Selective silencing of α-globin by the histone demethylase inhibitor IOX1: a potentially new pathway for treatment of β-thalassemia

Thalassemia is the world’s most common form of inherited anemia, and in economically undeveloped countries still accounts for tens of thousands of premature deaths every year. The accumulation of free excess α-globin chains in red blood cells and their precursors, as a result of the decreased production of β-globin, is believed to be the main pathophysiological mechanism leading to hemolytic anemia and ineffective erythropoiesis in β-thalassemia. Clinical genetic data accumulated over the last 30 years indicate that a natural reduction in α-globin chain output by 25-50%, resulting from con-inherited α-thalassemia, ameliorates the disease phenotype in patients with β-thalassemia. Herein, we have developed and performed a targeted small molecule screen to identify compounds which downregulate α-globin expression. This identified IOX1, a pan-histone demethylase inhibitor, which selectively downregulates α-globin expression without perturbing erythroid differentiation or general gene expression, more specifically β-like globin expression. Our data show that selective silencing of α-globin expression in erythroid cells is pharmacologically feasible, and IOX1 is a lead compound to developing new therapy to treat β-thalassemia through the novel pathway of downregulating α-globin expression.

We first optimized a serum-free, miniature erythroid differentiation system starting from primary human CD34+ cells, the exact type of cells we would ultimately like to target in vivo (Figure 1). This culture system produced a sufficient number of viable, relatively pure, and synchronous populations of human erythroid cells in vitro to enable us to perform high throughput screens (Figure 1A,B). CD34+ cells were differentiated in 96-well plates over 21 days along the erythroid lineage, and the morphology and immunophenotypic characteristics of the resultant cells faithfully recapitulated normal erythropoiesis (Figure 1C,D). These cells demonstrated a gradual expression of erythroid-specific cell surface proteins (Figure 1E-F) and the hemoglobin protein analysis confirmed the higher proportion of fetal hemoglobin (HbF) and adult hemoglobin (HbA) in cells differentiated from umbilical cord and adult CD34+ cells, respectively (Figure 1F).

We then validated the culture system using hydroxyurea and sodium butyrate, which were previously shown to alter globin gene expression. Erythroid cells incubated with these compounds demonstrated a dose dependent increase in the γ/β messenger ribonucleic acid (mRNA) ratio, consistent with previously reported data (Figure 1G,H). Next, we transfected erythroid cells with two validated small interfering RNAs targeting human α-globin RNA, which resulted in the expected knockdown of α-globin expression (Online Supplementary Figure S1). These observations confirm that the small-scale erythroid differentiation system which we have optimized is a valid tool to examine changes in globin gene expression in vitro.

Previous studies have revealed contrasting epigenetic environments containing the human α- and β-globin genes. The human α-globin gene cluster is located on chromosome 16, in a gene dense, early replicating, open chromatin environment and its promoter is associated with unmethylated CpG islands and, in non-erythroid cells, is enriched for H3K27me3 which signals transcriptional silencing. By contrast, in non-erythroid cells the β-globin gene is situated in a relatively gene sparse, late replicating, closed heterochromatin environment on chromosome 11, and the promoter of the β-globin gene is methylated rather than enriched for H3K27me3. Therefore, in the search for drugs which specifically alter expression of α-globin, we performed a selective screen, using a small molecule library of epigenetically active cell permeable compounds, potentially targeting these different epigenetic environments. This library contains a collection of 37 compounds that were designed to inhibit a wide range of epigenetic pathways (Online Supplementary Table S1). Erythroid cells were incubated for 72 hours with these compounds, and gene expression levels were obtained using Fluidigm high throughput quantitative polymerase chain reaction (qPCR) system. The primary screening criterion was downregulation of α-globin expression without altering β-globin expression, and an α/β globin mRNA ratio of less than 0.75 was considered as the cutoff for identifying high-scoring compounds. This screen identified four compounds that downregulate α-globin expression: histone demethylase (KDM) inhibitor, IOX1; histone deacetylase inhibitor, vorinostat; histone methyltransferase inhibitor, chaetocin and lysine-specific histone demethylase 1 inhibitor, tranylcypromine (Figure 2B and Online Supplementary Figures S3-S5). Of these compounds, the novel KDM inhibitor IOX1 provided the most promising results with the desired effects on globin gene expression. Chaetocin decreased the viability of erythroid cells at low concentrations and tranylcypromine markedly retarded erythroid differentiation, as evidenced by immature cell morphology and lack of expression of erythroid-specific cell surface proteins. Therefore these two compounds were not followed up further (Online Supplementary Figure S6). Vorinostat downregulated α-globin expression whilst inducing γ-globin expression (Online Supplementary Figure S7) and is currently under further investigation.

To further examine the effect of IOX1 on globin gene expression, we titrated the concentration of IOX1 with the developing erythroid cells. This confirmed initial observations: IOX1 caused a dose-dependent decrease in α-globin expression, whereas the expression of β-globin was largely unaffected (Online Supplementary Figures S8 and S9). The decrease in α/β-globin mRNA ratios was statistically significant at all doses tested (Figure 2C). We then analyzed the mRNA levels of all globin genes in erythroid cells treated with IOX1 using the nCounter Digital Analyzer (NanoString Technologies), which found that IOX1 significantly downregulated α- γ-, μ- and ζ-globin expression (Figure 2D). Interestingly, with the exception of γ-globin, IOX1 downregulated α- and other α-like globin genes (μ and ζ) situated in the α-globin locus, whereas the expression levels of β-like globin genes (β, δ and ε) were unaffected, suggesting that IOX1 acts selectively on the α-globin locus.

IOX1 reduced cell expansion by about 40% (fold expansion dropped from 18-fold to 11-fold) at 40μM concentration, but the proportion of viable cells remained unchanged over all dose levels (Figure 3A,B). This suggests that IOX1 has a mild inhibitory action on erythroid cell proliferation in vitro, although it does not adversely affect cellular viability. Morphologically, erythroid cells treated with a dose range of IOX1 differentiated in a sim-
ilar way to untreated cells, suggesting that IOX1 does not alter erythroid differentiation (Figure 3C). This was further confirmed by immunophenotypic cell surface marker expression, which demonstrated no significant differences in the expression levels of CD34, CD71 and CD235a between IOX1 treated and control cells (Figure 3D-G). This is of particular importance, as some other compounds that are currently being tested for the treatment of β-thalassemia via the upregulation of γ-globin and fetal hemoglobin alter erythroid cell differentiation.13

We then conducted microarray analysis to examine the possible effects of IOX1 on global erythroid gene expression. Using this microarray, which assayed over 47 000 transcripts, mRNA abundance of most of the genes were similar in IOX1 treated cells when compared to the control, with a very high correlation coefficient (R=0.992) (Figure 3H). In total, only 162 genes were differentially expressed between the two groups (Online Supplementary Tables S2 and S3). Next, we analyzed the expression levels and fold differences of 52 genes which were reported as essential for erythroid physiology (adopted from the publicly available online database, Hembre). Expression levels in IOX1 treated and untreated cells were not significantly different in all but one of the 52 genes, further confirming the minimal effects of IOX1 on erythroid physiology (Online Supplementary Table S4).

Figure 1. Characterization and validation of the small scale erythroid differentiation system used for small molecule screen. Human CD34+ hematopoietic stem and progenitor cells purified from umbilical cord or adult peripheral blood were cultured in a two-phase liquid culture system in a serum-free medium for 21 days. (A) Mean fold expansion during erythroid differentiation; error bars represent SD (n=3). (B) Mean percentage cell viability during erythroid differentiation; error bars represent SD (n=3). (C) Morphology of cells by cytothis stained using modified Wright’s stain at different time points (day 0-21), in culture representing different stages of erythroid differentiation, demonstrating progression through stages of pro-, basophilic and polychromatic to orthochromatic erythroblasts; scale bar – 10μm. (D) Representative flow cytometry plots of cells stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD71 and phycoerythrin (PE)-conjugated anti-CD235a antibodies, demonstrating sequential expression of CD71 followed by CD235a and subsequent loss of CD71. (E) Relative expression of α (HBA), β (HBB) and γ (HBG) globin mRNA levels quantified by qPCR and normalized to the housekeeping gene RPL13A at different time points in culture (adult blood CD34+ cells); error bars represent SD (n=3). (F) Hemoglobin subtypes of the erythroid cells differentiated from umbilical cord and adult CD34+ cells analyzed by isoelectric focusing. The samples were run against a commercial set of standards. (G and H) γ/β mRNA ratio after incubation of erythroid cells in a dose range of hydroxyurea and sodium butyrate. Compounds were added to the liquid culture medium on day 7 of erythroid cell differentiation (corresponding to the proerythroblast stage), and the cells were then incubated in a 5% CO2 atmosphere at 37°C for 72 hours. Data on erythroid cells differentiated from umbilical cord and adult CD34+ cells are presented in red and blue, respectively. mRNA: messenger ribonucleic acid; HbF: hemoglobin F; HbA: hemoglobin A; HbE: hemoglobin E; HbA2: hemoglobin A2; HbS: hemoglobin S; HbE: hemoglobin E; HbC: γ-globin; HBB: β-globin; HBG: γ-globin; HBA: α-globin;
Next we investigated the mechanism by which IOX1 exerts its effect on globin gene expression in erythroid cells. Gene ontology enrichment analysis performed on differentially regulated gene sets obtained by microarray analysis did not reveal a simple interpretation of how IOX1 specifically affects $\alpha$-globin expression. However, previous reports on IOX1 demonstrate that it acts as a broad range inhibitor of histone demethylase enzymes. Therefore, we examined the changes in the pattern of histone methylation in erythroid cells treated with IOX1. Western blot of histone protein extracts from IOX1 treated erythroid cells showed an increase in two repressive chromatin modifications, H3K27me3 and H3K9me3 (Figure 3I,J).

We then looked at the changes of these chromatin modifications at $\alpha$- and $\beta$-globin loci using chromatin immunoprecipitation (ChIP) assays (Figure 3K,L). In the untreated cells, H3K27me3 abundance at the $\beta$-globin promoter was similar to the level observed at the negative control region, whereas the level at the $\alpha$-globin promoter was higher, which is consistent with our previous findings in which we showed that human $\alpha$-globin expression may be reduced by polycomb-mediated repression. Furthermore, treatment with IOX1 increased H3K27me3 abundance at both the $\alpha$- and $\beta$-globin promoters, with a more pronounced change at the $\alpha$-promoter. In contrast, H3K9me3 was more abundant at the $\beta$-globin promoter compared to the $\alpha$-globin promoter in untreated cells, which further increased after IOX1 treatment. These observations suggest that the $\alpha$-globin silencing effect of IOX1 is likely to be mediated via the
inhibition of the KDM enzymes responsible for the removal of H3K27 methylation marks at the α-globin locus. KDM enzymes known to act at this site are KDM6A and KDM6B, and IOX1 inhibits these enzymes at various IC₅₀ values in vitro. During the initial compound screen, GSK-J4, a specific inhibitor of KDM6A/B, downregulated both α- and β-globin. However, a recent report suggests that GSK-J4 also inhibits KDM5 enzymes that demethylase H3K4me3, which might explain why it downregulated both α- and β-globin. Our attempts to phenocopy the effect of IOX1 by knocking down individual enzymes were not successful, suggesting the presence of additional KDM enzymes acting at the H3K27 locus or an alternative pathway of its action. However, this
should not preclude the use of IOX1 as a lead compound for reducing α-globin expression.

In conclusion, we have demonstrated that selective silencing of α-globin expression, without affecting the β-like globin expression or erythroid differentiation, is pharmacologically feasible. The histone demethylase inhibitor, IOX1, exerts the desired changes in erythroid cells and has potential as a lead compound to develop a novel therapy for β-thalassemia, which is still a life-limiting disease without a definitive cure.

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Acknowledgments: the authors would like to thank the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics (funded by Wellcome Trust grant reference 090532/Z/09/Z and MRC Hub grant G0900747/10707) for the generation of the Gene Expression data. We also acknowledge Jennifer Eglington of Oxford University Hospital NHS Trust for helping with the isoelectric focusing of hemoglobin.

Funding: this work was supported by grants to DRH by the UK Medical Research Council (grant number MC_UU_12025/1 unit programme MC_UU_12025/4) and the NIHR Oxford Biomedical Research Centre. SM is a Commonwealth Scholar, funded by the UK government. We also acknowledge funding from the Helmut Horten Foundation. The SGC is a registered charity (number 1097737) that receives funds from AbbVie, Bayer Pharma AG, Boehringer Ingelheim, Canada Foundation for Innovation, Eisheiman Institute for Innovation, Genome Canada, Innovative Medicines Initiative (EU/EFPIA) [ULTRA-DD grant no. 115766], Janssen, Merck & Co., Novartis Pharma AG, Ontario Ministry of Economic Development and Innovation, Pfizer, São Paulo Research Foundation-FAPESP, Takeda, and the Wellcome Trust [106169/Z/14/Z].

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doi:10.3324/haematol.2016.155655

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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