ARTICLE

In vitro and *in vivo* induction of fetal hemoglobin with a reversible and selective DNMT1 inhibitor

Aidan G. Gilmartin,[1] Arthur Groy,[1] Elizabeth R. Gore,[1] Charity Atkins,[1] Edward R. Long,[1] Monica N. Montoute,[1] Zining Wu,[1] Wendy Halsey,[1] Dean E. McNulty,[1] Daniela Ennulat,[1] Lourdes Rueda,[1] Melissa B. Pappalardi,[1] Ryan G. Kruger,[1] Michael T. McCabe,[1] Ali Raoof,[2] Roger Butlin,[2] Alexandra Stowell,[2] Mark Cockerill,[2°] Ian Waddell,[2°] Donald Ogilvie,[2°] Juan Luengo,[1°] Allan Jordan[2°] and Andrew B. Benowitz[1]

[1]GlaxoSmithKline, Collegeville, Pennsylvania, PA, USA and [2]Drug Discovery Unit, Cancer Research UK Manchester Institute, University of Manchester, Alderley Park, Manchester, UK

[°]*MC current address: MediTech Media, Manchester, UK; IW current address: Charles River Laboratories, Saffron Walden, UK; DO current address: Framingham Consulting Limited, Manchester, UK; JL current address: Prelude Therapeutics, Newark, DE, USA; AJ current address: Sygnature Discovery Limited, Nottingham, UK*

Ferrata Storti Foundation

Haematologica 2021
Volume 106(7):1979-1987

ABSTRACT

Pharmacological induction of fetal hemoglobin (HbF) expression is an effective therapeutic strategy for the management of β-hemoglobinopathies such as sickle cell disease. DNA methyltransferase (DNMT) inhibitors 5-azacytidine (5-aza) and 5-aza-2′-deoxycytidine (decitabine) have been shown to induce HbF expression in both preclinical models and clinical studies, but are not currently approved for the management of hemoglobinopathies. We report here the discovery of a novel class of orally bioavailable DNMT1-selective inhibitors as exemplified by GSK3482364. This molecule potently inhibits the methyltransferase activity of DNMT1, but not DNMT family members DNMT3A or DNMT3B. In contrast with cytidine analog DNMT inhibitors, the DNMT1 inhibitory mechanism of GSK3482364 does not require DNA incorporation and is reversible. In cultured human erythroid progenitor cells, GSK3482364 decreased overall DNA methylation resulting in derepression of the γ-globin genes *HBG1* and *HBG2* and increased HbF expression. In a transgenic mouse model of sickle cell disease, orally administered GSK3482364 caused significant increases in both HbF levels and in the percentage HbF-expressing erythrocytes, with good overall tolerability. We conclude that in these preclinical models, selective, reversible inhibition of DNMT1 is sufficient for the induction of HbF, and is well-tolerated. We anticipate that GSK3482364 will be a useful tool molecule for the further study of selective DNMT1 inhibition both *in vitro* and *in vivo*.

Introduction

Adult hemoglobin (HbA) is a tetramer composed of two α-globin and two β-globin polypeptide chains ($\alpha_2\beta_2$) with four coordinated heme molecules, which is encoded by genes *HBA1*, *HBA2* and *HBB*. Various mutations in the β-globin gene *HBB* cause the β-hemoglobinopathies sickle cell disease (SCD) and β-thalassemia, the most common heritable blood disorders in the world.[1] In sickle cell anemia, the primary form of SCD, a missense mutation in both alleles of HBB results in an E6V substitution, producing sickle hemoglobin ($\alpha_2\beta^S_2$; HbS). In its deoxygenated state, the E6V mutant β-globin proteins in the HbS tetramer enable hydrophobic interactions with mutant β-globin proteins in neighboring HbS tetramers, resulting in hemoglobin aggregates. These aggregates grow into rods that distort the cell into a characteristic sickle shape, increase erythroid cell rigidity, and ultimately result in cell membrane damage and

Correspondence:

ANDREW B. BENOWITZ
Andrew.B.Benowitz@GSK.com

Received: January 30, 2020.

Accepted: June 18, 2020.

Pre-published: June 25, 2020.

https://doi.org/10.3324/haematol.2020.248658

hemolysis. These changes in the sickle erythrocytes produce a cascade of effects that result in anemia, impaired blood flow, and painful vaso-occlusive events that ultimately cause tissue ischemia and long-term damage.[2]

During fetal development and until shortly after birth, erythrocytes preferentially express an alternative hemoglobin tetramer termed fetal hemoglobin ($\alpha_2\gamma_2$; HbF) that is composed of two γ-globin chains paired with α-globin chains rather than β-globin chains. The genes encoding for γ-globin, *HBG1* and *HBG2*, lack the mutation that causes SCD. Consequently, symptoms of SCD first manifest several months after birth following the "hemoglobin switch", the transition from HbF to HbA, or to HbS in the case of SCD patients.[3] During the transition from HbF to HbA/HbS, the genes encoding for γ-globin, *HBG1* and *HBG2*, are repressed by transcriptional complexes that include GATA1, TR2/TR4, MYB, KLF1, Sox6, BCL11A, LRF, DNMT1, and HDAC1/2.[4-6] The repressor complexes cause significant chromatin remodeling, controlled in part through increased DNA methylation of *HBG1* and *HBG2* gene promoters and demethylation of the *HBB* gene promoter.[7,8] Although HbF typically decreases to a few percent of total hemoglobin shortly after birth, HbF levels can remain elevated in a rare condition called hereditary persistence of HbF (HPFH) in which mutations prevent the normal repression of γ-globin.[9] When HPFH co-occurs with the mutations that cause SCD, elevated levels of HbF can prevent the aggregation of HbS and protect erythrocytes from sickling, significantly ameliorating the disease.[10]

To date, the most important pharmacological agent for the management of SCD remains the ribonucleotide reductase inhibitor hydroxyurea (HU), which benefits patients through increasing HbF expression and reducing the incidence of vaso-occlusive crises. Although HU mitigates the clinical severity of disease for many SCD patients, there are important limitations to the clinical utility of HU. Importantly, there is typically a narrow therapeutic window between the efficacious dose of HU for beneficial HbF induction and the maximum tolerated dose typically defined by acceptable myelosuppression. As a consequence, there are variable pharmacological responses to HU in many patients.[11-13] There is therefore a desire to identify alternative agents that safely and consistently induce HbF to therapeutic levels for the treatment of SCD.

The hypomethylating agent (HMA) 5-azacytidine (5-aza) is a cytidine analog that was first demonstrated to induce HbF in an anemic baboon model.[14] It was subsequently confirmed to increase HbF in investigational studies of patients with SCD and β-thalassemia[15-18] as well as in patients with myelodysplastic syndrome and acute myeloid leukemia.[19,20] Low doses of decitabine were also confirmed to increase HbF levels in SCD patients, in some cases exceeding the maximal HbF levels observed with HU.[18] Decitabine and 5-aza are inhibitors of DNA methyltransferases (DNMT), enzymes that establish and maintain the epigenetic pattern of DNA methylation that functions in chromatin condensation and gene silencing. The catalytically active members of the DNMT family are DNMT3A, DNMT3B, and DNMT1. DNMT3A and DNMT3B establish the *de novo* pattern of DNA methylation, while DNMT1 is the primary maintenance methyltransferase that propagates the pattern of DNA methylation to daughter cells during cell division.[21] In cultured human erythroid progenitor cells (EPC)[22-24] and *in vivo* models with monkeys,[25,26] treatments with either decitabine or 5-aza decreased methylation of multiple CpG sites in the promoters of *HBG1* and *HBG2*, resulting in increased γ-globin expression and elevated HbF levels. While they are effective inducers of HbF, the decitabine and 5-aza mechanism of action relies on incorporation into DNA, and they both carry drug label warnings for genotoxicity and cytotoxicity. Decitabine and 5-aza are currently approved only for use in myelodysplastic syndromes and acute myeloid leukemia, and they are not currently approved to treat β-hemoglobinopathies.

In the current work, we describe the identification of a novel class of orally-dosed, reversible DNMT1-selective inhibitors, exemplified by GSK3482364. This molecule caused decreased DNA methylation in cultured human EPC, resulting in increased γ-globin gene expression and increased HbF. In a murine model of SCD, orally dosed GSK3482364 decreased DNA methylation in bone marrow, and increased HbF expression in erythrocytes. Notably, although GSK3482364 and decitabine were comparable in their maximal effects on DNA methylation in cells, GSK3482364 treatment resulted in lower cytotoxicity in cultured cells as well as improved *in vivo* tolerability in preclinical models. These results indicate that selective, reversible inhibition of DNMT1 is sufficient for the induction of HbF, is well-tolerated *in vivo*, and that neither irreversible DNMT1 inhibition nor inhibition of DNMT3A or DNMT3B is required for this effect.

Methods

Erythroid progenitor cell culture

Cryopreserved human bone marrow $CD34^+$ cells (AllCells) were confirmed to be sourced ethically, and their research use was in accord with the terms of the informed consents under an Institutional Review Board/Ethics Commity approved protocol. Cells were cultured according to previously described methods[23] for 7 days to generate EPC. For compound treatment studies, cell culture plates were typically incubated for 3-5 days unless otherwise indicated. In order to investigate potential drug effects on erythroid maturation to reticulocytes, $CD34^+$ cells were cultured for 19 days in a three stage protocol that models maturation into reticulocytes with continuous compound treatment. Details for cell culture and methods for cellular assays can be found in the *Online Supplemental Appendix*.

Methylation-sensitive restriction endonuclease assays

Genomic DNA was extracted from EPC or bone marrow using Zymo Quick-gDNA kits (Zymo Research). Total DNA was measured on a NanoDrop (ThermoFisher), diluted, and split into tubes containing reaction buffer +/- methylation-sensitive HpaII (New England Biolabs) for a 1-hour reaction. Reaction products were then quantitated in a 50 μL SYBR Green quantitative polymerase chain reaction (qPCR) (Applied Biosystems). -53 base pair: Primer 1: 5'-GAACTGCTGAAGGGTGCT-3', Primer 2: 5'-GACAAGGCAAACTTGACCAATAG-3'.

In vivo studies

All studies were conducted in accordance with the GlaxoSmithKline (GSK) Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee either at GSK or by the ethical review process at the institution where the work was performed. Male and female human hemoglobin transgenic mice [B6;129-HBAtm1(HBA)Tow/*HBB*tm2(*HBG1,HBB**)Tow/J Mice] (Jackson Laboratories) were 6-8 weeks of age and grouped into

approximately sex balanced groups of five to six mice. Unless otherwise noted, mice were administered vehicle (10% DMA/90% PEG400) or GSK3482364 twice daily (b.i.d.) by oral gavage on weekdays with no doses on the intervening 2-day weekend, typically for a 12 days study. Alternatively, mice were administered vehicle (phospahate buffered saline [PBS]) or decitabine thrice weekly (Monday, Wednesday, Friday) subcutaneously (s.c.) for a total of six doses. At the end of the dosing period, blood was collected into EDTA tubes for analysis, and HbF was analyzed as detailed below. For bone marrow analysis, femurs from mice were flushed with Dulbecco's PBS, cells were centrifuged briefly to pellet, and cell pellets were processed for DNA methylation analysis (as above) or for RNA analysis (detailed in the *Online Supplemental Appendix*). For bone marrow histology assessment, sternums from treated animals were formalin-fixed, paraffin embedded, sectioned and stained with hematoxylin and eosin. Complete blood counts were conducted with an Advia Hematology Analyzer (Siemens).

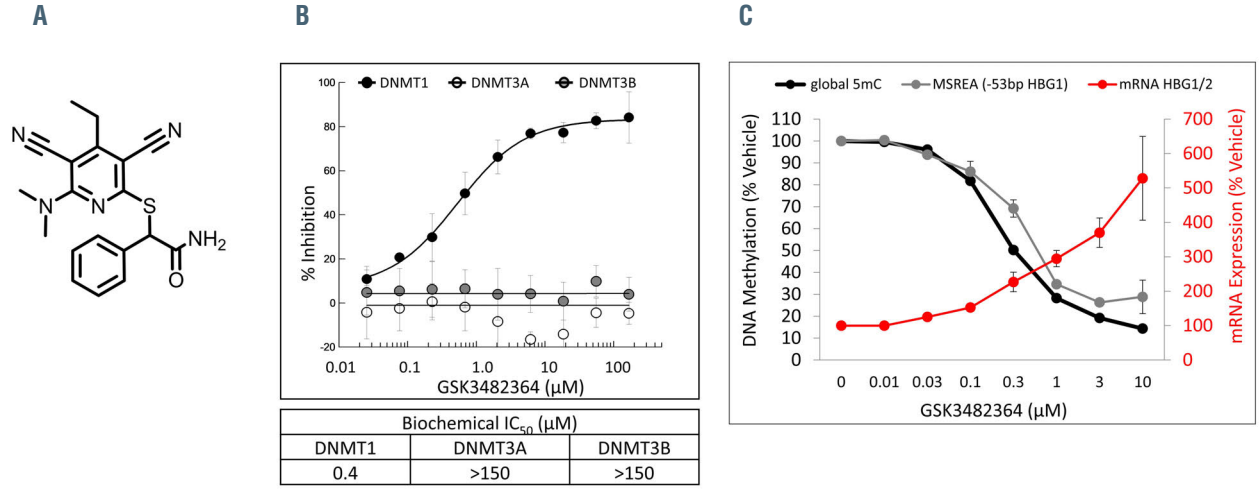
Fetal hemoglobin analysis by high-performance liquid chromatography or flow cytometry

Percentage HbF was determined by high-performance liquid chromatography (HPLC) using the D-10 Hemoglobin Analyzer (Bio-Rad). Percentage F-cells was determined by flow cytometry with a FACSCanto I (BD BioSciences) using a mouse monoclonal anti-human HbF antibody conjugated to allophycocyanin (APC) (Life Technologies). For analysis of human cell cultures, the nuclear stain Syto16 (Life Technologies) was used to distinguish EPC (Syto16^{high}) from enucleated reticulocytes (Syto16^{low}). For analysis of mouse whole blood samples, Syto16 was used to distinguish mature red blood cells (Syto16^{negative}) from reticulocytes (Syto16^{low}). Flow cytometry data were analyzed with FlowJo v7 software (Tree Star). One-way ANOVA of data was employed to determine significance of changes relative to vehicle treated samples (Graphpad Prism v7).

Results

A high-throughput screen was conducted to identify novel biochemical inhibitors of DNMT1 methyltransferase activity. Briefly, approximately 1.8 million compounds were assayed in a scintillation proximity assay measuring the transfer of a radioactive methyl group by recombinant DNMT1 to a hemi-methylated 40-mer DNA substrate (Stowell, A. *et al.* manuscript in preparation; Pappalardi, M. *et al.* manuscript submitted). Screening hits were further profiled to eliminate compounds that were also inhibitors of DNMT3A or DNMT3B or that were non-specific DNA binders. From this screen and a subsequent medicinal chemistry campaign that employed non-radioactive breaklight format methyltransferase assays [27] (see the *Online Supplementary Appendix*), a class of potent biochemical inhibitors of DNMT1 that does not inhibit either DNMT3A or DNMT3B was identified, as exemplified by GSK3482364[28] (Figure 1A and B). GSK3482364 was confirmed to be a reversible inhibitor of DNMT1 using a jump dilution protocol in which a preincubated complex of DNMT1 and compound was rapidly diluted 100-fold with the addition of substrates, and recovery of DNMT1 activity was established (Pappalardi, M. *et al.*). Since the mechanism of action of decitabine and 5-aza requires incorporation into DNA before covalently trapping the DNMT proteins,[29,30] these compounds do not show inhibition of DNMT1 in this biochemical assay.

In cultured EPC, GSK3482364 treatment produced dose-dependent DNA hypomethylation. Bone marrow derived CD34+ cells were expanded and differentiated over 7 days into EPC, previously characterized as expressing high CD71 and increasing CD235.[23] Day 7 EPC were treated with a dose range of GSK3482364 for 5 additional days, after which DNA was harvested. Compound effects on EPC DNA methylation were measured by enzymatically digesting DNA into nucleosides and measuring the ratio of methylcytosine to total cytosine by mass spectrometry. GSK3482364 treatment reduced 5-methylcytosine levels in a dose-dependent manner with an IC_{50} of 0.24 μM (Figure 1C). An additional orthogonal assay was also developed to measure the level of methylation on specific cytosines located at positions -53 basepair (bp) relative to transcription start sites of both *HBG1* and *HBG2*. As has been previously reported, these are among a number of highly



Biochemical IC_{50} (μM)		
DNMT1	DNMT3A	DNMT3B
0.4	>150	>150

Figure 1. Biochemical and cellular inhibition of DNMT1 with GSK3482364. (A) Structure of DNMT1 inhibitor GSK3482364. (B) Representative data for GSK3482364 activity in biochemical methyltransferase assays with DNMT1 (black), DNMT3A (white), or DNMT3B (gray). (C) Effect of GSK3482364 on 5-methylcytosine (black), *HBG1/HBG2* -53bp methylation (gray), and *HBG1/HBG2* mRNA (red) in erythroid progenitor cells treated for 5 days.

methylated cytosines in the promoters of *HBG1* and *HBG2* that become hypomethylated in response to DNMT1 gene silencing in EPC.[31,38] We have confirmed the hypomethylation of a number of the same methylcytosines in response to DNMT1 inhibition by bisulfite sequencing, including the -53 bp methylcytosine for *HBG1* (*Online Supplementary Figure S1*). Consistent with the findings in the global methylcytosine assay, methylation of the -53 bp HBG1/HBG2 cytosine residues was decreased for cells treated with GSK3482364 with an IC_{50} of 0.33 μM (Figure 1C).

In order to determine the effect of decreased *HBG1* and *HBG2* promoter methylation on gene expression, mRNA from treated cells was measured by reverse transcritase qPCR (RT-qPCR) with an assay that detects both *HBG1* and *HBG2* mRNA (99% genetic identity). After 5 days of treatment, GSK3482364 caused a dose-dependent increase in *HBG1/HBG2*, raising levels 5.3-fold compared to vehicle treated cells (Figure 1C). Increases in globin gene expression correlated inversely with DNA methylation levels; at the cellular IC50 of the -53 bp methylcytosine assay, 0.33 μM GSK3482364 treatment caused more than a 2-fold increase in *HBG1/HBG2* mRNA.

In order to further characterize the effect of GSK3482364 on cultured EPC, HbF protein expression was measured by enzyme-linked immunosorbent assay (ELISA). Day 7 EPC cultured in the presence of GSK3482364 for 5 additional days increased HbF 2-fold at 0.56 μM, and up to a maximum of 300% of vehicle-treated levels (Figure 2A). In comparison, decitabine treatment increased HbF 2-fold at 0.04 μM, and up to a maximum of 250% of vehicle. Notably, decitabine treatment reproducibly generated a bell-shaped curve response in the ELISA assay, consistent with cytotoxicity at high concentrations which was confirmed in parallel cell growth assays (Figure 2B). At high concentrations

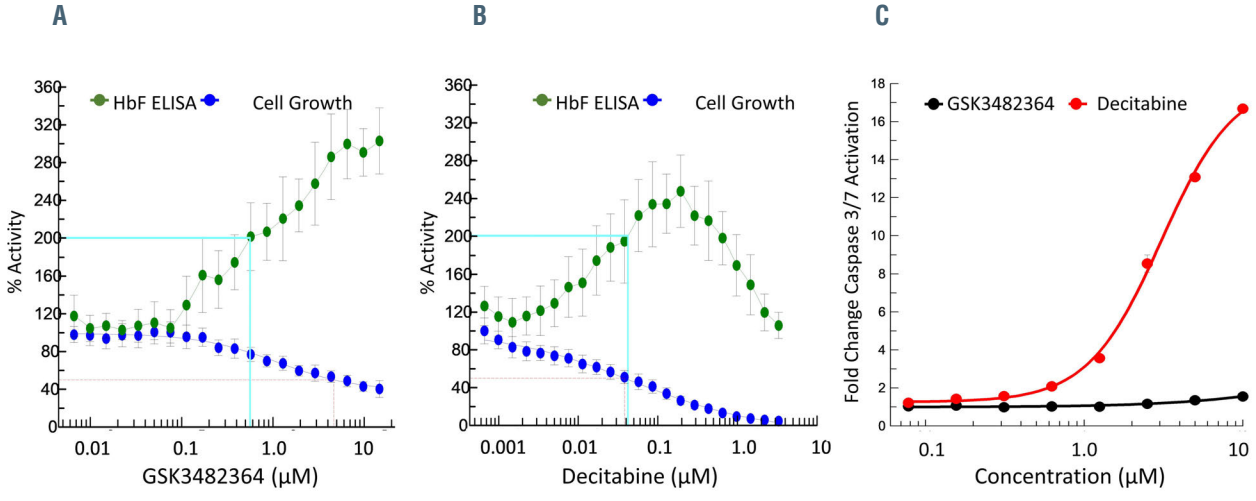


Figure 2. Effect of GSK3482364 and decitabine on fetal hemoglobin, cell growth, and caspase activation in erythroid progenitor cells. Erythroid progenitor cells (EPC) were treated for 5 days with GSK3482364 (A) or decitabine (B) and then assayed for change in fetal hemoglobin (HbF) by enzyme-linked immunosorbent assay (green) or cell growth (blue); data represent the mean +/- standard deviation for n=20 assays. Concentrations resulting in 200% HbF and 50% growth inhibition are indicated with light blue and gray lines, respectively. (C) Caspase-Glo assay measure caspase 3/7 activation in EPC after 3 days treatment with GSK3482364 (black) or decitabine (red).

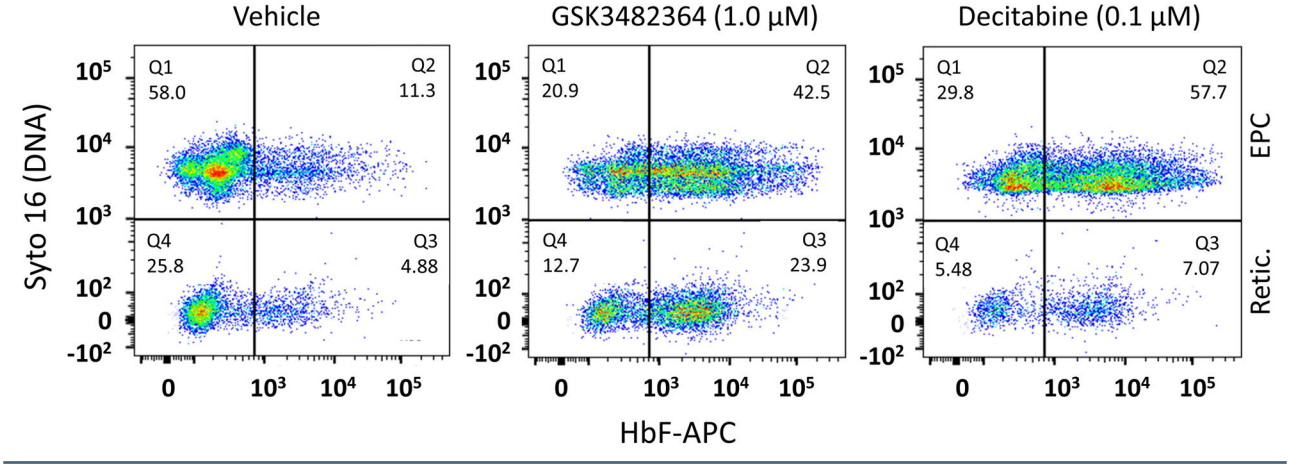


Figure 3. Effects of GSK3482364 and decitabine on fetal hemoglobin levels and reticulocyte differentiation after 18 days in cell culture. $CD34^+$ bone marrow hematopoietic stem cells were cultured for 18 days in the presence of GSK3482364 or decitabine, with three phases of media exchange promoting erythroid differentiation into reticulocytes. Day 18 cells were stained with Syto16 (nucleic acids) and anti-fetal hemoglobin (anti-HbF) (APC) antibody. Erythroid progenitor cells are $Syto16^{high}$ events that retain nuclei, and reticulocytes are $Syto16^{low}$ events that lack nuclei.

representing 25x the concentrations that increased HbF 2-fold, GSK3482364 and decitabine caused, respectively, 58% and 90% cell growth inhibition of EPC over 5 days of cell culture.

In order to better characterize the relative acute cytotoxicity of GSK3482364 and decitabine, caspase 3/7 activity was measured in EPC treated with compounds for 2 days. GSK3482364 and decitabine increased cleavage of a caspase 3/7 substrate by 1.5-fold and 16.5-fold, respectively (Figure 2C) at the highest test concentration of 10 μM. Comparing at equivalent relative potencies 18-fold above the concentration that causes 2-fold HbF induction, GSK3482364 (10 μM) and decitabine (0.7 μM) increased caspase activity by 50% and 120%, respectively. In agreement with the cell growth assays, these data indicate potentially differentiated cytotoxicity between the two compounds.

It has previously been observed that treatment with cytidine analog HMA including 5-aza, decitabine, and zebularine cause destabilization of DNMT1 and to a lesser extent DNMT3A and DNMT3B, through a ubiquitin-dependent mechanism.[32-36] There is a debate whether the effects of the cytidine analog HMA on DNMT3A and DNMT3B protein levels are due to direct inhibition and DNA adduct formation, or are instead an indirect outcome of DNMT1 inhibition. However, evidence that zebularine-incorporated oligonucleotides can trap and biochemically inhibit DNMT1, DNMT3A, and DNMT3B[37, 38] suggests that cytidine analog HMA may not be DNMT1-selective. In our study of EPC treated for 24 hours with decitabine, both DNMT1 and to a lesser extent DNMT3A levels were shown to decrease while DNMT3B was largely unaffected (*Online Supplementary Figure S2*). Treatment with GSK3482364 also caused a decrease in DNMT1 protein, although less than decitabine, and had no effect on either DNMT3A or DNMT3B levels. The mechanism by which GSK3482364 causes DNMT1 protein levels to decrease remains a matter of investigation.

In order to characterize longer treatment effects of GSK3482364 on cellular expansion and differentiation, bone marrow CD34+ cells were expanded and differentiated in a three-phase erythroid cell differentiation protocol[39] over 18 days in the presence of GSK3482364, decitabine, or vehicle. Fresh compound or vehicle were added at each stage of media exchange. On day 18, cells were stained with Syto16 to label nucleic acids and to distinguish mature enucleated reticulocytes from less mature erythroblasts. Cells were also stained with an anti-HbF antibody and analyzed by flow cytometry. On day 18, it was found that 31% of the vehicle-treated cells had matured into enucleated reticulocytes (Syto16^{low}), distinguishable from the less mature erythroblasts (Syto16^{high}). Consistent with HbF ELISA results, GSK3482364 treatment (1.0 μM) for 18 days caused a >4-fold increase in HbF-positive cells compared to vehicle (Figure 3). Moreover, the resulting fraction of cells maturing into reticulocytes was comparable to or slightly higher than vehicle treatment, indicating that treated cells were not arrested earlier in erythropoiesis. Decitabine (0.1 μM) treatment caused a similar increase in HbF-positive cells as GSK3482364 (1 μM) when compared to vehicle, but also caused a marked decrease in the proportion of cells maturing into reticulocytes, reflecting delayed or arrested cellular maturation at this concentration.

In order to measure the *in vivo* HbF induction activity of GSK3482364, the Townes mouse model of SCD[40] was employed in which mouse α- and β-globin genes were replaced with human genes *HBA1*, *HBG1*, and *HBB* including the E6V sickle mutation. Mice in this model express HbF during fetal development, repress HbF shortly after birth, and experience cell sickling and multiple organ pathologies analogous to sickle cell disease. GSK3482364 was administered orally (p.o.) to 6-8 week old mice b.i.d. at 10 or 50 mg/kg for 12 days (weekday dosing only). At the end of dosing, HbF levels were measured in whole blood by an HPLC method, and F-cells were measured by flow cytometry. Compared with vehicle treated animals, both dose levels of GSK3482364 caused significant increases in both HbF and F-cells (Figure 4). At the 50 mg/kg dose, HbF increased 10.3-fold and F-cells increased 8.4-fold relative to vehicle treatment.

In a subsequent study of murine SCD model mice treated with GSK3482364, blood was analyzed for HbF, and femoral bone marrow was harvested on day 12 to measure changes in DNA methylation and RNA expression. Treatment with GSK3482364 caused dose-dependent increases in HbF protein in whole blood by up to 9-fold *versus* vehicle treatment (Figure 5A). No further increase in HbF was observed between 33.3 mg/kg and 100 mg/kg doses, suggesting a plateau of activity that is consistent with a plateau of the exposure of GSK3482364 (data not shown). In bone marrow samples, globin gene expression and *HBG1* promoter methylation were evaluated. In order to assess effects on DNA methylation, the -53 bp *HBG1* methylation-sensitive restriction endonuclease assay was employed. Bone marrow from treated animals showed a dose-dependent decrease in methylation at this site by 25% compared to vehicle-treated animals (Figure 5A). In the same samples, *HBG1* mRNA levels increased in all dose groups by up to 29-fold (Figure 5B). In contrast, *HBB* and *HBA1* mRNA levels were not significantly changed in dose groups compared to baseline; this is consistent with the low baseline promoter methylation of these highly expressed genes.

In a parallel study, transgenic mice were dosed subcutaneously with decitabine at doses of 0.2, 0.4, or 0.8 mg/kg

Table 1. Effects of GSK3482364 twice daily *in vivo* on blood cell counts and bone marrow DNA methylation.

Study 1-GSK3482364	RBC (x10^6/μl)	*Platelets* (x10^3/μl)	*Neutrophils* (x10^3/μl)	*Lymphocytes* (x10^3/μl)	*Monocytes* (x10^3/μl)	*% Change DNA* Methylation
Vehicle	7.7 ± 1.0	819 ± 145	3.7 ± 2.1	14.9 ± 3.9	0.7 ± 0.5	0 ± 4.0
3.7 mg/kg	7.4 ± 0.3	757 ± 150	2.9 ± 2.4	12.6 ± 5.6	0.5 ± 0.2	-2.2 ± 2.7
11.1 mg/kg	8.1 ± 1.0	854 ± 107	2.4 ± 0.8	10.7 ± 3.3	0.5 ± 0.4	-14.7 ± 5.7**
33.3 mg/kg	7.0 ± 1.0	733 ± 90	3.2 ± 2.3	9.7 ± 1.8	0.4 ± 0.2	-25.4 ± 8.1**
100 mg/kg	7.6 ± 1.4	678 ± 210	2.3 ± 1.2	11.0 ± 4.9	0.3 ± 0.3	-23.9 ± 9.5**

Mean +/- standard deviation are shown. Asterisks indicate significance by 1-way ANOVA. (**P<0.001)

thrice weekly for a total of six doses and terminated after the final dose on day 12. In previous studies, lower doses of decitabine administered daily resulted in no significant HbF induction in a 2-week timeframe, while higher doses administered daily or every other day were not tolerated (*Online Supplemental Table S1*). In the current decitabine study, mean HbF levels were increased by up to 2-fold at the 0.4 mg/kg dose, and by up to 4.4-fold at the 0.8 mg/kg (Figure 5A). However, 0.8 mg/kg was not tolerated based on the death of two of six animals. Bone marrow samples from surviving decitabine-treated animals exhibited decreased -53 bp DNA methylation by 4%, 9%, and 13% respectively in the 0.2, 0.4, and 0.8 mg/kg dose groups (Figure 5A).

As further characterization of the effects of dosing with GSK3482364 in the SCD mouse model, complete blood counts were taken at all doses. Compared to vehicle-treated animals, GSK3482364-treated SCD mice had no remarkable changes in peripheral blood cell counts or red cell indices (Table 1). Consistent with peripheral blood cell counts, histopathology of sternal bone marrow from the high dose group of animals treated with GSK3482364 (100 mg/kg) demonstrated no overt abnormalities in marrow cellularity or hematopoietic cell composition (six of six mice; Figure 6). Notably, in the comparable 12 day study, dose levels of decitabine that caused HbF induction (≥0.2 mg/kg) also caused dose-dependent decreases in multiple blood cell counts (*Online Supplemental Table S2*), indicating that these dose regimens were all cytotoxic to some extent in the SCD mice. While lower doses of decitabine (0.1-0.2 mg/kg) administered either daily or every other day were poorly effective at inducing HbF in our previous 12 day studies (*Online Supplemental Table S1*), we acknowledge that alternative non-cytotoxic dose regimens or longer study durations might better optimize the efficacy and tolerability of decitabine in these mice.

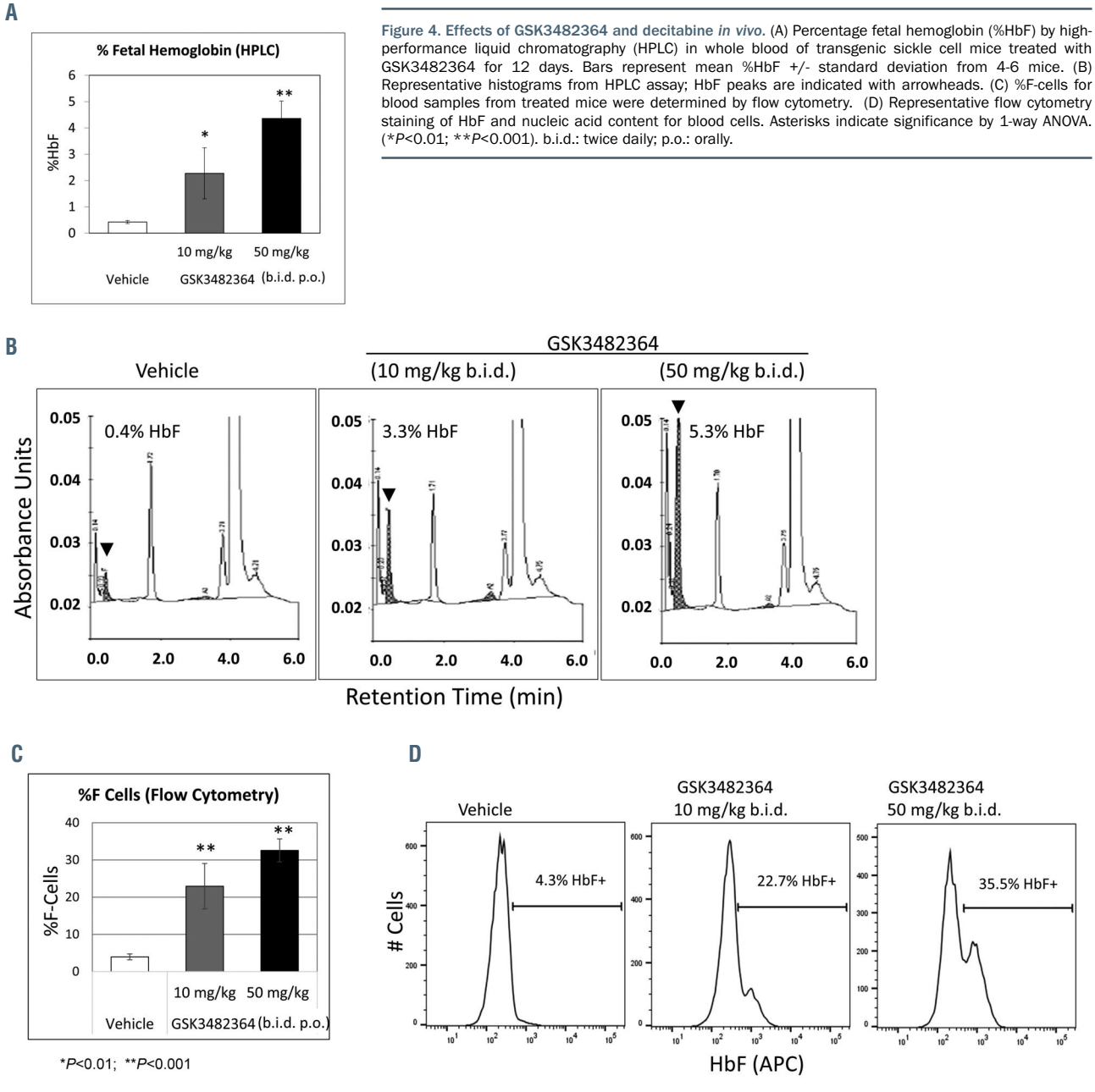
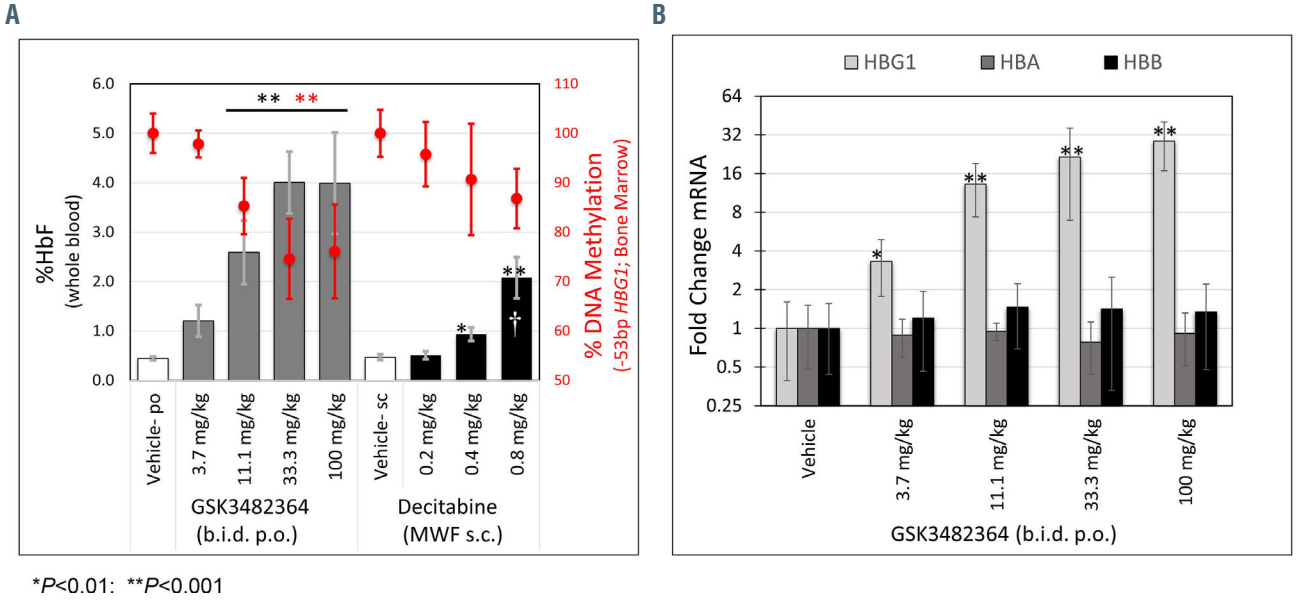


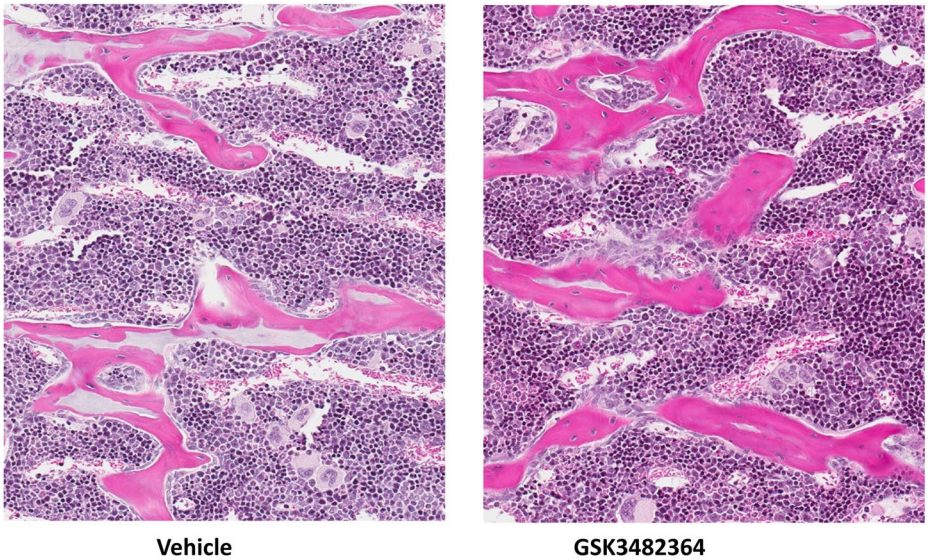
Figure 4. Effects of GSK3482364 and decitabine *in vivo*. (A) Percentage fetal hemoglobin (%HbF) by high-performance liquid chromatography (HPLC) in whole blood of transgenic sickle cell mice treated with GSK3482364 for 12 days. Bars represent mean %HbF +/- standard deviation from 4-6 mice. (B) Representative histograms from HPLC assay; HbF peaks are indicated with arrowheads. (C) %F-cells for blood samples from treated mice were determined by flow cytometry. (D) Representative flow cytometry staining of HbF and nucleic acid content for blood cells. Asterisks indicate significance by 1-way ANOVA. (*P<0.01; **P<0.001). b.i.d.: twice daily; p.o.: orally.

In order to further assess the effect of GSK3482364 on alternative dose schedules, SCD mice were dosed as previously described or on a reduced frequency schedule (b.i.d for 3 weekdays). Induction of HbF was observed to be approximately equivalent for mice dosed on both schedules (*Online Supplemental Figure S3A*). Since both dose regimens were equally efficacious, we next tested the effect of dosing GSK3482364 on the reduced frequency schedule for either 2 or 4 weeks to determine the effect of longer dose duration on efficacy and tolerability. Both 2- and 4-week dosing regimens were well-tolerated with no effects on body weight compared to control mice, and all mice exhibited significant gain in HbF (*Online Supplemental Figure S3B*). Following 4 weeks of dosing, there was a small increase in percentage HbF induction compared to animals dosed for only 2 weeks, but this increase was not found to be significant. As with previous studies, peripheral blood cell counts measured after 4 weeks indicated no remarkable differences from vehicle treated animals in any parameters. These results demonstrate that GSK3482364 can robustly induce HbF *in vivo* with intermittent dosing, and that it is well-tolerated for up to 4 weeks without causing evidence of myelosuppression in mice.



*P<0.01: **P<0.001

Figure 5. Effects of GSK3482364 and decitabine *in vivo*. (A) Bars represent mean percentage fetal hemoglobin (%HbF) +/- standard deviation (SD), measured by high-performance liquid chromatography (HPLC), in whole blood of sickle cell mice treated with GSK3482364 (twice daily [p.o.]) or decitabine (thrice weekly [s.c.]) for 12 days. Red circles represent mean +/- SD of DNA methylation (-53bp *HBG1* methylcytosine) from bone marrow of 4-6 mice. (B) Bone marrow was further evaluated for *HBG1*, *HBA*, and *HBB* mRNA levels by reverse transcriptase quantitative polymerase chain reaction. In all cases levels are normalized to vehicle-treated control levels. Asterisks indicate significance by 1-way ANOVA (*P<0.01; **P<0.001).



Vehicle

GSK3482364
100 mg/kg (b.i.d. p.o.)
-23.9% DNA methylation

Figure 6. Effects of GSK3482364 on bone marrow cellularity. Representative histopathology images (15x) of sternum bone marrow for mice following 12 days dosing with vehicle or GSK3482364. b.i.d.: twice daily; p.o.: orally.

Discussion

As potential agents for the management of SCD, the DNMT inhibitors 5-aza and decitabine have demonstrated significant induction of HbF levels in preclinical and clinical studies.[14, 18, 23, 41-45] The robust HbF induction caused by these compounds is explained both by the high proliferative index of bone marrow progenitor cells and by transitional changes in DNA methylation that they undergo during erythropoiesis. Changes in DNA methylation occur during key determining events in hematopoiesis.[46] During erythropoiesis a rapid decrease in global DNA methylation marks a commitment toward erythropoietin dependence and the expression of erythroid specific master regulators GATA1 and KLF1.[8, 47] As hematopoietic stem cells commit to erythropoiesis, DNMT1 becomes the dominantly expressed DNMT with the primary role of maintaining DNA methylation and regulating globin gene transcription.[8, 31, 48] For EPC in adult bone marrow, key cytosine residues in the promoter region of *HBG1* and *HBG2* become highly methylated while the β-globin *HBB* gene promoter is largely unmethylated, corresponding to the increased expression of β-globin and the repression of γ-globin expression.[31, 49]

Although the known HMA decitabine and 5-aza have proven to be valuable probes to study the biology of DNMT methylation, and partial and complete DNMT1 knockout studies in animals have established a critical role for DNMT1 in cellular differentiation and stem cell maintenance, it has not been previously possible to study the *in vitro* and *in vivo* cellular and hematopoietic effects of reversible and selective DNMT1 inhibition. GSK3482364 represents a novel class of DNMT1-selective inhibitors that are mechanistically distinct from other HMA. In cellular studies, GSK3482364 treatment caused DNA hypomethylation and HbF induction with maximal effects that were approximately equivalent to decitabine treatment. However, at concentrations of GSK3482364 and of decitabine that produced equivalent HbF induction, decitabine was observed to consistently cause more cell growth inhibition. In an *in vitro* model of erythropoiesis, expanding and differentiating from $CD34^+$ cells to enucleated reticulocytes, GSK3482364 and decitabine caused equivalent increases in HbF-cells, but GSK3482364 treatment resulted in a larger proportion of cells maturing into HbF expressing reticulocytes. In a transgenic mouse model of SCD, the effects of orally-dosed GSK3482364 on bone marrow DNA methylation and erythrocyte HbF elevation exceeded the corresponding effects of decitabine at tolerated doses over a 12-day period. Examination of complete blood counts and bone marrow cellularity from *in vivo* studies with GSK3482364 suggests that the effects of this compound on DNA methylation in the bone marrow were well-tolerated without evidence of other adverse hematological effects.

Chronic intermittent pharmacologic inhibition of DNMT1 does not appear to fully phenocopy the effects of permanent DNMT1 deletion. In our mouse studies, the limited impact on bone marrow cellularity or blood cell populations appears to indicate that repeat daily doses of GSK3482364 were tolerated by hematopoietic stem and progenitor cells. Notably, in our *in vivo* studies we did not observe the significant increase in platelets that has been reported in clinical studies with low dose decitabine and that is attributed to effects of hypomethylation in promoting megakaryocyte maturation.[18, 45, 50] Since the increase in platelets does not appear to be captured in our mouse model, we cannot currently draw any conclusions about potential differentiating effects on platelets for GSK3482364 or related compounds. Future studies in non-human primate models, where multiple HbF inducers were initially characterized, are warranted to address this and other questions about the optimal dosing regimens for this class of DNMT1 selective inhibitors. The differential cellular biology and *in vivo* pharmacology observed with GSK3482364 as compared to decitabine suggest that this may be a useful tool molecule to study the selective, reversible inhibition of DNMT1 in hematopoiesis and for the elevation of HbF in erythrocytes.

Disclosures

AGG, AG, EG, CA, EL, MM, ZW, WH, DM, DE, LR, MP, MTM, RGK, JL, and ABB are current or former employees of GlaxoSmithKline; AR, RB, AS, MC, DO, IW, and AJ are current or former employees of CRUK Manchester Institute; patent US20190194166 pertains to compounds discussed in this manuscript.

Contributions

AGG, AG, EG, CA, EL, MNM, ZW, WH, DM, DE, AS, MP, and ABB designed or performed experiments; AR, RB, MC, AJ, LR, DO,IW, MTM, RGK, and JL were involved in the identification and synthesis of the lead compound; AGG wrote the manuscript and all authors read and approved the final version of the manuscript.

Acknowledgments

The authors wish to thank Karen Evans, Rakesh Nagilla, Elizabeth Rivera, Karen Lynch, Denise Depagnier, Chris Traini, Leonard Azzarano, and Shanker K. Sundaram for contributions to this work. Additionally, we wish to thank Jerry Adams, Dirk Heerding, Chris Carpenter, Steve Pessagno, Philip Chapman, Charlotte Burt, Martyn Bottomley, Kristin Goldberg, and Chris Kershaw for their contributions to the identification of GSK3482364. Work conducted at the Cancer Research UK Manchester Institute was wholly funded by Cancer Research UK (Grant numbers C480/A1141 and C5759/A17098).

Funding

Work conducted at the Cancer Research UK Manchester Institute was wholly funded by Cancer Research UK (Grant numbers C480/A1141 and C5759/A17098).

References

1. Weatherall D. Beginnings: The Molecular Pathology of Hemoglobin. Molecular Hematology: Third Edition, 2010:1-18.
2. Kato GJ, Piel FB, Reid CD, et al. Sickle cell disease. Nat Rev Dis Primers. 2018;4:18010.
3. Sankaran VG, Xu J, Orkin SH. Advances in the understanding of haemoglobin switching: Review. Br J Haematol. 2010;149(2): 181-194.
4. Sankaran VG, Orkin SH. The switch from fetal to adult hemoglobin. Cold Spring Harb Perspect Med. 2013;3(1):a011643.
5. Suzuki M, Yamamoto M, Engel JD. Fetal globin gene repressors as drug targets for molecular therapies to treat the β-globinopathies. Mol Cell Biol. 2014;34(19):

3560-3569.
6. Masuda T, Wang X, Maeda M, et al. Transcription factors LRF and BCL11A independently repress expression of fetal hemoglobin. Science. 2016;351(6270):285-289.
7. Mabaera R, West RJ, Conine SJ, et al. A cell stress signaling model of fetal hemoglobin induction: what doesn't kill red blood cells may make them stronger. Exp Hematol. 2008;36(9):1057-1072.
8. Shearstone JR, Pop R, Bock C, et al. Global DNA demethylation during mouse erythropoiesis in vivo. Science. 2011;334(6057):799-802.
9. Higgs DR, Wood WG. Genetic complexity in sickle cell disease. Proc Natl Acad Sci U S A. 2008;105(33):11595-11596.
10. Perrine RP, Brown MJ, Clegg JB, Weatherall DJ, May A. Benign sickle-cell anaemia. Lancet. 1972;2(7788):1163-1167.
11. Lanzkron S, Strouse JJ, Wilson R, et al. Systematic review: hydroxyurea for the treatment of adults with sickle cell disease. Ann Intern Med. 2008;148(12):939-955.
12. Steinberg MH, Barton F, Castro O, et al. Effect of hydroxyurea on mortality and morbidity in adult sickle cell anemia: Risks and benefits up to 9 years of treatment. JAMA. 2003;289(13):1645-1651.
13. Maier-Redelsperger M, de Montalembert M, Flahault A, et al. Fetal hemoglobin and F-cell responses to long-term hydroxyurea treatment in young sickle cell patients. Blood. 1998;91(12):4472-4479.
14. DeSimone J, Heller P, Hall L, Zwiers D. 5-Azacytidine stimulates fetal hemoglobin synthesis in anemic baboons. Proc Natl Acad Sci U S A. 1982;79(14):4428-4431.
15. Dover GJ, Charache SH, Boyer SH, Talbot J, Smith KD. 5-Azacytidine increases fetal hemoglobin production in a patient with sickle cell disease. Prog Clin Biol Res. 1983;134:475-488.
16. Ley TJ, Anagnou NP, Noguchi CT, et al. DNA methylation and globin gene expression in patients treated with 5-azacytidine. Prog Clin Biol Res. 1983;134:457-474.
17. Lowrey CH, Nienhuis AW. Brief report: treatment with azacitidine of patients with end-stage +|- thalassemia. N Engl J Med. 1993;329(12):845-848.
18. Saunthararajah Y, Hillery CA, Lavelle D, et al. Effects of 5-aza-2′-deoxycytidine on fetal hemoglobin levels, red cell adhesion, and hematopoietic differentiation in patients with sickle cell disease. Blood. 2003;102(12):3865-3870.
19. Reinhardt D, Haase D, Schoch C, et al. Hemoglobin F in myelodysplastic syndrome. Ann Hematol. 1998;76(3-4):135-138.
20. Lubbert M, Ihorst G, Sander PN, et al. Elevated fetal haemoglobin is a predictor of better outcome in MDS/AML patients receiving 5-aza-2'-deoxycytidine (Decitabine). Br J Haematol. 2017;176(4):609-617.
21. Lyko F. The DNA methyltransferase family: a versatile toolkit for epigenetic regulation. Nat Rev Genet. 2018;19(2):81-92.
22. Bradner JE, Mak R, Tanguturi SK, et al. Chemical genetic strategy identifies histone deacetylase 1 (HDAC1) and HDAC2 as therapeutic targets in sickle cell disease. Proc Natl Acad Sci U S A. 2010;107(28):12617-12622.
23. Li H, Xie W, Gore ER, et al. Development of phenotypic screening assays for gamma-globin induction using primary human bone marrow day 7 erythroid progenitor cells. J Biomol Screen. 2013;18(10):1212-1222.
24. Torrealba-de Ron AT, Papayannopoulou T, Knapp MS, et al. Perturbations in the erythroid marrow progenitor cell pools may play a role in the augmentation of HbF by 5-azacytidine. Blood. 1984;63(1):201-210.
25. Akpan I, Banzon V, Ibanez V, et al. Decitabine increases fetal hemoglobin in Papio anubis by increasing gamma-globin gene transcription. Exp Hematol. 2010;38(11):989-993.e1.
26. Chin J, Singh M, Banzon V, et al. Transcriptional activation of the gamma-globin gene in baboons treated with decitabine and in cultured erythroid progenitor cells involves different mechanisms. Exp Hematol. 2009;37(10):1131-1142.
27. Wood RJ, McKelvie JC, Maynard-Smith MD, Roach PL. A real-time assay for CpG-specific cytosine-C5 methyltransferase activity. Nucleic Acids Res. 2010;38(9):e107.
28. Adams ND, Benowitz AB, Rueda Benede ML, et al. inventors. Substituted Pyridines as Inhibitors of DNMT1. Patent WO/2017/216727. 2017.
29. Santi DV, Norment A, Garrett CE. Covalent bond formation between a DNA-cytosine methyltransferase and DNA containing 5-azacytosine. Proc Natl Acad Sci U S A. 1984;81(22):6993-6997.
30. Oka M, Meacham AM, Hamazaki T, et al. De novo DNA methyltransferases Dnmt3a and Dnmt3b primarily mediate the cytotoxic effect of 5-aza-2′-deoxycytidine. Oncogene. 2005;24(19):3091-3099.
31. Mabaera R, Richardson CA, Johnson K, et al. Developmental- and differentiation-specific patterns of human γ- and β-globin promoter DNA methylation. Blood. 2007;110(4):1343-1352.
32. Ghoshal K, Datta J, Majumder S, et al. 5-Aza-Deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. Mol Cell Biol. 2005;25(11):4727-4741.
33. Robaina MC, Mazzoccoli L, Arruda VO, et al. Deregulation of DNMT1, DNMT3B and miR-29s in Burkitt lymphoma suggests novel contribution for disease pathogenesis. Exp Mol Pathol. 2015;98(2):200-207.
34. Palii SS, Van Emburgh BO, Sankpal UT, Brown KD, Robertson KD. DNA methylation inhibitor 5-aza-2′-deoxycytidine induces reversible genome-wide DNA damage that is distinctly influenced by DNA methyltransferases 1 and 3B. Mol Cell Biol. 2008;28(2):752-771.
35. Cheng JC, Yoo CB, Weisenberger DJ, et al. Preferential response of cancer cells to zebularine. Cancer Cell. 2004;6(2):151-158.
36. Patel K, Dickson J, Din S, et al. Targeting of 5-aza-2′-deoxycytidine residues by chromatin-associated DNMT1 induces proteasomal degradation of the free enzyme. Nucleic Acids Res. 2010;38(13):4313-4324.
37. Zhang Z-M, Lu R, Wang P, et al. Structural basis for DNMT3A-mediated de novo DNA methylation. Nature. 2018;554(7692):387-391.
38. Sledziewski A, Devos T, Kole R (inventors). Oligonucleotide inhibitors of DNA methyltransferases and their use in treating diseases. Patent WO2014011573. 2015.
39. Hu J, Liu J, Xue F, et al. Isolation and functional characterization of human erythroblasts at distinct stages: implications for understanding of normal and disordered erythropoiesis in vivo. Blood. 2013;121(16):3246-3253.
40. Wu LC, Sun CW, Ryan TM, et al. Correction of sickle cell disease by homologous recombination in embryonic stem cells. Blood. 2006;108(4):1183-1188.
41. Charache S, Dover G, Smith K, et al. Treatment of sickle cell anemia with 5-azacytidine results in increased fetal hemoglobin production and is associated with nonrandom hypomethylation of DNA around the gamma-delta-beta-globin gene complex. Proc Natl Acad Sci U S A. 1983;80(15):4842-4846.
42. Koshy M, Dorn L, Bressler L, et al. 2-Deoxy 5-azacytidine and fetal hemoglobin induction in sickle cell anemia. Blood. 2000;96(7):2379-2384.
43. Nienhuis AW, Ley TJ, Humphries RK, Young NS, Dover G. Pharmacological manipulation of fetal hemoglobin synthesis in patients with severe beta-thalassemia. Ann N Y Acad Sci. 1985;445:198-211.
44. Letvin NL, Linch DC, Beardsley GP, et al. Influence of cell cycle phase-specific agents on simian fetal hemoglobin synthesis. J Clin Invest. 1985;75(6):1999-2005.
45. Molokie R, Lavelle D, Gowhari M, et al. Oral tetrahydrouridine and decitabine for non-cytotoxic epigenetic gene regulation in sickle cell disease: a randomized phase 1 study. PLoS Med. 2017;14(9):e1002382.
46. Van Der Ploeg LHT, Flavell RA. DNA methylation in the human gamma delta beta-globin locus in erythroid and nonerythroid tissues. Cell. 1980;19(4):947-958.
47. Pop R, Shearstone JR, Shen Q, et al. A key commitment step in erythropoiesis is synchronized with the cell cycle clock through mutual inhibition between PU.1 and S-phase progression. PLoS Biol. 2010;8(9):e1000484.
48. Gautier E-F, Ducamp S, Leduc M, et al. Comprehensive proteomic analysis of human erythropoiesis. Cell Rep. 2016;16(5):1470-1484.
49. Mavilio F, Giampaolo A, Care A, et al. Molecular mechanisms of human hemoglobin switching: selective undermethylation and expression of globin genes in embryonic, fetal, and adult erythroblasts. Proc Natl Acad Sci U S A. 1983;80(22):6907-6911.
50. van den Bosch J, Lubbert M, Verhoef G, Wijermans PW. The effects of 5-aza-2'-deoxycytidine (Decitabine) on the platelet count in patients with intermediate and high-risk myelodysplastic syndromes. Leuk Res. 2004;28(8):785-790.