in anemic primates. **(A)** Plasma concentrations after RB7 was administered intravenously ($\circ$) or orally ($\bullet$) to baboon 5002. **(B)** Plasma concentrations of RB7 after administration of single oral doses at 25 mg/kg ($\circ$), baboon 6202; 50 mg/kg ($\bullet$), baboon 5002; or 100 mg/kg ($\square$), baboon 3301. **(C)** Plasma concentrations after administration of RB16 intravenously ($\circ$) or orally ($\bullet$) to baboon 5002. **(D)** Plasma concentrations of RB16 after administration of single doses at 10 mg/kg ($\circ$), baboon 6202; 25 mg/kg ($\bullet$), baboon 5002; or 50 mg/kg ($\square$), baboon 3301. **(E)** Pharmacokinetic analyses of RB16 after administration of the same dose in baboon 5002 ($\circ$), baboon 3601 ($\bullet$), or baboon 1504 ($\square$). Targeted plasma levels are concentrations which induce γ -globin in erythroid progenitor cultures, shown by the dotted line.

Fig. 6.

Effects of candidate compound RB16 on γ -globin mRNA gene expression in transgenic mice. Normalized γ -globin mRNA levels $\pm$ SEM in transgenic mice γ 8653 after administration of normal saline control, ST20 at 500 mg/kg, or RB16 at 50 mg/kg. Control values are shown by the dotted line. Statistical significance is denoted by * $p < 0.05$, control vs. ST20; ** $p < 0.001$, control vs. RB16, and ST20 vs. RB16.

Fig. 7.

Effects of the lead compounds RB7 and RB16 on γ -globin induction in anemic baboons. Pharmacodynamic assays in baboons that underwent phlebotomy to achieve stable anemia. Band intensities represent the levels of γ -globin mRNA and 18S rRNA in peripheral blood collected from baboons prior to (baseline; b) and during short course (days 2–7) and long course (days 6–20) administration of RB7 (**A**) or prior to (baseline; b) and during administration (days 4–24) of RB16 (**B**); relative levels represent γ -globin expression compared to baseline –day 0 of treatment, after normalized to 18S RNA levels. (**C**) γ -Globin chain synthesis before and during administration of RB7 in an anemic baboon. No detectable baseline synthesis of γ -globin chain was observed prior to administration of RB7 (top panel). Seven percent γ -globin chain synthesis was detected during treatment with RB7 (bottom panel). Closed symbols (●) designated ^3H CPM, and opened symbols (○) OD at 280 nm. Globin chain synthesis ratios are shown.

Table 1

Test compound designations, structures, and active concentrations in primary erythroid cultures

ID	Structure	Concentration, μM
AB		100
ST20		200
RB3		5
RB7		20–100

ID	Structure	Concentration, μM
RB9		100
RB12		5–10
RB13		5–20
RB14		40
RB15		30–60
RB16		30–50
RB17		10–20
RB19		5–10

ID	Structure	Concentration, μM
RB21		10–30
RB28		10–30
RB29		10
RB30		50

Table 2

Pharmacokinetic parameters of RB7 and RB16 in baboons

Treatment (compound, dose, route)	C _{max} (μM)	T _{max} (h)	t _{1/2} (h)	AUC _{last} (h*μM)	AUC _{inf} (h*μM)	F
RB7 50 mg/kg IV	266	0.50	18.5	951	4073	
RB7 50 mg/kg PO	118	1.00	119.5	501	8776 ^a	53% ^a
RB16 10 mg/kg IV	465	0.25	5.1	1643	2477	
RB16 10 mg/kg PO	387	0.50	3.5	1546	2005	81%

^aBased on AUC_{last}. Abbreviations: C_{max}: maximum plasma concentration observed during a dosing interval; T_{max}: observed time to reach the maximum plasma concentration; t_{1/2}: apparent terminal half-life; AUC_{last}: area under the concentration-time curve from time zero to the time of the last quantifiable concentration; AUC_{inf}: area under the concentration-time curve from time zero to infinite time; F: bioavailability; IV: intravenous; PO: oral.