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以藥物誘發胎兒血紅素治療重症鐮刀型貧血與β型地中海型貧血

Pharmacological induction of HbF for treating severe sickle cell disease and β-thalassemia

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誌謝

總覺得才剛結束三年的研替沒多久，轉眼間博士班也要畢業了。念博士班的這幾年很感謝沈哲鯤老師提供了非常豐富的資源讓我來進行研究，很多實驗因此可以很順利的進行下去。本篇論文可以順利的完成也要感謝每位論文指導委員在每年的進度報告時給的建議，讓我可以修正研究方向或是發現哪邊還有不足。其中尤其要感謝蔣維棠老師，蔣老師在實驗所需藥物的提供，藥物動力學的測試以及最後整合實驗資料發表於期刊時花了很大的功夫。

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摘要

利用治療藥物誘發胎兒血紅素再表現已被建議做為治療重症鐮刀型貧血或β型地中海型貧血等貧血的替代療法。Hydroxyurea (HU)是第一個通過美國 FDA 核可用來治療鐮刀型貧血的胎兒血紅素誘發藥物。然而 HU 對 25%的病人無效。在本實驗室之前的研究中已發現數個具有相同藥效基團的化合物可以誘發胎兒血紅素再表現，但由於不佳的水溶性和生物吸收效率限制了往臨床使用的發展性。為了研發出可作為藥物使用的化合物，我們進一步的探討其結構和活性之間的關係。在本篇研究中我們分成了兩個系列的化合物進行研究。我和中研院生醫所的蘇燦隆老師合作合成了系列一的化合物，SS-2394 是被挑選進一步研究的候選化合物。在之前的研究中已經了解了 SS-2394 誘發胎兒血紅素的效果以及其作用機制，在此我進一步利用 IPA 去探討其對細胞的整體影響。同時利用 β-YAC 基因轉殖小鼠和鐮刀型貧血小鼠測試 SS-2394 在體內的作用效果。另一方面我和國衛院的蔣維棠老師合作，利用之前已被報導過的胎兒血紅素誘發化合物 TN1 和 Compound II 的結構合成了 93 個系列二的新化合物。在此我們揭露系列二中更為有效的化合物 AS-28，可以在無細胞毒性的濃度下有效誘發胎兒血紅素的表現。我也探討了 AS-28 的作用機制以及利用 IPA 分析其對細胞的整體影響。此外口服餵食 AS-28 給鐮刀型貧血症的小鼠可以減緩貧血和其他相關症狀。本論文研究結果顯示 AS-28 有潛力發展成新一代治療重症鐮刀型貧血以及 β 型地中海型貧血的新藥。

關鍵字:胎兒血紅素;鐮刀型貧血;地中海型貧血;藥物;誘發物; 血紅蛋白疾病

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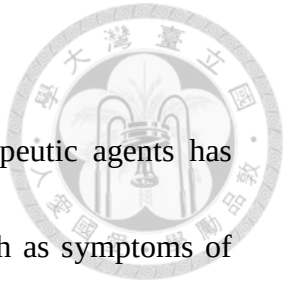

Abstract

Reactivation of fetal hemoglobin (HbF) expression by therapeutic agents has been suggested as an alternative strategy to modulate anemia, such as symptoms of severe β-thalassemia and sickle cell disease (SCD). Hydroxyurea (HU) is the first US FDA-approved HbF inducer for treating SCD. However, approximately 25% of the patients with SCD have no response to HU. In our previous study, we found several compounds bearing same pharmacophore can induce HbF. However the poor solubility and bioavailability limit the development of these inducers for clinical use. To develop drug-like compounds, further structure-activity relationship studies were conducted. In my thesis, I studies two classes of compounds. Class 1 compound, in cooperation with Dr. Tsann-Long Su in IBMS, Academia Sinica, **SS-2394** is the candidate compound, in which HbF induction ability and molecular mechanisms had been revealed in previous study. In this study, I further investigate the global effect of **SS-2394** by Ingenuity Pathway Analysis (IPA). The *in vivo* efficacy of **SS-2394** in β-YAC transgenic mice and SCD mice were evaluated. The other compounds categorized as Class 2 compound were from Dr. Weir-Torn Jiaang in NHRI, who synthesized 93 new compounds based on the structure of previous studies, and identified as HbF inducer, **TN1** and compound II. Herein, I found a more potent inducer from class 2, compound **AS-28**. It can efficiently induce HbF expression at

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non-cytotoxic concentrations. The molecular mechanisms of **AS-28** for regulation of HbF expression was also investigated. The global effects of **AS-28** were also applied for IPA. In addition, we demonstrated that oral administration of **AS-28** can ameliorate anemia and the related symptoms in sickle cell disease mice. The results of this study suggest that **AS-28** can be further developed as a novel agent for treating hemoglobinopathies, such as severe β-thalassemia and sickle cell disease.

Keywords: Fetal hemoglobin; Inducer; β-Thalassemia; Sickle cell disease (SCD); Compound; hemoglobinopathies

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關鍵字縮寫 Abbreviation

World Health Organization	WHO
Fetal hemoglobin	HbF
Adult hemoglobin	HbA
Sickle cell disease	SCD
Hydroxyurea	HU
Sickle hemoglobin	HbS
Histone deacetylase inhibitors	HDACi
Trichostatin A	TSA
Sodium butyrate	NaB
Food & Drug Administration	FDA
Hereditary ersistence of fetal hemoglobin	HPFH
Murine erythroleukemia cells	MEL cells
Peripheral blood mononuclear cells	PBMC
Reverse transcription quantitative polymerase chain reaction	RT-qPCR
Half maximal inhibitory concentration	CC_{50}
Effective concentration	EC
Ingenuity pathway analysis	IPA
Intraperitoneal	IP
Complete blood count	CBC
Intravenous	IV
Per oral	PO
Pharmacokinetics	PK
2-Hydroxypropyl-β-cyclodextrin	HP-β-CD
High performance liquid chromatography	HPLC
Red blood cells	RBC
Hemoglobin	HGB
Reticulocytes	RET
White blood cells	WBC
Neutrophils	NEUT
Percentage of HbF positive cells	F-cell %
Hematopoietic stem cell	HSC

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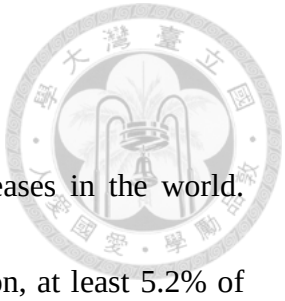

1. Introduction

Hemoglobinopathy is one of the most common inherited diseases in the world. According to prevalence estimates by the World Health Organization, at least 5.2% of the world's population exhibits hemoglobin disorders, accounting for approximately 3.4% of the deaths in children of less than 5 years of age [1]. It can be separated into two types of globin disorders: thalassemia (expression defects in globin chain synthesis) and structural disorders (in which the amino acid sequence is altered to produce abnormal hemoglobin). The two most common hemoglobinopathies, sickle cell disease (SCD) and β-thalassemia, are both adult hemoglobin (HbA) deficiencies that result in severe anemic syndromes. Approximately 4.5% of the global population suffers from either of these two diseases [2].

SCD is one of the most common monogenic disorders worldwide. Over 70% of SCD-affected newborns are from Africa, and it is estimated that more than 230,000 children with SCD are born in Africa every year. SCD is caused by a point mutation in the 6th amino acid of the β-globin gene resulting in a glutamic acid to valine alteration. Mutant β globin chains polymerize under hypoxia and change the erythrocytes into a sickle shape [3]. These sickle-shaped erythrocytes not only lose their functions to transport oxygen, but also obstruct the local capillaries to restrict blood flow. Consequently, vaso-occlusive crisis results in pain and organ damage (especially to the

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liver and heart). Moreover, these sickle-shaped erythrocytes lose their elasticity, leading to hemolytic crisis and anemia. With a high risk of early death, the life expectancy of patients with SCD is reported to be shortened to an average of 42–48 years [4]. β-thalassemia is a hereditary anemia caused by mutations or deletions within the β-globin gene that reduce the expression levels of β-globin chains. Owing to the lack of sufficient β-globin chains, the excess α-globin chains form toxic aggregates and bind to cell membranes, and this induces rapid apoptosis of the erythrocytes during early erythroblast development [5-7].

Three therapeutic strategies have been used to modulate anemia and the related symptoms of patients with severe hemoglobinopathy. The most common therapeutic strategy is regular transfusion throughout the remaining life span to support the functional HbA required for survival. However, long-term transfusion is accompanied with a high risk of iron overload, which can cause tissue damage and organ dysfunction. To avoid iron overload caused by transfusion, these chronically transfused patients must receive regular and expensive iron chelation treatment. Alternatively, bone marrow transplantation has been used for the treatment of severe SCD and β-thalassemia. Identification of a matched bone marrow donor is the primary limitation for this therapy. The potential for a potent immune response after transplantation has also been of concern. In addition to these two therapeutic strategies, pharmacological induction of

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fetal hemoglobin (HbF) expression is an alternative treatment for diminishing the syndromes of patients suffering from hemoglobin disorders [8, 9]. For patients with β-thalassemia, elevation of fetal γ-globin chain synthesis can balance the excess of α-globin chains by forming HbF, thereby modulating the severe anemia observed in these patients [10]. Moreover, increase in γ-globin chain synthesis can prevent the formation of sickle-shaped erythrocytes, as HbF directly inhibits polymerization of sickle hemoglobin in patients with SCD [11]. In clinical, the SCD patients coupled with hereditary persistence of fetal hemoglobin (HPFH) show the milder syndrome than normal SCD patients [12]. Thus, pharmaceutical induction of HbF in patients with hemoglobinopathies is a potentially useful therapeutic strategy. To date, several chemotherapeutic agents, such as trichostatin A and apicidin (histone deacetylase inhibitors; HDACi), 5′-azacytidine (DNA methyltransferase inhibitor), hydroxyurea (HU) (ribonucleotide reductase inhibitor), and sodium butyrate (NaB) and its derivatives (short-chain fatty acids, also known as HDACi), have been demonstrated to stimulate HbF production [13-18]. Among them, HU is the first effective therapeutic medicine approved by the US Food & Drug Administration for the treatment of SCD, which induces the expression of HbF for functional substitution of sickle hemoglobin in patients with SCD [19-22]. However, several side-effects of HU therapy have been reported, including leucopenia, thrombocytopenia, myelosuppression, and potential

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reproductive toxicity [23, 24]. In addition, at least 25% of patients with SCD are poor or non-responders to HU treatment [25]. Thus, identification of new agents that can induce the expression of endogenous embryonic/fetal globin chains is essential to provide an alternative therapeutic strategy for these patients. Other than three strategies I have mentioned above. In recent years, US-FDA proved the other three drugs (L-glutamine, Crizanlizumab tmca and Voxelotor) base on different strategies to ameliorate SCD symptoms. L-Glutamine is an amino acid which can be metabolized into glutamate, a precursor of glutathione. It can increase the antioxidative activity and may reduce the complications of SCD. Crizanlizumab tmca is a monoclonal antibody against human P-selectin. The P-selectin expressed in the endothelial cells and platelets which involves in the adhesion of sickle-shape RBC to blood vessel. Crizanlizumab tmca can block P-selectin and prevent the vaso-occlusive crisis in patients. Voxelotor can directly bind to HbS and increase the oxygen affinity of HbS. The increase of oxygen affinity can inhibit the polymerization of HbS [26, 27]. All the strategies including induction of HbF show the promising results to mitigate symptoms in SCD patients.

In our previous study, we identified six compounds that have the same pharmacophore (Compound II) as HbF inducers [28]. However, the poor solubility and bioavailability restrict the further drug development. I cooperated with Dr. Tsann-Long Su in IBMS, Academia Sinica to modify the pharmacophore of compound I-VI as class

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1 compound. **SS-2394** is the candidate compound I chose for further study. **SS-2394** shows higher γ-globin gene induction efficacy than HU. It also can induce γ-globin gene in HU non-response cells. The studies of molecular mechanisms and γ-globin gene induction efficacy in HU response or non-response cells of **SS-2394** have been revealed in my master thesis [29]. In this thesis, I further analyzed the global effect of **SS-2394** in primary human erythroid cells. I also investigate the *in vivo* efficacy of **SS-2394** in β–YAC transgenic mice and SCD mice.

In a previous study, Nam et al. [30] conducted a high-throughput screening and identified a potent HbF inducer, **TN1**, which induced HbF more potently than HU in KU812 and K562 leukemia cell lines. However, our studies found that **TN1** was not orally active and did not significantly increase γ-globin gene expression in primary erythroid cells. Both drawbacks could limit the future development of **TN1** for the treatment of patients with β-thalassemia and SCD in clinical trials. To continue to develop potent and orally active inducers, we used **TN1** and **compound II** as a lead to redesign the analogs as class 2 compound. This thesis has led to the discovery of **AS-28**, which can efficiently induce γ-globin gene expression at non-cytotoxic concentrations and shows oral activity in ameliorating anemia and the related symptoms in an SCD mouse model. In addition to **AS-28**, some analogs displayed similar HbF-inducing

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capabilities to those of **AS-28**. Further studies that explain the possible biological mechanism underlying **AS-28**-mediated re-activation of γ-globin gene expression and the global effect of **AS-28** in primary human erythroid cells are also discussed herein. These promising results demonstrate that **AS-28** or its analog relatives can be further developed as novel agents for treating hemoglobinopathies, such as β-thalassemia and sickle cell disease.

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2. Materials and methods

2.1. Primary erythroid cell culture

This study was conducted with the approval of the Human Subject Research Ethics Committee/IRB, Academia Sinica (AS-IRB01-16014). Peripheral blood samples were purchased from the Taipei Blood Center. The concentrated blood was diluted 1:5 (V/V) in phosphate-buffered saline (PBS) and distributed in a thin layer on Ficoll-paque PLUS (d = 1.007 g/mL) (GE Healthcare) in a SepMate column (STEMCELL). After centrifugation at 1,200 g for 10 min, the cells in the inter-phase region were collected. The collected cells were washed with PBS and centrifuged at low speed three times. The remaining mononuclear cells were expanded in Phase I medium containing SFEM (STEMCELL), 100 ng/ml SCF, 20 ng/mL IL-3, 20 ng/mL IL-6, and 100 ng/mL FLT3-L at 37 °C in an incubator with 5% CO_2 for 7 days. The expanded mononuclear cells were further differentiated in Phase II medium containing SFEM (STEMCELL), 20 ng/mL SCF, 5 ng/mL IL-3, 1 U/mL EPO, 2 μM dexamethasone for another 7 days. The differentiated status of cells was confirmed by Benzidine staining and Liu's stain according to previous published protocol [31, 32] and manufacturer's instructions respectively. The differentiated erythroid cells were treated with indicated compounds of different dosages with a seeding density of 5×10^5 cells/mL for another 3 days [28, 29, 31].

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2.2. RT-qPCR

After 3 days of compound treatment, total RNA was extracted by a Quick-RNA miniprep kit (Zymo) and reverse-transcription was performed using Maxima H Minus Reverse Transcriptase (ThermoFisher Scientific) according to the manufacturer's instructions. Quantitative PCR was performed on a LightCycler Nano system with SYBR green master mix following the manufacturer's instructions (Roche). The RT-qPCR data was normalized to the Cq number of β-actin and compared to a mock control. All primer sequences used for RT-qPCR are as follows:

γ-globin forward primer, 5′-CTTCCTTGGGAGATGCCAT-3′;

γ-globin reverse primer, 5′-GAATTCTTTGCCGAAATGGAT-3′;

β-globin forward primer, 5′-CTTTAGTGATGGCCTGGCT-3′;

β-globin reverse primer, 5′-CACTGGTGGGGTGAATTCT-3′;

BCL11A forward primer, 5′-TGGTATCCCTTCAGGACTAGGT-3′;

BCL11A reverse primer, 5′-TCCAAGTGATGTCTCGGTGGT-3′;

c-Myb forward primer, 5′-ACAGAAATACGGTCCGAAACG-3′;

c-Myb reverse primer, 5′-CCAATTCTCCCCTTTAAGTGC-3′;

NFE4 forward primer, 5′-CCAGAAAGCAGGCCACAGCA-3′;

NFE4 reverse primer, 5′-AGGGCCCCAGTAGGTGAGAT-3′;

NFE2L2 forward primer, 5′-TTCCCGGTCACATCGAGAG-3′;

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NFE2L2 reverse primer, 5′-TCCTGTTGCATACCGTCTAAATC-3′;

GATA1 forward primer, 5′-TTGTCAGTAAACGGGCAGGTA-3′;

GATA1 reverse primer, 5′-CTTGCGGTTTCGAGTCTGAAT-3′;

CPOX forward primer, 5′-GCTGGGGTGAGCATTTCTGTT-3′;

CPOX reverse primer, 5′-GCATGAGGATTCTTGGGGTGG-3′;

β-actin forward primer, 5′-CCTGAACCCCAAGGCCAACC-3′;

β-actin reverse primer, 5′-CAGGGATAGCACAGCCTGGA-3′;

mouse β-actin forward primer, 5′-CCTGTATGCCTCTGGTCGTA-3′;

mouse β-actin reverse primer, 5′-CTGTGTTGGCATAGAGGTCTTTA-3′.

2.3. Cell viability assay

Cell viability was assessed using AlamarBlue reagent (Invitrogen). After 3 days of treatment, 100 μL of treated cell culture was transferred into a 96-well plate and 1/10 volume of AlamarBlue reagent was added and the cell culture was incubated overnight at 37 °C. Cell viability was evaluated using a multi-label counter (Ex 530-560 nm, Em 590 nm) (PerkinElmer).

2.4. Western blot

After 3 days of treatment, total protein was extracted by modified RIPA (50 mM Tris-HCl pH7.8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM NaV_3O_4 and 1x complete EDTA-free

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Protease Inhibitor Cocktail). Histone was extracted by histone extraction buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1.5 mM PMSF and 0.2 N HCl) at 4 °C overnight. Centrifuge the extracts at 13,000 rpm for 5 minutes and collected the supernatant. Total 50 μg of protein extracts were separated by 10% (for BCL11A) or 15 % SDS-PAGE (for γ-globin and histone) and the resulting gel was blotted onto PVDF membrane. After blocking with 5% non-fat milk in TBST, the membrane was incubated overnight with primary antibody against total acetylated histone H4, total histone H4, γ-globin, BCL11A or β-actin at 4 °C. After incubating with horseradish peroxidase-conjugated secondary antibodies and washing the blot, the signals of indicated proteins were visualized by ECL (Omics Bio) following the manufacturer's protocol.

2.5 Pharmacokinetics

Male Sprague–Dawley rats weighing 300–400 g each (8–12 weeks old) were obtained from BioLASCO (Taiwan Co., Ltd, Ilan, Taiwan). The animal studies were performed according to NHRI institutional animal care and committee-approved procedures. Animals were surgically prepared with a jugular-vein cannula one day before dosing and fasted overnight (for approximately 18–20 h) before dosing. Water was available *ad libitum* throughout the experiment. Food was provided at 4 h after dosing. A single 2.0 mg/kg and 10 mg/kg dose of compound, as a PEG400/DMA (80/20,

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v/v) solution, was separately administered to groups of 3 rats each intravenously (IV) and oral gavage (PO), respectively. Each animal received 2 or 10 mL of the dosing solution per kg of body weight for IV and PO, respectively. At 0 (before dosing), 2, 5 (IV only), 15, and 30 min and at 1, 2, 4, 6, 8, and 24 h after dosing, a blood sample (0.15 mL) was collected from each animal through the jugular-vein cannula and stored in ice (0–4 °C). Immediately after collecting the blood sample, 150 mL of physiological saline (containing 30 Units of heparin per ml) was injected into the rat through the jugular-vein cannula. Plasma was separated from the blood by centrifugation (14,000 g for 15 min at 4 °C in a Beckman Model AllegraTM 6R centrifuge) and stored in a freezer (-20 °C). All samples were analysed for the parent drug by LC-MS/MS. Data were acquired through selected reaction ion monitoring. Plasma concentration data were analyzed with non-compartmental method.

2.6. Animal studies

2.6.1. β-YAC transgenic mouse studies

This animal study was performed according to Academia Sinica institutional animal care and utilization committee (IACUC)-approved protocol. β-YAC transgenic mice were the gift from Dr. Keiji Tanimoto, University of Tsukuba [33]. The mice were bred at the animal facility, in Institute of Molecular Biology, Academia Sinica. Mice of 6–8 weeks old were used for experiments. Treatment groups received PBS plus **SS-2394**

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(5 mg/kg), which was administered by IP injection once daily for 5 days a week for 3 weeks. HU (100 mg/kg in PBS) as comparison group was administered by IP injection once daily for 5 days a week for 3 weeks.

2.6.1.1. Total blood RNA extraction and RT-qPCR

Total blood from treated mice was collected by submandibular blood collection once a week. Total RNA was extracted by Total RNA miniprep. Purification kit (GeneMark) and reverse-transcription was performed using Maxima H Minus Reverse Transcriptase (ThermoFisher Scientific) according to the manufacturer's instructions. Quantitative PCR was performed on a LightCycler Nano system with SYBR green master mix following the manufacturer's instructions (Roche). The RT-qPCR data was normalized to the Cq number of β-actin and compare to the week 0.

2.6.2. SCD mouse studies

2.6.2.1. ***SS-2394***

This animal study was performed according to Academia Sinica institutional animal care and utilization committee (IACUC)-approved protocol. Sickle cell disease mice (B6; 129-Hba$^{tm1(HBA)Tow}$, Hbb$^{tm2(HBG1,HBB*)Tow}$/J) were purchased from Jackson Laboratory [34] and were bred at the AS Core, in Academia Sinica. Mice of 6–8 weeks old were used for experiments. Treatment groups received vehicle (PBS) plus **SS-2394** (10 mg/kg and 20 mg/kg), which was administered by IP injection once daily for 28

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days. HU (100 mg/kg in PBS) as comparison group was administered by IP injection once daily for 5 days a week for 4 weeks.

2.6.2.2. ***AS-28*** *and* ***AS-61***

Treatment 1 groups received vehicle [20% (2-Hydroxypropyl)-β-cyclodextrin] plus **AS-28** (50 mg/kg, 75 mg/kg and 100 mg/kg), which was administered by oral gavage twice per day, 5 days per week, for 4 weeks. Treatment 2 groups received vehicle [20% (2-Hydroxypropyl)-β-cyclodextrin] plus **AS-28** (100 mg/kg and 150 mg/kg) or **AS-61** (50 mg/kg), which was administered by oral gavage once daily for 28 days.

2.6.3. Complete blood count analysis

Blood was collected by submandibular blood collection and analysis was performed using a ProCyte Dx automatic analyzer.

2.6.4. F-cell quantitation

Wash 10 μL of whole blood twice with PBS and then fix it with 0.05% glutaraldehyde for 10 min. After fixation, the cells were washed twice with PBS and then permeabilized by adding cold 0.1% Triton X-100/PBS for 5 min. Cells were washed twice with 0.1% BSA/PBS and stained with PE-HbF antibody (BD) for 40 min at room temperature (with protection from light). Finally, the cells were washed three times with 0.1% BSA/PBS and passed through a 0.3 μM cell strainer (Falcon). F-cell % was analyzed by flow cytometry (LSRII-18P, BD) [28].

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2.6.5. Hemoglobin high-performance liquid chromotography (HPLC)

Lysed 50 μL of whole blood in 200 μL ddH_2O and centrifuged it for 5 min at 13,000 rpm to extract the hemolysate. HPLC was performed according to a previously described protocol [31].

2.6.6. Blood smear

For hypoxia treatment, whole blood was incubated in a hypoxic incubator chamber (3% O_2) for 30 min. We used 2 μL of whole blood to make blood smears on slides. Slides were air-dried and then stained with Liu's stain [32] before quantifying the percentage of sickle-shaped cells.

2.7. RNA-Seq

RNA from 3 set of **SS-2394, AS-28** treated-cells and mock control cells were extracted by Quick-RNA miniprep kit (Zymo). The library preparation, sequencing (illumina Novasq/Hiseq 4000, Read length: PE150) were done by TOOLS according to their manufacturer's instructions. The following analysis was done by TOOLS and Bioinformatics core in Institute of Molecular Biology, Academia Sinica.

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2.8. Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM) for at least 3 independent experiments. Statistical analysis of the raw data was performed by the 2-tailed student t test. A probability of less than 0.05 ($P < 0.05$) was considered significant.

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3. Results

3.1 Screen and identify the HbF inducing parental compounds.

In our previous study, we setup a high-throughput system to screen the HbF inducing compounds. Adult murine erythroleukemia (MEL) cell line barring the dual-fluorescence reporter (γ globin promoter-directed DsRed and β-globin promoter-directed EGFP, Figure 1a, 1b) were treated with compounds (compound library from NHRI) and screen the DsRed signal to identify the HbF inducing compounds. Total of 10,000 compounds were tested and identified six compounds can induce the DsRed fluorescence in MEL cells. Next, we set up primary human erythroid cultures from adult normal donors to validate the γ-globin gene activation abilities of these six compounds. PBMCs were isolated from the peripheral blood of adult normal donors, and conducted for two-phase *in vitro* differentiation. Differentiation status of primary human erythroid cells were verified by Benzidine staining and Liu's stain. Benzidine staining showed that after 14 days of *in vitro* differentiation, hemoglobin (heme-containing protein), can be detected (blue color) in cells. Liu's stain revealed that most of cells were differentiated into orthochromatic erythroblasts (Figure 2, 3). The dosage dependence of the compounds I to VI on γ-globin gene activation in the primary human erythroid cells and their inhibitory effects on cell proliferation are shown in Figure 4. The studies of high-throughput screening and identified compounds I to VI as

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HbF inducers have been published on Journal of Molecular and Cellular Biology [28]. Although compounds I to VI can induce γ-globin gene expression, the poor solubility and bioavailability (Table 1) limit the further drug development. Base on the pharmacophore of compounds I to VI, I cooperated with Dr. Tsann-Long Su in IBMS, Academia Sinica and Dr. Weir-Torn Jiaang in NHRI to modify and synthesize new class of compounds (class 1 and class 2 respectively).

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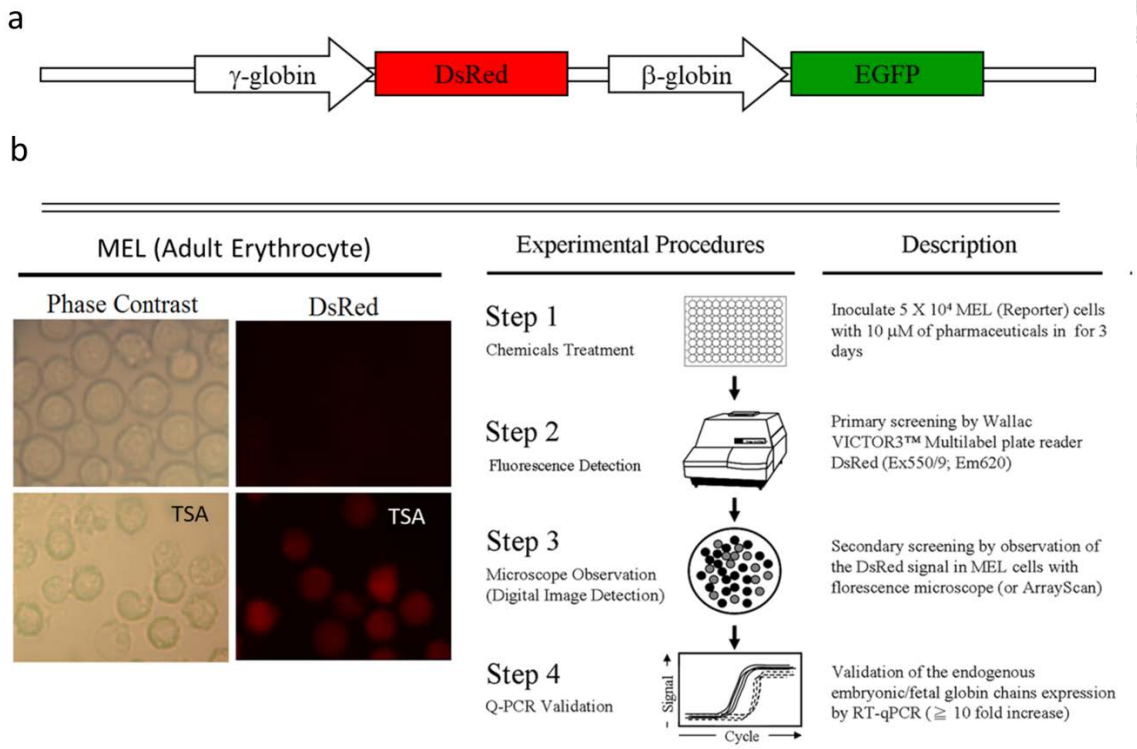

Figure 1. High throughput screening flow chart (a) General construction of reporter (b) Phase-contrast and DsRed florescence images of cells stably transfected with the dual-fluorescence reporter plasmid Top row, MEL cells; second row, MEL cells treated with TSA for 3 days. In step 1, ~10,000 compounds were tested for their ability to activate the γ globin promoter in MEL cells cultured on 96-well plates. In steps 2 and 3, elevation of DsRed fluorescence was detected by a fluorescence reader and further confirmed by a digital image detector. In step 4, the activation of the endogenous mouse embryonic and/or fetal globin genes as induced by the compound(s) was verified by RT-qPCR analysis. HU and NaB were used as reference compounds [28].

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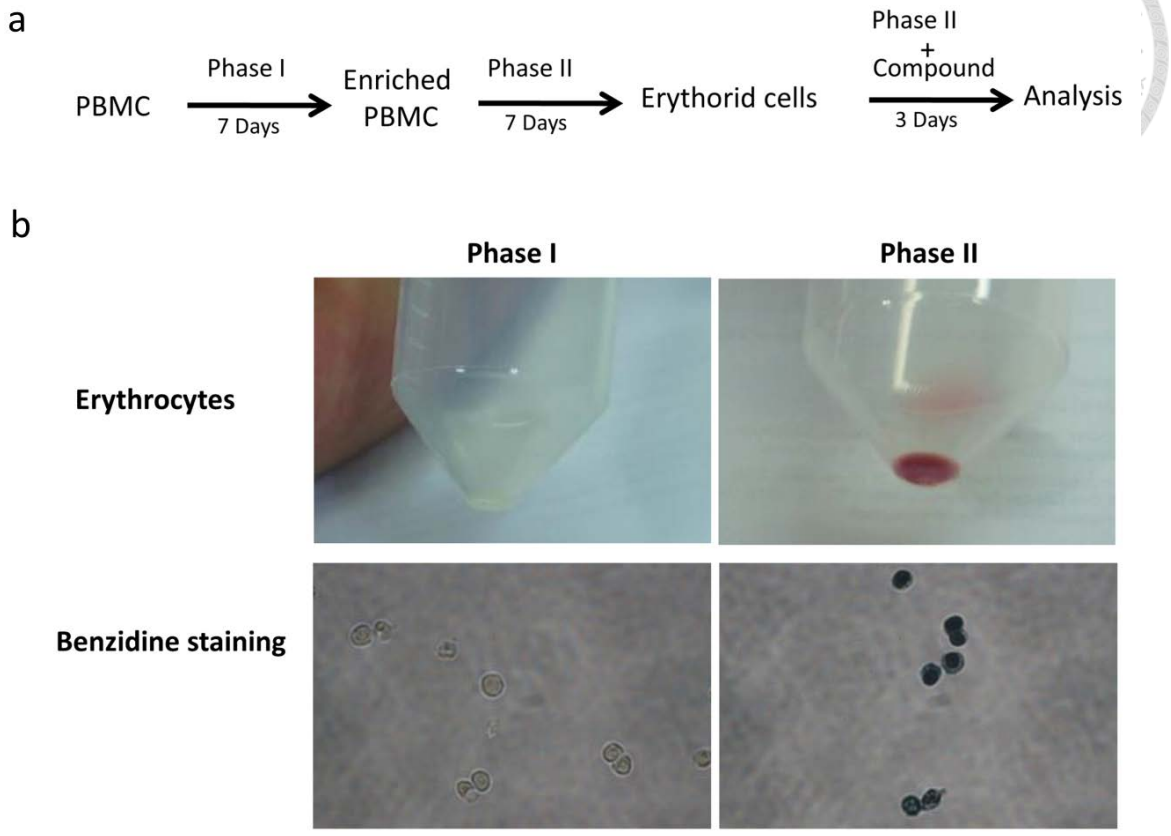

Figure 2. Procedure of primary human erythroid culture systems and Benzidine staining of hemoglobin. (a) Briefly describe the procedure of primary human erythroid culture systems (b) After 14 days differentiation, cell pellet turns to red color. Benzidine staining in phase I and Phase II cells to detect the hemoglobin accumulation (Benzindine will be oxidized by heme and become blue color) [29].

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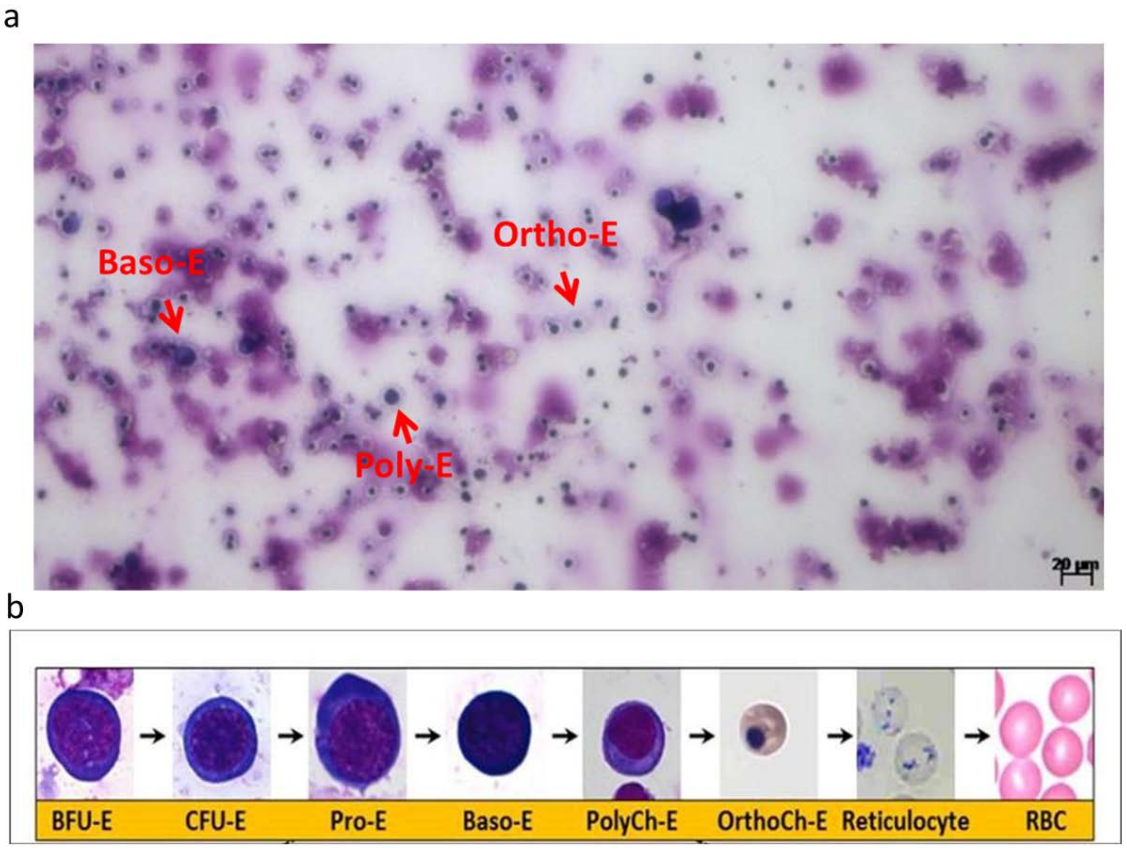

Figure 3. Liu's stain to determine the differential stage of primary human erythroid cells after 17 days *in vitro* differentiation. (a) Seeding 4x10^5 cells on chamber slide and incubate for 30 minutes. Remove culture medium and wash by PBS once then perform Liu's stain according to the manufacturing instruction and take photos by AxioImager Z1. (b) Lineage of erythroid differentiation [35]. Arrow indicates the different cell lineage.

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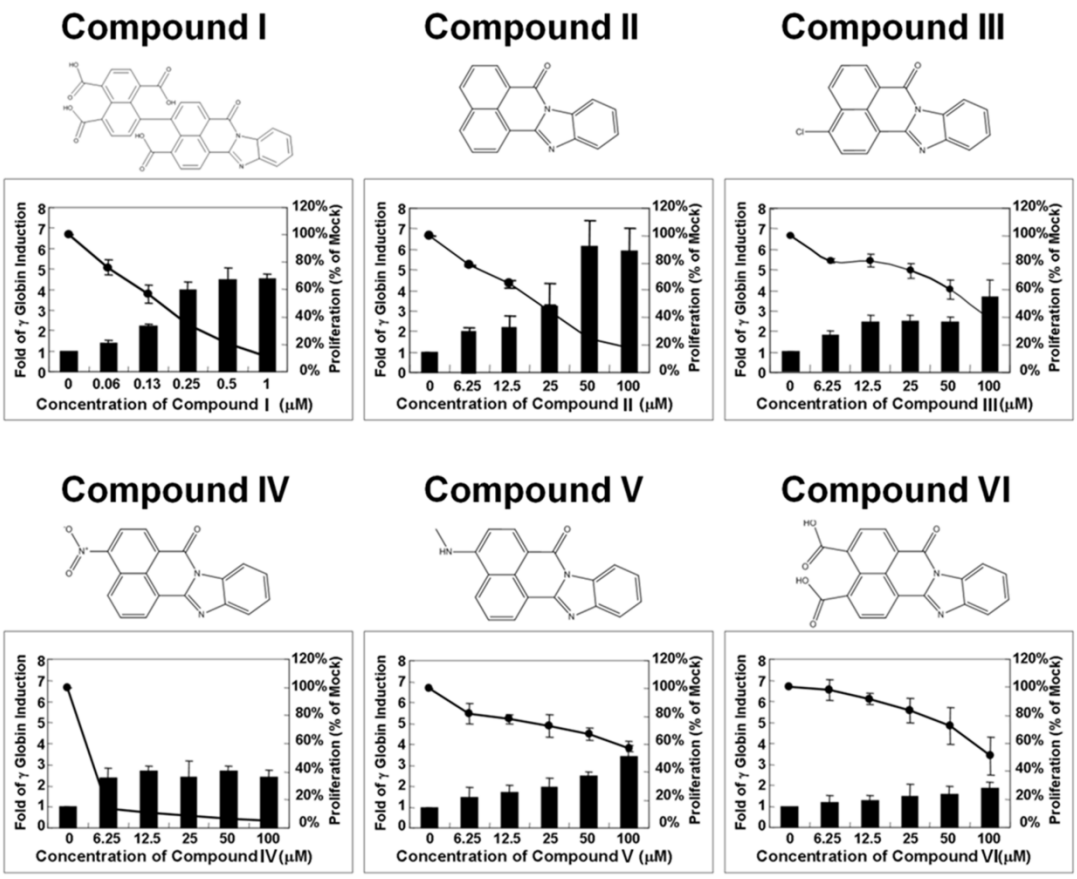

Figure 4. Induction of γ-globin gene and the inhibition of cell proliferation by compound I-VI. Primary human erythroid cells were treated with different dosages of compound I-VI for 3 days. The induction of γ-globin mRNAs and proliferation rate were detected by RT-qPCR and AlamarBlue assay, respectively [28].

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Table 1. Pharmacokinetic profiles of compound II in rat.

Compound	IV				PO			
	$T_{1/2}$ (h)	CL (mL/(min·kg))	V_{ss} (L/kg)	$AUC_{(0\text{-inf})}$ (ng·h/mL)	$T_{1/2}$ (h)	C_{max} (ng/mL)	$AUC_{(0\text{-inf})}$ (ng·h/mL)	F (%)
II[a]	5.8	3.9	8.4	928.1	5.75	3.6	122	2.34

PO, per oral. [a] Dose: IV 2.07 mg/kg; PO 19.3 mg/kg.

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3.2 In vitro γ-globin gene induction capability studies of class 1 compounds

Base on the pharmacophore of Compound II, Dr. Tsann-Long Su in IBMS, Academia Sinica designed total 27 compounds as class 1 compound. The γ-globin gene induction capabilities and their inhibitory effects on cell proliferation of these compounds were test in primary human erythroid cells. By using this assay, total 6 of 27 compounds were identified as HbF inducing compounds. In order to evaluate the therapeutic potentials of these six compounds in comparison to HU, the CC_{50} (the half maximal inhibitory concentration) and the EC (effective concentration) of each compounds were calculated (Table 2). The CC_{50} values indicate the concentrations of the individual compounds inhibit the proliferation rates of the cells by 50%. The EC values are defined as the concentrations of compounds that induce the γ-globin gene expression by 1.9-fold, the fold of the γ-globin gene induction by HU at CC_{50}. The ratio of CC_{50} to EC (CC_{50}/EC) is used to evaluate the benefits of these potential HbF-inducing compounds in comparison to HU (the ratio CC_{50}/EC was set as 1) (Table 2). According to the highest value of therapeutic index (CC_{50}/EC = 2.9), **SS-2394** was chose as candidate compound for further investigation (Structure of **SS-2394** is shown in Figure 5a). The dose-dependent γ-globin induction and inhibitory of cell proliferation are shown in Figure 5b.

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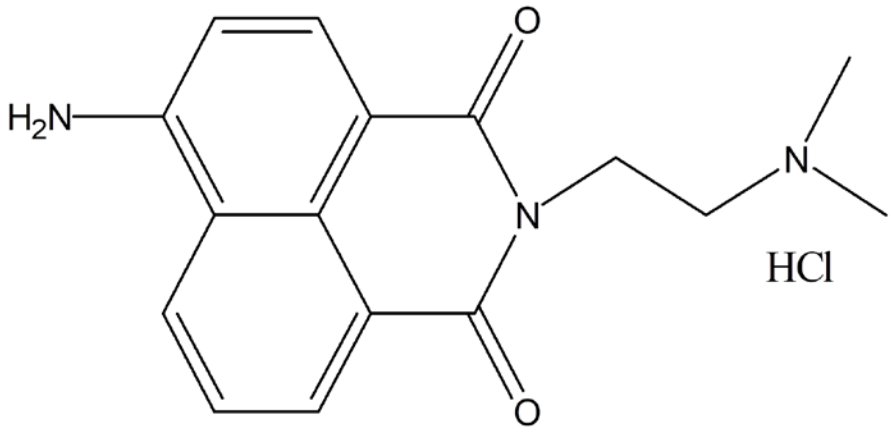
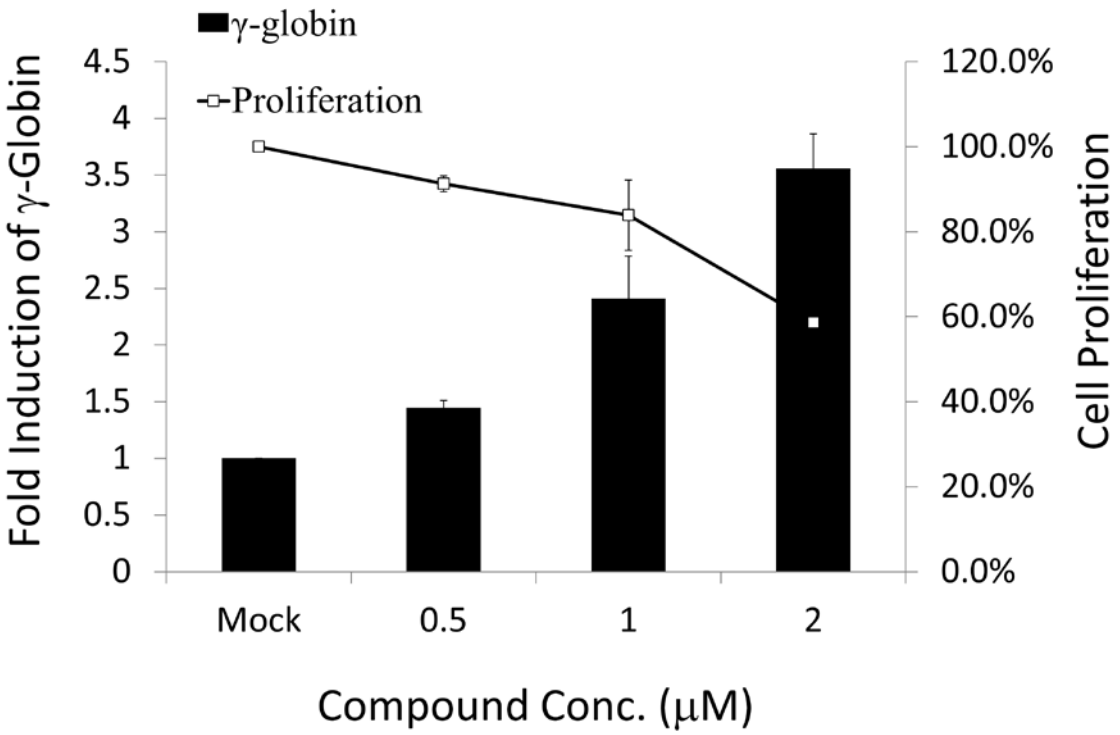
a



SS-2394

b



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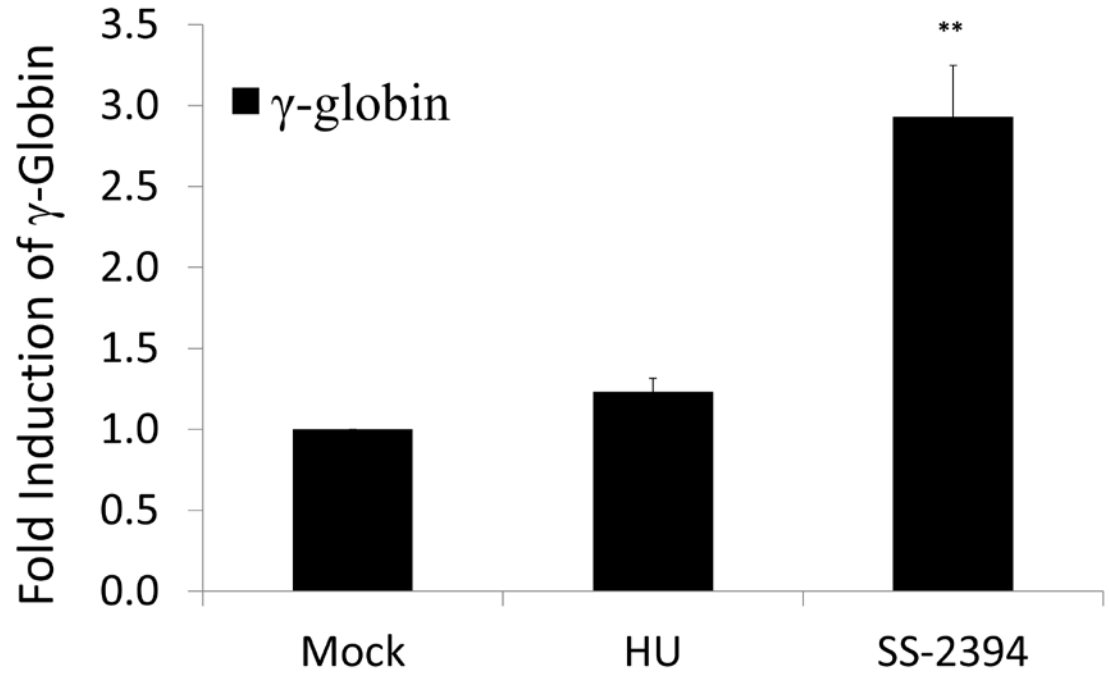

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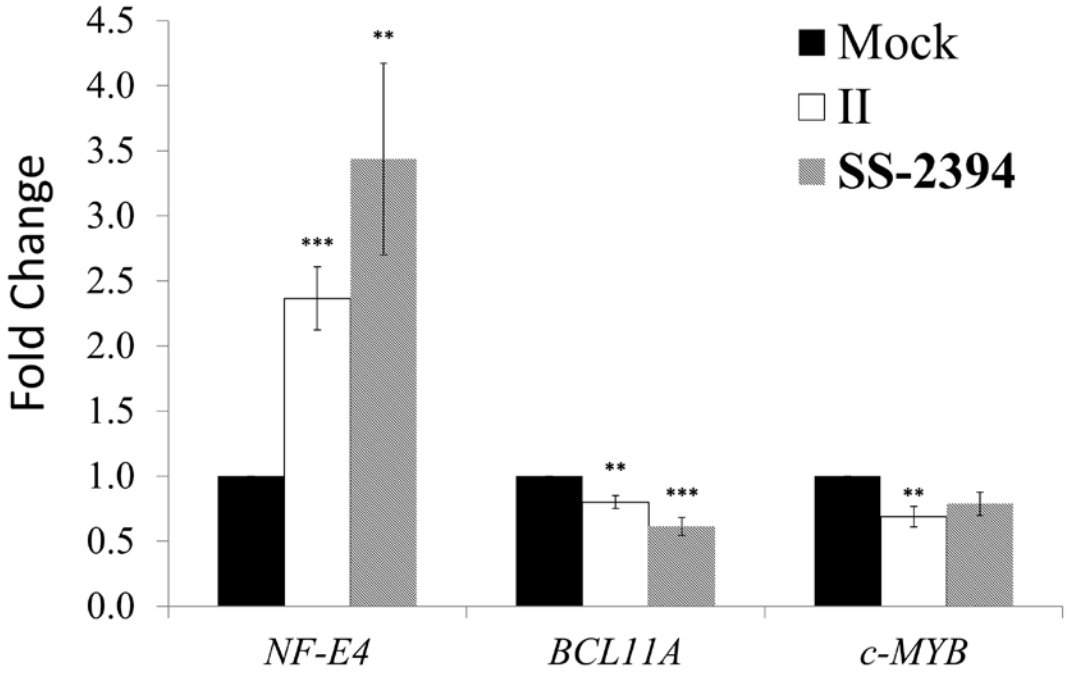

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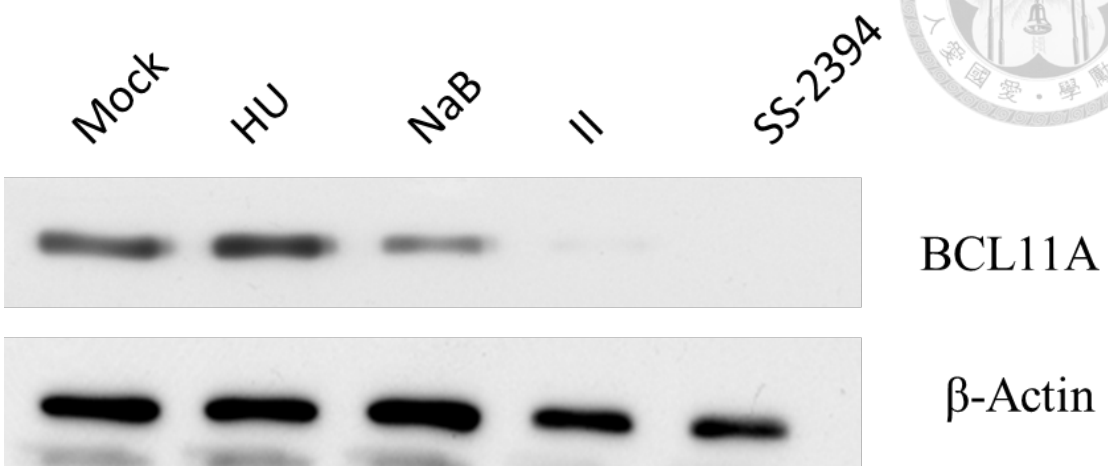

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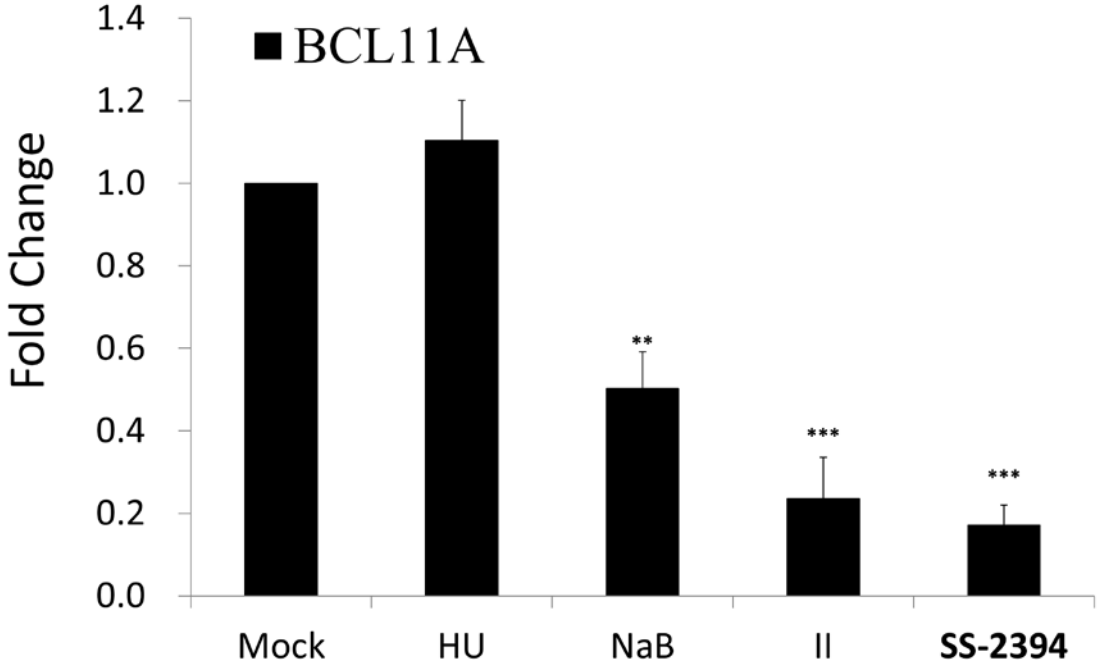

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Figure 5. γ-globin gene induction capability and molecular mechanisms of SS-2394.

(a) The structure of SS-2394. (b) Primary human erythroid cells were treated with different dosages of **SS-2394** for 3 days. The induction of γ-globin mRNAs and proliferation rate were detected by RT-qPCR and AlamarBlue assay, respectively (c) The primary human erythroid cells were treated with **SS-2394 (**2.3 μM**)** and HU **(147** μM**)** for 3 days. The induction of γ-globin mRNAs was detected by RT-qPCR. The induction folds of γ-globin gene in HU-treated cells which were similar to Mock control defined as HU non-response cells. (d) Primary human erythroid cells were treated with Compound II (22 μM), SS-2394 (2.3 μM) for 3 days. The expression level changes in *NF-E4*, *BCL11A*, and *c-MYB* mRNAs were detected by RT-qPCR. (e) Protein extracts (50 μg) from compound treated-cells and mock control cells were loaded for western blot. Antibody against BCL11A was used to detect the expression level change after compound treatment. β-Actin was used as an internal control. (f) Quantification of western blot results was normalized with β-actin and compared to that of the mock control. All data are from at least three independent experiments and presented as means ± SEM (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$) [29].

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Table 2. Therapeutic index of class 1 compounds.

Compound	CC_{50}(μM)	EC(μM)	CC_{50} / EC	γ-globin Induction Fold at CC_{50}
II	21.8	2.3	9.7	3.0
A2	0.3	2.9	0.1	1.1
E1	2.3	7.8	0.3	1.3
B1	10.7	4.7	2.3	4.1
B4	5.0	2.2	2.3	3.3
SS-2394	2.3	0.8	2.9	4.0
E3	11.2	7.4	1.5	2.7
Hydroxyurea	146.5	146.5	1	1.9
Sodium butyrate	221.6	NA	NA	1.3

CC, cytotoxic concentration; EC, effected concentration; NA, not available [29].

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*3.3 Compound **SS-2394** activates γ-globin expression in HU non-response primary human erythroid cells*

According to previous report, at least 25 % SCD patients are poor or non-response to HU treatment. I found the normal blood sample from Taipei blood bank also can separate into HU response or non-response groups. After 3 days of compound treatment, the γ-globin gene induction folds were evaluated by RT-qPCR. The induction folds of γ-globin gene in HU-treated cells which were similar to Mock control defined as HU non-response cells. The RT-qPCR result showed that **SS-2394** still can induce γ-globin gene up to 2.9-fold in HU non-response cells (Figure 5c).

*3.4 Molecular mechanisms for γ-globin gene induction by **SS-2394***

It has been reported that several transcriptional factors are involved in the regulation of the γ-globin gene expression. *NF-E4* was reported to form transcriptional activation complex which were recruited to the γ-globin promoter and turned on the γ-globin gene expression in the primary human erythroid cells [36]. *c-Myb* was showed to involve in erythropoiesis and as a γ-globin gene repressor [37]. In addition, *BCL11A* was found to be a developmental stage-specific repressor of γ-globin gene [38]. To clarify the regulatory factors involved in modulation of the γ-globin gene expression by **SS-2394**, the mRNA levels of these transcription factors were estimated by RT-qPCR. According to the RT-qPCR data, **SS-2394** can inhibit *BCL11A* (0.6-fold). In addition,

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SS-2394 also increases *NF-E4* (3.4-fold) (Figure. 5d). However, the expression of γ-globin gene repressor *c-Myb* was only significant decreased in compound II treated cells. I further analyzed the protein level of BCL11A in compounds-treated primary erythroid cells by western blot (Figure 5e, 5f). A dramatic reduction of BCL11A was revealed either in compound II or **SS-2394** treated cells.

*3.5. Global effect studies of **SS-2394** in primary human erythroid cells*

In order to figure out the effect of **SS-2394** on primary human erythroid cells, RNA from three sets of **SS-2394** treated cells and Mock control cells were collected and send for the RNA-seq analysis. Differentially expressed genes (DEGs) were selected (fold change >1.4, $p < 0.05$) and applied for Ingenuity Pathway Analysis (IPA). Top 5 canonical pathways were show in Table 3. The results show that **SS-2394** involved in DNA repair and cell cycle pathways which indicate **SS-2394** may have toxic effect in primary human erythroid cells.

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Table 3. Top 5 canonical pathways affected by SS-2394 treatment in primary human erythroid cells.

Name	**p-value**	**Overlap**
Cell Cycle Control of Chromosomal Replication	7.68E-10	23.2% (13/56)
DNA Double-Strand Break Repair by Homologous Recombination	1.49E-05	35.7% (5/14)
Role of BRCA1 in DNA Damage Response	2.67E-05	12.5% (10/80)
ATM Signaling	2.71E-05	11.3% (11/97)
Mismatch Repair in Eukaryotes	3.12E-05	31.2% (5/16)

DEGs were selected from RNA-seq data (fold change >1.4, p <0.05) and analyzed by IPA.

*3.6. In vivo efficacy studies of **SS-2394** in β–YAC transgenic mice and SCD mice.*

In order to evaluate the *in vivo* HbF induction efficacy, β–YAC transgenic mice were used to perform the intraperitoneal injection (IP) and examine the effect of **SS-2394**. One dose of **SS-2394** (5 mg/kg) was IP injected into β–YAC transgenic mice once daily for 5 days a week. Blood samples were collected once a week for 3 weeks. Total RNA from peripheral blood were extracted and detected the γ-globin gene level by RT-qPCR (Figure 6). The result showed that **SS-2394** can induce γ-globin gene expression to 1.8–fold. HU (100 mg/kg) was IP injected into β–YAC transgenic mice

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once daily for 5 days a week as control. To examine the efficacy of **SS-2394** to ameliorate the anemia symptoms, two dose of **SS-2394** (10 mg/kg and 20 mg/kg) were IP injected to 6- to 8-week-old SCD mice once daily. After 28 days treatment, blood samples from the treated SCD mice were collected for several biochemical analyses (Table 4). Complete blood cell assessment showed the toxic effect on decreasing the red blood cell number and hemoglobin amount at high concentration of **SS-2394** (20 mg/kg) (Table 4). In addition, the **SS-2394**-treated groups showed a slightly decrease in the white blood cell and neutrophil number in SCD mice. Although we did not observe the improvement in percentage of F-cells and HbF, the sickle cell percentage in blood smear were decreased (Table 4 and Figure 7). In the group treated with HU (100 mg/kg, intraperitoneal injection once daily, the toxic effects on decreasing the red blood cell number and hemoglobin amount were observed (Table 4). Moreover, the HU-treated group showed a slightly decrease in the white blood cell and neutrophil number in SCD mice. However, I did not find improvement in percentage of F-cells and HbF which is consistent with the percentages of sickle-shaped cells in the blood smear (Table 4 and Figure 7d).Taken together, **SS-2394** showed the HbF induction capability in β–YAC transgenic mice not in the SCD mice. But **SS-2394** still can decrease the sickle cell percentage in SCD mice.

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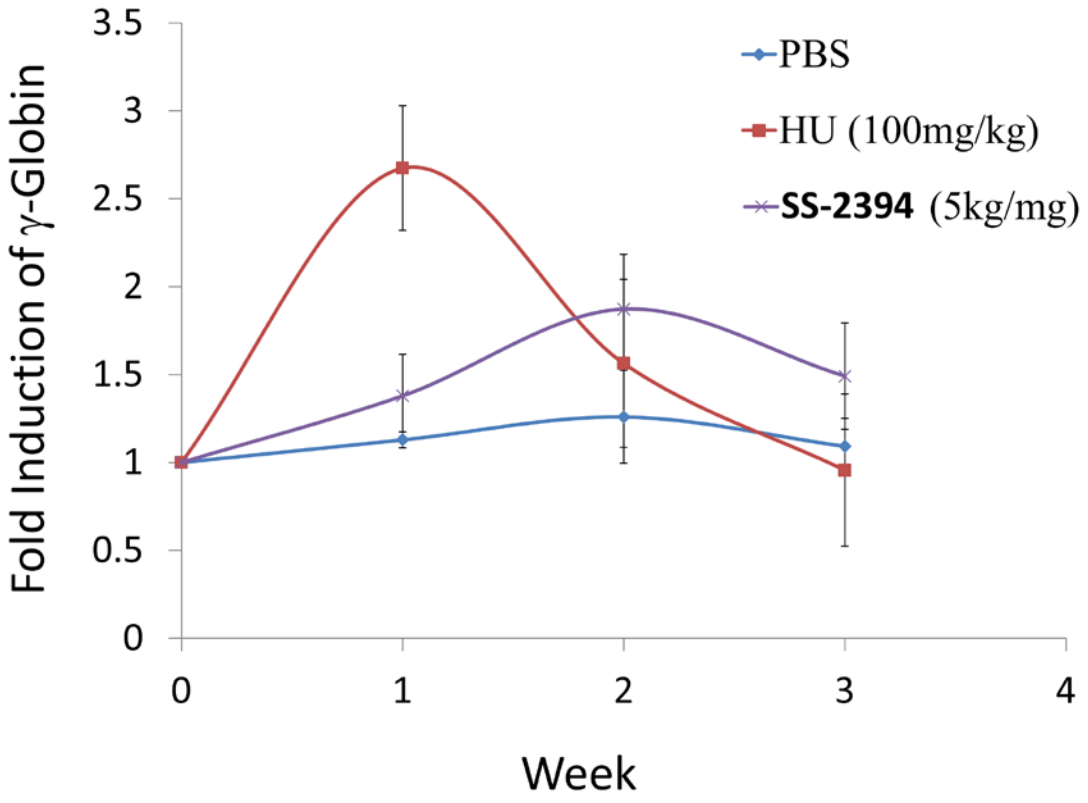

Figure 6. **γ-globin gene induction fold in the peripheral blood of compound-treated β-YAC transgenic mice.** Mice were IP injected with PBS, HU (100mg/kg) and **SS-2394** (5 mg/kg) once daily, 5 days a week for 3 weeks. Blood was collected by submandibular blood collection once a week and extracted the total RNA. γ-globin gene induction fold was detected by RT-qPCR and compare to the week 0. Each group contained three mice and data presented as means ± SEM.

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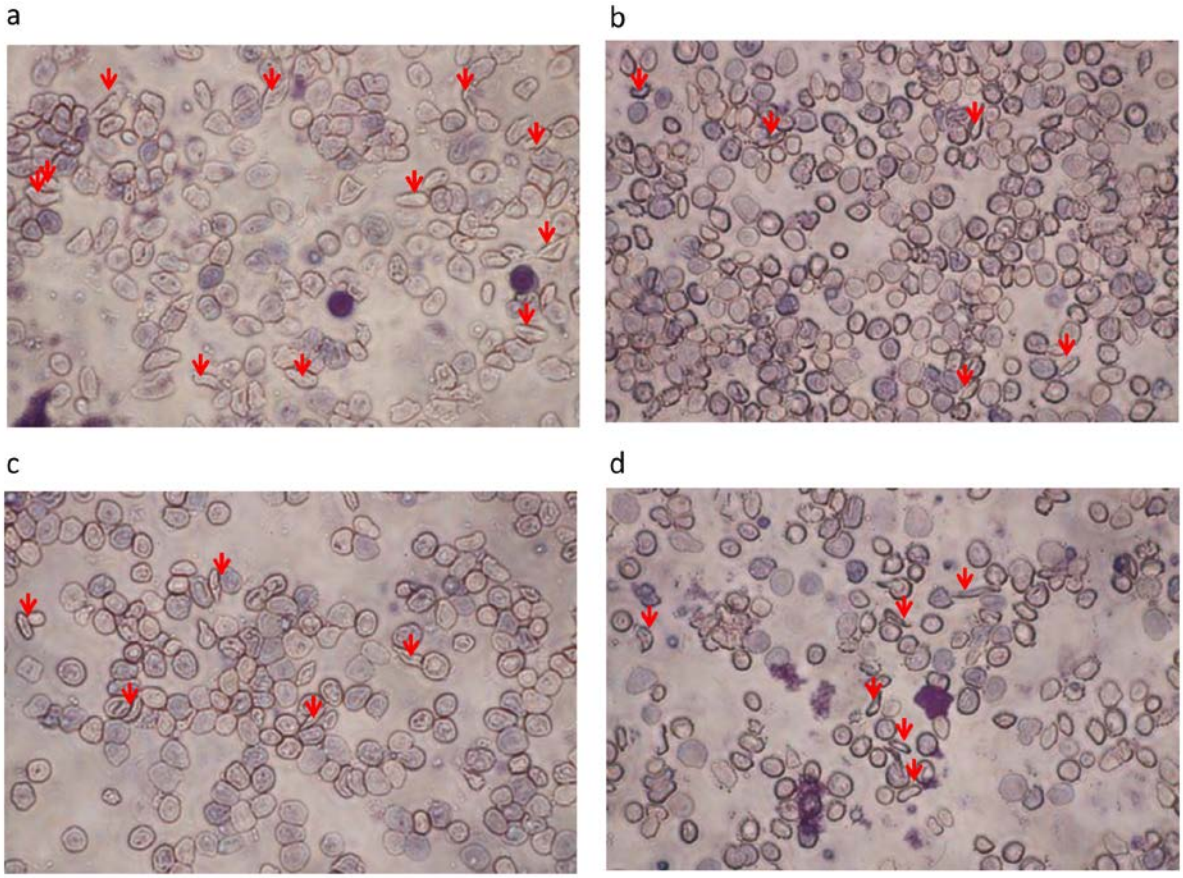

Figure 7. **Blood smear of compound (SS-2394 and HU)-treated mice.** Blood was collected by submandibular blood collection and treated with hypoxia (3% O_2) for 30 min. Blood (2.0 μL) from each sample was used to prepare the blood smear on the slide. The slides were air-dried and then stained with Liu's stain. (a) Mock (b) **SS-2394** (IP 10 mg/kg) (c) **SS-2394** (IP 20 mg/kg) (d) HU (IP 100 mg/kg). Each group contained at least three mice. Arrows indicate the sickle-shape cells.

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Table 4. Antianemic effect of SS-2394 against SCD mice

Blood test[a]	Mock	**SS-2394** IP[b] 10 mg/kg	**SS-2394** IP 20 mg/kg	HU IP 100 mg/kg
RBC (M/uL**)**	7.86±0.17	7.48±0.31	6.97±0.23*	6.34±0.41**
HGB (g/dL)	7.93±0.21	7.41±0.37	6.83±0.24**	6.7±0.31*
RET (%)	45.68±4.47	47.87±2.91	53.23±3.52	50.92±1.58
WBC (K/uL)	57.72±14.31	41.1±7.13	24.28±4.49*	23.02±3.45
NEUT (K/uL)	43.67±14.08	13.52±5.37	10.67±0.93*	9.75±1.13
HbF (%)	1.42±0.17	1.42±0.14	1.2±0.11	1.3±0.19
F-cell (%)	0.24±0.02	0.22±0.02	0.20±0.02	0.23±0.03
Sickle cell (%)	16.7±1.0	12.3±0.6**	12.7±0.4**	14.7±0.4

[a] RBC, red blood cells; HGB, hemoglobin; RET, reticulocytes; WBC, white blood cells; NEUT, neutrophils; F-cell %, percentage of HbF positive cells s; HbF, fetal hemoglobin.

[b] IP, intraperitoneal. All data are from at least three mice and presented as means ± SEM. (*: $P < 0.05$, **: $P < 0.01$)

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3.7 In vitro γ-globin gene induction capability studies of class 2 compounds.

Class 2 compounds were synthesized base on the pharmacophore of TN1 and compound II, in cooperation with Dr. Weir-Torn Jiaang in NHRI. Total of 93 compounds including **TN1** were tested in a primary human erythroid cell culture system (Figure 8). Using this assay, CC_{50}, maximal fold of γ-globin gene induction with no cytotoxicity and the therapeutic index (The ratio of CC_{50} to EC is used to evaluate the benefits of these compounds in comparison to HU) of the tested compounds were evaluated. As shown in Table 5, the maximal γ-globin gene induction level of **TN1**, as previous identified HbF inducer [30], is only 1.4-fold. Moreover, the therapeutic index of **TN1** is not available. It cannot induce γ-globin gene expression up to 1.9-fold (the fold of the γ-globin gene induction by HU at its CC_{50}.). In a total of 93 compounds, six compounds show better therapeutic index than HU (Table 5). Among them, **AS-28** is an analog of **TN1** which has the highest γ-globin gene induction fold (3.8-fold) with no cytotoxicity. Therefore, compound **AS-28** was selected for further biological activity, pharmacokinetic, and *in vivo* efficacy studies.

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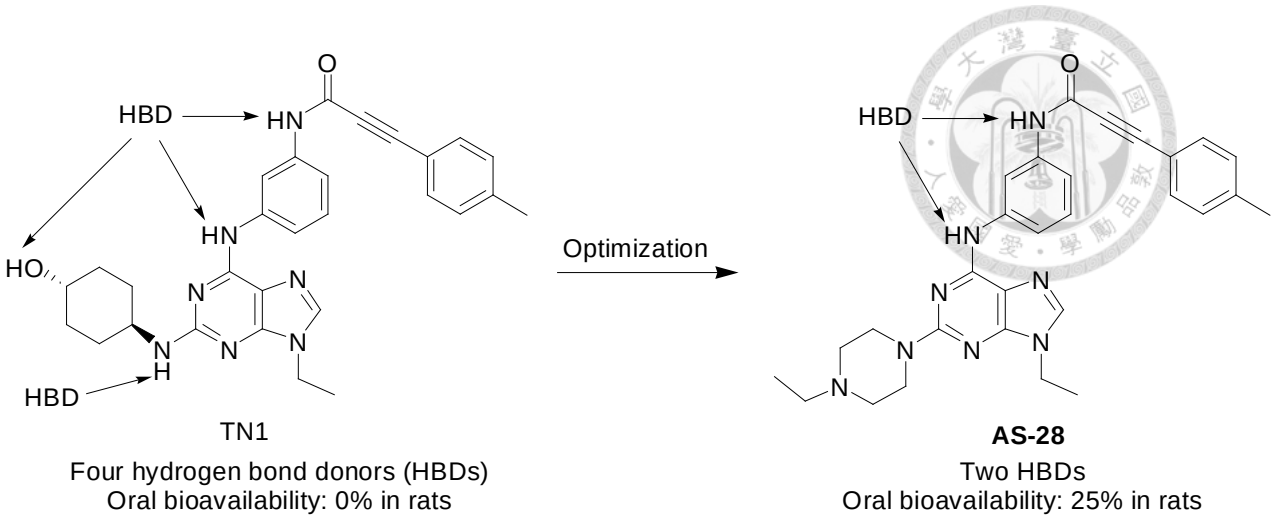

Figure 8. Identification of orally available TN1 analog AS-28 as an inducer of fetal hemoglobin

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Table 5. Therapeutic index of class 2 compounds

Compound	CC_{50}(μM)	EC(μM)	CC_{50} / EC	Max γ-globin Induction Fold with No Cytotoxicity	γ-globin Induction Fold at CC_{50}
II	21.8	2.3	9.7	NA	3.0
TN1	0.4	NA	NA	1.4	1.4
AS-28	2.7	0.48	5.7	**3.8**	2.7
AS-29	3.9	0.9	4.3	3.4	2.3
AS-61	1.5	0.2	8.1	2.5	4.2
AS-78	7.3	2.7	2.7	NA	NA
AS-85	4.6	0.7	6.8	1.8	2.0
AS-93	0.6	0.3	2.3	2.3	NA
Butyric Acid	221.6	NA	NA	NA	1.3
Hydroxyurea	146.5	146.5	1.0	NA	1.9

CC, cytotoxic concentration; EC, effected concentration; NA, not available.

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*3.8 Dose-dependent γ-globin gene induction by **AS-28***

I evaluated the efficacy of γ-globin gene induction by **AS-28** treatment using a primary human erythroid cell culture system. After 2 weeks of *in vitro* erythroid cell differentiation, cells were treated with **AS-28** for 3 days and then harvested for reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis, cell viability assay, and western blot analysis. Fold change of γ-globin mRNA expression in primary human erythroid cell cultures was significantly increased by **AS-28** treatment in a dose-dependent manner (working concentrations ranging from 0.06 μM to 2 μM, Figure 9a). The maximal induction (4.8-fold) of the γ-globin gene by **AS-28** was observed at the concentration of 2.0 μM. Higher concentrations (4.0 μM) of **AS-28** treatment resulted in cell cytotoxicity, which reduced γ-globin induction. Importantly, primary human erythroid cells treated with 1.0 μM **AS-28** activated γ-globin mRNA expression by 3.8-fold without inducing significant cytotoxicity. I further evaluated the protein level of γ-globin. I found the γ-globin protein levels in **AS-28**-treated cells were higher than those in the mock control (Figures 9b and 9c). In contrast, the specificity of **AS-28** for inducing the β-globin and γ-globin genes was verified. I found that after treat with 0.5 μM **AS-28**, it can specifically induce γ-globin gene expression but not that of β-globin (Figure 9d). However, HU induced similar levels of β-globin and γ-globin. Specificity is important for treating patients with SCD. The increase in the expression of

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the mutant β-globin gene is not helpful for this disease. The RNA and protein results show that **AS-28** can induce γ-globin gene expression without cytotoxicity and demonstrate the specificity to induce higher expression of the γ-globin gene than that of the β-globin gene. These results indicate the dosing range and safety of **AS-28** could be better than that of HU.

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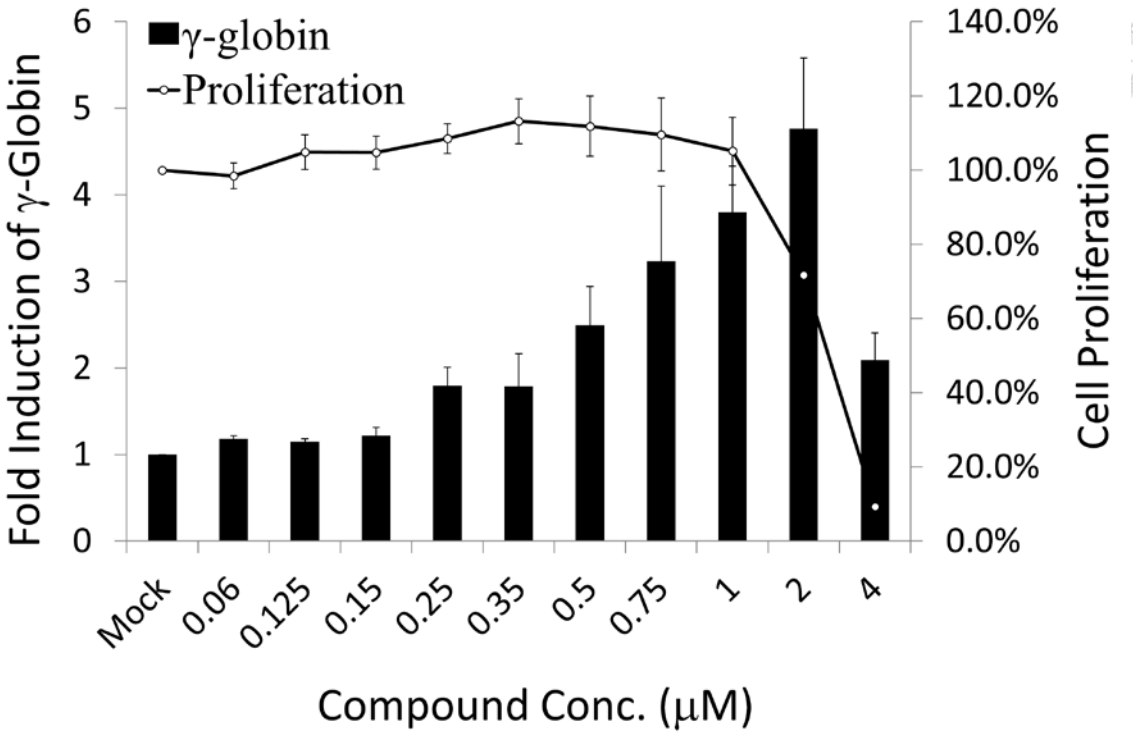

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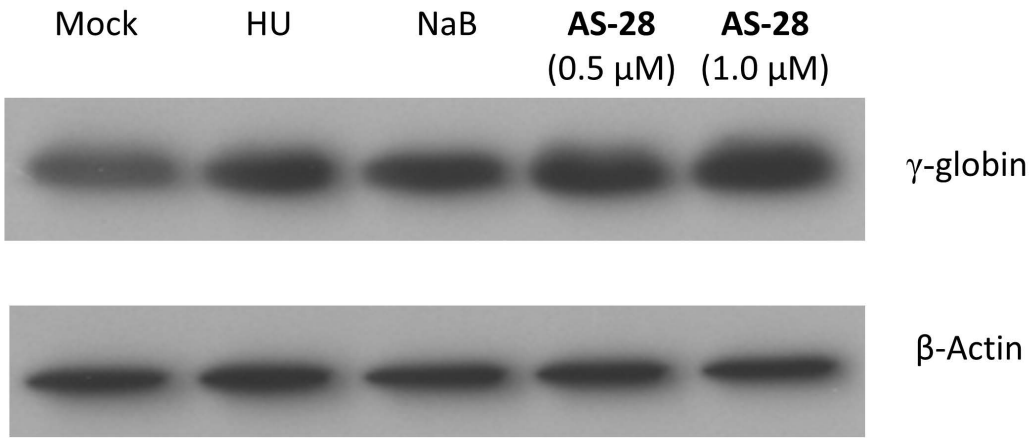

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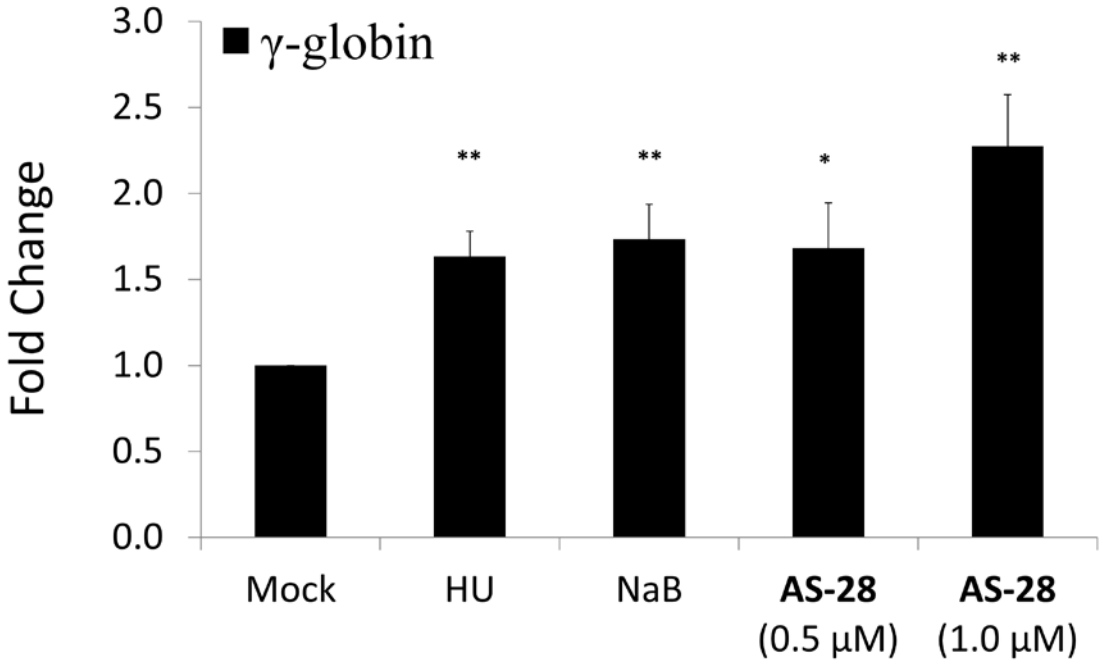

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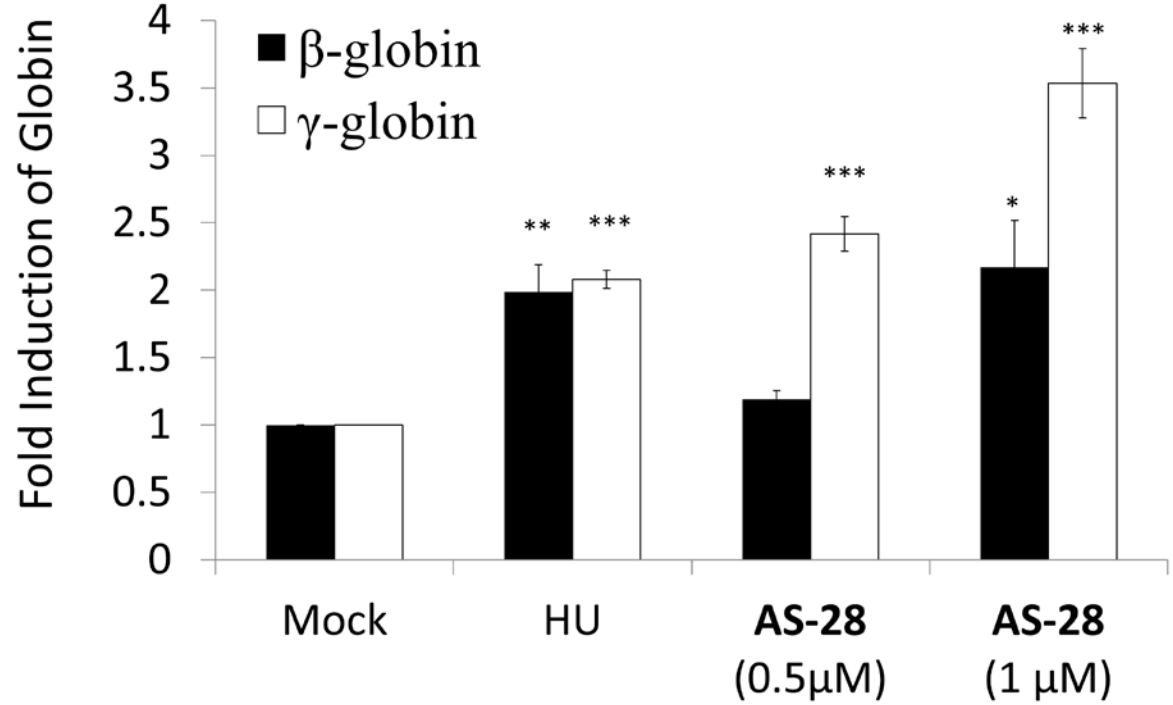

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Figure 9. Dose-dependent γ-globin gene induction and proliferation rate by AS-28.

(a) Primary human erythroid cells were treated with different dosages of **AS-28** for 3 days. The induction of γ-globin mRNAs and proliferation rate were detected by RT-qPCR and AlamarBlue assay, respectively. (b) Primary human erythroid cells were treated with HU (147 μM), NaB (222 μM), and **AS-28** (0.5 and 1.0 μM) for 3 days. After 3 days of treatment the cells were harvested for protein extraction. Protein extracts (50 μg) from compound-treated cells and mock control cells were loaded for western blot. Antibody against γ-globin was used to detect the expression change after compound treatment. β-Actin was used as an internal control. (c) Quantification of western blot results was normalized with β-actin and compared to that of the mock control (d) Fold induction of β-globin and γ-globin mRNAs was detected by RT-qPCR. All data are from at least three independent experiments and presented as means ± SEM (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

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*3.9. Compound **AS-28** activates γ-globin gene expression in HU non-response primary human erythroid cells*

A previous study has demonstrated that the *in vitro* differentiated human erythroid cells can represent the response or non-response effect of HU on patients [39]. My previous studies already found that the normal blood obtained from the Taipei blood bank can also separate into HU response or non-response groups [28, 29]. As there are currently limited therapeutic options for patients with SCD who are poorly- or non-response to HU, I next examined the HbF-inducing capability of **AS-28** in primary human erythroid cells that are non-response to HU treatment. As shown in Figure 10, **AS-28** treatment (0.5 μM) could elevate γ-globin mRNA expression at least 2-fold in HU non-response cells with only minor cytotoxicity. This outcome demonstrates that **AS-28** could be developed as a next-generation therapeutic agent for the treatment of a broad spectrum of patients with hemoglobinopathies, possibly including patients with HU low/non-responsive SCD.

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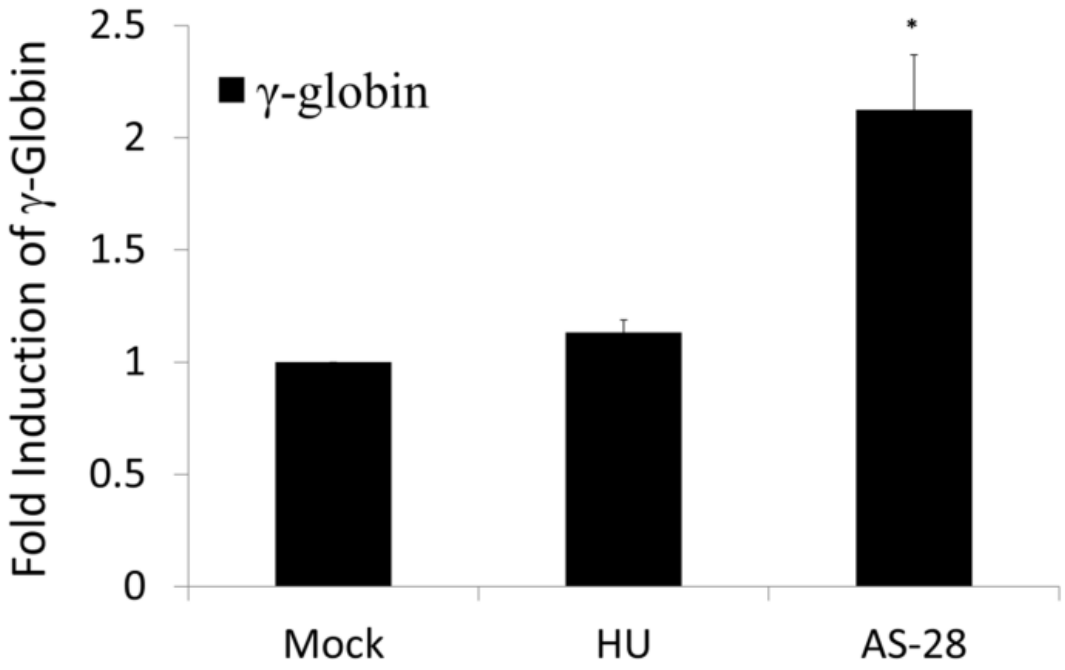

Figure 10. **Compound AS-28 induces γ-globin gene expression in HU non-response primary erythroid cells.** The primary human erythroid cells were treated with **AS-28 (**0.5 μM**)** and HU **(147** μM**)** for 3 days. The induction of γ-globin mRNAs was detected by RT-qPCR. The induction folds of γ-globin gene in HU-treated cells which were similar to Mock control defined as HU non-response cells. All data are from at least three independent experiments and presented as means ± SEM (*: $P < 0.05$).

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*3.10. Compound **AS-28** is not an HDACi*

Several HDACi have been suggested to be HbF-inducing reagents, such as NaB, apicidin, and trichostatin A. It is believed that the hyper-acetylation of histone by HDACi treatments would contribute to the activation of γ-globin gene expression. To reveal whether the epigenetic modification of histone H4 was manipulated by **AS-28** treatment, primary human erythroid cells were treated with HU, NaB, or **AS-28** for 3 days, and the acetylation status of cellular histone H4 was analyzed. Our data showed that the global acetylation status of histone H4 (total acH4) was significantly enhanced in NaB-treated primary erythroid cells. However, different from NaB, the amount of global acetylated histone H4 was not dramatically altered in **AS-28**-treated cells. This indicated that **AS-28** was likely not a histone deacetylase inhibitor (Figure 11).

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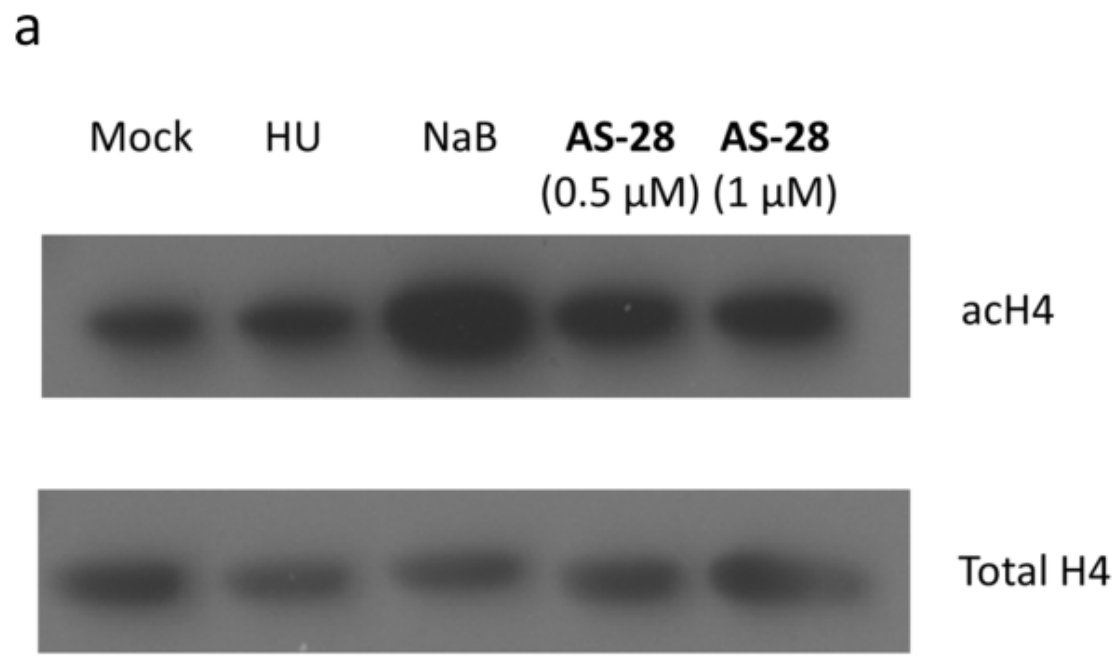

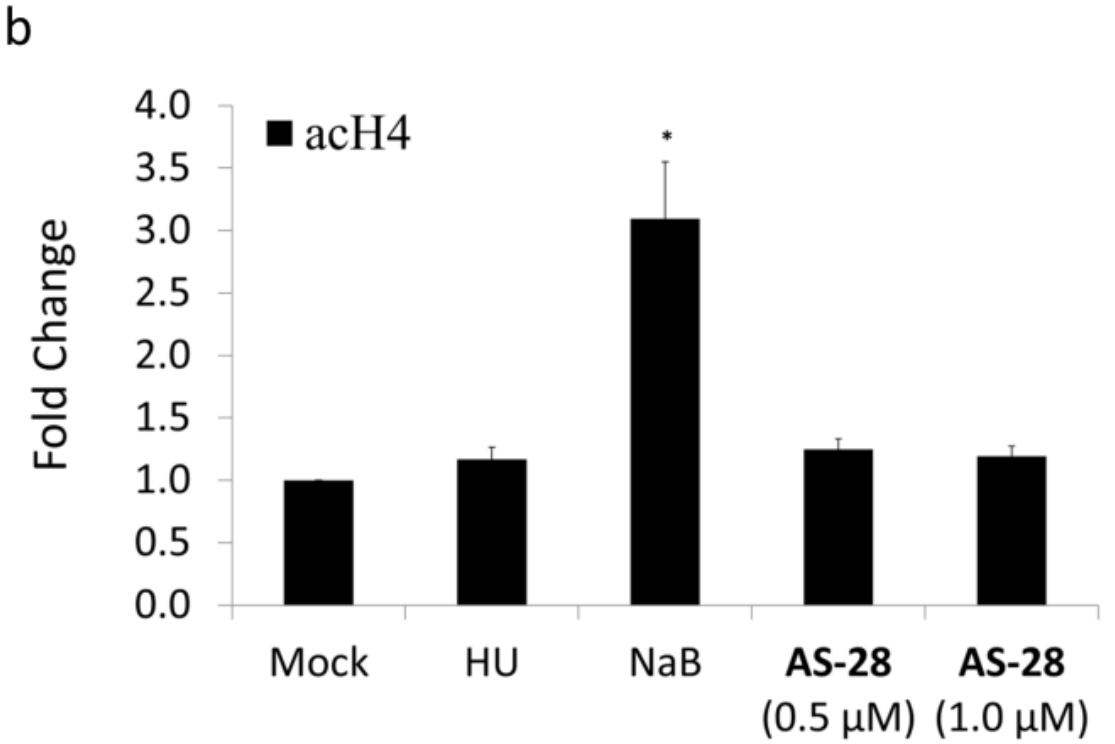

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Figure 11. Compound AS-28 is not a histone deacetylase inhibitor. (a) Primary human erythroid cells were treated with HU (147 μM), NaB (222 μM), and **AS-28** (0.5 and 1.0 μM) for 3 days. After 3 days of treatment, cells were harvested for histone extraction. Histone extracts (50 μg) from compound-treated cells and mock control cells were loaded for western blot. Antibody against total acetylated histone H4 (acH4) was used to detect the acetylation status change after compound treatment. Total histone H4 (Total H4) was used as an internal control. (b) Quantification of western blot results was normalized with total H4 and compared to that of the mock control. All data are from at least three independent experiments and presented as means ± SEM (*: $P < 0.05$).

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*3.11. Molecular mechanisms for γ-globin gene induction by **AS-28***

To identify the molecular mechanisms of **AS-28**, I performed RNA-seq analysis to reveal the gene expression pattern between mock and **AS-28**-treated cells. Previous studies have demonstrated that the mechanism of the globin switch during development was very complicated. Many transcription factors assemble either activation or inhibition complexes to manipulate this process. From the RNA-seq results, I found that the expression levels of several transcription factors (*GATA1*, *NFE2L2*, and *c-MYB*) were changed and the results were validated by RT-qPCR (Figure 12a). *GATA1* has been reported as a general activator of β-type globin genes [40]. *NFE2L2* has been proved to directly bind to the γ-globin promoter to activate γ-globin gene expression [41, 42]. In contrast, previous studies suggest that *c-MYB* may act as a repressor of γ-globin gene expression [43, 44]. According to the RT-qPCR data, **AS-28** can induce *GATA1* (1.6-fold and 2.3-fold) and *NFE2L2* (1.7-fold and 2.3-fold) at 0.5 and 1.0 μM, respectively. In addition, **AS-28** also inhibits *c-MYB* (0.7-fold and 0.6-fold) (Figure. 12a). In summary, **AS-28** manipulates γ-globin expression through these three regulators. However, the detailed mechanisms need further study. Other than these regulators, I found **AS-28** increased the expression level of *CPOX* (1.8-fold and 2.2-fold, Figure 12a) which is involved in the heme-biogenesis pathway [45]. Compound **AS-28** may contribute to heme-biogenesis during globin synthesis. I further detected the

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protein level of BCL11A after **AS-28** treatment. BCL11A has been reported as a key repressor of the γ-globin gene during the globin switch from fetal to adult age [38, 46]. Knock down of BCL11A in primary cells can dramatically reactivate the expression of γ-globin [38]. As shown in Figures 12b and 12c, treatment with higher concentration of **AS-28** (1.0 μM) obviously downregulated the protein level of BCL11A. NaB has been reported to inhibit BCL11A as a positive control [47].

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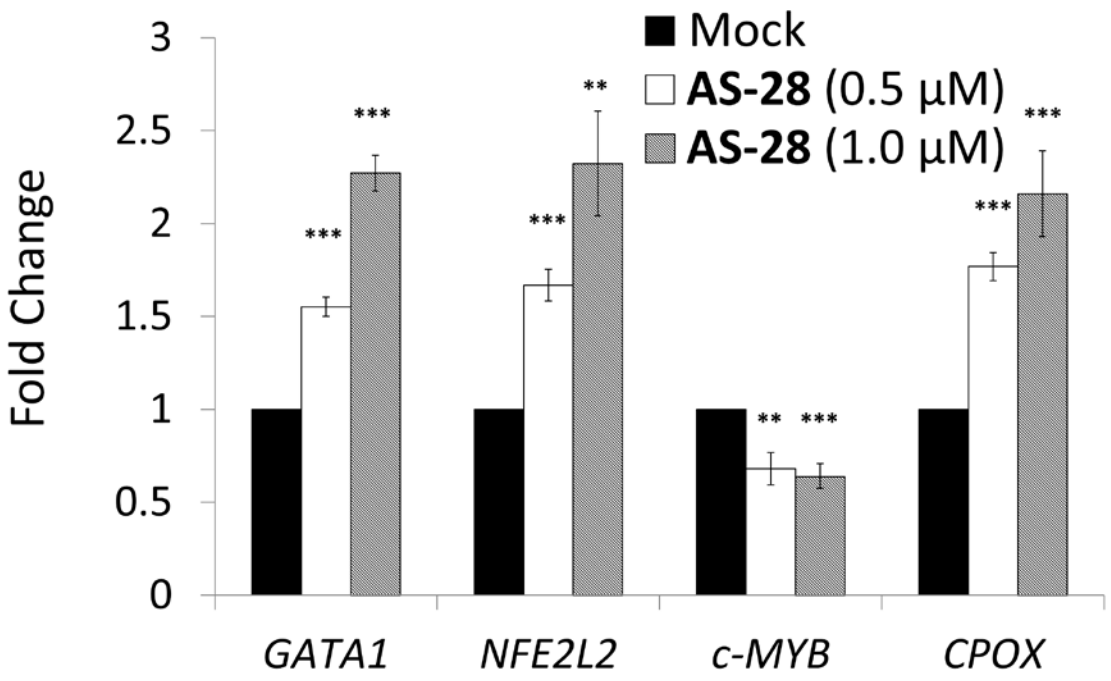

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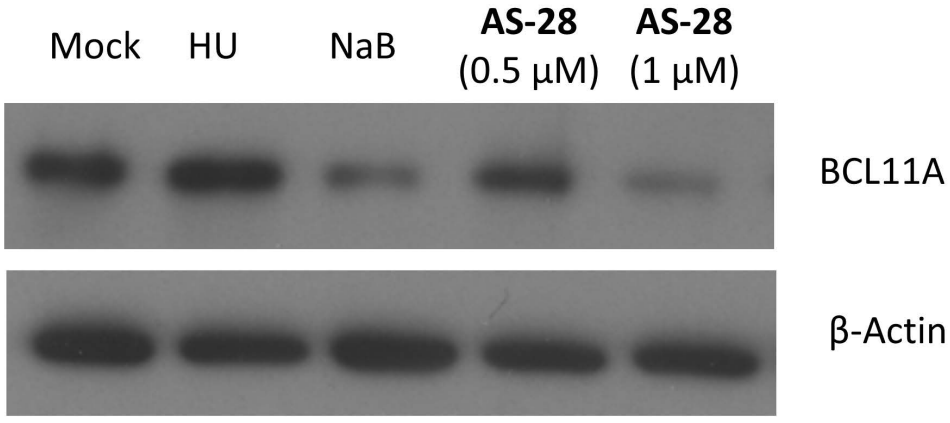

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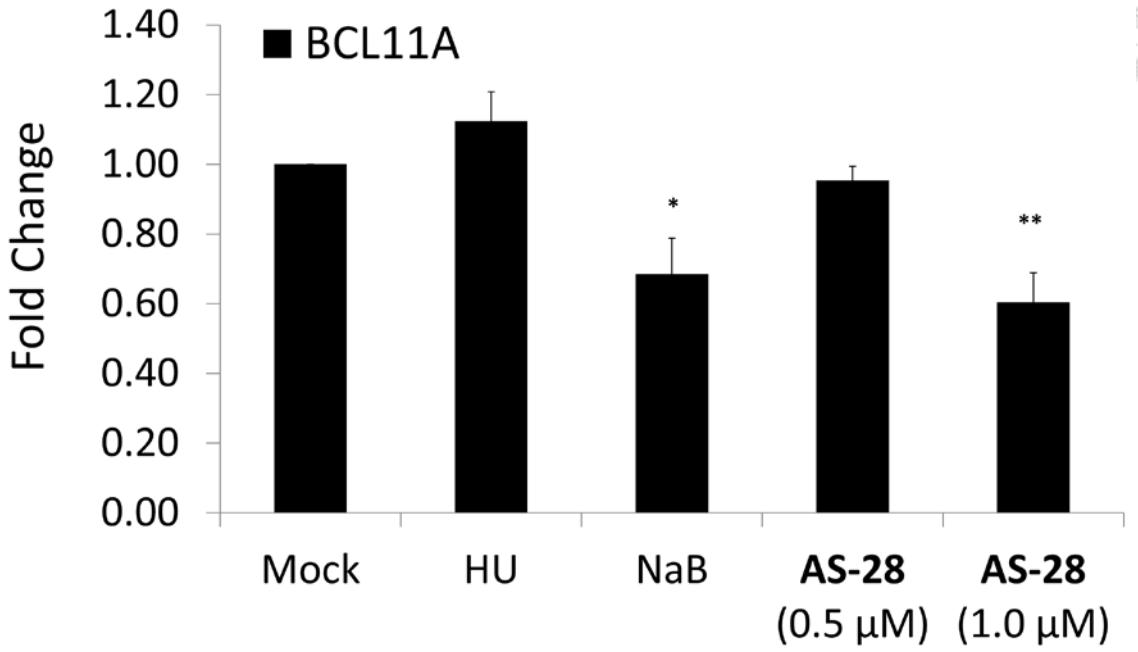

Figure 12. **Compound AS-28 elevated expression levels of the γ-globin-related genes (*GATA1, NFE2L2, MYB, CPOX,* and *BCL11A*).** (a) Primary human erythroid cells were treated with HU (147 μM), NaB (222 μM), and **AS-28** (0.5 and 1 μM) for 3 days. The expression level changes in *GATA1*, *NFE2L2*, *MYB*, and *CPOX* mRNAs were detected by RT-qPCR (b) Protein extracts (50 μg) from compound treated-cells and mock control cells were loaded for western blot. Antibody against BCL11A was used to detect the expression level change after compound treatment. β-Actin was used as an internal control. (c) Quantification of western blot results was normalized with β-actin and compared to that of the mock control. All data are from at least three independent experiments and presented as means ± SEM (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

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*3.12. Global effect studies of **AS-28** in primary human erythroid cells*

To reveal the global effect of **AS-28** on human primary erythroid cells, the DEGs from RNA-seq data (fold change ≥ 1.4 and $p < 0.05$) were selected and analyzed by Ingenuity Pathways Analysis (IPA). As show in Table 6, the top 5 canonical pathways affected by **AS-28** treatment of the primary human erythroid cells does not include the cell toxicity-related pathways, ex. cell cycle arrest, DNA repair, DNA damage, etc. This result suggests that **AS-28** is feasible for further drug development.

Table 6. Top 5 canonical pathways affected by AS-28 treatment in primary human erythroid cells.

Name	**p-value**	**Overlap**
Colanic Acid Building Block Biosynthesis	6.07E-05	21.4% (3/14)
GDP-mannose Biosynthesis	4.66E-04	33.3% (2/6)
UDP-N-acetyl-D-galactosamine Biosynthesis II	1.68E-03	18.2% (2/11)
Sertoli Cell-Sertoli Cell Junction Signaling	3.47E-03	2.8% (5/179)
Phagosome Maturation	7.77E-03	2.9% (4/138)

DEGs were selected from RNA-seq data (fold change >1.4, p <0.05) and analyzed by IPA.

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*3.13. Pharmacokinetic studies of compounds **TN1** and **AS-28***

Table 7 shows the pharmacokinetic properties of **TN1** and **AS-28** evaluated in male Sprague–Dawley rats. Compound **TN1** exhibited long half-life (10.2 h), high plasma clearance (CL = 60.9 mL/(min·kg)), and moderate volumes of distribution (V_{ss} = 3.8 L/kg) after intravenous (IV, 2.0 mg/kg) administration; however, oral bioavailability (F%) of **TN1** was not observed. On the contrary, **AS-28** showed moderate half-life (7.2 h), moderate plasma clearance (CL = 8.2 mL/(min·kg)), and low volumes of distribution (V_{ss} = 0.3 L/kg) after intravenous (5.0 mg/kg) administration. A single 20 mg/kg oral dose of **AS-28** was administered as a solution containing 22% hydroxypropyl-β-cyclodextrin in water and absorbed with a moderate half-life in rats ($t_{1/2}$ = 3.8 h). The C_{max} (2730 ng/mL) and AUC (8996 ng·h/mL) were high and the oral bioavailability was 25%. After demonstrating favorable pharmacokinetic properties and potent induction of γ-globin gene expression in primary human erythroid cells, **AS-28** was appropriate for continued *in vivo* investigation to determine the antianemic activity in an SCD mouse model.

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Table 7. **Pharmacokinetic profiles of compounds TN1 and AS-28 in rats.**

Compound	IV				PO			
	$T_{1/2}$ (h)	CL (mL/(min·kg))	V_{ss} (L/kg)	$AUC_{(0\text{-inf})}$ (ng·h/mL)	$T_{1/2}$ (h)	C_{max} (ng/mL)	$AUC_{(0\text{-inf})}$ (ng·h/mL)	F (%)
TN1[a]	10.2	60.9	3.8	610	ND	ND	ND	ND
AS-28[b]	7.2	8.2	0.3	8862	3.8	2730	8996	25

[a] Dose: IV 2.0 mg/kg; PO 10 mg/kg. [b] Dose: IV 5.0 mg/kg; PO 20 mg/kg. PO, per oral. ND, not detected.

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*3.14. In vivo efficacy studies of **AS-28** in SCD mice*

To further evaluate the therapeutic potential of **AS-28** for treating hemoglobinopathies, a mouse model of SCD was used to examine the effects of **AS-28** on relieving the symptoms of anemia in this disease model. Three doses of **AS-28** (50, 75 and 100 mg/kg) were administered orally to 6- to 8-week-old SCD mice twice daily for 5 days a week. After 4 weeks of treatment, blood samples from the treated SCD mice were collected for several biochemical analyses (Table 8). Complete blood cell assessment showed that **AS-28** treatment slightly increased red blood cell numbers and hemoglobin levels in peripheral blood samples of SCD mice compared to those of mock-treated control mice. Reticulocyte, white blood cell, and neutrophil values were equivalent between **AS-28**-treated and mock-treated SCD mice. HPLC analysis revealed that **AS-28** significantly increased the percentage of HbF in SCD mice, which is consistent with our finding of an elevated percentage of F-cells in these mice, as detected by flow cytometry. A diagnostic indicator of SCD is the characteristic sickle-shaped morphology of red blood cells under hypoxia. A previous study demonstrated that over-expression of γ-globin can reduce the percentage of sickle-shaped red blood cells in the peripheral blood of patients with SCD [48]. I assessed the percentages of sickle-shaped cells in smears of SCD mouse blood under hypoxia and found that this parameter was significantly decreased upon treatment with

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AS-28 (Figure 13b, 13c and 13d), indicating that **AS-28** treatment might decrease the risk of vaso-occlusive crisis in SCD mice. In order to know whether the higher dose of AS-28 will have better effect or not, I increase the dosage to 150 mg/kg and change the dosing period to once daily for 28 days. In this experiment, I also pretest the *in vivo* efficacy of second candidate compound, AS-61. As shown in Table 9, higher dose of AS-28 (150 mg/kg) can increase higher red blood cell numbers and hemoglobin levels. However, the HbF percentage, F-cell percentage and sickle cell percentage did not show obvious different between high and low dose AS-28-treated groups (Table 9 and Figure 14). Surprisingly, AS-61 showed the similar results to AS-28. Moreover, AS-61 can decrease the other diagnostic indicator of SCD, RET percentage, in SCD mice (Table 9). Taken together, the *in vivo* experiments in SCD mice suggest that **AS-28** might be a potential drug candidate for β-thalassemia and SCD.

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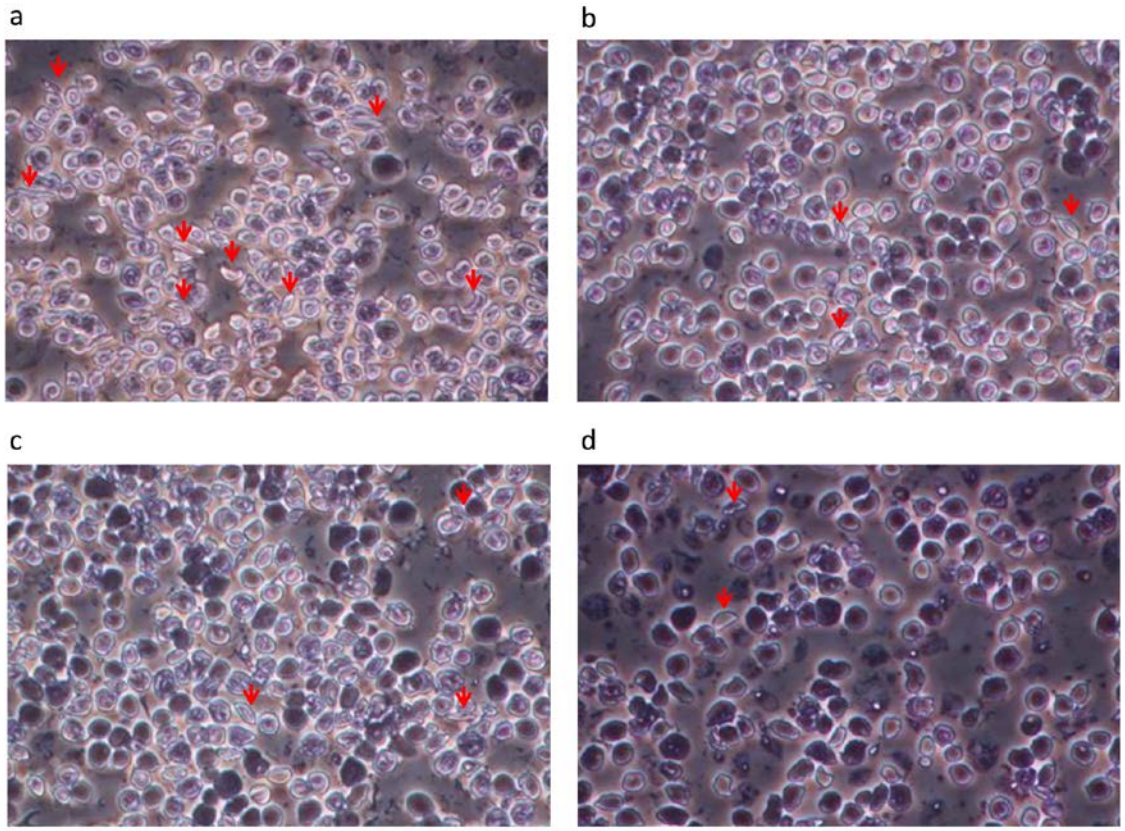

Figure 13. **Blood smear of compound (AS-28)-treated mice.** Blood was collected by submandibular blood collection and treated with hypoxia (3% O_2) for 30 min. Blood (2.0 μL) from each sample was used to prepare the blood smear on the slide. The slides were air-dried and then stained with Liu's stain. (a) Mock (b) **AS-28** (PO 50 mg/kg) (c) **AS-28** (PO 75 mg/kg) (d) **AS-28** (PO 100 mg/kg). Each group contained at least three mice. Arrows indicate the sickle-shape cells.

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Table 8. **Antianemic effect of AS-28 against SCD mice.**

Blood test	Mock	**AS-28** PO 50 mg/kg	**AS-28** PO 75 mg/kg	**AS-28** PO 100 mg/kg
RBC (M/uL**)**	7.23±0.75	7.78±0.58	7.89±0.43	8.05±0.62
HGB (g/dL)	7.46±0.7	8.15±0.68	8.23±0.41	8.05±0.7
RET (%)	57.34±3.67	58.98±3.18	57.51±3.55	54.66±2.3
WBC (K/uL)	34.15±5.85	35.85±3.37	35.75±3.43	44.12±6.63
NEUT (K/uL)	13.39±4.65	15.05±2.89	12.15±2.73	15.64±3.47
HbF (%)	0.20±0.02	0.28±0.02*	0.23±0.03	0.30±0.03*
F-cell (%)	1.63±0.11	2.15±0.14****	2.1±0.26	2.42±0.12***
Sickle cell (%)	16.78±1.9	10.0±1.4****	8.1±0.9**	8.8±0.8**

RBC, red blood cells; HGB, hemoglobin; RET, reticulocytes; WBC, white blood cells; NEUT, neutrophils; F-cell %, percentage of HbF positive cells s; HbF, fetal hemoglobin. PO, per oral. All data are from at least three mice and presented as means ± SEM.

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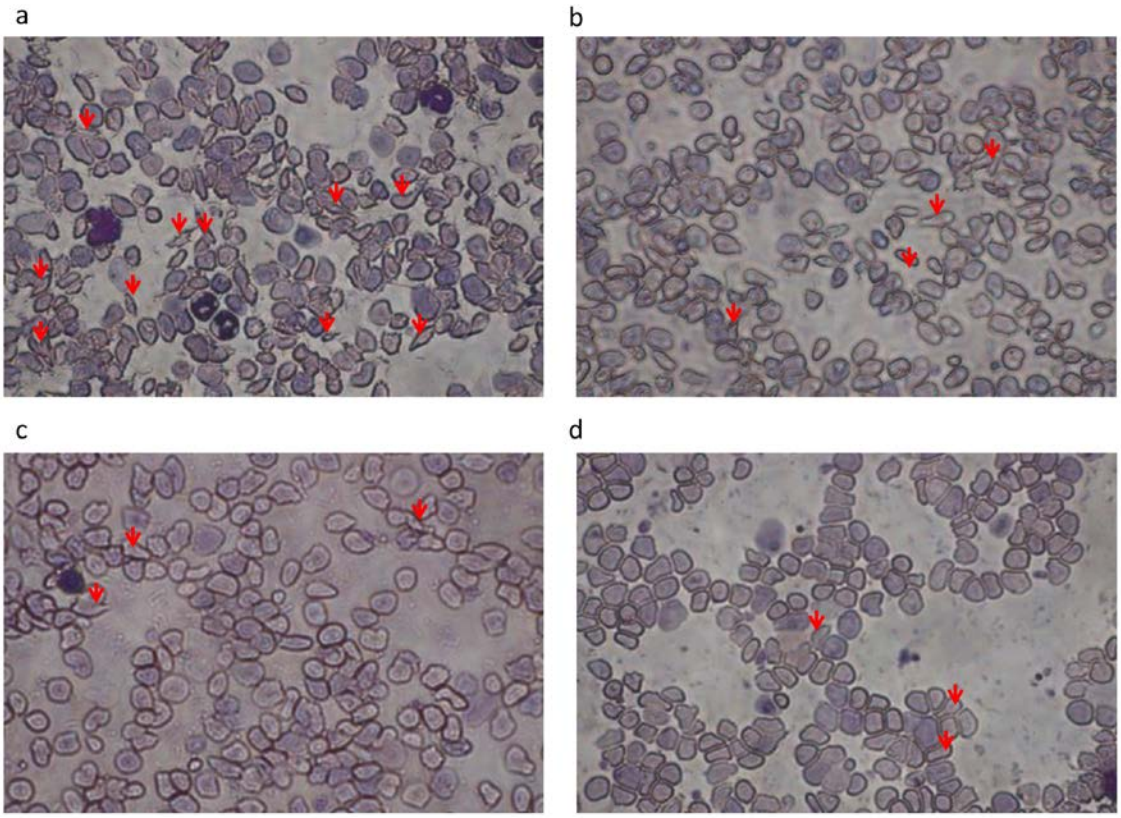

Figure 14. Blood smear of compound (AS-28 and AS-61)-treated mice. Blood was collected by submandibular blood collection and treated with hypoxia (3% O_2) for 30 min. Blood (2.0 μL) from each sample was used to prepare the blood smear on the slide. The slides were air-dried and then stained with Liu's stain. (a) Mock (b) **AS-28** (PO 100 mg/kg) (c) **AS-28** (PO 150 mg/kg) (d) **AS-61** (PO 50 mg/kg). Each group contained at least three mice. Arrows indicate the sickle-shape cells.

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Table 9. **Antianemic effect of AS-28 and AS-61 against SCD mice.**

Blood test	Mock	**AS-28** PO 100 mg/kg	**AS-28** PO 150 mg/kg	**AS-61** PO 50 mg/kg
RBC (M/uL**)**	6.36±0.52	7.42±0.54	8.22±0.5	7.91±0.13*
HGB (g/dL)	6.63±0.72	8.18±0.6	9.03±0.61	8.13±0.19
RET (%)	61.7±2.14	62.43±4.15	57.97±1.09	44.66±3.89*
WBC (K/uL)	41.38±7.54	62.99±22.56	52.99±18.5	50.41±17.71
NEUT (K/uL)	12.75±2.53	30.46±13.66	23.83±12.57	25.18±14.06
HbF (%)	1.08±0.28	1.49±0.07	1.84±0.12	1.80±0.15
F-cell (%)	0.22±0.02	0.31±0.04	0.30±0.08	0.29±0.03
Sickle cell (%)	19.3±1.19	13.7±0.61**	14.1±0.32*	12.3±0.7**

RBC, red blood cells; HGB, hemoglobin; RET, reticulocytes; WBC, white blood cells; NEUT, neutrophils; F-cell %, percentage of HbF positive cells s; HbF, fetal hemoglobin. PO, per oral.

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4. Discussion

4.1 Screening system of γ-globin inducing compounds and six candidate compounds.

Several fluorescence-based (or luciferase-based) reporter systems have been used to assess the activation of γ-globin promoter which affected by HbF-inducing compounds. Several HbF-inducing compounds were identified from these reporter systems [30, 49, 50]. However, the therapeutic effects of these compounds in HU non-response cells are presently unknown. In our previous study, we had setup a simple dual-fluorescence reporter system in MEL cells (Figure. 1) to carry out high-throughput screening for novel γ-globin inducing compounds. Among the 10,000 compounds, six candidate compounds (Compound I-VI) had been identified. By using the primary human erythroid cells, the γ-globin inducing abilities of six compounds had been confirmed (Figure 4). Most interestingly, compound II (the pharmacophore of six compounds) is able to activate γ-globin gene expression in HU non-response primary human erythroid cells. However, the asymmetric structure (difficulty of isoform isolation), physical properties (water-insoluble) and poor bioavailability (Table 1) limit the further drug development of these compounds. In order to modify the structure to improve the disadvantage of these six compounds, class 1 and class 2 compounds were designed and synthesized in cooperation with Dr. Tsann-Long Su in IBMS, Academia Sinica and Dr. Weir-Torn Jiaang in NHRI respectively.

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4.2 ***SS-2394*** *a candidate compound from class 1 compound.*

Base on the pharmacophore of compound II, class 1 compounds were designed as symmetric compounds to prevent the difficulty of isoform isolation (Figure 5a). The hydrochloride salt dramatically improves the solubility of several class 1 compounds. However, the therapeutic index shows that none of class 1 compounds are higher than compound II. Consider to the γ-globin gene induction fold at CC_{50} is higher than compound II and the therapeutic index is highest in class 1 compound (Table 2), **SS-2394** was chose for further investigation.

The beneficial effects of HU treatment, including reductions of acute chest syndrome and the painful crisis, seem to contribute by the increase of HbF levels in patients [19-21]. However, many SCD patients are poorly or none responsive to HU treatment [25]. Interestingly, **SS-2394** shows the same capability as compound II to induce the γ-globin gene in HU non-response primary human erythroid cells.

The molecular basis studies for the mode of actions of **SS-2394** on γ-globin gene activation revealed that **SS-2394** decrease either mRNA or protein level of the γ-globin repressor BCL11A (Figure 5d-f). In recent studies, down-regulation of BCL11A was proposed as a promising therapeutic strategy for induction of HbF to treat SCD and β-thalassemia patients. Either CRISPR/Cas9 editing or lentiviral transduction to down-regulate BCL11A in CD34+ HSC and transplanted to patients can mitigate the

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disease symptoms [51, 52]. It suggests that down-regulation of BCL11A play an important role in **SS-2394** mediated γ-globin gene reactivation.

However, global effect analysis by IPA shows that **SS-2394** treatment affects several cell cycle and DNA repair pathways (Table 3). It suggests that **SS-2394** may have toxic effects in primary erythroid cells. The toxic effects of **SS-2394** may come from its original reported functions. The structure of **SS-2394** had been reported as DNA intercalator and topoisomerase II inhibitor [53, 54].

In vivo studies of **SS-2394** show opposite effects in β-YAC transgenic mice and SCD mice (Figure 6, Table 4). **SS-2394** can induce γ-globin mRNA in β-YAC transgenic mice but not increase the percentage of F-cell and percentage of HbF in SCD mice. In previous reports of HU in β-YAC transgenic mice and SCD mice show the similar results, HU can induce percentage of F-cell in β-YAC transgenic mice but fail to increase percentage of HbF in SCD mice [22, 49]. The opposite results may be due to the difference of the γ-globin regulation elements in mice. β-YAC transgenic mice bears whole human β-locus region but SCD mice only knock-in the γ-globin and β-globin genes into mice adult globin (β_{maj} and β_{min}) region.

In summary, the global effect analysis of **SS-2394** revealed that **SS-2394** has toxic effects in primary human erythroid cells. But **SS-2394** can increase γ-globin level in β-YAC transgenic mice and decrease the sickle cell percentage in SCD mice. This

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suggested that **SS-2394** still has potential for further studies.

4.3 ***AS-28*** *a potent candidate compound from class 2 compound*

Starting with the well-known HbF inducer **TN1**'s core scaffold and with the rational design approach, I successfully identified a preclinical candidate **AS-28,** which significantly increases the expression of the γ-globin gene and dramatically improves oral bioavailability compared with that of compound **TN1** (Table 5, 7).

Importantly, the *in vitro* assay demonstrated that **AS-28** can efficiently induce γ-globin expression at a non-toxic concentration in primary erythroid cells and HU non-response primary erythroid cells (Figure 9, 10). The potential to induce γ-globin expression in HU non-response primary erythroid cells is similar to the class 1 candidate **SS-2394**. Both of them could be the candidate compounds for treating HU non-response patients.

Molecular basis studies showed that **AS-28** does not alter the acetylation status of histone H4 in comparison to that by NaB. Furthermore, RNA-seq analysis demonstrated that **AS-28** reduces the expression level of *c-MYB,* which is a repressor of γ-globin and increases the expression levels of *GATA1* and *NFE2L2,* which are believed to be involved in γ-globin activation. However, only high dose of AS-28 (1.0 μM) can significantly decrease the protein level of BCL11A. These results reflect the mechanism of fold induction in γ-globin with **AS-28** treatment. Compound **AS-28** at 1.0 μM

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induced a higher level of γ-globin than that by **AS-28** at 0.5 μM (Figure 9, 12).

In addition, the global effect analysis of AS-28 did not show AS-28 involved in toxic pathways. The IPA result shows that AS-28 affect the synthesis of membrane component of erythroid cells (Table 6). Nevertheless, further studies are required to determine the mechanisms in detail for the effects of **AS-28** on regulating γ-globin expression and synthesis of membrane component of erythroid cells.

Furthermore, efficacy studies *in vivo* demonstrated that **AS-28**, which is safe, orally active, and well-tolerated, provides dose-dependent relief from anemia symptoms in SCD mice. Surprisingly, pretest result of AS-61 in SCD mice shows the promising feedback. Taken together, the excellent *in vitro* and *in vivo* antianemia activities of **AS-28** suggest that AS-28 could be a next-generation drug candidate that is worthy of clinical evaluation for the treatment of β-thalassemia and SCD, especially for patients who are resistant to HU. The studies of AS-28 had been published on European Journal of Medicinal Chemistry. [55]

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6. Appendices

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Pharmacological Induction of Human Fetal Globin Gene in Hydroxyurea-Resistant Primary Adult Erythroid Cells

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Pharmacological induction of the fetal γ globin gene and the consequent formation of HbF (α_2/γ_2) in adult erythroid cells are one feasible therapeutic strategy for sickle cell disease (SCD) and severe β-thalassemias. Hydroxyurea (HU) is the current drug of choice for SCD, but serious side effects limit its clinical use. Moreover, 30 to 50% of patients are irresponsive to HU treatment. We have used high-throughput screening to identify benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one and its derivatives (compounds I to VI) as potent γ globin inducers. Of the compounds, I to V exert superior γ globin induction and have better therapeutic potential than HU, likely because of their activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway and modulation of expression levels and/or chromosome binding of γ globin gene regulators, including BCL11A, and chromatin structure over the γ globin promoter. Unlike sodium butyrate (NaB), the global levels of acetylated histones H3 and H4 are not changed by compound II treatment. Remarkably, compound II induces the γ globin gene in HU-resistant primary human adult erythroid cells, the p38 signaling pathway of which appears to be irresponsive to HU and NaB as well as compound II. This study provides a new framework for the development of new and superior compounds for treating SCD and severe β-thalassemias.

The expression of α-like globin chains (ζ [embryonic] and α [fetal/adult]) and β-like globin chains (ε [embryonic], γ [fetal], and β [adult]) is under temporal control during human development. Genetic defects such as deletions or mutations inside the globin gene loci result in abnormal expression of the hemoglobins and consequent hemoglobinopathies (1). β-Thalassemia and sickle cell disease (SCD) are the two most common hemoglobinopathies (2). Patients with severe β-thalassemia are defective in generation of the adult β globin chain and become profoundly anemic when the hemoglobin switch is completed from HbF (α_2/γ_2) to HbA (α_2/β_2). With the generation of functional HbA impaired, patients with β-thalassemia major require regular blood transfusion to replenish their HbA for survival. On the other hand, patients with SCD produce mutated sickle hemoglobin HbS (α_2/β^s_2), which polymerizes under low-oxygen conditions and distorts the red blood cells into the characteristic sickle shape (3).

Pharmacologic induction of HbF expression has been suggested to be a useful strategy for modulation of anemia and related symptoms in severely hemoglobinopathic patients (4–6). The elevation of fetal γ globin chain synthesis balances the excess of α globin chain by formation of HbF, thus modulating severe anemia in β-thalassemia major patients and directly inhibiting the polymerization of HbS in SCD patients (7, 8). So far, several chemotherapeutic agents, such as the histone deacetylase inhibitors (HDACi), e.g., trichostatin A (TSA), apicidin, sodium butyrate (NaB), etc.; short-chain fatty acid (SCFA) derivatives, e.g., 2,2-dimethylbutyrate (HQK-1001), NaB, etc.; DNA methyltransferase inhibitors, e.g., 5′-azacytidine; and ribonucleotide reductase inhibitors, e.g., hydroxyurea (HU), have been shown to be able to stimulate HbF production (9–15). Among them, HU is the only U.S. FDA-approved therapeutic drug for the treatment of SCD despite its side effects, including leucopenia, thrombocytopenia, myelosuppression, and potential reproductive toxicity (16, 17). However, a notable proportion of hemoglobinopathic patients have poor outcomes or no response to HU treatment (18, 19). A search for new γ globin-inducing agents for treating the hemoglobinopathies, including β-thalassemia major and SCD, is thus warranted.

In this study, we used a simple dual-fluorescence reporter to screen for potential HbF-inducing compounds and investigated the possible mechanisms underlying the reactivation of the γ globin gene. Benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one as well as several of its derivatives was identified to potently increase the fetal γ globin gene expression in the adult erythroid cells. These heterocyclic compounds modulate the expression of several γ globin regulators as well as the activation of p38 mitogen-activated protein kinase (MAPK) signaling, both of which are known to contribute to γ globin gene activation in adult erythroid cells (20–24). Significantly, this series of compounds, as exemplified by compound II, could induce γ globin gene expression in HU-resistant as well as HU-responsive human adult erythroid cells. Therefore, these compounds can be considered highly promising for development into a new generation of therapeutic drugs for SCD and β-thalassemia major.

MATERIALS AND METHODS

Ethics statement. This study was conducted with the approval of the Human Subject Research Ethics Committee/IRB, Academia Sinica

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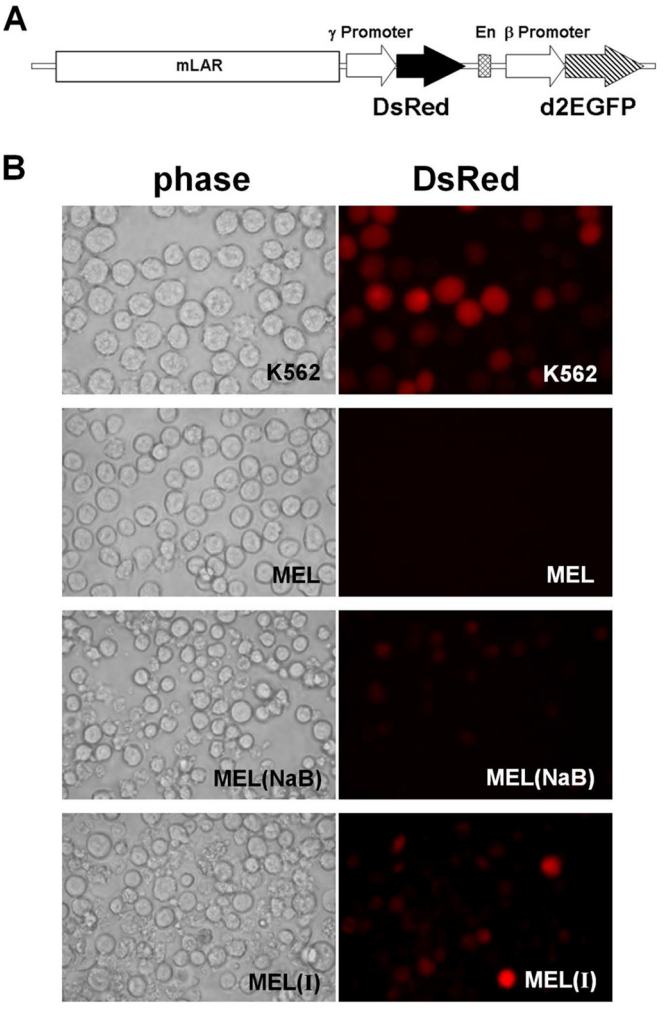

FIG 1 High-throughput screening to find compounds capable of inducing the fetal globin gene. (A) Physical map of the dual-fluorescence reporter. (B) Phase-contrast and DsRed florescence images of cells stably transfected with the dual-fluorescence reporter plasmid. Top row, K562 cells; second row, MEL cells; third row, MEL cells treated with NaB; bottom row, MEL cells treated with compound I for 3 days.

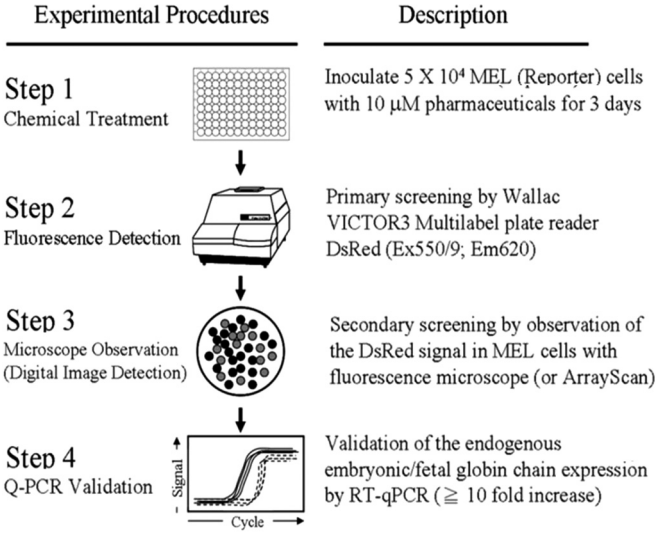

FIG 2 Experimental procedures for the high-throughput screening. In step 1, ~10,000 heterocyclic compounds were tested for their ability to activate the γ globin promoter in MEL cells cultured on 96-well plates. In steps 2 and 3, elevation of DsRed fluorescence was detected by a fluorescence reader and further confirmed by a digital image detector. In step 4, the activation of the endogenous mouse embryonic and/or fetal globin genes as induced by the compound(s) was verified by RT-qPCR analysis. HU and NaB were used as reference compounds.

(AS-IRB01-10039). Written informed consent was obtained from all blood donors.

Chemical compounds. A chemical library used for the screening in this study was from the National Health Research Institutes (NHRI) at Taiwan and contains 10,000 heterocyclic compounds with drug-like structures. Compound II was purchased from ChemDiv.

Reporter constructs. The reporter plasmid pmLAR-Gp-DsRed-Bp-d2EGFP was constructed by multiple steps of a subcloning process. In brief, an 8,003-bp mini-LAR (mLAR) excised from pLAR-β (25) and a 1,622-bp β globin promoter (Bp) generated by PCR amplification were cloned into pd2EGFP-1 to generate pmLAR-Bp-d2EGFP. A 1,377-bp γ globin promoter (Gp) was subcloned into pDsRed-Monomer-C1 to generate pGp-DsRed-C1. The Gp-DsRed fragment was then excised from the pGp-DsRed-C1 plasmid and reinserted between the mLAR and Bp-d2EGFP of pmLAR-Bp-d2EGFP. A 759-bp enhancer-containing fragment 3′ to the ^{A}γ globin gene (26) was generated by PCR and inserted downstream of the DsRed gene.

Cell culture. Mouse erythroleukemic MEL cells (27) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 20% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin. Human erythroleukemic K562 cells (28) were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 1% penicillin, and 1% streptomycin. All cells were incubated in a 37°C chamber under a 5% CO_2 humidified atmosphere. For establishment of the stable cell lines, MEL cells or K562 cells were transfected with the reporter pmLAR-Gp-DsRed-Bp-d2EGFP and selected with 700 μg/ml of neomycin. Preparation of the primary human adult erythroid cells followed the standard procedure of the two-phase liquid culture method (20). The peripheral blood from healthy blood donors was provided by the Taipei Blood Center. In brief, the culture was initiated by maintaining the purified peripheral blood mononuclear cells in the first-phase culture medium, the serum-free expansion medium (SFEM) with 1× cc100 cytokine mix (100 ng/ml Flt-3 ligand, 100 ng/ml stem cell factor [SCF], 20 ng/ml interleukin-3 [IL-3], 20 ng/ml IL-6) (StemSpan), for 7 days. In the phase II culture, cells were kept in the differentiation medium (SFEM with 20 ng/ml SCF, 1 U/ml erythropoietin [EPO], 5 ng/ml IL-3, 2 μM dexamethasone) for another 7 days

FIG 3 Induction of γ globin gene expression by six heterocyclic compounds with a common core structure. (A) Six heterocyclic compounds with identical core structures (benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one) were examined with respect to their effects on the induction of γ globin gene expression and survival rates of the primary human adult erythroid cells. The relative fold induction of the γ globin gene (bars) by these compounds was analyzed by RT-qPCR (the mock control was set as 1). The relative rates of cell survival (the curves) were derived from use of the alamarBlue reagent (with the mock control set as 100%). (B) Flow cytometry and morphological analyses of compound II-treated cells. (Left panel) Differentiated primary human adult erythroid cells were treated with compound II for 3 days and then labeled with FITC-conjugated anti-CD71 and PE-conjugated anti-CD235a monoclonal antibodies. The percentage of cells in the Q3 region ($CD71^{low}$ $CD235a^{high}$) representing late orthochromatophilic erythroblasts and reticulocytes was determined by flow cytometry. (Right panel) The morphology of compound II-treated cells was analyzed by a Liu staining assay.

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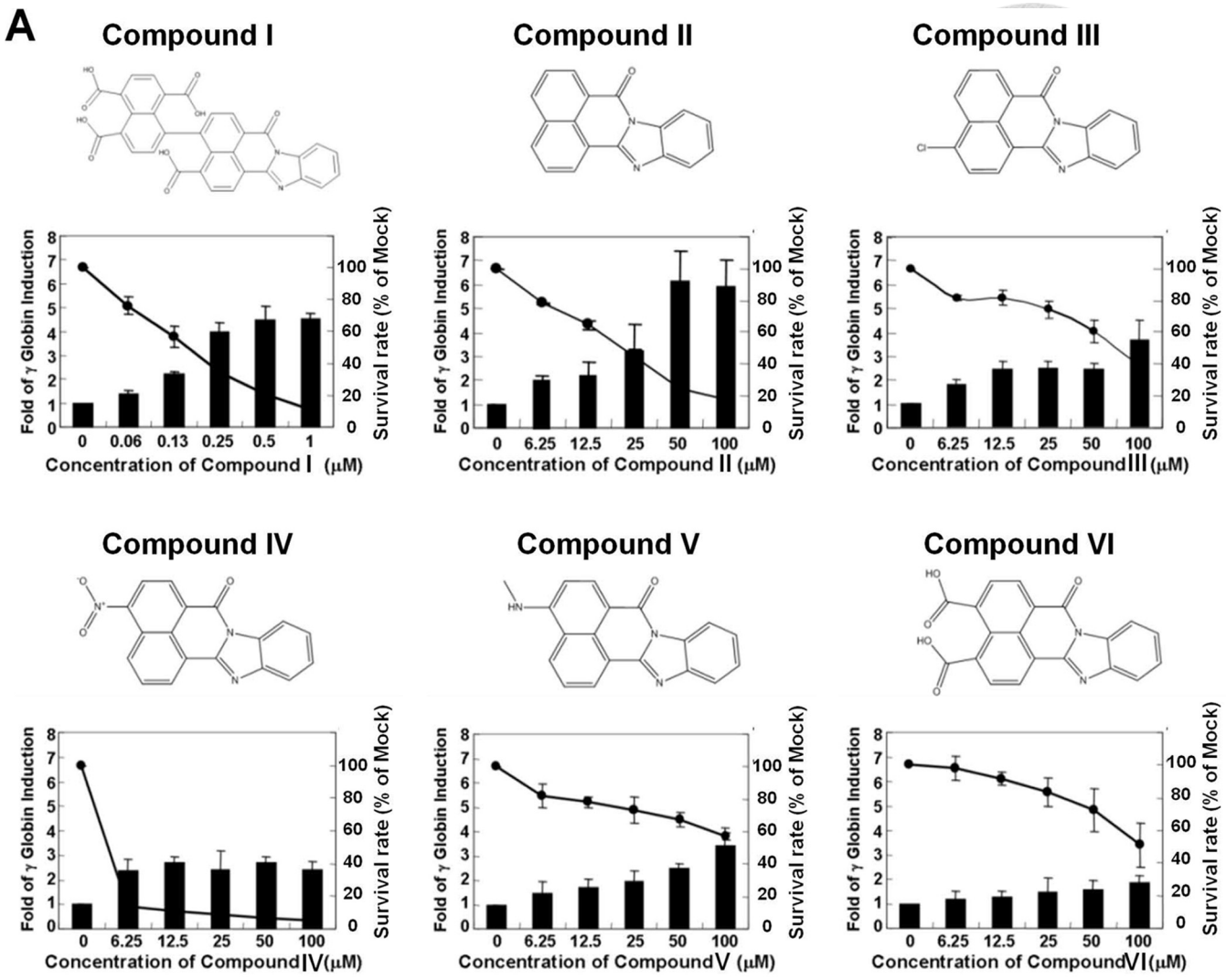

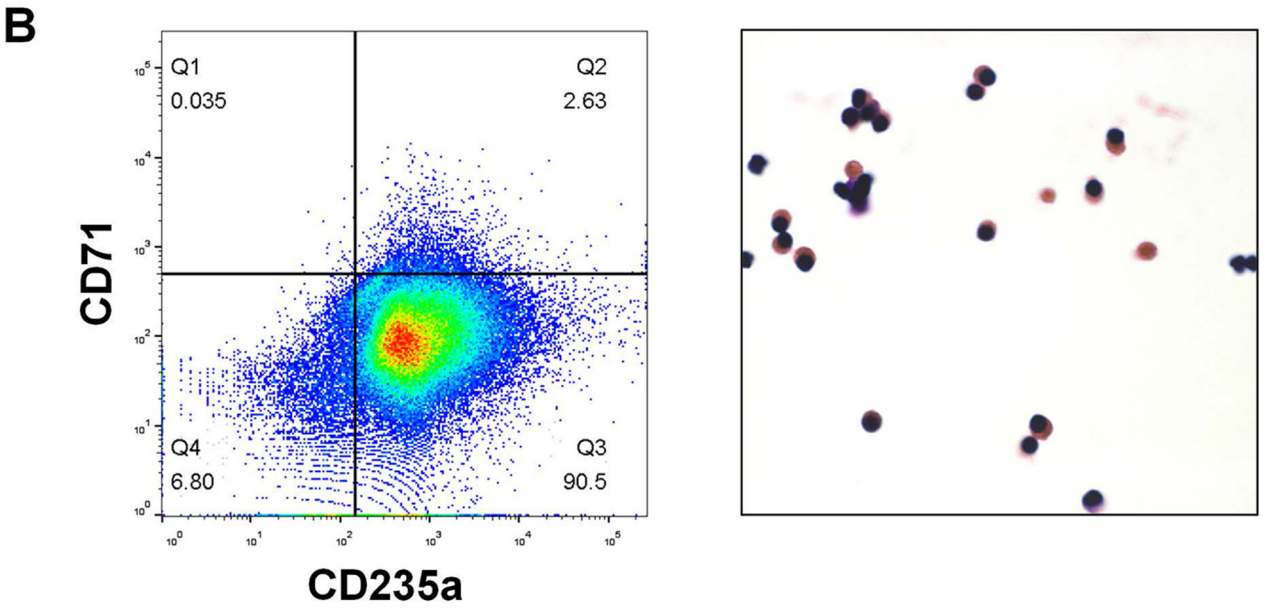

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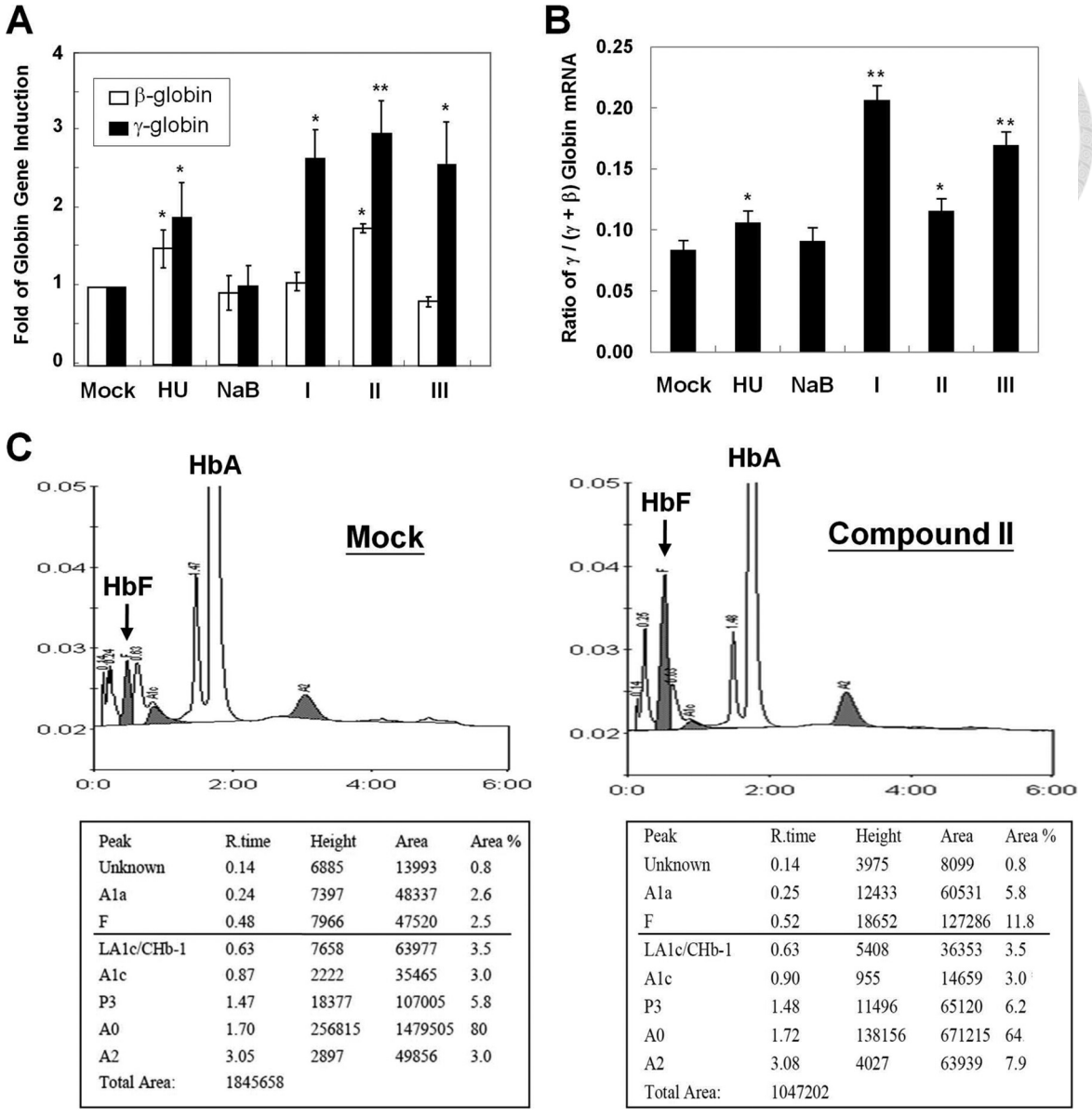

Peak	R.time	Height	Area	Area %
Unknown	0.14	6885	13993	0.8
A1a	0.24	7397	48337	2.6
F	0.48	7966	47520	2.5
LA1c/CHb-1	0.63	7658	63977	3.5
A1c	0.87	2222	35465	3.0
P3	1.47	18377	107005	5.8
A0	1.70	256815	1479505	80
A2	3.05	2897	49856	3.0
Total Area:	1845658			

Peak	R.time	Height	Area	Area %
Unknown	0.14	3975	8099	0.8
A1a	0.25	12433	60531	5.8
F	0.52	18652	127286	11.8
LA1c/CHb-1	0.63	5408	36353	3.5
A1c	0.90	955	14659	3.0
P3	1.48	11496	65120	6.2
A0	1.72	138156	671215	64
A2	3.08	4027	63939	7.9
Total Area:	1047202			

FIG 4 Comparison of the γ and β globin levels in the primary human adult erythroid cells treated with different γ globin-inducing agents. (A) The cells were treated with different compounds at the their CC_{50}s, HU (147 μM), NaB (222 μM), and heterocyclic compounds I (0.2 μM), II (22 μM), and III (75 μM), respectively, for 3 days. The total mRNAs were extracted, and the relative fold inductions of the β and γ globin mRNAs were determined by relative RT-qPCR analysis. (B) For calculation of the ratio of γ/(γ + β) globin mRNAs in the compound-treated primary human adult erythroid cells, the amounts of γ and β globin mRNAs were estimated by using an absolute RT-qPCR method. All data are presented as means ± SEM ($n = 3$) (*, $P < 0.05$; **, $P < 0.01$, by t test). (C) Hemolysates were prepared from mock control primary erythroid cells (left panel) or compound II-treated cells (right panel) on day 10 of differentiation, and the presence of HbF and HbA was revealed by hemoglobin HPLC. Hemoglobins and proteins in hemolysates were separated by HPLC, and the proportions of each peak are shown. The position of the HbF peak is labeled with an arrow in the chromatogram. The table below the chromatogram shows the raw data for the retention time, height, area, and area percentage of each peak: F (hemoglobin F/HbF), LA1c/CHb-1 (labile A1c), A1c (glycated hemoglobin), A0 (hemoglobin A0/HbA0), and A2 (hemoglobin A2/HbA2).

at a density of 0.1×10^6 to 1×10^6 cells/ml. The differentiated primary erythroid cells were then treated with the indicated chemical compound(s) in the differentiation medium for 3 days and harvested for further analysis. The morphology of the compound-treated primary erythroid cells was analyzed by the Liu staining assay, a rapid and simple histologic stain modified from the Romanowsky stain (29). The culture medium and compound(s) were refreshed every 2 or 3 days. To pretest the responsiveness of a primary erythroid culture to HU treatment, one-half of each peripheral blood sample was differentiated into primary erythroid cells and then treated with HU (147 μM) for 3 days. Total mRNA was then harvested for analysis of the γ globin gene induction by reverse transcription-quantitative PCR (RT-qPCR). For high-performance liquid chromatography (HPLC) analysis, the cells were treated with 10 μM compound II at the beginning of phase II culture and continuously cultured for 10 days. The hemolysates were prepared from cells using osmotic lysis in water with three freeze-thaw cycles, and debris was removed by centrifugation. HPLC was carried out in the clinical laboratories of National Taiwan University Hospital using clinically calibrated standards for the human hemoglobins.

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Robotic screening of chemical compounds. MEL cells carrying the dual-fluorescence reporter were treated with 10 μM individual compounds for 3 days and primarily scanned for DsRed intensity using a Wallac Victor3 1420 multilabel counter (excitation, 550/9; emission, 620). The coefficient of variation (% CV) value of 8% and a z-factor value of 0.6 confirmed the quality of the screening. NaB and 0.1% dimethyl sulfoxide (DMSO) were used as positive and negative controls, respectively. The secondary screening was performed using a digital image detector, the Cellomics Arrayscan 3.0 system. The expression levels of the endogenous globin genes in the positive hits from the high-throughput screening were subsequently analyzed by RT-qPCR analysis. HU and NaB were used as reference compounds.

RT-qPCR analysis. Total RNAs were isolated from the cells by using the RNAspin minikit (GE Healthcare). cDNA synthesis was carried out using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR was then performed using SYBR green PCR master mix (Applied Biosystems) and the ABI 7500 real-time system. All data were analyzed after normalization to the expression level of the human β-actin gene. All primer sequences used for RT-qPCR are as follows: ε globin forward primer, 5′-GCAAGAAGGTGCTGACTTC-3′; ε globin reverse primer, 5′-CCTTGCCAAAGTGAGTAGC-3′; γ globin forward primer, 5′-CTTCCTTGGGAGATGCCAT-3′; γ globin reverse primer, 5′-GAATTCTTTGCCGAAATGGAT-3′; δ globin forward primer, 5′-CTTTAGTGATGGCCTGGCT-3′; δ globin reverse primer, 5′-GATAGGCAGCCTGCATTTG-3′; β globin forward primer, 5′-CTTTAGTGATGGCCTGGCT-3′; β globin reverse primer, 5′-CACTGGTGGGGTGAATTCT-3′; BCL11A forward primer, 5′-TGGTATCCCTTCAGGACTAGGT-3′; BCL11A reverse primer, 5′-TCCAAGTGATGTCTCGGTGGT-3′; c-Myb forward primer, 5′-ACAGAAATACGGTCCGAAACG-3′; c-Myb reverse primer, 5′-CCAATTCTCCCCTTTAAGTGC-3′; NFE4 forward primer, 5′-CCAGAAAGCAGGCCACAGCA-3′; NFE4 reverse primer, 5′-AGGGCCCCAGTAGGTGAGAT-3′; CP2 forward primer, 5′-ACAAACTTCTCAGGGGCAGA-3′; CP2 reverse primer, 5′-GTTAACCTTGGACGCACCAT-3′; β-actin forward primer, 5′-CCTGAACCCCAAGGCCAACC-3′; β-actin reverse primer, 5′-CAGGGATAGCACAGCCTGGA-3′.

Flow cytometry analysis. Primary human adult erythroid cells were labeled with fluorescein isothiocyanate (FITC)-conjugated anti-CD71 (BD Biosciences; catalog no. 555536) and phycoerythrin (PE)-conjugated anti-CD235a (BD Biosciences; catalog no. 555570) in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) at 25°C for 20 min in the dark. Then, the cells were washed twice in PBS with 0.1% BSA, followed by resuspension with 1 ml PBS with 0.1% BSA and filtration by cell strainer. The cells were analyzed on a BD LSRII-18P flow cytometer within 1 h after staining. Unstained cells were used as a negative control.

Western blot analysis and antibodies. For whole-cell extract preparation, the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing a complete protease inhibitor cocktail (Roche) for 30 min on ice. For isolation of the total cellular histones, the primary human adult erythroid cells were lysed in histone extraction buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 1.5 mM phenylmethylsulfonyl fluoride [PMSF], and 0.2 N HCl) at 4°C overnight and then neutralized by adding 0.375 M Tris-HCl (pH 8.0). Twenty micrograms of the whole-cell extract or histone extract was separated on SDS-PAGE gels, transferred onto nitrocellulose paper, and incubated with different primary antibodies. Anti-histone H3 (catalog no. 05-499), anti-acetylated histone H3 (catalog no. 16-599), anti-histone H4 (catalog no. 05-858), anti-acetylated histone H4 (catalog no. 06-866), anti-dimethyl-histone H3 (Lys4) (catalog no. 07-030), anti-dimethyl-histone H3 (Lys9) (catalog no. 17-681), and anti-trimethyl-histone H3 (Lys9) (catalog no. 07-442) were from Millipore. The mouse anti-BCL11A monoclonal antibody (ab-19487) was from Abcam. Rabbit anti-p38 MAPK antibody (catalog no. 9212s) and rabbit anti-phospho-p38 MAPK (T180/Y182) antibody (catalog no. 92118) were from Cell Signaling. Anti-β globin chain antibody (sc-21757) and anti-γ globin chain antibody (sc-21756) were purchased from Santa Cruz.

ChIP assay. The chromatin immunoprecipitation (ChIP) assay followed the general protocol (30). The cell extracts from cross-linked primary human adult erythroid cells were immunoprecipitated with the addition of purified rabbit IgG and antibodies against BCL11A, H3K4me2, H3K9me2 and H3K9me3, respectively. The immunocomplexes were subjected to a reversal of cross-links at 67°C for 4 h and treated with proteinase K at 45°C overnight. The DNA samples recovered were analyzed by quantitative PCR (qPCR) in the ABI 7500 real-time system. All experiments were performed in triplicate. The primer sequences used in the ChIP assay are as follows: HS3 forward primer,5′-ATAGACCATGAGTAGAGGGCAGAC-3′; HS3 reverse primer, 5′-TGATCCTGAAAACATAGGAGTCAA-3′; ^{A}γ promoter forward primer, 5′-TTACTGCGCTGAAACTGTGG-3′; ^{A}γ promoter reverse primer, 5′-CAGTGGTTTCTAAGGAAAAGTGC-3′; ^{A}γ (+3)kb forward primer, 5′-GAACAGAGCAGCACACTT-3′; ^{A}γ (+3)kb reverse primer, 5′-TGAACAATAGTCCATGTCAAATCCT-3′; δ (−1)kb forward primer, 5′-GCAACAGAAGCCCAGCTATT-3′; δ (−1)kb reverse primer, 5′-GTGGCATGGTTTGATTTGTG-3′; β promoter forward primer, 5′-TGGTATGGGGCCAAGAGATA-3′; β promoter reverse primer, 5′-TGCTCCTGGGAGTAGATTGG-3′.

Statistical analysis. All data are from 3 to 7 independent experiments, and they are presented as means ± standard errors of the means (SEM). Statistical significance of the difference between experimental and control groups was determined by the two-tailed Student *t* test.

RESULTS

Setup of a high-throughput screening system to survey embryonic/fetal globin gene expression-inducing chemical compounds. To identify novel compounds capable of inducing the expression of the fetal γ globin gene, we constructed a dual-fluorescence reporter as a screening tool for high-throughput screening (Fig. 1A). Consistent with previous findings (31, 32), the expression of the γ globin promoter-directed DsRed fluorescence was detectable only in the human fetal/embryonic erythroid K562 cells but not in mouse adult erythroid MEL cells (top two rows of panels, Fig. 1B). Moreover, reactivation of the γ globin promoter, as revealed by the induction of DsRed fluorescence, could be achieved by treating MEL cells with the HbF-inducing agent NaB (third row of panels, Fig. 1B). These data suggest that MEL cells carrying the dual-fluorescence reporter could be used to screen for novel HbF-inducing compounds. A total of 10,000 compounds were tested for their ability to induce γ globin promoter-directed DsRed fluorescence in MEL cells by following the high-throughput screening flow chart in Fig. 2. Six heterocyclic compounds, compounds I to VI (Fig. 3A), induced DsRed fluorescence in MEL cells, as exemplified by compound I (bottom row of panels, Fig. 1B). Consistent with the reporter assay, RT-qPCR analysis showed that the levels of the endogenous mouse embryonic/fetal globin genes (βh1 and Ey) were induced by 7-fold and 50-fold, respectively, in compound I-treated MEL cells (data not shown).

Induction of endogenous γ globin gene expression and modulation of globin gene regulators in primary human adult erythroid cells by heterocyclic compounds. We set up primary human erythroid cultures from adult normal donors and confirmed the globin gene expression pattern over time throughout the differentiation process (data not shown). The data are consistent with the profiles reported by Xu et al. (33). The dosage dependence of the heterocyclic compounds I to VI on γ globin gene activation in the primary human erythroid cells and their inhibitory effects on cell survival are shown in Fig. 3A. Interestingly, these heterocyclic compounds shared an identical core structure,

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i.e., that of compound II (benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one). As shown in Fig. 3B, 90% of the compound II-treated primary erythroid cells remained orthochromatic erythroblasts, as validated by flow cytometry and Liu staining analyses. Relative fold inductions of the γ and β globin genes in the primary human adult erythroid cells by compounds I, II, and III at their half-maximal inhibitory concentrations (CC_{50}), as estimated by RT-qPCR analysis, were compared to those by HU and NaB. As seen in Fig. 4A, compounds I, II, and III were more potent γ globin inducers than either HU or NaB. The ratios of γ/(γ + β) globin mRNAs were calculated from the absolute quantification and are shown in Fig. 4B. HU and compound I to III treatment significantly increased the ratio of γ/(γ + β) globin mRNAs in comparison to that of the mock control. Although compound II had the highest γ globin gene induction among the tested compounds, it could only modestly increase the ratio of γ/(γ + β) globin mRNAs due to the nonspecific activation of the β globin mRNA expression. Notably, although NaB treatment failed to increase the γ globin mRNA expression at its CC_{50} (222 μM) (Fig. 4A), it could elevate the level of the γ globin mRNA at a higher concentration, e.g., 500 μM (data not shown). Finally, while cells treated with a combination of 22 μM compound II and 147 μM HU did not show better γ globin gene induction than the cells treated with compound II or HU alone, combination treatment with 22 μM compound II and 500 μM NaB resulted in additive elevation of the γ globin mRNA expression (data not shown).

The induction level of HbF in compound II-treated primary erythroid culture was also analyzed by hemoglobin high-performance liquid chromatography (HPLC). As seen in Fig. 4C, a substantially elevated fraction representing HbF (increasing from 2.5% to 11.8%) was detected, indicating that the induction of γ globin mRNA was accompanied by an increase in HbF level. The proportion of hemoglobin A2 (HbA2 [A2]) was also increased (from 3% to 7.9%), whereas the proportion of the adult hemoglobin (HbA0 [A0]) was significantly decreased from 80% to 64% upon compound II treatment (Fig. 4C).

A number of transcription factors have been identified to serve as either activators or repressors of globin gene transcription, including GATA1 (34), NF-E2 (35), EKLF (36), YY1 (37), TR2/TR4 (38), NF-E4 (39), RREB1 (40), and BCL11A (20). Among these factors, BCL11A has been suggested to be a critical repressor of γ globin gene expression, and its downregulation in primary adult erythroid cells has been suggested to lead to the activation of HbF expression (20). Moreover, inactivation of BCL11A in SCD transgenic mice was shown to correct the pathological defects of SCD through inducing a high level of HbF (41). As shown in Fig. 5A, the mRNA levels of BCL11A and c-Myb were diminished, while that of NF-E4 was upregulated in a dosage-dependent manner upon treatment of the primary erythroid culture with compound I, II, or III. On the other hand, in NaB-treated erythroid cells, there was a decrease in only the BCL11A mRNA level. Furthermore, HU treatment did not cause a change in the mRNA level of BCL11A, c-Myb, or NF-E4 (top 3 panels, Fig. 5A). No significant change in the mRNA level of CP2 (bottom panel of Fig. 5A), TR2, TR4, or GATA-1 (data not shown) could be observed upon treatment by any of the heterocyclic compounds tested.

Western blotting of BCL11A in the different compound-treated erythroid cultures (Fig. 5B) was consistent with the RNA data in Fig. 5A. In particular, the amount of the BCL11A protein was drastically reduced in the primary human adult erythroid cells treated with compounds I to III and, to a lesser extent, NaB (Fig. 5B). In interesting contrast, similar to the RT-qPCR analysis in Fig. 5A, no obvious reduction in the level of the BCL11A protein could be detected in the primary human adult erythroid cells upon HU treatment (Fig. 5B).

Regarding the NaB effect, downregulation of BCL11A by RNA interference (RNAi) (20) or NaB (42) was shown to elevate the level of γ globin mRNA. However, we did not see a significant change of the γ globin mRNA level upon treatment with NaB at its CC_{50} (Fig. 4A), despite downregulation of the level of BCL11A protein (Fig. 5B). This inconsistency could be due to the differences of the assay conditions, e.g., the different amounts of the chemicals used, the different cell types, the different time points and lengths of the NaB treatment, etc. In particular, 600 μM NaB was used to treat K562 cells by Chen et al. (42), while we used 222 μM NaB in the analyses described for Fig. 4A and 5B. Notably though, as mentioned above, the level of γ globin mRNA in the primary erythroid culture could be elevated by a higher concentration, i.e., 500 μM, of NaB (data not shown).

Also, NaB did not change the level of γ globin mRNA at its CC_{50} (222 μM), but it elevated the level of the γ globin chain (compare Fig. 4A and 5B). This observation was consistent with a previous report that NaB could increase the efficiency of γ globin chain translation without change of the γ globin mRNA level (43).

Decreased binding of BCL11A in the human β globin gene locus in compound II-treated primary human adult erythroid cells. The binding profile of the BCL11A factor within the human β-like globin gene locus was studied previously (20, 33). As seen in Fig. 5C, the chromatin immunoprecipitation (ChIP) assay showed that BCL11A indeed bound to 5′ HS3, a region 3 kb downstream of the ^{A}γ globin gene, and a region 1 kb upstream of the δ globin gene of the human β-like globin gene locus in the primary human adult erythroid cells (white bars, Fig. 5C). However, BCL11A binding at these regions was significantly reduced upon compound II treatment (black bars, Fig. 5C). These data suggest that a lower level of BCL11A in the compound II-treated

FIG 5 Changes in the levels of γ globin gene regulators in primary human adult erythroid cells treated with compound I, II, or III in comparison to HU and NaB. (A) Dosage dependence of the effects of HU, NaB, and compounds I to III on the mRNA levels of different γ globin gene regulators. The primary human adult erythroid cells were treated with different dosages of the indicated compounds for 3 days. The relative fold inductions of BCL11A, c-Myb, NF-E4, and CP2 mRNAs were then determined by relative RT-qPCR analysis. The expression levels of these genes in the mock control cells were set as 1. (B) Western blotting of BCL11A, β globin, γ globin, and β-actin in primary human adult erythroid cells. The total proteins were extracted from the cells, and the expression patterns of BCL11A, the β globin chain, and the γ globin chain were determined by Western blotting (upper panels). The expression of β-actin was used as a loading control. The intensities of the Western blot signals of the BCL11A, β globin chain, and γ globin chain of different samples were quantified using densitometry analysis software and compared in the bar graph. (C) ChIP analysis of BCL11A binding on the human β-like globin locus in primary human adult erythroid cells before (Mock) and after (II) treatment with compound II. ChIP of the primary human adult erythroid cells was carried out using the BCL11A antibody. IgG was used as the control. The PCR-assayed regions of the β-like globin locus included HS3, the ^{A}γ globin promoter, a region at 3 kb downstream of Aγ, a region at 1 kb upstream of the δ globin gene, and the β globin promoter. All data are presented as means ± SEM ($n = 3$) (*, $P < 0.05$; **, $P < 0.01$, by *t* test).

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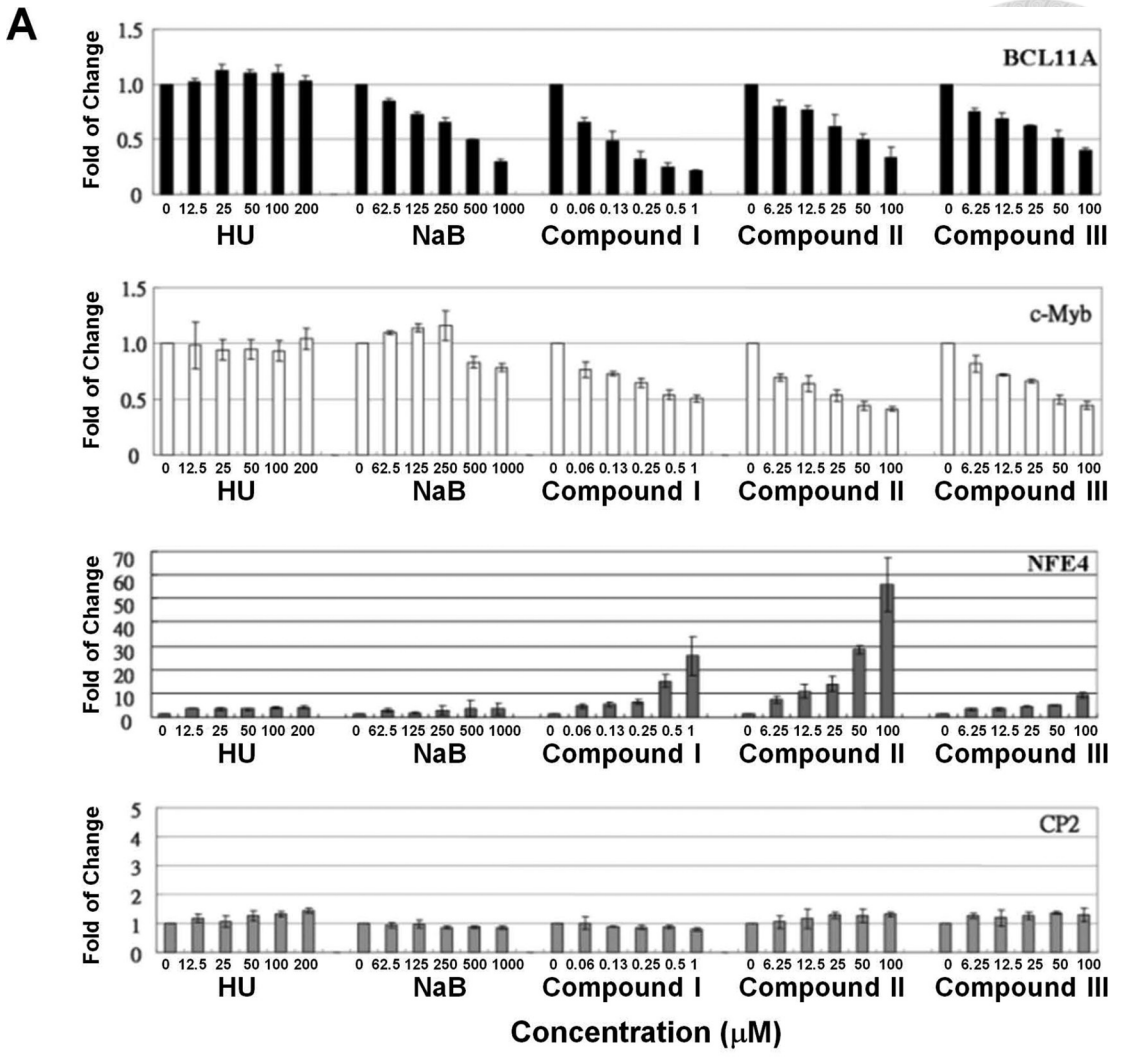

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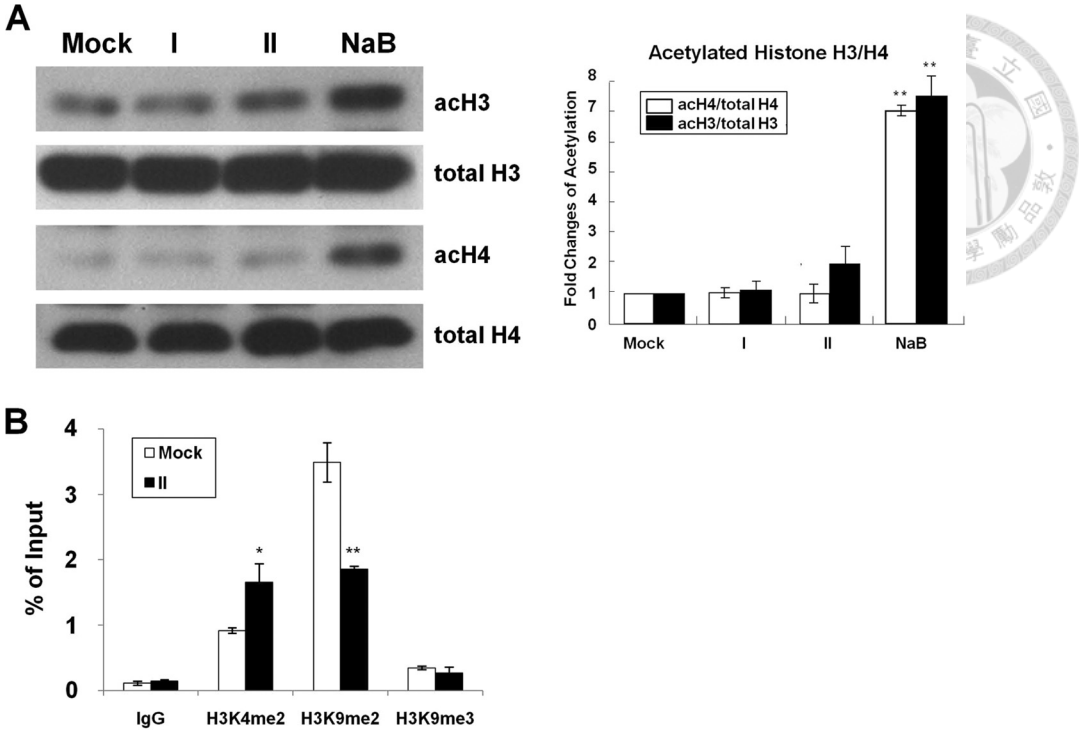

FIG 6 Global acetylation patterns of histones H3 and H4 in primary human adult erythroid cells treated with compound I or II or NaB. (A) Primary human adult erythroid cells were treated with compound I or II or NaB at its CC_{50} for 3 days, and the total cellular histones were prepared by the acid extraction method. The acetylation patterns of the histones were analyzed by Western blotting using antibodies against histone H3, histone H4, acetylated histone H3 (acH3), and acetylated histone H4 (acH4) (left panels). The changes of the acetylation of H3 and H4 are shown on the right. (B) Methylation statuses of H3K4 and H3K9 around the $^{A}\gamma$ globin promoter. ChIP analysis with use of anti-H3K4me2, anti-H3K9me2, or anti-H3K9me3 was carried out to examine the distributions of H3K4me2, H3K9me2, and H3K9me3 on the human $^{A}\gamma$ globin promoter in the primary human adult erythroid cells before (Mock) and after (II) compound II treatment. IgG was used as the control. All data are presented as means ± SEM ($n = 3$) (*, $P < 0.05$; **, $P < 0.01$, by t test).

erythroid cells also results in inefficient recruitment of this γ globin repressor to the β-like globin locus, thus relieving the γ globin gene from transcriptional repression.

No change in the global acetylation levels of histones H3 and H4 in primary human adult erythroid cells treated with compound I or II. Treatment of cells with TSA or NaB leads to global changes in the histone modifications, in particular in acetylation, and subsequently induces γ globin gene expression (12). We thus next examined and compared the acetylation status of histones H3/H4 in the primary human adult erythroid cells treated with compound I or II or NaB, which is a class I and II HDACi. The global levels of acetylated histone H3 (acH3) and acetylated histone H4 (acH4) were significantly enhanced in the NaB-treated cells but not upon treatment with compounds I and II (Fig. 6A) or HU (data not shown), suggesting that compounds I and II and HU are not HDACi and do not globally change the acetylation status of histones H3 and H4. Possible changes of the methylation statuses of lysine 4 on histone H3 (H3K4) and lysine 9 on histone H3 (H3K9) around the $^{A}\gamma$ globin promoter were also examined. As expected, the level of H3K4me2 was enriched on the $^{A}\gamma$ globin promoter upon activation of the γ globin gene by compound II. On the other hand, the level of histone H3 with dimethylated lysine 9 (H3K9me2) was significantly decreased after compound II treatment (Fig. 6B).

Therapeutic potential of compounds I to VI in comparison with NaB and HU. To evaluate the therapeutic potential of compounds I to VI in comparison with other HbF inducers, we determined the half-maximal inhibitory concentration (CC_{50}) and the effective concentration (EC) of each compound. The CC_{50} value is the concentration of a compound at which the cell survival rate is reduced by 50%. The EC value was defined as the concentration of a compound that induced the γ globin gene expression by 1.9-fold, the fold of the γ globin gene induction by HU at its CC_{50}. The CC_{50}/EC ratio was used to determine whether the compound tested has better therapeutic potential than that of HU, the CC_{50}/EC ratio of which was set as 1. As shown in Table 1, compounds I to V exhibited better therapeutic potentials (CC_{50}/EC) than HU. It should be noted that compounds I to V also induced γ globin gene expression to higher levels than HU at their CC_{50} (Table 1). Among all the compounds, compound II had superior therapeutic potential, with a CC_{50}/EC ratio of 9.7 and a relatively higher γ globin fold induction (3.0) at its CC_{50} (Table 1).

To evaluate the specificities of globin gene activation by compounds I to III, the amounts of the individual β-like globin mRNAs were measured by absolute RT-qPCR. As shown in Table 2, the proportions of the ε and γ globin mRNAs were both significantly increased by these compounds. On the other hand, the proportions of the β globin mRNA were notably reduced after drug treatment. In comparison to HU and NaB, compounds I to III were more effective γ globin inducers, increasing the proportions of the γ globin mRNA from 8.0% to between 10.7% and 19.6% in the drug-treated primary human adult erythroid cells while reducing the β globin mRNA proportions from 87.2% to between 75.2% and 81.4% (Table 2).

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TABLE 1 Comparison of CC_{50} and γ globin gene-inducing abilities of different compounds[c]

Compound	CC_{50} (μM)[a]	EC (μM)[b]	CC_{50}/EC	γ globin induction fold (CC_{50}) (mean ± SEM)
I	0.17	0.12	1.4	2.7 ± 0.4*
II	22	2.3	9.7	3.0 ± 0.2**
III	75	9.3	8.1	2.6 ± 0.8*
IV	3.6	1.9	1.9	2.2 ± 0.7
V	135	25.8	5.2	3.4 ± 1.4
VI	103	106	1.0	1.9 ± 0.4
Butyric acid	222	NA[d]	NA	1.3 ± 0.2
Hydroxyurea	147	147	1.0	1.9 ± 0.3*

[a] CC_{50}, half-maximal inhibitory concentration; the concentration of a compound at which the cell survival rate was reduced by 50%.
[b] EC, effective concentration; the concentration of a compound that induces the γ globin gene expression by 1.9-fold (the fold induction of the γ globin gene by HU at its CC_{50}).
[c] The primary human erythroid cells were treated with NaB, HU, or compounds I to VI individually. The CC_{50}, the EC, and the ratio of CC_{50} to EC (CC_{50}/EC) of compounds I to VI were calculated and compared together with those of NaB and HU. Data are presented as the means from three independent experiments. Statistical significance of the difference between experimental and control groups was determined by the two-tailed Student *t* test (*, $P < 0.05$; **, $P < 0.01$).
[d] NA, not available.

TABLE 2 Relative expression levels of individual β-like globin genes in primary human erythroid cells treated with different compounds[a]

Compound	% expression of gene (mean ± SEM) ($n = 3$) for:			
	ε globin	γ globin	δ globin	β globin
Mock	0.1 ± 0.0	8.0 ± 0.7	4.7 ± 0.2	87.2 ± 1.0
HU	0.2 ± 0.0	9.9 ± 0.8	6.8 ± 0.4	83.1 ± 1.3
NaB	0.3 ± 0.0	8.8 ± 1.1	3.2 ± 0.2	87.8 ± 1.2
Compound I	0.6 ± 0.2	19.6 ± 1.3	4.7 ± 0.1	75.2 ± 0.9
Compound II	0.3 ± 0.0	10.7 ± 1.0	7.6 ± 0.6	81.4 ± 1.2
Compound III	0.3 ± 0.1	15.6 ± 1.2	7.6 ± 0.4	76.5 ± 1.0

[a] Primary human erythroid cells were treated with HU (147 μM), NaB (222 μM), compound I (0.2 μM), compound II (22 μM), or compound III (75 μM), respectively, for 3 days, and the expression level of each globin mRNA was estimated by absolute RT-qPCR. The proportions of the individual β-like globin mRNAs among the total β-like globin mRNAs are shown.

Induction of γ globin gene expression in HU-resistant erythroid cells by compound II. Previous studies have suggested that the increase in HbF levels in primary erythroid cultures treated with HU was comparable to the HU responsiveness of patients who received HU treatment (44). To evaluate whether compound II could induce the γ globin gene in HU-resistant cells, we established primary human erythroid cell cultures from the peripheral blood samples of 10 different normal blood donors and divided them into two groups according to their HU responsiveness based on pretested culture response. The dose responses of the two groups of primary human erythroid cells to HU were compared in Fig. 7A. Out of the 10 cultures, the γ globin gene expression in 7 of them, including those shown already in Fig. 3 to 5, could be upregulated by HU (Fig. 7B, HU-responsive). However, the other 3 erythroid cultures were resistant to HU induction of the γ globin gene (Fig. 7B, HU-resistant). Remarkably, compound II could induce γ globin gene transcription in all of these 10 primary human erythroid cell cultures (Fig. 7B). Notably, similarly to the HU-responsive erythroid cultures (Fig. 5A), the mRNA levels of BCL11A and NF-E4 in the HU-resistant primary human erythroid cultures were decreased and increased, respectively, upon treatment with compound II, and they were unaltered by HU (Fig. 7C).

The activation of the p38 MAPK signaling pathway has been suggested to mediate the fetal γ globin gene expression in response to NaB or TSA treatment (23, 24). To see whether the p38 MAPK signaling pathway was involved in the reactivation of the γ globin gene expression by the compounds described above, we examined the phosphorylation status of p38 MAPK in the primary human adult erythroid cells. HU, NaB, and compound II all significantly increased the phosphorylation level of p38 MAPK in the HU-responsive erythroid cells (left panel of Fig. 7D). In interesting contrast, the phosphorylation level of p38 MAPK was not notably changed in the HU-resistant erythroid cells upon treatment with these compounds (right panel of Fig. 7D). To further explore the possible role of p38 MAPK activation in relation to the HU responsiveness of the erythroid cells, the HU-responsive erythroid cells were pretreated with a selective p38 MAPK inhibitor, SB203580 (SB), and then the fold induction of γ globin by HU, NaB, and compound II, respectively, was analyzed. As shown in Fig. 7E, after inactivation of the p38 MAPK signaling by SB203580, HU failed to elevate the γ globin mRNA level whereas compound II could still induce γ globin expression (compare the right 4 bars of Fig. 7E). Similar results were observed in the HU-resistant cells upon pretreatment with SB203580 (data not shown). Taken together, the data in Fig. 7 show that the HU resistance of erythroid cells with respect to γ globin gene activation is associated with a lack of activation of p38 MAPK. Since treatment with compound II but not HU leads to significant decrease of BCL11A and increase of NF-E4 in all of the primary human erythroid cultures (Fig. 5A and 7C), the former agent could still induce the γ globin gene in the HU-resistant erythroid cells.

DISCUSSION

Several fluorescence-based (or luciferase-based) reporter systems have been used previously for the assessment of the activation of γ globin promoter as affected by HbF-inducing agents to explore new therapeutic agents for treating severe β-thalassemia and SCD (31, 32, 45–47). Several short-chain fatty acid (SCFA) derivatives that are able to induce γ globin have been identified by one of these studies (48). Also, the capability of SCFAs to induce γ globin gene expression and F-reticulocytes has been evaluated in transgenic mice and baboons (48–50). Among them, 2,2-dimethylbutyrate (HQK-1001) has been found to be the most potent oral γ globin gene inducer and is now being tested for use in SCD, β-thalassemia intermedia, and HbE/β-thalassemia in phase II clinical trials (15, 51–54). However, the therapeutic effects of HQK-1001 in patients who are irresponsive to HU treatment are presently unknown. In the present study, we have used a simple MEL cell-based dual-fluorescence reporter system (Fig. 1) to carry out high-throughput screening for novel γ globin-inducing compounds (Fig. 2). Six asymmetrical heterocyclic benzo[*de*]benzo[4,5]imidazo[2,1-*a*]isoquinolin-7-one derivatives (compounds I to VI) capable of inducing the γ globin gene have been identified (Fig. 3A). Taking compound II (core structure of the six) as an example, we have investigated the mode of action of this series of compounds on γ globin gene activation by using primary human erythroid cultures. HPLC

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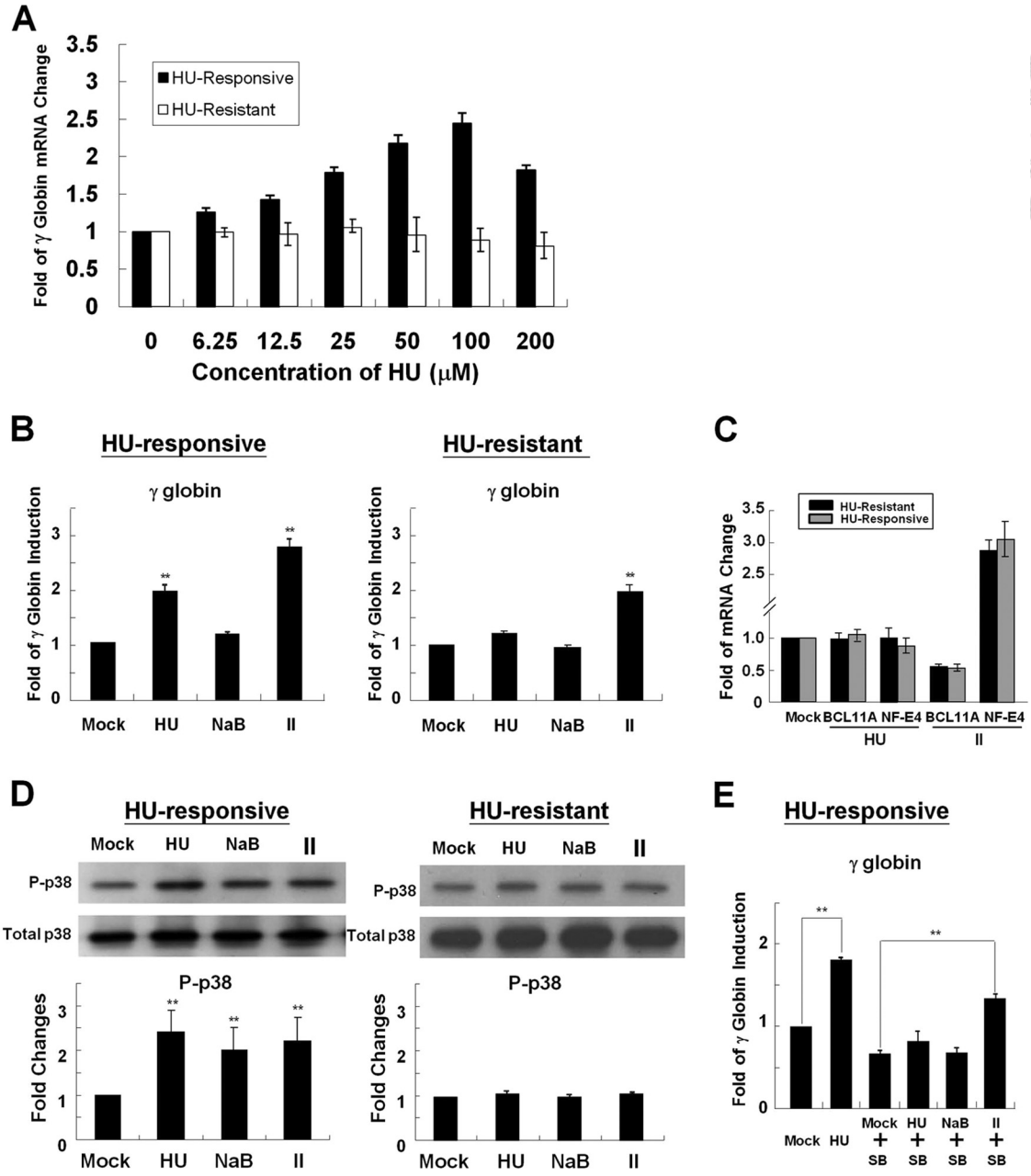

FIG 7 Molecular basis of inducibility of γ globin gene activation in HU-responsive and HU-resistant erythroid cells. (A) HU dosage responses of the HU-responsive and HU-resistant primary human erythroid cells. HU responses of the primary human adult erythroid cells from different normal blood donors were pretested first as described in Materials and Methods, and the cells were divided into HU-responsive and the HU-resistant groups. The 2 groups of the primary human adult erythroid cells were then treated with different dosages of HU for 3 days. After the HU treatment, the total mRNAs were extracted for RT-qPCR analysis and the relative induction levels of the γ globin genes in the 2 groups of cells were calculated and compared. The expression level of the γ globin gene in the mock control cells was set as 1. All data are presented as means ± SEM ($n = 3$). (B) Average fold induction of the γ globin mRNA in 7 independent HU-responsive and 3 independent HU-resistant primary human erythroid cell cultures treated with HU, NaB, or compound II. Note that the level of γ globin mRNA in the HU-resistant erythroid cells could be enhanced by treatment with compound II but not HU or NaB. Mock, no treatment. (C) Relative levels of BCL11A and NF-E4 mRNAs in HU-responsive and HU-resistant primary erythroid cells with or without drug treatment. The primary erythroid cells were treated with HU or compound II for 3 days, and the expression levels of BCL11A and NF-E4 mRNAs were determined by relative RT-qPCR. The expression levels of these genes in the mock control cells were set as 1. (D) Phosphorylation status of p38 MAPK in HU-responsive and HU-resistant primary erythroid cells with or without drug treatment. Upper panels, Western blot patterns of phosphorylated p38 MAPK (P-p38) and total p38. Bottom panels, effects of HU, NaB, and compound II on the relative levels of P-p38. Mock, no treatment. (E) Inducibility of the γ globin gene activation in HU-responsive primary human adult erythroid cell cultures treated with the p38 MAPK inhibitor SB (SB203580). The cultured erythroid cells were preincubated with or without SB (10 μM) for 1 h before treatment with HU, NaB, or compound II at its CC_{50} for 3 days. The fold induction of γ globin mRNAs was then analyzed by relative RT-qPCR. Each bar is the average of data from 3 independent HU-responsive primary human erythroid cell cultures. All data are presented as means ± SEM ($n = 3$ or 7) (**, $P < 0.01$, by t test).

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analysis shows that compound II is very stable after 3 days of culture (data not shown). Most interestingly, we have found that compound II is able to activate γ globin gene expression even in HU-resistant cells.

Several experiments have provided interesting insights into the possible underlying mechanisms of γ globin gene induction by the heterocyclic compounds in relation to the modulation of the phosphorylation status of p38 MAPK and the expression levels of γ globin transcription regulators (Fig. 5A and 7D). First, we have found that the phosphorylation level of p38 MAPK is significantly increased by compound II as well as HU and NaB in the primary human erythroid cells that are HU responsive (left panel of Fig. 7D). Previous studies have suggested that HU induces γ globin transcription through elevation of the level of circulating nitric oxide (NO), which stimulates p38 MAPK activation (55–57). Furthermore, inhibition of p38 MAPK activation prevents HU-induced γ globin gene expression in K562 cells (58). Interestingly, NaB and TSA also increase the γ globin synthesis through activation of p38 MAPK signaling (59). Since many HbF-inducing agents stimulate the stress-activated p38 MAPK, a cell stress signaling model for fetal globin induction has been proposed (60). Compound II thus may induce the γ globin gene in part through this cell stress signaling pathway as well.

Second, as shown in Fig. 5A, in addition to p38 MAPK, the induction of γ globin gene expression by the currently identified compounds is also accompanied by a significant increase of the activator NF-E4 and decreases of the repressors BCL11A and c-Myb, respectively. The levels of these γ globin regulators have been previously demonstrated by others to modulate γ globin gene expression in either K562 cells or primary human erythroid cells (20–22, 41). Among them, BCL11A appears to be the primary repressor of the γ globin gene, and its downregulation elevates the fetal γ globin gene expression *in vitro* in primary human adult erythroid cell culture and *in vivo* in genetically engineered mice (20, 61). The requirement for BCL11A in chromatin loop formation and modulation of γ globin gene silencing has been studied by chromosome conformation capture (3C) assay (33). Several erythroid regulators, including GATA1, FOG1, and SOX6, and the nucleosome remodeling/deacetylase complex (NuRD) have been found to interact with BCL11A and may be involved in regulation of the hemoglobin switch (20, 33). In addition, KLF1 regulates the expression of BCL11A and modulates the γ to β globin gene switching (62). Interestingly, compound II decreases the levels of BCL11A mRNA and BCL11A protein in the primary human erythroid cells (Fig. 5A and B). Significantly, the decrease of BCL11A upon compound II treatment is associated with the lower binding of the repressor at several regions of the human β-like globin locus (Fig. 5C), which has been suggested to play an important role in γ globin gene regulation (20). Thus, reactivation of the γ globin gene expression in adult erythroid cells by compound II appears to involve the modulations of two different pathways, i.e., regulation of the mRNA levels of γ globin transcription regulators and activation of the cellular p38 MAPK signaling to phosphorylate p38. Notably, decrease of BCL11A gene expression upon compound II treatment does not decrease the level of β globin gene expression (Fig. 5), which is consistent with a previous report that the promoter activity of the β globin gene remains unchanged in cells with RNAi-mediated knockdown of BCL11A (32). Thus, transient knockdown of BCL11A expression appears to affect the expression of only the γ, but not the β, globin gene.

The beneficial effects of HU treatment, including reductions in the rate of pain crisis and acute chest syndrome, seem to be attributable to the increase in HbF levels in patients (63–65). The requirement for HbF induction for clinical benefits during HU therapy is also supported by studies of the sickle cell mouse model (66). However, many hemoglobinopathic patients are poorly responsive or unresponsive to HU therapy, but the molecular mechanisms underlying this resistance to HU have been unclear. In particular, HU can increase HbF in only approximately half of SCD patients (18). In addition, around 30% of β-thalassemia patients are found to be nonresponders to HU treatment (19). Although multiple single nucleotide polymorphisms (SNPs) have been found to be associated with the HbF response to HU, the regulatory pathway(s) responsible for the HU-resistance of γ globin gene activation has remained unknown. We have investigated the activation of p38 MAPK signaling and its correlation with HU responsiveness of the primary erythroid cells (Fig. 7D). It appears that either of two intracellular changes contributes to the reactivation of the γ globin gene in compound-treated adult erythroid cells: (i) activation of p38 MAPK and (ii) alterations in the amounts of the γ globin gene regulators, i.e., reduction of the repressor BCL11A and increase of the activator NF-E4 (Fig. 5 and 7C). In HU-responsive erythroid cells, HU induces the first change but not the second. On the other hand, compound II induces both changes (Fig. 7C and D). Thus, the level of the γ globin gene expression in adult erythroid cells treated with compound II is higher than that in HU-treated cells (Fig. 7B). In HU-resistant erythroid cells, HU does not induce either of the two changes (Fig. 7C and D). Although compound II is not able to activate the p38 MAPK pathway in HU-resistant cells either (right panel of Fig. 7D), it can still induce alterations of the amounts of the γ globin regulators (Fig. 7C) and consequently reactivate the γ globin gene in these cells (right panel of Fig. 7B), albeit to a lesser extent than in the HU-responsive cells (compare the 2 panels of Fig. 7B). However, whether the lower sensitivity of p38 MAPK signaling to stress plays a causative role in the irresponsiveness of HU-resistant erythroid cells to γ globin gene induction needs to be further investigated.

In summary, our high-throughput screening and the following analyses have identified a series of new compounds that can induce γ globin gene expression in the primary human erythroid cells derived from normal adult blood donors. These compounds appear to be excellent lead compounds for further development of universal drugs for treating SCD and severe β-thalassemias. First, compound II shows a better therapeutic potential (CC_{50}/EC) as well as a higher γ globin-inducing capability at its CC_{50} than the other HbF-inducing agents tested (Table 1). Second, this series of compounds displays a higher activity of the γ globin gene induction than the other compounds tested (Table 2). Third, compound II does not change the global acetylation status of histones H3 and H4 (Fig. 6A), while it modulates the levels of H3K4me2 and H3K9me2 on the γ globin promoter (Fig. 6B). Fourth, compound II induces the γ globin gene transcription in HU-resistant as well as HU-responsive primary human erythroid cells through their capability to modulate the expression levels of several γ globin regulators, including the repressor BCL11A. Finally, combined use of compound II or its derivatives with a reagent(s) capable of activating the p38 MAPK pathway in HU-resistant erythroid cells would be of great therapeutic potential for treatment of SCD and severe β-thalassemias. However, further studies

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are warranted to evaluate the therapeutic effects of compound II and its derivatives in patients with SCD and severe β-thalassemias.

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We declare no conflict of interest.

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Research paper

Potent and orally active purine-based fetal hemoglobin inducers for treating β-thalassemia and sickle cell disease

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ABSTRACT

Reactivation of fetal hemoglobin (HbF) expression by therapeutic agents has been suggested as an alternative treatment to modulate anemia and the related symptoms of severe β-thalassemia and sickle cell disease (SCD). Hydroxyurea (HU) is the first US FDA-approved HbF inducer for treating SCD. However, approximately 25% of the patients with SCD do not respond to HU. A previous study identified TN1 (**1**) as a small-molecule HbF inducer. However, this study found that the poor potency and oral bioavailability of compound **1** limits the development of this inducer for clinical use. To develop drug-like compounds, further structure-activity relationship studies on the purine-based structure of **1** were conducted. Herein, we report our discovery of a more potent inducer, compound **13a**, that can efficiently induce γ-globin gene expression at non-cytotoxic concentrations. The molecular mechanism of **13a,** for the regulation HbF expression, was also investigated. In addition, we demonstrated that oral administration of **13a** can ameliorate anemia and the related symptoms in SCD mice. The results of this study suggest that **13a** can be further developed as a novel agent for treating hemoglobinopathies, such as β-thalassemia and SCD.

1. Introduction

Hemoglobinopathy is one of the most common inherited diseases in the world. According to prevalence estimates by the World Health Organization, at least 5.2% of the world's population exhibits hemoglobin disorders, accounting for approximately 3.4% of the deaths in children of less than 5 years of age [1]. It can be separated into two types of globin disorders: thalassemia (expression defects in globin chain synthesis) and structural disorders (in which the amino acid sequence is altered to produce abnormal hemoglobin). The two most common hemoglobinopathies, sickle cell disease (SCD) and β-thalassemia, are both adult hemoglobin (HbA) deficiencies that result in severe anemic syndromes. Approximately 4.5% of the global population suffers from either of these two diseases [2].

SCD is one of the most common monogenic disorders worldwide. Over 70% of SCD-affected newborns are from Africa, and it is estimated that more than 230,000 children with SCD are born in Africa every year. SCD is caused by a point mutation in the 6th amino acid of the β-globin gene (resulting in a glutamic acid to valine alteration). Mutant β globin chains polymerize under hypoxia and change the erythrocytes into a sickle shape [3]. These sickle-shaped erythrocytes not only lose their functions to transport oxygen, but also obstruct the local capillaries to restrict blood flow. Consequently, vaso-occlusive crisis results in pain and organ damage (especially to the liver and heart). Moreover, these sickle-shaped erythrocytes lose their elasticity, leading to hemolytic crisis and anemia. With a high risk of early death, the life expectancy of patients with SCD is reported to be shortened to an average

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Abbreviations

WHO	World Health Organization
HbF	fetal hemoglobin
SCD	sickle cell disease
HU	hydroxyurea
HbS	sickle hemoglobin
HDACi	histone deacetylase inhibitors
TSA	trichostatin A
NaB	sodium butyrate
FDA	Food & Drug Administration
PKIs	protein kinase inhibitors
HBD	hydrogen bond donor
4-DMAP	4-dimethylaminopyridine
RT-qPCR	reverse transcription quantitative polymerase chain reaction
PAMPA	parallel artificial membrane permeability assay
IV	intravenous
PO	per oral
RBC	red blood cells
HGB	hemoglobin
RET	reticulocytes
WBC	white blood cells
NEUT	neutrophils
F-cell %	percentage of HbF positive cells
HbF	fetal hemoglobin
IP	intraperitoneal injection

of 42–48 years [4]. β-Thalassemia is a hereditary anemia caused by mutations or deletions within the β-globin gene that reduce the expression levels of β-globin chains. Owing to the lack of sufficient β-globin chains, the excess α-globin chains form toxic aggregates and bind to cell membranes, and this induces rapid apoptosis of the erythrocytes during early erythroblast development [5–7].

Three therapeutic strategies have been used to modulate anemia and the related symptoms of patients with severe hemoglobinopathy. The most common therapeutic strategy is regular transfusion throughout the remaining life span to support the functional HbA required for survival. However, long-term transfusion is accompanied with a high risk of iron overload, which can cause tissue damage and organ dysfunction. To avoid the iron overload caused by transfusion, these chronically transfused patients must receive regular and expensive iron chelation treatment. Alternatively, bone marrow transplantation has been used for the treatment of severe SCD and β-thalassemia. Identification of a matched bone marrow donor is the primary limitation for this therapy. The potential for a potent immune response after transplantation has also been of concern. In addition to these two therapeutic strategies, pharmacological induction of fetal hemoglobin (HbF) expression is an alternative treatment for diminishing the syndromes of patients suffering from hemoglobin disorders [8,9]. For patients with β-thalassemia, elevation of fetal γ-globin chain synthesis can balance the excess of α-globin chains by forming HbF, thereby modulating the severe anemia observed in these patients [10]. Moreover, increase in γ-globin chain synthesis can prevent the formation of sickle-shaped erythrocytes, as HbF directly inhibits polymerization of sickle hemoglobin (HbS) in patients with SCD [11]. Thus, pharmaceutical induction of HbF in patients with hemoglobinopathies is a potentially useful therapeutic strategy.

To date, several chemotherapeutic agents, such as trichostatin A and apicidin (histone deacetylase inhibitors; HDACi), 5′-azacytidine (DNA methyltransferase inhibitor), hydroxyurea (HU) (ribonucleotide reductase inhibitor), and sodium butyrate (NaB) and its derivatives (short-chain fatty acids, also known as HDACi), have been demonstrated to stimulate HbF production [12–17]. Among them, HU is the first effective therapeutic medicine approved by the US Food & Drug Administration (FDA) for the treatment of SCD, which induces the expression of HbF for functional substitution of HbS in patients with SCD [18–21]. However, several side-effects of HU therapy have been reported, including leucopenia, thrombocytopenia, myelosuppression, and potential reproductive toxicity [22,23]. In addition, at least 25% of patients with SCD are poor or non-responders to HU treatment [24]. Thus, identification of new agents that can induce the expression of endogenous embryonic/fetal globin chains is essential to provide an alternative therapeutic strategy for these patients. In recent years, the FDA has approved three new drugs (L-glutamine, crizanlizumab-tmca and voxelotor) based on different strategies to ameliorate SCD symptoms. L-Glutamine is a precursor of glutathione and can be metabolized into glutamate. It can increase the antioxidative activity and may reduce the complications of SCD. Crizanlizumab-tmca is a monoclonal antibody against human P-selectin. The P-selectin is expressed in the endothelial cells and platelets which involves in the adhesion of sickle-shape RBC to blood vessel. Crizanlizumab-tmca can block P-selectin and prevent the vaso-occlusive crisis in patients. Voxelotor can directly bind to HbS and increases the oxygen affinity of HbS. The increase of oxygen affinity can inhibit the polymerization of HbS [25,26]. All the strategies including induction of HbF show promising results in mitigating the symptoms of SCD.

In a previous study, Nam et al. [27] conducted a high-throughput screening and identified a potent HbF inducer, TN1 (**1**), which induced HbF more potently than HU in KU812 and K562 leukemia cell lines. Western blot analysis indicated that treatment with 100 nM compound **1** resulted in 2.9- and 3.7-fold increase in γ-globin expression in KU812 cells and K562 cells, respectively. However, our studies found that this inducer **1** was not orally active and did not significantly increase γ-globin gene expression in primary erythroid cells (1.4-fold at 100 nM). Both the drawbacks could limit the future development of **1** for the treatment of patients with β-thalassemia and SCD in clinical trials. Compound **1** was originally developed as a protein kinase inhibitor (PKI) [28]. Nearly all of FDA-approved small molecule PKIs are orally active. An analysis of the hydrogen-bond donor (HBD) count for these approved PKIs revealed that the number of HBDs ranged from 1 to 4, with an average value of 2.1 [29]. We suppose that the lack of oral absorption for **1** may be because of the presence of more HBDs in the molecule [30]. To continue to develop potent and orally active inducers, we used **1** as a lead to redesign a second series of 2,6-diamino-substituted purine **1** analogs (Fig. 1). The approach can remove two hydrogen-bond donors in the water-soluble *trans*-4-aminocyclohexanol. In this study, the structure-activity relationships, *in vitro* properties, and pharmacokinetics of a series of purine **1** analogs are reported. This study has led to the discovery of *N*-ethylpiperazine, **13a**, which can efficiently induce γ-globin gene expression at non-cytotoxic concentrations and shows oral activity in ameliorating anemia and the related symptoms in an SCD mouse model. In addition to **13a**, some analogs displayed similar HbF-inducing capabilities to those of **13a**. Further studies that explain the possible biological mechanism underlying **13a**-mediated reactivation of γ-globin gene expression are also discussed herein.

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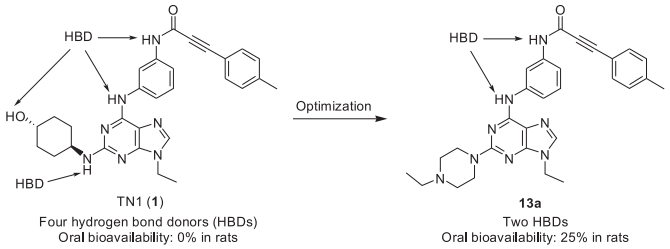

Fig. 1. Identification of orally available TN1 analog **13a** as an inducer of fetal hemoglobin.

These promising results demonstrate that **13a** or its analog relatives can be further developed as novel agents for treating hemoglobinopathies, such as β-thalassemia and SCD.

2. Results and discussion

2.1. Chemistry

The 2-amino-6-anilino-purine derivatives **13**, **14,** and **19**–**22** described in Table 1 can be prepared according to previously described methods (Scheme 1) [27,28]. Treatment of 3-nitroaniline **2** or methyl 3-aminobenzoate **3** with 2,6-dichloropurine **4** in the presence of 1-pentanol at 85 °C yielded 6-monosubstituted purine derivatives **5** or **6**, respectively. *N*-ethylation of purine **5** or **6** was performed by reaction with EtI in the presence of K_2CO_3 as the base that gave the desired *N*-9 alkylated isomer **7** or **8** with high selectivity and in a good isolated yield. The treatment of **7** or **8** with 2° amines (neat) at 150 °C or with 4-(2-hydroxyethyl)morpholine in the presence of 4-dimethylaminopyridine (4-DMAP) and diglyme, and microwaved at 200 °C yielded 4,6-disubstituted purines **9**–**10**. The reduction of the nitro group (**9**) by catalytic hydrogenation of Pd/C or hydrolysis of the methyl ester group (**10**) by NaOH in aqueous MeOH yielded the intermediate amine **11** or acid **12**, which was followed by formation of amides with carboxylic acids or amines using coupling reagents and produced target compounds **13a**-**h** or **14**, respectively. Condensation of amine **11** with 4-nitrophenyl chloroformate followed by reacting with 1-(4-fluorophenyl)piperazine provided the urea **13i**. The general synthetic route for the preparation of final products quinazoline **19**, thiazolo[5,4-d]pyrimidine **20**, purine **21,** and furo[2,3-d]pyrimidine **22** is similar to that of **13** (Scheme 1). Reacting the final compounds **13a**-**b** and **13d** with hydrochloride in MeOH yielded their hydrochloride salts.

2.2. In vitro pharmacology

As shown in Table 1, the target compounds were tested in a primary human erythroid cell culture system. Using this assay, maximal fold of γ-globin gene induction by the tested compound was evaluated. The first compound, **1**, which is a known inducer of HbF [27], increased the γ-globin gene expression by 1.4-fold at a concentration of 0.1 μM, but significantly reduced the cell proliferation rate by 39% at the same concentration (cell viability = 61%). Removal of the *trans*-4-aminocyclohexanol group of **1** to give **21** dramatically increased the expression of γ-globin to 4.1-fold at a higher concentration of 15 μM with low antiproliferative activity at that concentration. Further studies were carried out to examine the effects of various structural scaffolds on γ-globin gene induction. No improvement in induction fold was observed when the purine core structure (**1** or **21**) was replaced by quinazoline (**19**), thiazolo [5,4-d]pyrimidine (**20),** or furo[2,3-d]pyrimidine (**22**). Compounds **19** and **22** showed less than 2-fold increase of γ-globin gene, and compound **20** had deleterious effects on the regulation of γ-globin gene expression (0.87-fold) and cell viability (19% at 10 μM). Next, we examined the effects of water-solubilizing substituents on the secondary position of the purine ring and compared the potencies of compounds **13a**-**13f** with *trans*-4-aminocyclohexanol analog **1** and unsubstituted analog **21**. *N*-ethylpiperazine (**13a**), *N*-(2-fluoroethyl)piperazine (**13b**), and *N*-(2-hydroxyethyl)piperazine (**13c**) were significantly more potent HbF inducers (>4.5-fold) than **1** (1.4-fold). At the concentration of maximal induction, **13a** exhibited the lowest cytotoxicity (72% cell viability at 2.0 μM) in comparison to that of **13b** and **13c** (50% and 58% cell viability at 5.0 μM and 1.25 μM, respectively). When the nitrogen was removed from the ring system, *N*,*N*-dimethylpiperidin-4-amine (**13d**) and racemic 3-(dimethylamino)pyrrolidine (**13e**) were apparently less potent (<2-fold induction) than the unsubstituted (**21**, 4.1-fold induction) compound. Compound **13f** bearing a morpholine group tethered by a two-carbon ether induced a moderate 2.6-fold increase in γ-globin gene expression at a low concentration of 0.4 μM (cell viability = 78%).

Having demonstrated the importance of the purine core structure and water-solubilizing groups, extensive structure-activity relationships studies were conducted by modifying the terminal amide pharmacophore (**13g**-**i** and **14a**-**b**). A significant drop in induction fold was observed when the R″ substituent of **13a** was changed from an alkyne group (4.8-fold) to a vinyl group (**13g**, 2.3-fold), cyclopropyl group (**13h**, 1.1-fold), or piperazinyl group (**13i**, 2.2-fold). Among them, urea **13i** had the strongest antiproliferative activity (8% cell viability) at the concentration of maximal induction (10 μM) in comparison to that of **13g** (128% cell viability at 1.25 μM) and **13h** (77% cell viability at 1.25 μM). When the third position of aniline was changed from the amino group (**13**) to the carboxyl group (**14**), racemic *trans*-2-phenylcyclopropylbenzamide **14a** induced a slight (1.3-fold) increase in γ-globin gene expression. In contrast, biphenyl-4-yl-benzamide **14b** slightly decreased γ-globin gene expression by 0.87-fold in the controls. Obviously, compounds **13g**-**i** and **14a**-**b** were substantially less potent than **13a** in the induction of γ-globin gene expression. These results indicate that the terminal amide group (**13a**) with an ethynylbenzene at R" (Table 1) is an important pharmacophore of the molecule. Among the compound **1** analogs listed in Table 1, four compounds (**21** and **13a**-**c**) are significantly more potent target inducers (>4.0-fold) than **1** (1.4-fold). Especially, **13a** exhibited the highest (4.8-fold) γ-globin gene induction at a low concentration of 2.0 μM with acceptable cytotoxicity at that concentration. Therefore, compound **13a** was selected for further biological activity, pharmacokinetic, and *in vivo* efficacy studies.

2.3. Dose-dependent γ-globin gene induction by *13a*

We evaluated the efficacy of γ-globin gene induction by **13a** treatment using a primary human erythroid cell culture system. After 2 weeks of *in vitro* erythroid cell differentiation, cells were treated with **13a** for 3 days and then harvested for reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis, cell viability assay, and western blot analysis. Fold change of γ-globin mRNA expression in primary human erythroid cell cultures was significantly increased by **13a** treatment in a dose-dependent manner (working concentrations ranging from 0.06 μM to 2 μM, Fig. 2a). The maximal induction (4.8-fold) of the γ-globin gene by **13a** was observed at the concentration of 2.0 μM. Higher concentrations (4.0 μM) of **13a** treatment resulted in cell cytotoxicity, which reduced γ-globin induction. Importantly, primary human erythroid cells treated with 1.0 μM **13a** activated γ-globin mRNA expression by 3.8-fold without inducing significant cytotoxicity. In

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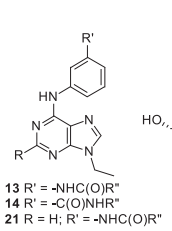
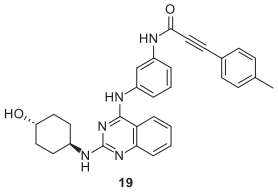
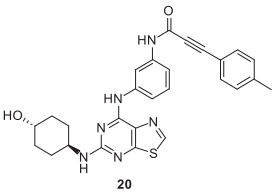
Table 1
γ-Globin fold induction in compound-treated human primary erythroid cell cultures.

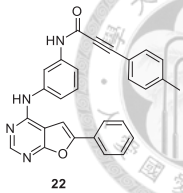




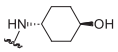
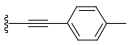
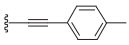
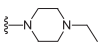
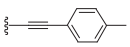
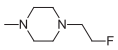
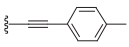
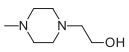
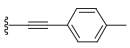
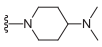
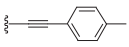
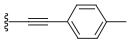
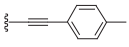
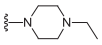
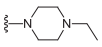
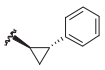
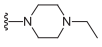
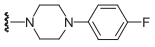
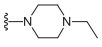
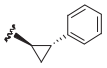
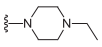
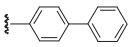
19



20



22

Compd	R	R″	Biological activity		
			Concentration (μM)	Viability (%)	Fold induction of γ-globin gene
1			0.1	61	1.4
21	H		15	75	4.1
19	–	–	1.25	46	1.8
20	–	–	10	19	0.87
22	–	–	10	103	1.2
13a			2.0	72	4.8
13b			5.0	50	4.6
13c			1.25	58	4.6
13d			0.625	75	1.7
13e			1.25	53	1.5
13f			0.4	78	2.6
13g			1.25	128	2.3
13h			1.25	77	1.1
13i			10	8	2.2
14a			0.83	71	1.3
14b			0.625	105	0.84

addition, the dose-dependent effects of HU and NaB were also evaluated (Supporting material, Fig. S1). HU (100 μM) and NaB (250 μM) provided maximal γ-globin inducing activities of 2.5- and 1.3-fold, respectively, in primary human erythroid cells. The CC_{50} values of HU and NaB (CC_{50} = 147 and 222 μM, respectively) were determined for the next studies. We further evaluated the protein level of γ-globin. We found the γ-globin protein levels in **13a**-treated cells were higher than those in the mock control (Fig. 2b and c). In contrast, we verified the specificity of **13a** for inducing the γ-globin and β-globin genes. We found that after 0.5 μM **13a** treatment, it can specifically induce γ-globin gene expression but not that of β-globin (Fig. 2d). However, HU induced similar levels of γ-globin and β-globin. Specificity is important for treating patients with SCD. The increase in the expression of the mutant β-globin gene is not helpful for this disease. The RNA and protein results show that **13a** can induce γ-globin gene expression without cytotoxicity and demonstrate the specificity to induce higher expression of the γ-globin gene than that of the β-globin gene. These results indicate the dosing range and safety of **13a** could be better than that of HU.

*2.4. Compound **13a** activates γ-globin expression in HU-resistant primary human erythroid cells*

A previous study has demonstrated that the *in vitro* differentiated human erythroid cells can represent the response or non-response effect of HU on patients [31]. Our studies found that the normal blood obtained from the Taipei blood bank can also separate into HU response or non-response groups. As there are currently limited therapeutic options for patients with SCD who are

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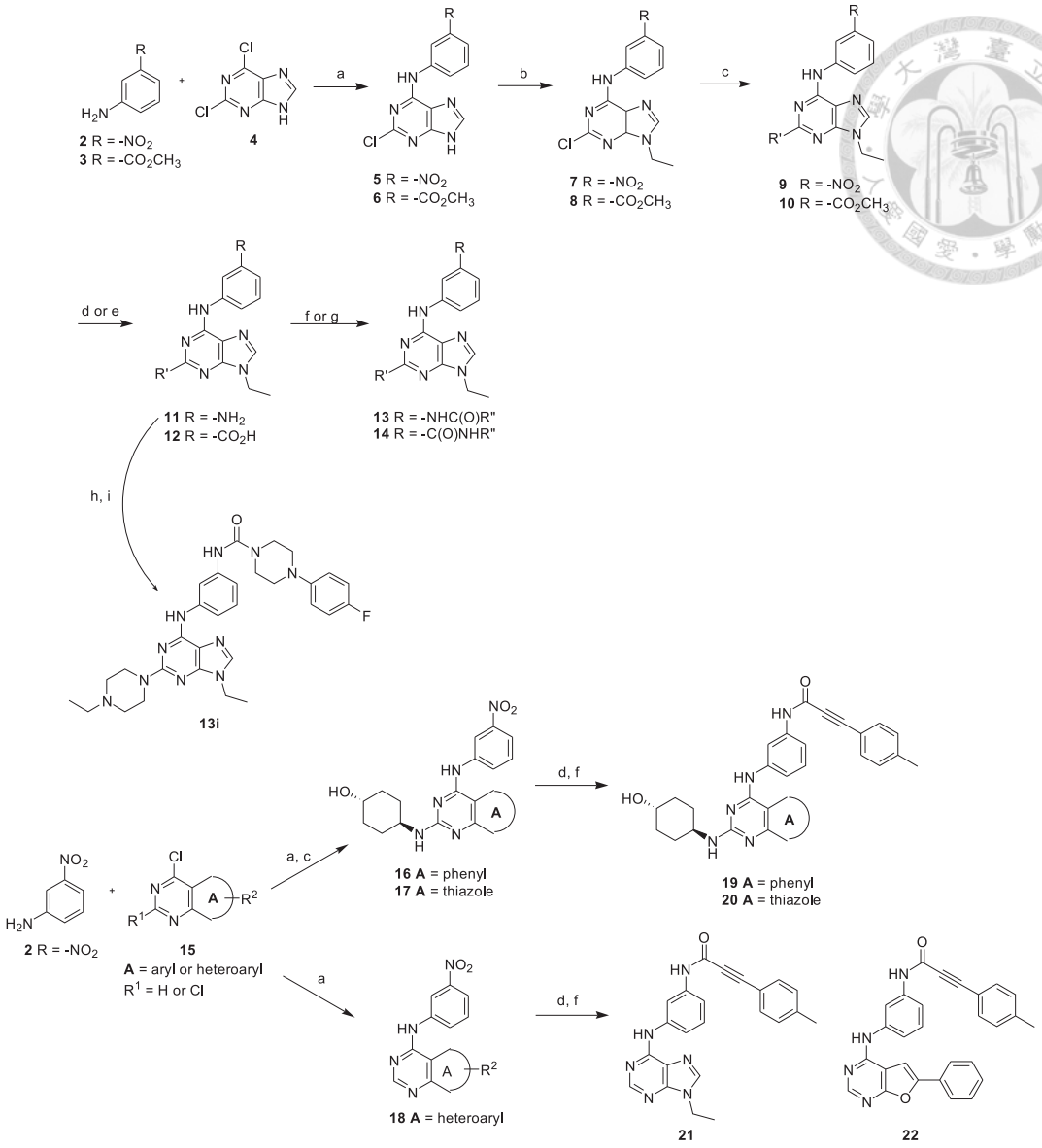

Scheme 1. Reagents and conditions: (a) 1-pentanol, 85 °C, overnight, 82%; (b) K_2CO_3, EtI, DMF, rt, 2 h, 90%; (c) for **1**, **19,** or **20**, *trans*-4-aminocyclohexanol, diglyme, 150 °C, 2 days; for **13** and **14** except for **13f**, 2° amines (neat), 150 °C, 2 h; for **13f**, 4-(2-hydroxyethyl)morpholine, 4-DMAP, diglyme, 200 °C, microwave, 1.5 h, 10%; (d) for **9** and **16**–**18**, H_2, 10% Pd/C, 6 N HCl, CH_3OH, 95%; (e) for **10**, MeOH, 1 N NaOH aqueous; (f) for **11** and reduced **16**–**18**, R″COOH, HATU, TEA, DMF, rt; (g) for **12**, R″NH_2, HATU, TEA, DMF, rt; (h) (i) 4-nitrophenyl chloroformate, pyridine, rt, 2 h, (ii) 1-(4-fluorophenyl)piperazine, pyridine, 40 °C, 1 h; (i) HCl, CH_3OH.

poorly- or non-responsive to HU, we next examined the HbF-inducing capability of **13a** in primary human erythroid cells that are non-responsive to HU treatment. As shown in Fig. 3, **13a** treatment (0.5 μM) could elevate γ-globin mRNA expression at least 2-fold in HU-resistant cells with only minor cytotoxicity. This outcome demonstrates that **13a** could be developed as a next-generation therapeutic agent for the treatment of a broad spectrum of patients with hemoglobinopathies, possibly including patients with HU low/non-responsive SCD.

2.5. Compound *13a* is not an HDACi

Several HDACi have been suggested to be HbF-inducing reagents, such as NaB, apicidin, and trichostatin A. It is believed that the hyper-acetylation of histone by HDACi treatments would contribute to the activation of γ-globin gene expression. To reveal whether the epigenetic modification of histone H4 was manipulated by **13a** treatment, primary human erythroid cells were treated with HU, NaB, or **13a** for 3 days, and the acetylation status of cellular histone H4 was analyzed. Our data showed that the global acetylation status of histone H4 (total acH4) was significantly enhanced in NaB-treated primary erythroid cells. However, different from NaB, the amount of global acetylated histone H4 was not dramatically altered in **13a**-treated cells. This indicated that **13a** was likely not a histone deacetylase inhibitor (Fig. 4).

2.6. Molecular mechanisms for γ-globin gene induction by *13a*

To identify the molecular mechanisms of **13a**, we performed RNA-seq analysis to reveal the gene expression pattern between mock and **13a**-treated cells. Previous studies have demonstrated that the mechanism of the globin switch during development was very complicated. Many transcription factors assemble either

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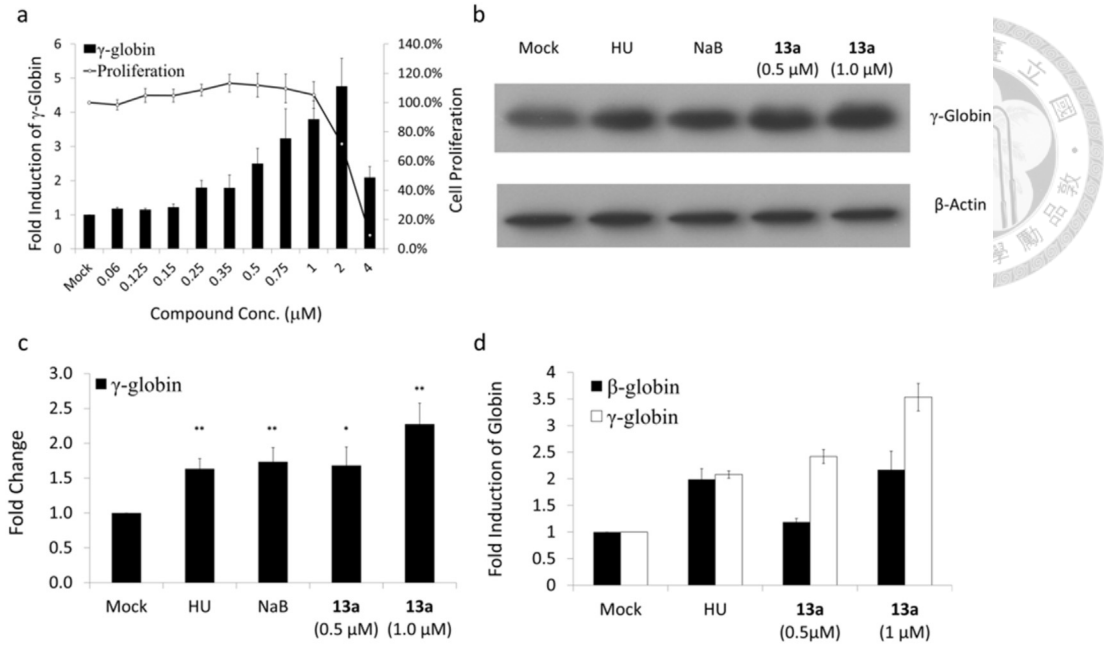

Fig. 2. Dose-dependent γ-globin gene induction and proliferation rate by **13a**. (a) Primary human erythroid cells were treated with different dosages of **13a** for 3 days. The induction of γ-globin mRNAs and proliferation rate were detected by relative RT-qPCR and AlamarBlue assay, respectively. (b) Primary human erythroid cells were treated with HU (147 μM), NaB (222 μM), and **13a** (0.5 and 1.0 μM) for 3 days. After 3 days of treatment the cells were harvested for protein extraction. Protein extracts (50 μg) from compound-treated cells and mock control cells were loaded for western blot. Antibody against γ-globin was used to detect the expression change after compound treatment. β-Actin was used as an internal control. (c) Quantification of western blot results was normalized with β-actin and compared to that of the mock control (d) Fold induction of γ-globin and β-globin mRNAs was detected by RT-qPCR. All data are from at least three independent experiments and presented as means ± SEM.

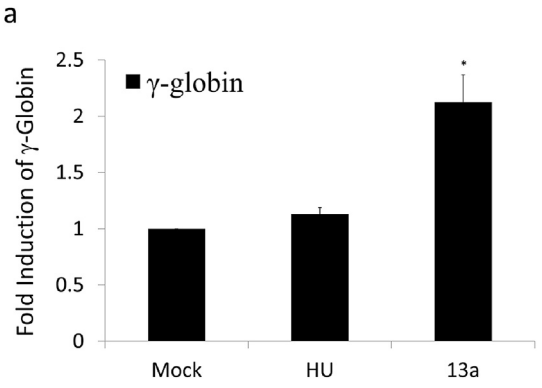

Fig. 3. Compound **13a** induces γ-globin gene expression in HU-resistant primary erythroid cells. The primary human erythroid cells were treated with **13a** (0.5 μM) and HU (**147** μM) for 3 days. The induction of γ-globin mRNAs was detected by relative RT-qPCR and HU-resistant samples identified. All data are from at least three independent experiments and presented as means ± SEM.

activation or inhibition complexes to manipulate this process. From the RNA-seq results, we found that the expression levels of several transcription factors (*GATA1*, *NFE2L2*, and *c-MYB*) were changed and the results were validated by RT-qPCR (Fig. 5a). *GATA1* has been reported as a general activator of β-type globin genes [32]. *NFE2L2* has been proved to directly bind to the γ-globin promoter to activate γ-globin gene expression [33,34]. In contrast, previous studies suggest that *c-MYB* may act as a repressor of γ-globin gene expression [35,36]. According to the RT-qPCR data, **13a** can induce *GATA1* (1.6-fold and 2.3-fold) and *NFE2L2* (1.7-fold and 2.3-fold) at 0.5 and 1.0 μM, respectively. In addition, **13a** also inhibits *c-MYB* (0.7-fold and 0.6-fold). In summary, **13a** manipulates γ-globin expression through these three regulators. However, the detailed mechanisms need further study. Other than these regulators, we found **13a** increased the expression level of *CPOX* (1.8-fold and 2.2-fold, Fig. 5a) which is involved in the heme-biogenesis pathway [37]. Compound **13a** may contribute to heme-biogenesis during globin synthesis. We further detected the protein level of BCL11A after **13a** treatment. BCL11A has been reported as a key repressor of the γ-globin gene during the globin switch from fetal to adult age [38,39]. Knock down of BCL11A in primary cells can dramatically reactivate the expression of γ-globin [38]. As shown in Fig. 5b and c, treatment with higher concentration of **13a** (1.0 μM) obviously downregulated the protein level of BCL11A. NaB has been reported to inhibit BCL11A as a positive control [40]. These results reflect the mechanism of fold induction in γ-globin with **13a** treatment. Compound **13a** at 1.0 μM induced a higher level of γ-globin than that by **13a** at 0.5 μM.

2.7. Pharmacokinetic studies of compounds *1* and *13a*

Table 2 shows the pharmacokinetic properties of **1** and **13a** evaluated in male Sprague–Dawley rats. Compound **1** exhibited long half-life (10.2 h), high plasma clearance (CL = 60.9 mL/(min·kg)), and moderate volumes of distribution (V_{ss} = 3.8 L/kg) after intravenous (IV, 2.0 mg/kg) administration; however, oral bioavailability (F%) of **1** was not observed. On the contrary, **13a** showed moderate half-life (7.2 h), moderate plasma clearance (CL = 8.2 mL/(min·kg)), and low volumes of distribution (V_{ss} = 0.3 L/kg) after intravenous (5.0 mg/kg) administration. A single 20 mg/kg oral dose of **13a** was administered as a solution containing 22% hydroxypropyl-β-cyclodextrin in water and absorbed with a moderate half-life in rats ($t_{1/2}$ = 3.8 h). The C_{max} (2730 ng/mL) and AUC (8996 ng h/mL) were high and the oral bioavailability was 25%. To clarify possible factors that caused the differences in oral bioavailability between compounds **1** and **13a**,

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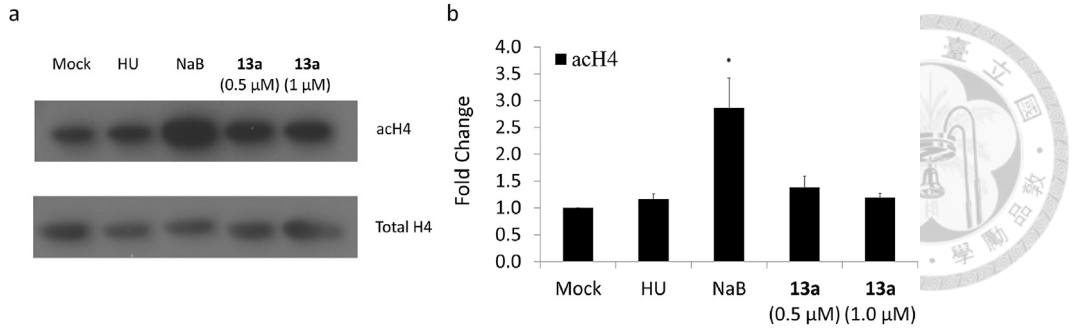

Fig. 4. Compound 13a is not a histone deacetylase inhibitor. (a) Primary human erythroid cells were treated with HU (147 μM), NaB (222 μM), and **13a** (0.5 and 1.0 μM) for 3 days. After 3 days of treatment, cells were harvested for histone extraction. Histone extracts (50 μg) from compound-treated cells and mock control cells were loaded for western blot. Antibody against total acetylated histone H4 (acH4) was used to detect the acetylation status change after compound treatment. Total histone H4 (Total H4) was used as an internal control. (b) Quantification of western blot results was normalized with total H4 and compared to that of the mock control. All data are from at least three independent experiments and presented as means ± SEM.

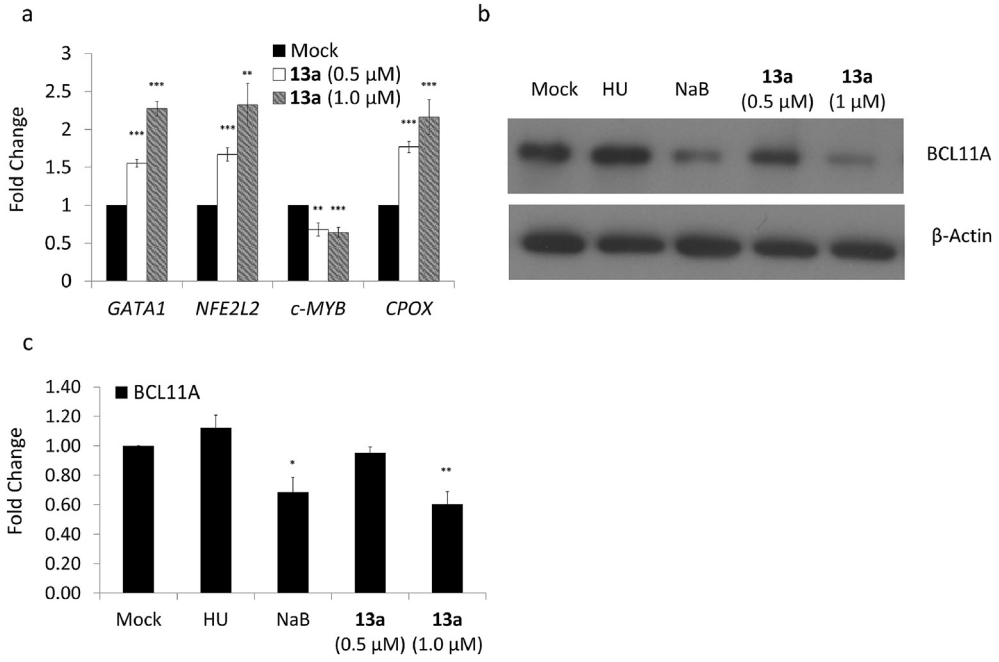

Fig. 5. Compound **13a** elevated expression levels of the γ-globin-related genes (*GATA1*, *NFE2L2*, *MYB*, *CPOX*, and *BCL11A*). (a) Primary human erythroid cells were treated with HU (147 μM), NaB (222 μM), and **13a** (0.5 and 1 μM) for 3 days. The expression level changes in *GATA1*, *NFE2L2*, *MYB*, and *CPOX* mRNAs were detected by relative RT-qPCR (b) Protein extracts (50 μg) from compound treated-cells and mock control cells were loaded for western blot. Antibody against BCL11A was used to detect the expression level change after compound treatment. β-Actin was used as an internal control. (c) Quantification of western blot results was normalized with β-actin and compared to that of the mock control. All data are from at least three independent experiments and presented as means ± SEM.

Table 2
Pharmacokinetic profiles of compounds **1** and **13a** in rats.

Compound	IV				PO[a]			
	$T_{1/2}$ (h)	CL (mL/(min·kg))	V_{ss} (L/kg)	$AUC_{(0\text{-inf})}$ (ng·h/mL)	$T_{1/2}$ (h)	C_{max} (ng/mL)	$AUC_{(0\text{-inf})}$ (ng·h/mL)	F (%)
1[b]	10.2	60.9	3.8	610	ND[d]	ND[d]	ND[d]	ND[d]
13a[c]	7.2	8.2	0.3	8862	3.8	2730	8996	25

[a] PO, per oral.
[b] Dose: IV 2.0 mg/kg; PO 10 mg/kg.
[c] Dose: IV 5.0 mg/kg; PO 20 mg/kg.
[d] ND, not detected.

both compounds were examined for their water solubility and permeability. Water solubility study (pH = 7.4) revealed that the solubilities of compounds **1** and **13a** were <10 ng/mL and 3380 ng/mL, respectively. Parallel artificial membrane permeability assay (PAMPA) indicated that the $logP_{app}$ values of compounds **1** and **13a** was −5.57 and −7.76, respectively. These data suggested that both compounds had low permeability; however, compound **13a** had higher water solubility (>300-fold) than that of compound **1**.

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Furthermore, *in vivo* rat pharmacokinetic data (Table 2) showed that compound **13a** (CL = 13 mL/(min·kg)) has lower total body clearance than that of compound **1** (CL = 60.9 ml/(min·kg)), indicating that compound **13a** was more stable than compound **1**. After demonstrating favorable solubility, pharmacokinetic properties and potent induction of γ-globin gene expression in primary human erythroid cells, **13a** was appropriate for continued *in vivo* investigation to determine the antianemic activity of **13a** in an SCD mouse model.

2.8. *In vivo efficacy studies of **13a** in SCD mice*

To further evaluate the therapeutic potential of **13a** for treating hemoglobinopathies, a mouse model of SCD (generated by the laboratory of Prof. Tim Townes, University of Alabama) was used to examine the effects of **13a** on relieving the symptoms of anemia in this disease model. Two doses of **13a** (50 and 100 mg/kg) were administered orally to 6- to 8-week-old SCD mice twice daily for 5 days a week. After 4 weeks of treatment, blood samples from the treated SCD mice were collected for several biochemical analyses (Table 3). Complete blood cell assessment showed that **13a** treatment slightly increased red blood cell numbers and hemoglobin levels in peripheral blood samples of SCD mice compared to those of mock-treated control mice. Reticulocyte, white blood cell, and neutrophil values were equivalent between **13a**-treated and mock-treated SCD mice. HPLC analysis revealed that **13a** significantly increased the percentage of HbF in SCD mice, which is consistent with our finding of an elevated percentage of F-cells in these mice, as detected by flow cytometry. A diagnostic indicator of SCD is the characteristic sickle-shaped morphology of red blood cells under hypoxia. A previous study demonstrated that over-expression of γ-globin can reduce the percentage of sickle-shaped red blood cells in the peripheral blood of patients with SCD [41]. We assessed the percentages of sickle-shaped cells in smears of SCD mouse blood under hypoxia and found that this parameter was significantly decreased upon treatment with **13a** (Fig. 6b and c), indicating that **13a** treatment might decrease the risk of vaso-occlusive crisis in SCD mice. In the group treated with HU (100 mg/kg, intraperitoneal injection once daily, the toxic effects on decreasing the red blood cell number and hemoglobin amount were observed (Table 3). Moreover, the HU-treated group showed a slightly decrease in the white blood cell and neutrophil number in SCD mice. However, we did not find improvement in percentage of F-cells and HbF which is consistent with the percentages of sickle-shaped cells in the blood smear (Table 3 and Fig. 6d). Taken together, the *in vivo* experiments in SCD mice suggest that **13a** might be a potential drug candidate for β-thalassemia and SCD.

3. Conclusion

Starting with the well-known HbF inducer **1**'s core scaffold and with the rational design approach, we successfully identified a preclinical candidate **13a,** which significantly increases the expression of the γ-globin gene and dramatically improves oral bioavailability compared with that of compound **1**. Importantly, the *in vitro* assay demonstrated that **13a** can efficiently induce γ-globin expression at a non-toxic concentration in primary erythroid cells and HU-resistant primary erythroid cells. Molecular mechanistic studies showed that **13a** does not alter the acetylation status of histone H4 in comparison to that by NaB. Furthermore, RNA-seq analysis demonstrated that **13a** reduces the expression level of *c-MYB,* which is a repressor of γ-globin and increases the expression levels of *GATA1* and *NFE2L2,* which are believed to be involved in γ-globin activation. The western blot result shows that the level of important γ-globin repressor BCL11A was decreased. Nevertheless, further studies are required to determine the detailed mechanisms for the effects of **13a** on regulating γ-globin expression. Furthermore, efficacy studies *in vivo* demonstrated that **13a**, which is safe, orally active, and well-tolerated, provides dose-dependent relief from anemia symptoms in SCD mice. Taken together, the excellent *in vitro* and *in vivo* antianemia activities of **13a** suggest that it could be a next-generation drug candidate that is worthy of clinical evaluation for the treatment of β-thalassemia and SCD, especially for patients who are HU-resistant.

4. Experimental

4.1. *Chemistry*

4.1.1. *General methods*

All commercial chemicals and solvents are reagent grade and were used without further treatment unless otherwise noted. ^{1}H NMR spectra were obtained with a Varian Mercury-300 or a Varian Mercury-400 spectrometer. Chemical shifts were recorded in parts per million (ppm, δ) and were reported relative to the solvent peak or TMS. LC/MS data were measured on an Agilent MSD-1100 ESI-MS/MS System. High-resolution mass spectra (HRMS) were measured with a Thermo Finnigan (TSQ Quantum) electrospray ionization (ESI) mass spectrometer. Flash column chromatography was done using silica gel (Merck Kieselgel 60, No. 9385, 230–400 mesh ASTM). Reactions were monitored by TLC using Merck 60 F_{254} silica gel glass backed plates (5 × 10 cm); zones were detected visually under ultraviolet irradiation (254 nm) or by spraying with phosphomolybdic acid reagent (Aldrich) followed by heating at 80 °C. All starting materials and amines were commercially available unless otherwise indicated. The purity of compounds was determined by a Hitachi 2000 series HPLC system and a Waters Acquity UPLC/BSM with PhotoDiode Array detector. Except for compound **13f**, purity of all

Table 3
Antianemic effect of **13a** against SCD mice.

Blood test[a]	Mock	13a PO[b] 50 mg/kg	13a PO 100 mg/kg	HU IP[c] 100 mg/kg
RBC (M/uL)	7.23 ± 0.75	7.78 ± 0.58	8.05 ± 0.62	6.34 ± 0.41
HGB (g/dL)	7.46 ± 0.7	8.15 ± 0.68	8.05 ± 0.7	6.7 ± 0.31
RET (%)	57.34 ± 3.67	58.98 ± 3.18	54.66 ± 2.3	50.92 ± 1.58
WBC (K/uL)	34.15 ± 5.85	35.85 ± 3.37	44.12 ± 6.63	23.02 ± 3.45
NEUT (K/uL)	13.39 ± 4.65	15.05 ± 2.89	15.64 ± 3.47	9.75 ± 1.13
HbF (%)	0.20 ± 0.02	0.28 ± 0.02*	0.30 ± 0.03*	0.23 ± 0.03%
F-cell (%)	1.63 ± 0.11	2.15 ± 0.14**	2.42 ± 0.12***	1.3 ± 0.19%
Sickle cell (%)	16.78 ± 1.9	10.0 ± 1.4*	8.8 ± 0.8**	14.7 ± 0.4%

[a] RBC, red blood cells; HGB, hemoglobin; RET, reticulocytes; WBC, white blood cells; NEUT, neutrophils; F-cell %, percentage of HbF positive cells s; HbF, fetal hemoglobin.
[b] PO, per oral.
[c] IP, intraperitoneal injection.

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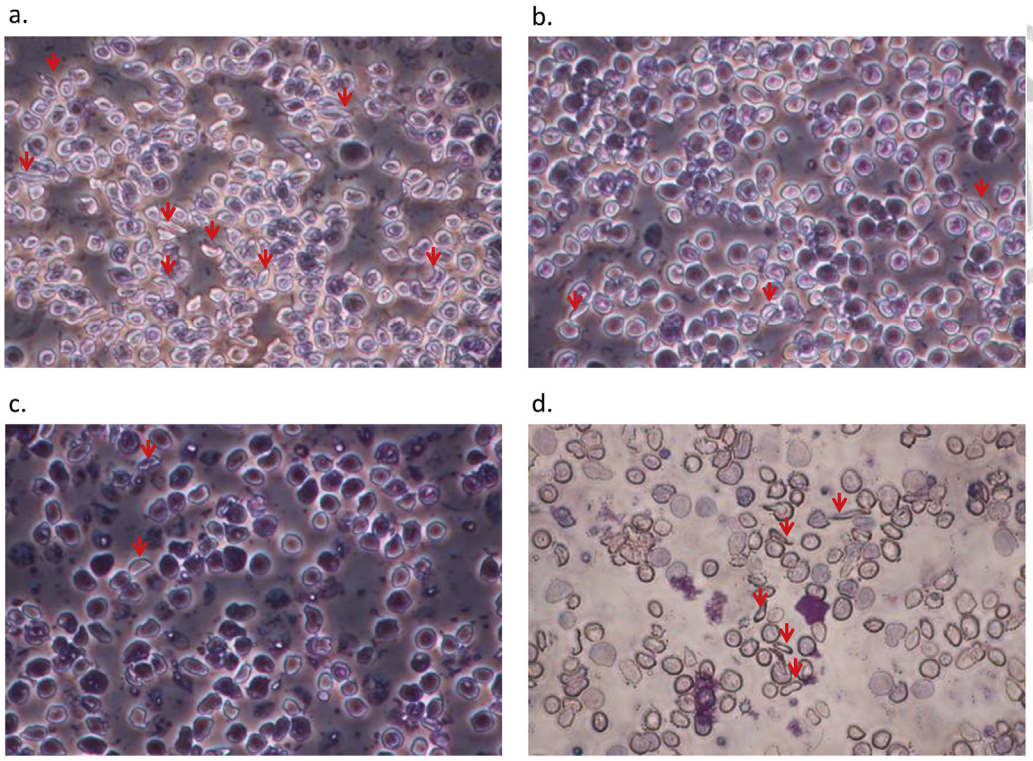

Fig. 6. Bloods smear of compound-treated mice. Blood was collected by submandibular blood collection and treated with hypoxia (3% O_2) for 30 min. Blood (2.0 μL) from each sample was used to prepare the blood smear on the slide. The slides were air-dried and then stained with Liu-stain. (a) Mock (b) **13a** (PO 50 mg/kg) (c) **13a** (PO 100 mg/kg) (d) HU (IP 100 mg/kg). Each group contained at least three mice. Arrows indicate the sickle-shape cells.

the other target compounds shown in Table 1 was over 95% based on a reverse phase C_{18} column (Agilent ZORBAX Eclipse XDB-C18 5 μm, 4.6 mm × 150 mm, Condition A) and a UPLC reverse phase C_{18} column (Waters Acquity BEH-C18, 2.1 mm (ID) x 50 mm (L), 1.7 μm particle size, Condition B) under the following gradient elution condition: Mobile phase A-acetonitrile (10%–90%, 0–45 min) and mobile phase B-10 mM NH_4OAc aqueous solution containing 0.1% formic acid (90%–10%, 0–45 min) for Condition A and Mobile phase A-acetonitrile (10%–90%, 0–6.5 min) and mobile phase B-2.0 mM NH_4OAc aqueous solution containing 0.1% formic acid (90%–10%, 0–6.5 min) for Condition B. The flow-rate was 0.5 ml/min and 0.6 mL/min for Condition A and Condition B, respectively. The injection volume was 20 μL and 5.0 μL for Condition A and Condition B, respectively. The system operated at 25 °C. Peaks were detected at λ = 254 nm.

Except for **13f**, Compounds **13**–**14** and **19**–**22** were prepared following the previous publication with some modifications [27,28].

*4.1.2. N-[3-({9-ethyl-2-[(trans-4-hydroxycyclohexyl)amino]-9H-purin-6-yl}amino)phenyl]-3-(4-methylphenyl)prop-2-ynamide (**1**)*

^{1}H NMR (300 MHz, DMSO-d_6): δ 10.70 (s, 1H), 9.43 (s, 1H), 8.19 (s, 1H), 7.86 (s, 1H), 7.78 (d, J = 2.4 Hz, 1H), 7.56 (d, J = 8.1 Hz, 2H), 7.30 (d, J = 8.1 Hz, 2H), 7.22 (d, J = 5.4 Hz, 2H), 6.32 (bs, 1H), 4.49 (d, J = 4.5 Hz, 1H), 4.03 (q, J = 7.5, 6.9 Hz, 2H), 3.69 (bs, 1H), 3.31 (bs, 1H), 2.41 (s, 3H), 2.08–1.75 (m,4H), 1.38 (t, J = 7.2 Hz,3H), 1.26 (t, J = 9.9 Hz, 4H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 15.7, 21.6, 31.1, 34.8, 37.9, 49.9, 69.1, 84.4, 85.1, 112.8, 114.2, 116.9, 117.1, 128.8, 130.1, 132.6, 138.2, 138.6, 140.9, 141.1, 150.8, 152.4, 158.8; MS (ES$^+$) m/z calcd. for $C_{29}H_{31}N_7O_2$: 509.3; found: 510.2 (M + H$^+$); HRMS (ESI) calcd. for $C_{29}H_{32}N_7O_2$: 509.2539; found: 510.2619 (M + H$^+$); HPLC (condition A) t_R = 27.76 min, 96.5%; UPLC (condition B) t_R = 2.21 min, 97.1%.

*4.1.3. N-(3-{[9-ethyl-2-(4-ethylpiperazin-1-yl)-9H-purin-6-yl]amino}phenyl)-3-(4-methylphenyl)prop-2-ynamide dihydrochloride (**13a**)*

^{1}H NMR (300 MHz, DMSO-d_6): δ 10.92 (s, 1H), 10.77 (bs, 1H), 10.39 (s, 1H), 9.20 (s, 1H), 8.83 (s, 1H), 7.56 (d, J = 8.0 Hz, 2H), 7.44 (d, J = 8.4 Hz, 1H), 7.38–7.25 (m, 3H), 7.18 (d, J = 8.0 Hz, 1H), 4.80 (d, J = 13.6 Hz, 2H), 4.23 (q, J = 7.2 Hz, 2H), 3.56–3.43 (m, 4H), 3.18–3.11 (m, 2H), 3.03 (q, J = 10.6 Hz, 2H), 2.37 (s, 3H), 1.46 (t, J = 7.4 Hz, 3H), 1.28 (t, J = 7.6 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 11.7, 15.6, 21.6, 38.1, 44.1, 51.9, 52.3, 84.4, 85.0, 111.0, 112.8, 114.3, 117.0, 128.9, 130.1, 132.6, 138.8, 139.1, 140.8, 141.1, 150.7, 152.0, 158.5; MS (ES$^+$) m/z calcd. for $C_{29}H_{32}N_8O$: 508.3; found: 509.2 (M + H$^+$); HRMS (ESI) calcd. for $C_{29}H_{33}N_8O$: 508.2699; found: 509.2770 (M + H$^+$); HPLC (condition A) t_R = 26.66 min, 99.1%; UPLC (condition B) t_R = 1.96 min, 98.2%.

*4.1.4. N-[3-({9-ethyl-2-[4-(2-fluoroethyl)piperazin-1-yl]-9H-purin-6-yl}amino)phenyl]-3-(4-methylphenyl)prop-2-ynamide dihydrochloride (**13b**)*

^{1}H NMR (300 MHz, DMSO-d_6): δ 11.35 (bs, 1H), 10.94 (s, 1H), 10.50 (s, 1H), 8.94 (s, 1H), 8.61 (s, 1H), 7.55 (d, J = 7.8 Hz, 2H), 7.43 (d, J = 7.8 Hz, 1H), 7.39–7.30 (m, 1H), 7.32 (d, J = 8.1, 8.1 Hz, 2H), 7.16 (d, J = 8.4 Hz, 1H), 5.05–4.60 (m, 4H), 4.22 (q, J = 7.1 Hz, 2H), 3.67–3.36 (m, 4H), 3.29–3.05 (m, 2H), 2.37 (s, 3H), 1.46 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.7, 21.6, 41.7, 51.5, 56.2, 80.1, 84.3, 85.3, 111.7, 115.1, 116.3, 116.9, 129.6, 130.1, 132.7, 137.8, 139.4, 139.6, 141.1, 149.4, 150.3, 159.9; MS (ES$^+$) m/z calcd. for $C_{29}H_{31}FN_8O$: 526.3; found: 527.3 (M + H$^+$); HRMS (ESI) calcd. for $C_{29}H_{32}FN_8O$: 526.2605; found: 527.2687 (M + H$^+$); HPLC (condition A) t_R = 26.15 min, 96.9%; UPLC (condition B) t_R = 1.96 min, 95.3%.

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*4.1.5. N-[3-({9-ethyl-2-[4-(2-hydroxyethyl)piperazin-1-yl]-9H-purin-6-yl}amino)phenyl]-3-(4-methylphenyl)prop-2-ynamide (**13c**)*

^{1}H NMR (400 MHz, DMSO-d_6): δ 10.76 (s, 1H), 9.58 (s, 1H), 8.38 (s, 1H), 7.96 (s, 1H), 7.60−7.48 (m, 3H), 7.31 (d, J = 8.0 Hz, 2H), 7.25 (t, J = 8.0 Hz, 1H), 7.12 (d, J = 8.0 Hz, 1H), 4.63 (bs, 1H), 4.08 (q, J = 7.2 Hz, 2H), 4.02−3.28 (m, 6H), 2.91−2.43 (m, 6H), 2.37 (s, 3H), 1.40 (t, J = 7.2, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 15.6, 21.6, 38.1, 44.1, 53.1, 57.9, 60.1, 84.4, 85.0, 112.7, 114.2, 114.4, 117.0, 128.9, 130.1, 132.6, 138.8, 139.1, 140.8, 141.1, 150.8, 152.0, 158.6; MS (ES$^+$) m/z calcd. for $C_{29}H_{32}N_8O_2$: 524.3; found: 525.3 (M + H$^+$); HRMS (ESI) calcd. for $C_{29}H_{33}N_8O_2$: 524.2648; found: 525.2726 (M + H$^+$); HPLC (condition A) t_R = 24.54 min, 98.2%; UPLC (condition B) t_R = 1.83 min, 97.4%.

*4.1.6. N-[3-({2-[4-(dimethylamino)piperidin-1-yl]-9-ethyl-9H-purin-6-yl}amino)phenyl]-3-(4-methylphenyl)prop-2-ynamide dihydrochloride (**13d**)*

^{1}H NMR (400 MHz, DMSO-d_6): δ 10.96 (s, 1H), 10.87 (s,1H), 10.61 (s, 1H), 9.15 (s, 1H), 8.42 (s, 1H), 7.56 (d, J = 8.0 Hz, 2H), 7.52 (d, J = 8.4 Hz, 1H), 7.35 (d, J = 8.0 Hz, 1H), 7.31 (d, J = 8.0 Hz, 2H), 7.23 (d, J = 8.4 Hz, 1H), 4.23 (q, J = 7.2 Hz, 2H), 3.52−3.40 (m, 1H), 3.44 (q, J = 7.2 Hz, 2H), 2.95 (t, J = 12.4 Hz, 2H), 2.69 (d, J = 5.2 Hz, 6H), 2.37 (s, 3H), 2.13 (d, J = 10.4 Hz, 2H), 1.61 (qd, J = 12.4, 3.6 Hz, 2H), 1.47 (t, J = 7.3 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.6, 21.6, 26.0, 43.1, 62.9, 84.3, 85.3, 112.0, 115.3, 116.4, 116.9, 129.5, 130.1, 132.7, 137.3, 139.3, 139.6, 141.1, 149.3, 150.5, 150.9, 159.3; MS (ES$^+$) m/z calcd. for $C_{30}H_{34}N_8O$: 522.3; found: 523.3 (M + H$^+$); HRMS (ESI) calcd. for $C_{30}H_{35}N_8O$: 522.2856; found: 523.2886 (M + H$^+$); HPLC (condition A) t_R = 25.50 min, 99.8%; UPLC (condition B) t_R = 1.95 min, 98.9%.

*4.1.7. N-[3-({2-[3-(dimethylamino)pyrrolidin-1-yl]-9-ethyl-9H-purin-6-yl}amino)phenyl]-3-(4-methylphenyl)prop-2-ynamide (**13e**)*

^{1}H NMR (300 MHz, DMSO-d_6): δ 11.31 (bs, 1H), 10.97 (s, 1H), 10.54 (s, 1H), 9.09 (s, 1H), 8.76 (bs, 1H), 7.62−7.40 (m, 1H), 7.56 (d, J = 7.8 Hz, 2H), 7.38−7.30 (m, 1H), 7.32 (t, J = 8.0 Hz, 2H), 7.17 (d, J = 7.5 Hz, 1H), 4.21 (q, J = 7.2 Hz, 2H), 4.18−3.30 (m, 5H), 2.98−2.70 (m, 6H), 2.55−2.20 (m, 2H), 2.37 (s, 3H), 1.46 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 15.6, 21.6, 30.4, 38.0, 44.5, 46.2, 51.5, 65.5, 84.4, 84.9, 112.8, 113.8, 114.2, 116.7, 117.1, 128.8, 130.1, 132.6, 138.3, 138.6, 141.0, 141.1, 150.7, 151.9, 152.2, 157.3; MS (ES$^+$) m/z calcd. for $C_{29}H_{32}N_8O$: 508.3; found: 509.2 (M + H$^+$); HRMS (ESI) calcd. for $C_{29}H_{33}N_8O$: 508.2699; found: 509.2868 (M + H$^+$); HPLC (condition A) t_R = 24.94 min, 98.9%; UPLC (condition B) t_R = 1.87 min, 97.8%.

*4.1.8. N-[3-({9-ethyl-2-[2-(morpholin-4-yl)ethoxy]-9H-purin-6-yl}amino)phenyl]-3-(4-methylphenyl)prop-2-ynamide (**13f**)*

^{1}H NMR (300 MHz, DMSO-d_6): δ 10.87 (s, 1H), 9.97 (s, 1H), 8.35 (s, 1H), 8.19 (s, 1H), 7.70−7.58 (m, 3H), 7.50−7.22 (m, 4H), 4.47 (t, J = 6.0 Hz, 2H), 4.18 (q, J = 7.2 Hz, 2H), 3.70−3.50 (m, 4H), 2.72 (t, J = 5.9 Hz, 2H), 2.60−2.50 (m, 4H), 2.42 (s, 3H), 1.46 (t, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 10.9, 17.2, 34.1, 49.5, 52.9, 60.2, 62.2, 78.7, 81.5, 107.0, 110.3, 111.6, 112.0, 112.3, 124.8, 124.9, 127.9, 133.7, 134.5, 134.8, 136.3, 146.7, 146.8, 147.9, 156.8; MS (ES$^+$) m/z calcd. for $C_{29}H_{31}N_7O_3$: 525.3; found: 526.3 (M + H$^+$); HRMS (ESI) calcd. for $C_{29}H_{32}N_7O_3$: 525.2488; found: 526.2564 (M + H$^+$); HPLC (condition A) t_R = 25.29 min, 92.4%; UPLC (condition B) t_R = 1.87 min, 90.9%.

*4.1.9. (2E)-N-(3-{[9-ethyl-2-(4-ethylpiperazin-1-yl)-9H-purin-6-yl]amino}phenyl)-3-phenylprop-2-enamide (**13g**)*

^{1}H NMR (300 MHz, DMSO-d_6): δ 10.13 (s, 1H), 9.52 (s, 1H), 8.44 (s, 1H), 7.96 (d, J = 2.1 Hz, 1H), 7.72−7.54 (m, 3H), 7.50−7.38 (m, 4H), 7.32−7.18 (m, 2H), 6.88 (d, J = 15.9 Hz, 1H), 4.08 (q, J = 6.9 Hz, 2H), 3.92−3.60 (m, 4H), 3.42−3.18 (m, 2H), 2.58−2.30 (m, 4H), 1.40 (t, J = 7.2 Hz 3H), 1.03 (t, J = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 12.1, 15.6, 38.1, 44.6, 52.1, 52.7, 112.4, 113.9, 114.3, 116.4, 122.9, 128.1, 128.8, 129.5, 130.2, 135.2, 138.9, 139.6, 140.3, 140.8, 152.0, 158.7, 163.8; MS (ES$^+$) m/z calcd. for $C_{28}H_{32}N_8O$: 496.3; found: 497.2 (M + H$^+$); HRMS (ESI) calcd. for $C_{28}H_{33}N_8O$: 496.2699; found: 497.2697 (M + H$^+$); HPLC (condition A) t_R = 21.17 min, 97.2%; UPLC (condition B) t_R = 1.77 min, 96.4%.

*4.1.10. N-(3-{[9-ethyl-2-(4-ethylpiperazin-1-yl)-9H-purin-6-yl]amino}phenyl)-2-phenylcyclopropanecarboxamide (**13h**)*

^{1}H NMR (300 MHz, DMSO-d_6): δ 10.25 (s, 1H), 9.55 (s, 1H), 8.50 (s, 1H), 8.01 (s, 1H), 7.50 (d, J = 8.1 Hz, 1H), 7.42−7.63 (m, 2H), 7.60−7.43 (m, 4H), 7.13 (d, J = 7.8 Hz, 1H), 4.14 (q, J = 7.1 Hz, 2H), 3.85−3.76 (m, 4H), 2.59−2.42 (m, 5H), 2.48−2.39 (m, 2H), 2.19 (quintet, J = 4.2 Hz, 1H), 1.55 (quintet, J = 4.4 Hz, 1H), 1.46 (t, J = 7.2 Hz, 3H), 1.05 (t, J = 7.5 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 10.7, 15.6, 16.0, 25.4, 27.1, 38.1, 43.2, 51.7, 111.9, 113.5, 114.6, 115.9, 126.3, 126.4, 126.6, 128.9, 139.2, 139.6, 140.7, 141.2, 151.8, 152.1, 158.3, 170.1; MS (ES$^+$) m/z calcd. for $C_{29}H_{34}N_8O$: 510.3; found: 511.3 (M + H$^+$); HRMS (ESI) calcd. for $C_{29}H_{35}N_8O$: 510.2856; found: 511.2943 (M + H$^+$); HPLC (condition A) t_R = 21.82 min, 98.1%; UPLC (condition B) t_R = 1.84 min, 98.2%.

*4.1.11. N-(3-{[9-ethyl-2-(4-ethylpiperazin-1-yl)-9H-purin-6-yl]amino}phenyl)-4-(4-fluorophenyl)piperazine-1-carboxamide (**13i**)*

To a solution of **11i** (50 mg, 0.14 mmol) in pyridine (1 mL) at room temperature was added 4-nitrophenyl chloroformate (31 mg, 0.15 mmol). After the reaction mixture was stirred for 2 h, most of solvent was removed under reduced pressure and the residue was dried in vacuo to give as a yellow foamy solid which was used in the next step without further purification (69 mg). A mixture of a yellow foamy intermediate (69 mg, 0.13 mmol) and 1-(4-fluorophenyl) piperazine (117 mg, 0.65 mmol) in pyridine (1 ml) was heated at 40 °C under an argon atmosphere for 1 h. Most of solvent was removed under reduced pressure and the residue was diluted with CH_2Cl_2 (10 mL), washed with saturated sodium bicarbonate. The organic layer was separated, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by chromatography on silica gel (5% MeOH/CH_2Cl_2) to give the desired product as a pale brown solid (32 mg, 43%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.38 (s, 1H), 8.59 (s, 1H), 8.32 (s, 1H), 7.93 (s, 1H), 7.30 (d, J = 7.8 Hz, 1H), 7.16−6.95 (m, 6H), 4.07 (q, J = 7.2 Hz, 2H), 3.82−3.60 (m, 4H), 3.72−52 (m, 4H), 3.18−2.98 (m, 4H), 2.58−2.32 (m, 4H), 2.42−2.18 (m, 2H), 1.39 (t, J = 7.2 Hz, 3H), 0.99 (t, J = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 11.8, 15.3, 38.2, 44.2, 44.5, 50.3, 52.4, 52.8, 111.3, 114.1, 114.6, 115.5, 115.8, 118.4, 118.5, 129.1, 137.2, 139.4, 140.1, 147.6, 151.7, 154.9, 159.1; MS (ES$^+$) m/z calcd. for $C_{30}H_{37}FN_{10}O$: 572.3; found: 573.3 (M + H$^+$); HRMS (ESI) calcd. for $C_{30}H_{38}FN_{10}O$: 572.3136; found: 573.3196 (M + H$^+$); HPLC (condition A) t_R = 20.87 min, 98.8%; UPLC (condition B) t_R = 1.77 min, 96.7%.

*4.1.12. 3-{[9-ethyl-2-(4-ethylpiperazin-1-yl)-9H-purin-6-yl]amino}-N-(2-phenylcyclopropyl)benzamide (**14a**)*

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.39 (s, 1H), 7.78 (s, 1H), 7.74 (s, 1H), 7.56 (s, 1H), 7.46−7.36 (m, 2H), 7.32−7.21 (m, 5H), 6.45 (s, 1H), 4.13 (q, J = 7.3 Hz, 2H), 3.94−3.88 (m, 4H), 3.15−3.07 (m, 1H), 2.60−2.51 (m, 4H), 2.45 (q, J = 7.3 Hz, 2H), 2.22−2.13 (m, 1H), 1.51 (t, J = 7.3 Hz, 3H), 1.34 (q, J = 6.9 Hz, 1H), 1.29−1.23 (m, 1H), 1.13 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 12.3, 15.6, 15.7, 24.5, 33.7, 38.1, 44.7, 52.2, 52.8, 114.3, 119.9, 120.9, 123.2, 126.1, 126.3, 128.6, 128.7, 135.2, 139.1, 140.6, 141.9, 151.9, 152.1, 158.7, 168.2; MS (ES$^+$) m/z calcd. for $C_{29}H_{34}N_8O$: 510.3; found: 511.3 (M + H$^+$); HRMS (ESI)

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calcd. for $C_{29}H_{35}N_8O$: 510.2856; found: 511.2929 (M + H^+); HPLC (condition A) t_R = 23.75 min, 97.1%; UPLC (condition B) t_R = 1.75 min, 95.2%.

*4.1.13. N-(biphenyl-4-yl)-3-{[9-ethyl-2-(4-ethylpiperazin-1-yl)-9H-purin-6-yl]amino}benzamide (**14b**)*

^{1}H NMR (400 MHz, CD_3OD-d_4): δ 8.94 (s, 1H), 7.90 (d, *J* = 2.4 Hz, 1H), 7.80 (s, 1H), 7.78 (d, *J* = 2.4 Hz, 1H), 7.67–7.58 (m, 6H), 7.52–7.46 (m, 1H), 7.45–7.40 (m, 2H), 7.36–7.28 (m, 1H), 4.18 (q, *J* = 6.5 Hz, 2H), 4.1–3.8 (m, 4H), 2.72–2.64 (m, 4H), 2.62–2.54 (m, 2H), 1.49 (t, *J* = 5.8 Hz, 3H), 1.09 (t, *J* = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 11.7, 15.6, 38.2, 44.2, 51.9, 52.2, 114.4, 120.1, 120.9, 121.4, 123.3, 126.7, 127.3, 127.5, 128.9, 129.4, 135.6, 135.9, 139.3, 140.2, 140.7, 152.0, 152.1, 158.5, 166.6; MS (ES$^+$) *m/z* calcd. for $C_{32}H_{34}N_8O$: 546.3; found: 547.3 (M + H^+); HRMS (ESI) calcd. for $C_{32}H_{35}N_8O$: 546.2856; found: 547.2942 (M + H^+); HPLC (condition A) t_R = 28.17 min, 98.5%; UPLC (condition B) t_R = 2.14 min, 96.4%.

*4.1.14. N-[3-({2-[(trans-4-hydroxycyclohexyl)amino]quinazolin-4-yl}amino)phenyl]-3-(4-methylphenyl)prop-2-ynamide (**19**)*

^{1}H NMR (300 MHz, DMSO-d_6): δ 10.79 (s, 1H), 9.32 (bs, 1H), 8.34 (d, *J* = 8.4 Hz, 1H), 8.20 (s, 1H), 7.75 (d, *J* = 7.2 Hz, 1H), 7.56 (d, *J* = 7.8 Hz, 2H), 7.52 (s, 1H), 7.42–7.20 (m, 5H), 7.10 (t, *J* = 7.6 Hz, 1H), 6.33 (bs, 1H), 4.50 (d, *J* = 4.2 Hz, 1H), 3.85–3.58 (m, 1H), 2.37 (s, 3H), 1.98–1.77 (m, 4H), 1.50–1.08 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.6, 31.1, 34.8, 48.4, 69.0, 84.4, 85.2, 113.9, 115.0, 117.0, 118.4, 120.7, 123.6, 128.9, 130.1, 130.5, 132.7, 133.1, 138.9, 140.5, 141.1, 150.8, 152.9, 158.6; MS (ES$^+$) *m/z* calcd. for $C_{30}H_{29}N_5O_2$: 491.2; found: 492.2 (M + H^+); HRMS (ESI) calcd. for $C_{30}H_{30}N_5O_2$: 491.2321; found: 492.2392 (M + H^+); HPLC (condition A) t_R = 26.30 min, 95.5%; UPLC (condition B) t_R = 1.97 min, 95.7%.

*4.1.15. 3-p-Tolyl-propynoic acid {3-[5-(4-hydroxycyclohexylamino) thiazolo[5,4-d]pyrimidin-7-ylamino]phenyl}amide (**20**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.41 (s, 1H), 8.30 (s, 1H), 7.93 (s, 1H), 7.66 (s, 1H), 7.57 (s, 1H), 7.49 (d, *J* = 8.0 Hz, 2H), 7.32 (t, *J* = 8.2 Hz, 1H), 7.19 (d, *J* = 8.0 Hz, 2H), 7.15 (s, 1H), 5.04 (d, *J* = 8.0 Hz, 1H), 3.96–3.88 (m, 1H), 3.72–3.64 (m, 1H), 2.39 (s, 3H), 2.24–2.18 (m, 2H), 2.07–2.02 (m, 2H), 1.64–1.44 (m, 2H), 1.46–1.23 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.6, 30.9, 34.7, 68.9, 84.4, 85.2, 113.8, 115.0, 117.1, 117.8, 128.9, 130.1, 132.6, 138.8, 140.3, 141.0, 145.1, 150.8, 153.9, 159.5; MS (ES$^+$) *m/z* calcd. for $C_{27}H_{26}N_6O_2S$: 498.2; found: 499.2 (M + H^+); HRMS (ESI) calcd. for $C_{27}H_{27}N_6O_2S$: 499.1916; found: 499.1949 (M + H^+); HPLC (condition A) t_R = 30.61 min, 100.0%; UPLC (condition B) t_R = 2.38 min, 99.8%.

*4.1.16. 3-p-Tolyl-propynoic acid [3-(9-ethyl-9H-purin-6-ylamino) phenyl]amide (**21**)*

^{1}H NMR (400 MHz, DMSO-d_6): δ 10.84 (s, 1H), 9.91 (s, 1H), 8.38 (s, 1H), 8.34 (s, 1H), 8.26 (s, 1H), 7.60 (d, *J* = 7.6 Hz, 1H), 7.53 (d, *J* = 8.0 Hz, 2H), 7.36 (d, *J* = 7.2 Hz, 1H), 7.30–7.22 (m, 3H), 4.24 (q, *J* = 7.2 Hz, 2H), 2.35 (s, 3H), 1.42 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 15.7, 21.6, 38.7, 84.4, 85.1, 112.9, 114.8, 117.1, 117.4, 120.4, 129.0, 130.1, 132.7, 138.9, 140.5, 141.1, 141.9, 150.0, 150.9, 152.1, 152.4; MS (ES$^+$) *m/z* calcd. for $C_{23}H_{20}N_6O$: 396.2; found: 397.1 (M + H^+); HRMS (ESI) calcd. for $C_{23}H_{21}N_6O$: 397.1777; found: 397.1780 (M + H^+); HPLC (condition A) t_R = 31.47 min, 99.7%; UPLC (condition B) t_R = 2.39 min, 99.4%

*4.1.17. 3-(4-methylphenyl)-N-{3-[(6-phenylfuro[2,3-d]pyrimidin-4-yl)amino]phenyl}prop-2-ynamide (**22**)*

^{1}H NMR (300 MHz, DMSO-d_6): δ 10.90 (s, 1H), 9.95 (s, 1H), 8.45 (s, 1H), 8.20 (s, 1H), 7.84 (d, *J* = 7.8 Hz, 2H), 7.73 (d, *J* = 7.2 Hz, 1H), 7.62 (s, 1H), 7.60-7,50 (m, 4H), 7.44 (d, *J* = 7.4 Hz, 1H), 7.44–7.28 (m, 4H), 2.37 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.6, 84.4, 85.3, 99.6, 104.6, 112.3, 114.9, 116.9, 124.8, 129.4, 129.5, 129.6, 129.8, 130.1, 132.7, 139.2, 140.3, 141.1, 150.9, 151.6, 153.6, 154.9, 166.6; MS (ES$^+$) *m/z* calcd. for $C_{28}H_{20}N_4O_2$: 444.2; found: 445.4 (M + H^+); HRMS (ESI) calcd. for $C_{29}H_{20}N_4O_2$: 444.1586; found: 445.1656 (M + H^+); HPLC (condition A) t_R = 38.33 min, 96.3%; UPLC (condition B) t_R = 3.19 min, 97.6%.

4.2. Biology

4.2.1. Primary erythroid cell culture

This study was conducted with the approval of the Human Subject Research Ethics Committee/IRB, Academia Sinica (AS-IRB01-16014). Peripheral blood samples were purchased from the Taipei Blood Center. The concentrated blood was diluted 1:5 (V/V) in phosphate-buffered saline (PBS) and distributed in a thin layer on Ficoll-paque PLUS (d = 1.007 g/mL) (GE Healthcare) in a Sep-Mate column (STEMCELL). After centrifugation at 1200 g for 10 min, the cells in the inter-phase region were collected. The collected cells were washed with PBS and centrifuged at low speed three times. The remaining mononuclear cells were expanded in Phase I medium containing SFEM (STEMCELL), 100 ng/ml SCF, 20 ng/mL IL-3, 20 ng/mL IL-6, and 100 ng/mL Flt3-L at 37 °C in an incubator with 5% CO_2 for 7 days. The expanded mononuclear cells were further differentiated in Phase II medium containing SFEM (STEMCELL), 20 ng/mL SCF, 5 ng/mL IL-3, 1 U/mL EPO for another 7 days. The differentiated erythroid cells were treated with indicated compounds of different dosages with a seeding density of 5×10^5 cells/mL for another 3 days [42,43].

4.2.2. Quantitative RT-PCR

After 3 days of compound treatment, total RNA was extracted by a Quick-RNA miniprep kit (Zymo) and reverse-transcription was performed using Maxima H Minus Reverse Transcriptase (ThermoFisher Scientific) according to the manufacturer's instructions. Quantitative PCR was performed on a LightCycler Nano system with SYBR green master mix following the manufacturer's instructions (Roche). The relative quantitative RT-PCR data was normalized to the Cq number of β-actin and compared to a mock control.

4.2.3. Cell viability assay

Cell viability was assessed using AlamarBlue reagent (Invitrogen). After 3 days of treatment, 100 μL of treated cell culture was transferred into a 96-well plate and 1/10 volume of AlamarBlue reagent was added and the cell culture was incubated overnight at 37 °C. Cell viability was evaluated using a multi-label counter (Ex 530–560 nm, Em 590 nm) (PerkinElmer).

4.2.4. Western blot analysis

After 3 days of treatment, total protein was extracted by modified RIPA (50 mM Tris-HCl pH7.8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM NaV_3O_4 and 1x complete EDTA-free Protease Inhibitor Cocktail). Histone was extracted by histone extraction buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1.5 mM PMSF and 0.2 N HCl) at 4 °C overnight. Centrifuge the extracts at 13,000 rpm for 5 min and collected the supernatant. We separated 50 μg of protein extracts by 10% (for BCL11A) or 15% SDS-PAGE (for γ-globin and histone) and the resulting gel was blotted onto PVDF membrane. After blocking with 5% non-fat milk in TBST, the membrane was incubated overnight with primary antibody against total acetylated histone H4, total histone H4, γ-globin, BCL11A or β-actin at 4 °C. After incubating with horseradish peroxidase-conjugated secondary antibodies and washing the blot, the signals of indicated proteins were visualized by ECL (Omics Bio) following the manufacturer's protocol.

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4.3. Pharmacokinetics

4.3.1. Rat pharmacokinetics assay in vivo

Male Sprague–Dawley rats weighing 300–400 g each (8–12 weeks old) were obtained from BioLASCO (Taiwan Co., Ltd, Ilan, Taiwan). The animal studies were performed according to NHRI institutional animal care and committee-approved procedures. Animals were surgically prepared with a jugular-vein cannula one day before dosing and fasted overnight (for approximately 18–20 h) before dosing. Water was available *ad libitum* throughout the experiment. Food was provided at 4 h after dosing. A single 2.0 mg/kg and 10 mg/kg dose of compound, as a PEG400/DMA (80/20, v/v) solution, was separately administered to groups of 3 rats each intravenously (IV) and oral gavage (PO), respectively. Each animal received 2 or 10 mL of the dosing solution per kg of body weight for IV and PO, respectively. At 0 (before dosing), 2, 5 (IV only), 15, and 30 min and at 1, 2, 4, 6, 8, and 24 h after dosing, a blood sample (0.15 mL) was collected from each animal through the jugular-vein cannula and stored in ice (0–4 °C). Immediately after collecting the blood sample, 150 mL of physiological saline (containing 30 Units of heparin per ml) was injected into the rat through the jugular-vein cannula. Plasma was separated from the blood by centrifugation (14,000 g for 15 min at 4 °C in a Beckman Model AllegraTM 6R centrifuge) and stored in a freezer (−20 °C). All samples were analyzed for the parent drug by LC-MS/MS. Data were acquired through selected reaction ion monitoring. Plasma concentration data were analyzed with non-compartmental method.

4.3.2. Determination of water solubility

Approximately **1** mg of compounds **1** and **13** were weighed, mixed with 1 mL of water, and gently shaken at room temperature for 24 h. The mixture were then be filtered through 0.22 μm PVDF disc filter (Millipore, MA, USA) to obtain a clear aqueous solution. The compounds **1** and **13** concentrated in the aqueous solution were determined by an LC/MS methods.

4.3.3. Determination of permeability with PAMPA assay

Test solutions (150 μL) of compounds **1** and **13** (10 μM) were added to the donor well of a 96 well plate, while the acceptor wells were filled with 300 μL of PBS. Donor and acceptor plates were assembled and incubated at room temperature for 16 h. The permeation of compound across an artificial membrane with 1% Lecithin (0.45 μm PVDF, Millipore, MA, USA) was quantified by LC/MS after 16 h incubation at room temperature.

4.4. Efficacy in SCD mouse model

4.4.1. Animal studies

Sickle cell disease mice (B6; 129-Hba$^{tm1(HBA)Tow}$, Hbb$^{tm2(HBG1,HBB*)Tow}$/J) were purchased from Jackson Laboratory [44] and were bred at the AS Core, Academia Sinica. This animal study was performed according to Academia Sinica institutional animal care and utilization committee (IACUC)-approved protocol. Mice of 6–8 weeks old were used for experiments. Treatment groups received vehicle [20% (2-Hydroxypropyl)-β-cyclodextrin] plus **13a** (50 mg/kg and 100 mg/kg), which was administered by oral gavage twice per day, 5 days per week, for 4 weeks. HU (100 mg/kg in PBS) as comparison group was administered by intraperitoneal injection once daily for 5 days a week for 4 weeks.

4.4.2. Complete blood count analysis

Blood was collected by submandibular blood collection and analysis was performed using a ProCyte Dx automatic analyzer.

4.4.3. F-cell quantitation

We washed 10 μL of whole blood twice with PBS and then fixed it with 0.05% glutaraldehyde for 10 min. After fixation, the cells were washed twice with PBS and then permeabilized by adding cold 0.1% Triton X-100/PBS for 5 min. Cells were washed twice with 0.1% BSA/PBS and stained with PE-HbF antibody (BD) for 40 min at room temperature (with protection from light). Finally, the cells were washed three times with 0.1% BSA/PBS and passed through a 0.3 μM cell strainer (Falcon). F-cell % was analyzed by flow cytometry (LSRII-18P, BD) [43].

4.4.4. Hemoglobin high-performance liquid chromotography (HPLC)

We lysed 50 μL of whole blood in 200 μL ddH_2O and centrifuged it for 5 min at 13,000 rpm to extract the hemolysate. HPLC was performed according to a previously described protocol [42].

4.4.5. Blood smear

For hypoxia treatment, whole blood was incubated in a hypoxic incubator chamber (3% O_2) for 30 min. We used 2 μL of whole blood to make blood smears on slides. Slides were air-dried and then stained with Liu-stain [45] before quantifying the percentage of sickle-shaped cells.

4.4.6. RNA-Seq

RNA from 3 set of **13a** treated-cells and mock control cells were extracted by Quick-RNA miniprep kit (Zymo). The library preparation, sequencing (illumina Novasq/Hiseq 4000, Read length: PE150) were done by TOOLS according to their manufacturer's instructions. The following analysis was done by TOOLS and Bioinformatics core in Institute of Molecular Biology, Academia Sinica.

4.4.7. Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM) for at least 3 independent experiments. Statistical analysis of the raw data was performed by the 2-tailed student *t*-test. A probability of less than 0.05 ($P < 0.05$) was considered significant. (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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