Tenofovir disoproxil fumarate stimulates HbF production in K562 cells and β-YAC transgenic mice: A therapeutic approach for γ-globin induction

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Abstract

Pharmacologic induction of fetal hemoglobin (HbF) is an effective strategy for treating β-hemoglobinopathies like β-thalassemia and sickle cell anemia by ameliorating disease severity. Hydroxyurea is the only FDA-approved agent that induces HbF, but significant non-responders and requirement for frequent monitoring of blood counts for drug toxicity limit clinical usefulness. Therefore, this study relates preclinical investigation of Tenofovir disoproxil fumarate (TDF) as a potential HbF inducing agent, using human erythroid cell line and a β-YAC mouse model. Erythroid induction of K562 cells was studied by the benzidine/H2O2 reaction, total hemoglobin production was estimated by plasma hemoglobin assay kit, and γ-globin gene expression by RT-qPCR, whereas, fetal hemoglobin production was estimated by flow cytometry and immunofluorescence microscopy. We observed significantly increased γ-globin gene transcription and HbF expression mediated by TDF in K562 cells. Subsequent treatment of β-YAC transgenic mice with TDF confirmed HbF induction in vivo through an increase in γ-globin gene expression and in the percentage of HbF positive red blood cells. Moreover, TDF showed no cytotoxic effect at HbF inducing concentrations. These data support the potential development of TDF for the treatment of hematological disorders, including β-thalassemia and sickle cell anemia.

1 INTRODUCTION

The β-thalassemias and sickle cell disease (SCD), genetic disorders affecting the β-chain of adult hemoglobin A (HbA), are serious anemias and comprise a growing global health burden (Atweh et al., 2003; Weatherall, 2010). Fetal hemoglobin (HbF, α2γ2, HBG) is an endogenous hemoglobin present in all humans, which is normally suppressed in infancy. Pharmacological augmentation of fetal hemoglobin (γ-globin) production, to replace the defective or missing β-globin chains, is a recognized therapeutic approach, as augmentation in HbF and F-cell levels reduce the ineffective erythropoiesis, and consequently the anemia, in β-thalassemia or reduce the severity of SCD (Perrine, Pace & Faller, 2014; Resar, Segal, Fitzpatric, Friedmann, Brusilow & Dover, 2002; Vichinsky, MacKlin, Wave, Lorey & Olivieri, 2005). In β-hemoglobinopathies, HbF levels >20-30%, are required to ameliorate most of the clinical complications, as fetal globin (γ-globin) chains inhibit excess α-globin chain accumulation in RBC’s membrane and polymerization of sickle hemoglobin, preventing many pathologic consequences.

Pharmacologic reactivation of HbF expression offers a broadly applicable treatment approach for global diseases. Several classes of therapeutic agents induce fetal globin and HbF, including chemotherapeutic agents (Hydroxyurea (HU), 5-azacytidine, and Decitabine), short-chain fatty acids (SCFAs) and derivatives (SC-FADs), histone deacetylase (HDAC) inhibitors, LSD-1 inhibitors, and factors that regulate globin translation.
(Bradner et al., 2010; Charache et al., 1995; Reich et al., 2000; Saunthararajah et al., 2003; Steinberg & Rodgers, 2001). Some of these therapeutics have clearly shown proof-of-principle, but, except for HU, have required parenteral administration, large doses, or require further clinical trials (Charache et al., 1995; Wang et al., 2011). After demonstrated efficacy in the Multicenter Hydroxyurea Study, in 1998 hydroxyurea (HU) became the only FDA-approved drug proven to induce HbF in sickle cell anemia but not in β-thalassemia (Strouse & Heeney, 2012; Ware & Aygun, 2009). While Hydroxyurea (HU) benefits many children and approximately half of adults (Bohacek, Boosalis, McMartin, Faller & Perrine, 2006; Taher et al., 2010). However, significant non-responders and requirement for frequent monitoring of blood counts for bone marrow toxicity limit clinical usefulness. Therefore, additional therapeutic agents would benefit those patients who are Hydroxyurea intolerant or nonresponsive to HU treatment (Perrine, Pace & Faller, 2014).

To address the need for additional safe and effective oral HbF inducers, we investigated an orally active drug Tenofovir disoproxil fumarate (Figure 1) for its potential to induce HbF. It has been widely used for long-term treatment of human immunodeficiency virus (HIV) and chronic hepatitis B (CHB) infections in adult patients (Buti & Homs, 2012; Pham & Gallant, 2006). Tenofovir is nucleotide analog of adenosine 5'-monophosphate and acts by inhibiting viral reverse-transcriptase (Pham & Gallant, 2006). Its favorable pharmacokinetic profile Oral dosing route, and reported better-tolerated safety profile have propelled it to become a first-line treatment for HIV or used in combination with other antiretroviral drugs (Durand-Gasselin et al., 2009; Hawkins, Veikley, Claire III, Guyer, Clark & Kearney, 2005; Van Rompay et al., 2012). Tenofovir’s primary route of elimination is renal, where it is excreted largely via glomerular filtration and proximal tubular secretion (Fernandez-Fernandez et al., 2011; Goicoechea et al., 2008). The toxicity related to the TDF are dose dependent renal toxicity due to accumulation of TDF in proximal renal tubules (Cihlar, Ho, Lin & Mulato, 2001).

Tenofovir's primary route of elimination is renal, where it is excreted largely via glomerular filtration and proximal tubular secretion (Fernandez-Fernandez et al., 2011; Goicoechea et al., 2008). The toxicity related to the TDF are dose dependent renal toxicity due to accumulation of TDF in proximal renal tubules (Cihlar, Ho, Lin & Mulato, 2001).

TDF is previously reported with improved levels of hemoglobin, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) (Pornprasert et al., 2009), but the effects of TDF on HbF was not previously tested. Therefore, in current study we used K562 cells to demonstrate the effect of TDF on erythroid differentiation, proliferation, hemoglobinization, γ-globin gene expression, and fetal hemoglobin production. Furthermore, preclinical examination of TDF in β-YAC transgenic mice confirmed that TDF increased HbF levels and the percent of HbF positive cells (F-cells) in vivo. These findings support the potential development of TDF as an HbF inducer for β-thalassemia and SCD.

2 MATERIALS AND METHODS

Active pharmaceutical ingredient (API) of Tenofovir Disoproxil Fumarate (TDF) (MW: 635.515) with purity >95% was obtained from Drug bank of Dr. Panjwani Center for Molecular Medicine and Drug Research, ICCBS, University of Karachi. API of TDF was made available in two lots. The first lot was reconstituted in Milli-Q water for cell culture while the second lot was reconstituted normal saline solution for in vivo studies to avoid potential toxicities in mice.

2.1 Cell culture and reagents

The human K562 erythroleukemia cells (CCL 243), obtained from ATCC, were cultured in RPMI-1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (Sigma, Canada), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma, USA) at 37 °C and 5% CO2 (Bianchi et al., 2000; Rowley, Ohlsson-Wilhelm & Farley, 1985; Viola et al., 2008). The 20mM of stock solutions of TDF were prepared in Milli-Q water (Sigma, France). 20mM stock solution of hydroxyurea (Sigma, USA) was solubilized in water, and stored at -20 deg.C. For erythroid differentiation studies, cells were seeded at density of 4x104 cells/ml and treated in dose and time dependent manner to determine active concentration in K562 cells. Medium was not changed during incubation period with compounds.
2.2 Benzidine assay for hemoglobinization

Concentration dependent-induced hemoglobin production in K562 cells was determined by benzidine assay. Hemoglobinized cells were detected by a specific reaction of hemoglobin with a benzidine-hydrogen peroxide reagent, based on the peroxidase-like activity of heme group of hemoglobin molecule (Viola et al., 2008). At day 5 of the treatment the cells were washed with phosphate buffer saline, and resuspended in 50 μl of PBS, and 50 μl of freshly prepared benzidine-hydrogen peroxide H₂O₂ solution containing 0.2% benzidine (Perkin Chemicals, China) in 0.5M glacial acetic acid (Lab Scan, Thailand) and 10% H₂O₂ (Sigma, Germany), and incubated in dark for 5 min at room temperature (Bianchi et al., 2000; Viola et al., 2008). Cells were then observed under light microscope (Micros, Austria) and percent of benzidine positive cell were calculated as (stained cells/total number of cells) x 100.

2.3 Cell growth and viability determination

Cell viability and growth kinetics were studied by determining the number of viable cells per milliliter with a hemocytometer using trypan blue dye exclusion method. Equal volume of cell suspension and 4% trypan blue solution were mixed, and cells were counted under light microscope using haemacytometer (Marienfeld, Germany) (Bianchi et al., 2000; Strober, 1997). The alamarBlue fluorescence assay was used to measure the cytotoxicity induced by TDF and HU (Nociari, Shalev, Benias & Russo, 1998; O’brien, Wilson, Orton & Pognan, 2000). Briefly, harvested cells were seeded at density of 4 × 10⁴ cells/ml on 96-well plate and treated in dose dependent manner and incubated for 48 h at 37 °C. The treated culture was incubated with 0.01% of resazurin reagent (alamarBlue) (Sigma, USA) in PBS for 4 h at 37 °C. The fluorescence was measured at excitation λ 560 nm and emission λ 590 nm using Microplate Reader (Varioskan LUX, USA) with SkanIt™ Software version 4.1.

2.4 Hemoglobin quantitation assay

For hemoglobin quantification, the harvested cells on day 5 of the treatment were washed thoroughly with ice cold PBS, an equal number of cells were resuspended in lysis buffer (0.2% Triton X-100 in 100mM potassium phosphate at pH 7.8) for 20 min (Witt, Sand & Pekrun, 2000; Zhang, Cho & Wong, 2007). Cell lysate was centrifuged at 250 g for 15 min, 50 μl of supernatant was used to determine the concentration of hemoglobin by using the sigma hemoglobin assay kit (Sigma, USA), according to the manufacturer’s protocol. Optical density (OD) was taken at λ 400 nm by Microplate Reader (SpectraMax, USA) and hemoglobin concentration was calculated as mean ± S.D. of three independent experiments.

2.5 Reverse transcription-quantitative PCR (RT-qPCR) analysis

The cells were treated with the TDF at different concentrations 50, 25, 12.5 μM and HU at 200 μM, respectively, on six-well plate and incubated for 5 days under standard growth conditions as for untreated group. At day five of treatment, total RNA from each experimental group was extracted by guanidinium/phenol-chloroform method using Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. RNA integrity was checked on 1.5% agarose gel. 1 μg of total RNA from each experimental group was used for cDNA synthesis by reverse transcription using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific Baltics, UAB) with the aid of 1 μl of Random Hexamer, 200 U RevertAid M-MuLV RT, 4 μl of 5X Reaction Buffer, 1 μl RNase Inhibitor and 2 μl of 10mM dNTP Mix. The reaction was initially incubated for 5 min at 25 °C followed by 60 min at 42 °C finally the reaction was terminated by heating at 70 °C for 5 min.

Quantitative real-time polymerase chain reaction (RT-qPCR) assay (Bianchi et al., 2000; Lampronti, Bianchi, Borgatti, Fibach, Prus & Gambari, 2003) of γ-globin gene was carried out by VeriQuest SYBR Green qPCR Master Mix (2X) with Fluorescein (affymetrix, USA) using Rotor-Gene Q 2.3.1.49 (QIAGEN, Hilden, Germany). Primer3web version 4.1.0 (Untergasser et al., 2012) was used for designing primers, for γ-globin
gene forward primer 5'-TTCTGGCAGAAGATGGT-3' and reverse 5'-AGCTCTGAATCATGGGCAGT-3', primers for internal control Gene human GAPDH (glyceraldehyde 3-phosphate dehydrogenase) forward 5'-CCAGAACATCATCCCTGCCT-3' and reverse 5'-CCTGCTTCACCACCTTCTTG-3', and primers for internal control Gene mouse GAPDH forward 5'-TGACGTGCCGCTGGAGAAA-3' and reverse 5'-AGTGTAGCCCAAGATGCCCTTCAG-3'. For real time PCR reaction 2 μl of cDNA template, 10 μl of Syber Green master mix, and 1 μl of each forward and reverse gene specific primer were used in final reaction volume of 20 μl. The thermal cycling conditions used were: 10 min at 95 °C for initial activation of Taq DNA polymerase, for PCR amplification; denaturation at 95 °C for 15 s, annealing at 60 °C for 30s and extension at 72 °C for 30 s, for 40 cycles.

The relative fold change in gene expression in treatment group, compared with untreated control was calculated as $2^{-\Delta\Delta Ct}$ method (Iacomino, Medici & Russo, 2008; Lampronti, Bianchi, Borgatti, Fibach, Prus & Gambari, 2003). Where ΔCt is the difference between Ct (cycle threshold) value of target gene and reference gene and ΔΔCt represent the difference between the ΔCt of treatment group and ΔCt of untreated group. Each sample was normalizes to GAPDH reference gene as an internal control.

2.6 Flow cytometry analysis

To measure percent of HbF positive cells (F-cells) through flow cytometry, as described previously (Shi, Cui, Engel & Tanabe, 2013). Briefly, 2×10^5 treated, and untreated K562 cells were taken at day 5 of the treatment. F-cells from mice and K562 cells were washed with 0.1% BSA/PBS, fixed with freshly papered 0.05% glutaraldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 3 min. Cells were incubated with 5 μl of fluorescein isothiocyanate (FITC)-conjugated anti-HbF antibody, MHFH01 (Life Technologies, Frederick) in 80 μl 0.1% BSA/PBS for 15 min at room temperature. Stained cells were washed thoroughly then analyses with BD FACSCanto II Flow cytometer (BD Bioscience, USA) though FL1 channel. The results were analyzed using Flowing Software version 2.5.1 (Finland). The population of F-cells (cells containing HbF) was represented as percent F-cells and fluorescence intensity of the merged histograms of three replicates.

2.7 Immunofluorescence microscopy

Immunocytochemistry of HbF was performed with modifications, the washed cells with PBS were fixed with 500 μl of 4% paraformaldehyde (PFA) for 20 min, and permeabilized with 0.1% Triton X-100 solution at room temperature. Immunostaining was done at 4 °C overnight with anti-HbF fluorescein isothiocyanate (FITC)-conjugated antibody, MHFH01 (Life Technologies, Frederick) at dilution 1:200. Cells were washed with PBS and counter stained the nuclei with 300 μl of 300 nM DAPI (4',6-diamidino-2-phenylindole) (Invitrogen, USA). Images were taken with fluorescence microscope (Nikon Eclipse 90i, Japan) using NIS-Elements software (Nikon, Japan). The data was quantified as mean fluorescence intensity/cell (Arbitrary Unit) using ImageJ 1.51 K (NIH, USA).

2.8 β-ΨΑ´ τρανσγενις μουστε τρεατμεντ προτοσολ

All animal studies were performed according to the guide for the care and use of laboratory animals, from the US Department of Health and Human Services, Institute of Laboratory Animal Resources, Washington, DC, 1985. β-YAC transgenic mouse model was used after confirmation of DNA genotyping, containing the full-length 82 kb human β-globin gene locus including the LCR and surrounding region (Figure S3). The five functional human globin genes 5’-ε-γ-δ-β-3’ are present and undergo normal developmental regulation with the γ-globin gene silenced shortly after birth (Peterson et al., 1993). β-YAC mice 5–6 months old were administered TDF dissolved in normal saline (50, 100 and 200 mg/kg/day) or HU (200 mg/kg/day) by intraperitoneal injections, 6 days/week for 4 weeks. We treated five mice per group with 3 males and 2 females; an untreated saline water control group was also analyzed. We collected blood by tail bleed for analyses and the level of F-cells were performed by flow cytometry using anti-HbF fluorescein isothiocyanate (FITC)-conjugated antibody, MHFH01 (Life Technologies, Frederick). Whereas, at week 4 the mice were
sacrificed to isolate the bone marrow for γ-globin mRNA expression in β-YAC transgenic mice. Expression of γ-globin gene was carried out, as previously described.

2.9 Statistical analysis

All statistics were carried out by software GraphPad prism 6 (GraphPad Software, USA). The statistical significances were calculated using one-way or two-way ANOVA, and P values cut off < 0.05 were considered statistically significant. The data are represented as mean ± standard error of the means (SEM).

3 RESULTS

3.1 TDF induces K562 erythroid differentiation

Studies were conducted in K562 cells to determine the ability of TDF to induce erythroid differentiation. K562 cells display characteristics of erythroid cells and these cells are useful for initial drug screening and discovery of potential HbF inducers (Bianchi et al., 2000). Erythroid differentiation effect of TDF was evaluated and summarized in Table 1. Almost all the concentrations have shown some level of erythroid differentiation effect on K562 cells ranges from 3.125-100 μM in dose dependent manner. The hemoglobin containing cells were appeared as benzidine reactive cells (dark green) and non-erythroid cell as benzidine-unreactive (color-less) as illustrated in Figure 2. We found that HU, a known HbF inducer as positive control showed maximal hemoglobin phenotype in K562 cells at 200 μM (EC50=54 μM), with significant increase in cellular size, when the cell proliferation was partly affected (Figure S1 and S2) (Ali et al., 2019). Initially, the dose dependent erythroid differentiating effect of the most effective compounds on K562 cells showed that, TDF at concentration of 12.5 μM induced erythroid differentiation with 45 ± 8.9 percent increase in benzidine positive cells. Similarly, at concentration 25 μM showed 65 ± 6.1 percent increase, while at 50 μM concentration showed 57 ± 6.1 percent increase in the population of benzidine positive cells as represented in Figure 2e. Further experimentation optimized maximal erythroid differentiation effects of TDF at active concentrations 50, 25, and 12.5 μM, without change in cell size, and set as active concentrations for subsequent experiments (Figure 2a-c). A seven days’ time dependent erythroid differentiation kinetics of most active concentrations of TDF showed that, at day 0, the proportions of benzidine positive cells were found for 50, 25, and 12.5 μM were 7.6 ± 1.8, 5.8 ± 1.1, and 4.3 ± 2.5 percent, respectively, and for untreated control was 7 ± 3.5 percent. With the passage of time, TDF displayed a significant increase in erythroid differentiation of K562 cells. The percentage of hemoglobinized cells at day 7 was, 39.48 ± 1.5, 54.15 ± 4.33, and 31.15 ± 8.96 percent, for active concentrations 50, 25, 12.5 μM, respectively, as compared to untreated control (12.89 ± 1.84 %), as shown in Figure 2f.

3.2 Effect on cells growth and viability

The dose dependent anti-proliferative effect of the TDF was expressed as percent of untreated control and represented as mean ± S.D. of at least three independent experiments Figure 3a. There is no significant inhibitory effect on cells viability of TDF at concentration [?]50 μM (CC50=66.46 μM). Whereas, HU showed no significant cytotoxicity at 50 μM or less (CC50=72 μM) as compared to untreated control shown in. Time dependent cell proliferation kinetics of TDF 50, 25, 12.5 μM and HU were conducted using optimal induction concentrations and no significant inhibitory effect of test compounds on cells viability and growth kinetics as compared to untreated control was observed, while HU showed a decrease in cell proliferation rate (Figure 3b), with effect on cell morphology, as shown in Supplementary Figure 2c. Moreover, CC50/EC50 ratio (therapeutic index (TI)) was calculated to determine the safety index to compare the efficacy and cytotoxic potential of TDF and HU. TDF exhibited TI of 26.37. These high therapeutic index values provided the evidence of safety and effectiveness of TDF, while not being toxic to cells as compare to HU (TI=1.33). So,
this is very important that, TDF induced erythroid differentiation at significantly lower concentrations than cytotoxic activity.

### 3.3 TDF promotes hemoglobin accumulation

Total hemoglobin concentration was determined as miligram/deciliter after 5 days incubation with TDF 50, 25, 12.5, 6.25 μM and HU 200 μM. TDF 25 μM showed highest significant accumulation of hemoglobin with the concentration of 11.79 ± 1.59 mg/dL, whereas TDF 50, 12.5, 6.25 μM and HU 200 μM showed 7.57 ± 2.05, 9.20 ± 1.58, 5.5 ± 1.58 and 6.83 ± 1.05 mg/dL as compared to untreated control as shown in Figure 4. These results provide evidence that erythroid induction of K562 cells in the presence of TDF is associated with increased hemoglobin expression.

### 3.4 ΤΔΦ ινδυςες γ-γλοβιν τρανσςριπτιον ανδ ΗβΦ εξπρεσσιον ιν Κ562 ςελς

Studies reported on the induction of α-globin transcription in K562 human erythroleukemia cells by variety of chemical inducers (Rowley, Ohlsson-Wilhelm, Farley & LaBella, 1981). Therefore, our initial studies were conducted in K562 cells to determine the ability of TDF to induce γ-globin transcription and HbF expression. K562 cells were treated with TDF for 48 h and γ-globin gene transcription was analyzed by RT-qPCR. We observed a significantly increase γ-globin mRNA levels of 7.14 ± 1.64, 6.02 ± 1.57-fold and 3.71 ± 1.01-fold by TDF 50, 25 and 12.5 μM, respectively, compare to 5.57 ± 0.92 -fold induction by HU (Figure 5). The next set of studies determined the effects of TDF on HbF protein expression by flow cytometry. Similar to mRNA levels, treatment with TDF 50, 25, 12.5 and 6.25 μM increased the F-cells to a maximum of 53.07% compared to 2.31% in untreated controls (Figure 6a and b). To substantiate HbF protein levels, we performed immunofluorescence microscopy confirming a dose-dependent increase in HbF (p < 0.05) by TDF in comparison with increase in induction by HU. The illustrated images are shown an increase in HbF production, quantify as mean fluorescence intensity per cell of FITC-conjugated anti-HbF antibody (Figure 7). In general, an increase in HbF level, along with γ-globin mRNA level, was observed which demonstrated the ability of TDF as fetal hemoglobin inducer.

### 3.5 ΤΔΦ ινδυςες γ-γλοβιν τρανσςριπτιον ανδ ΗβΦ εξπρεσσιον ιν β-ΨΑ?’ τρανςγενις μις

Studies have shown drug mediated HbF induction in cell culture systems, these findings do not always translate in vivo . Therefore, our further preclinical studies evaluated the potential of TDF to induce HbF using β-YAC transgenic mice, in which γ-globin to β-globin switching occurs during development (Peterson et al., 1993). To gain insights into the effects of TDF on γ-globin mRNA expression, we measured γ-globin mRNA level by RT-qPCR. As shown in Figure 8, treatment with 200 mg/kg TDF increased γ-globin mRNA expression 11.46-fold at week 4, compared to a 5.26-fold increase for HU, while, 50 mg/kg and 100 mg/kg showed 4.17-fold and 5.86-fold, respectively. It simply suggests TDF stimulated γ-globin mRNA expression in β-YAC transgenic mice.

We next analyzed the ability of TDF to induce HbF expression in vivo . As shown in Figure 9 the F-cells increased 3.21-fold (33.64%) and 3.79-fold (39.63%) in mice treated with 50 mg/kg/ and 100 mg/kg TDF, while 200 mg/kg increased F-cells 5.0-fold (52.35%), as compare to saline control F-cells (10.45%). To validate the effects of TDF on phenotypic expression of HbF positive cells, we measured HbF by FITC-conjugated anti-HbF antibody by immunofluorescence microscopy. The illustrated images are shown an increase in HbF production, quantify as mean fluorescence intensity per cell FITC-conjugated anti-HbF antibody (Figure 10). These findings support the ability of TDF to induce HbF in vivo in β-YAC transgenic mice.
4 DISCUSSION

Over the last three decades, numerous pharmacologic agents have been tested and shown to display HbF inducing properties in vitro, but few have translated into clinical efficacy. However, HbF induction by small molecules is an important therapeutic approach for treatment of the β-hemoglobinopathies and continues to be an intense area of investigation. Agents such as 5-azacytidine (Ley et al., 1983), decitabine (Saunthararajah et al., 2003) arginine butyrate (Atweh et al., 1999; Perrine et al., 1993) and short chain fatty acid derivatives (Fucharoen et al., 2013; Reid et al., 2014) were shown to induce HbF in clinical trials. These drugs act by diverse mechanisms including inhibition of DNA methyl transferases and histone deacetylases, enhanced DNA binding of transcription factors and cell signaling activation (Molokie et al., 2017). The use of HU is limited by a significant nonresponder rate, the need for close monitoring of blood counts for bone marrow toxicity, infertility, and patient concerns with taking a chemotherapy class agent (Sahoo et al., 2017; Wang et al., 2011). Thus, development of additional safe and effective oral agents that induce HbF alone or in combination with Hydroxyurea (HU), offer the potential for improved outcomes in β-hemoglobinopathies.

Studies reported on the induction of γ-globin transcription in K562 human erythroleukemia cells by variety of chemical inducers (Rowley, Ohlsson-Wilhelm, Farley & LaBella, 1981). K562 cell line isolated from a patient with chronic myelogenous leukemia in blast crisis is the most extensively used in vitro model for putative identification of new fetal hemoglobin inducers and to study their molecular mechanism, as well the kinetics of erythroid differentiation (Lozzio & Lozzio, 1975). Therefore, our initial studies were conducted in K562 cells to determine the ability of Tenofovir disoproxil fumarate (TDF) to induce γ-globin transcription and HbF expression. K562 cells display characteristics of erythroid cells including expression of the ε, γ and α globin genes (Cioe, McNab, Hubbell, Meo, Curtis & Rovera, 1981; Rutherford, Clegg, Higgs, Jones, Thompson & Weatherall, 1981) and these cells are useful for initial drug screening and discovery of potential HbF inducers (Bianchi, Chiarabelli, Borgatti, Mischiati, Fibach & Gambari, 2001).

In this study, we have preclinically evaluated TDF in comparison with HU for its potential to induce HbF production. We demonstrated the effect of TDF on human erythroleukemia cells (K562), including erythroid differentiation, proliferation, hemoglobinization, γ-globin gene expression, and fetal hemoglobin production. Under standard cell-growth conditions, the K562 cell line presents a low number of Hb synthesizing cells, while culturing in the presence of inducing agents can exhibit an increase in hemoglobinized cells, and characteristics of erythroid differentiation (Bianchi et al., 2000; Park, Choi, Jeong, Han & Kim, 2001; Rowley, Ohlsson-Wilhelm, Farley & LaBella, 1981; Rowley, Ohlsson-Wilhelm & Farley, 1985) These cells preferentially induce γ-globin gene expression and fetal hemoglobin, when exposed to inducing agents (Bianchi et al., 2000; Kohmura, Miyakawa, Kawai, Ikeda & Kizaki, 2004; Qian, Chen, Zhao, Guo & Qian, 2013; Rutherford, Clegg & Weatherall, 1979).

First, the erythroid induction of K562 cells was studied by the benzidine/H₂O₂ reaction (Ng & Ko, 2014; Perrine et al., 1993; Viola et al., 2008), and the results demonstrated that K562 cells undergo the erythroid differentiation with significant increase in benzidine positive cells following treatment with TDF, without any inhibitory effect on cell proliferation and growth kinetics. The extent of percent benzidine positive cells is proportional to increase in hemoglobinization of cells. Plasma hemoglobin assay kit was used to determine the total hemoglobin expression, and a substantial increase in hemoglobin concentration was observed. The effect of TDF on γ-globin gene expression was studied using RT-qPCR. Our finding demonstrated that erythroid differentiation was associated with induction of γ-globin mRNA production. Increase in γ-globin production is associated with increased fetal hemoglobin level. Flow cytometry and immunocytochemistry were used to complement the augmented γ-globin mRNA with HbF production. Immunophenotyping of HbF shows a correlation between increased fetal hemoglobin production and γ-globin mRNA expression.

To translate new HbF inducers into clinical trials requires evidence of efficacy in preclinical animal models. The β-YAC mouse model has been used to test different agents for their capacity to induce HbF in vivo. The in vivo safety of oral TDF was previously explored in female BALB/c mice model; mice were treated for 4 weeks with up to 1000 mg/kg without toxicity (Ng et al., 2015). The β-YAC is a transgenic mouse model
containing the full-length 81 kb human β-globin gene locus including the LCR and surrounding region. The functional genes of the human β-globin locus are arranged 5’ to 3’ in the order in which they are expressed during development: 5’-ε-γ-δ-β-3’. Upstream of the ε-globin gene is the locus control region (LCR), a set of four erythroid specific and developmentally stable DNase I hypersensitive sites (Forrester, Thompson, Elder & Groudine, 1986; Grosveld, Blom van Assendelft, Greaves & Kollias, 1987; Tuan, Solomon, Li & London, 1985), designated 5’ HS1 to 5’ HS4, that confer high level, integration site-independent, copy number-dependent expression on globin genes in cells of the erythroid lineage (Grosveld, Blom van Assendelft, Greaves & Kollias, 1987). Another developmentally stable DNase I hypersensitive site, 3’ HS1, is found 20 kb downstream from the β-globin gene (Forrester, Thompson, Elder & Groudine, 1986; Grosveld, Blom van Assendelft, Greaves & Kollias, 1987; Tuan, Solomon, Li & London, 1985). The developmental control of the genes of the β-locus has been investigated with various approaches, mainly using transgenic mice.

We treated β-YAC mice, 6 days per week for 4 weeks and subsequently demonstrated the ability of TDF to activate γ-globin transcription and HbF expression in comparison with HU and saline control. The mild increase in hematocrite counts and increase in hemoglobin suggest the safety of TDF, which support potential in vivo efficacy of TDF. We demonstrated the ability of TDF to activate γ-globin transcription and HbF expression in comparison with HU without affecting β-globin gene transcription. Even though β-YAC mice undergo hemoglobin switching in same order as occurs in human development, and serve as an excellent preclinical model for drug screening to evaluate the efficacy of new HbF inducing drugs. Limitations of this model included the lack of anemia and oxidative stress present in SCD and β-thalassemia. Anemic mice or baboons provide additional animal models to test pharmacological agents for their potential to induce HbF. Therefore, additional studies in these models will provide evidence for clinical safety and efficacy of TDF.

5 CONCLUSION

The TDF can mediate HbF induction inferred from increased in erythroid differentiation, γ-globin mRNA and HbF expression in human Erythroleukemia cells without producing any cytotoxic effect. The ability of TDF to activate γ-globin transcription and induce HbF production in β-YAC transgenic mice underlines benefits as a therapeutic candidate for developing new fetal hemoglobin inducer. However, the data presented open new avenues for further study on HbF induction in erythroid precursor cells, obtained from β-thalassemia and sickle cell anemia patient.

CONFLICT OF INTEREST STATEMENT

Authors declare no competing interest

AUTHORS’ CONTRIBUTION

SGM proposed the subject, designed the study, provided conceptual and technical guidance, along with the laboratory equipment and expertise to conduct analysis, and actively participated in manuscript writing. FK actively performed all the experiments, statistical analysis interpreted data and wrote the manuscript. HA actively participated in method optimization and performed the experiments. All authors read and review the manuscript.

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