Pomalidomide augments fetal hemoglobin production without the myelosuppressive effects of hydroxyurea in transgenic sickle cell mice

Short title: Pomalidomide regulates HbF in sickle cell mice

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Abstract

Pharmacological induction of fetal hemoglobin (HbF) expression is an effective treatment strategy for sickle cell disease (SCD) and β-thalassemia. Pomalidomide is a potent structural analog of thalidomide and member of a new class of IMiDs® immunomodulatory drugs. Recent reports demonstrated that pomalidomide reduced or eliminated transfusion requirements in certain hematological malignancies and induced HbF ex vivo in CD34+ progenitor cells from healthy and SCD donors. We investigated the effects of pomalidomide on erythropoiesis and hemoglobin synthesis in a transgenic mouse model of SCD. We found that eight weeks of treatment with pomalidomide induced modest increases of HbF with similar efficacy as hydroxyurea. However, in stark contrast to hydroxyurea’s myelosuppressive effects, pomalidomide augmented erythropoiesis and preserved bone marrow function. Surprisingly, combinatory therapy with both drugs failed to mitigate hydroxyurea’s myelotoxic effects and caused loss of HbF induction. These findings support further evaluation of pomalidomide as a novel therapy for SCD.
Introduction

Substantial experimental and clinical evidence support the development of targeted therapies for the induction of fetal hemoglobin in patients with SCD.\textsuperscript{1-6} Hydroxyurea, a ribonucleotide reductase inhibitor, is the most thoroughly investigated and only FDA approved treatment for adult patients with SCD that augments HbF expression and reduces clinical complications.\textsuperscript{7,8} Despite this progress, there is an unabated need for targeted HbF inducing therapies because many patients fail to respond to hydroxyurea. Pomalidomide and lenalidomide are proprietary thalidomide analogs, which belong to a novel class of IMiDs\textsuperscript{®} immunodulatory drugs. Both compounds recently emerged from clinical trials as highly promising treatments for various hematological cancers and chronic inflammatory conditions.\textsuperscript{9-11} Surprisingly, both IMiDs compounds were also found to restore erythropoiesis and to reduce or eliminate blood transfusion dependency in patients suffering from multiple myeloma and myelodysplastic syndromes.\textsuperscript{12-15} Furthermore, a recent ex vivo study demonstrated that pomalidomide and lenalidomide not only stimulated the proliferation of CD34+ progenitor cells and total hemoglobin production, but also upregulated HbF synthesis by a transcriptional mechanism.\textsuperscript{16} In this system, pomalidomide enhanced HbF synthesis more potently than lenalidomide and hydroxyurea and acted synergistically in combination with hydroxyurea. These results prompted the current study to evaluate pomalidomide’s hematological properties in a relevant in vivo model of SCD.
Methods

Mice

Knockout-transgenic sickle cell mice were bred at Georgia Health Sciences University according to institutional guidelines. Treatment groups consisted of vehicle (saline; n=8); pomalidomide (10 mg/kg; n=9); hydroxyurea (100 mg/kg; n=7); pomalidomide (10 mg/kg) + high-dose hydroxyurea (100 mg/kg; C/HD; n=8); pomalidomide (10 mg/kg) + low-dose hydroxyurea (10 mg/kg; C/LD; n=8). Pomalidomide (Celgene; Summit NJ) and hydroxyurea (Sigma) were mixed in saline and injected i.p. daily (Mon-Fri) for eight weeks. C57BL/6 mice were used for specific control experiments. Details of the mouse model, drug preparation and dose selection are included in Supplemental data. The study was approved by GHSU's Animal Care and Use Committee.

Pharmacokinetic analysis

See supplemental data for detailed information.

Complete blood count and RBC indices

Blood was collected by intracardiac puncture from ketamine/xylazine anesthetized mice in vacutainer EDTA tubes (Becton-Dickinson) and CBC analyzed with the CBC-Diff™ Veterinary Hematology System (Heska Corporation). Reticulocyte counts were determined by supravital staining with methylene blue.

Fetal hemoglobin analysis

Hemoglobin analysis from mouse hemolysates was done by analytic HPLC using a weak cation-exchange column SynChropak CM-300 (Eprogen INC) on the Waters Empower 32 HPLC system (Millipore Corporation).
F-cells

F cells were analyzed by flow cytometry using the Caltag fetal hemoglobin test kit (Caltag Laboratories) according to the manufacturer’s instructions. Flow cytometry was performed using the BD FACSCalibur system (BD Biosciences). HbF/F-cell (pg/cell) was calculated using mean cell hemoglobin (MCH) x %HbF / %F-cell.

Histology

Formalin-fixed bone marrow sections (4 microns) of the proximal femur were stained with H&E (Richard-Allan Scientific) and analyzed by two blinded investigators for the M:E ratio.

Statistics

Data are presented as mean ± SEM. Groups were analyzed by One-Way Analysis of Variance (ANOVA) followed by Student-Newman-Kuels. A p value < 0.05 was considered significant.
Results and discussion

Pomalidomide was well tolerated by sickle cell mice without signs of toxicity and with similar absorption and elimination properties compared to BL/6 control mice (supplemental Figure 2). Pomalidomide increased the level of HbF expression from 6.24% at baseline to 9.51% after eight weeks of treatment, which was comparable to hydroxyurea (Figure 1A) and similar to the magnitude of HbF induction observed in adult sickle cell patients treated with hydroxyurea in the MSH trial.\textsuperscript{7,8} It is important to note that the human beta globin cluster transgene in our model only encodes for the $\alpha_y$ globin gene sequence. Considering that pomalidomide is known to transcriptionally activate both $\alpha_y$ and $\gamma_y$ globin genes, this transgene design may have resulted in underreporting of pomalidomide’s \textit{in vivo} HbF activity.\textsuperscript{16,17} The increase in HbF expression in the pomalidomide and hydroxyurea groups was accompanied by a higher HbF content per F-cell without parallel increases in the F-cell percentage (Figure 1A). This lack of an F-cell response is most likely related to the high pretreatment F-cell values of \textgreater 50\% which is in agreement with the observed inverse relationship between the F-cell response to hydroxyurea and pretreatment F-cell values in a clinical trial of young patients with SCD.\textsuperscript{18} Motivated by the synergistic HbF-inducing activity of pomalidomide and hydroxyurea in \textit{ex vivo} CD34$^+$ progenitor cells, we conducted combinatory treatments and surprisingly observed a virtual loss of HbF induction above control levels (Figure 1A). We tested a combination of pomalidomide with a lower dose of hydroxyurea (10 mg/kg) to rule out compound toxicity as the cause of HbF inhibition. Interestingly, this regimen recovered bone marrow function but continued to block HbF production. The reason for this loss of HbF activity in the combined treatment groups is unclear, but could be related to the greater complexity of regulatory signals in the \textit{in vivo} microenvironment or differences between the $\beta$-globin gene clusters in the two systems.
Ineffective erythropoiesis is a contributory factor to anemia in SCD, albeit to a much lesser extent than in β-thalassemia syndromes. We found that pomalidomide, in addition to modulating HbF expression, expanded the erythron and improved the efficiency of erythropoiesis as evidenced by a trend toward higher reticulocyte counts (Figure 1B-C; Table 1). Because of the physical constraints of the mouse bone marrow compartment, the spleen in sickle mice functions as the major hematopoietic organ and becomes massively enlarged. Pomalidomide significantly raised the peripheral RBC count, caused further increases in spleen weight, and decreased the myeloid to erythroid (M:E) ratio in bone marrow and spleen. Plasma free hemoglobin levels in the pomalidomide group were not different from controls indicating that gains in the peripheral RBC counts were not secondary to a protective effect of HbF production on F cell survival. However, we noted that expansion of the erythroid lineage was associated with significantly reduced RBC mean corpuscular volumes (MCV) and only small increases in total hemoglobin levels. These findings suggest residual defects in hemoglobin production possibly secondary to iron restricted erythropoiesis or the mild β-thalassemic phenotype in this model. In contrast, hydroxyurea treatment was associated with sharply lower reticulocyte counts, a significant increase in the M:E ratio in both hematopoietic organs, and a reduction of spleen weights to less than one-half of control values. Bone marrow megakaryocyte counts appeared unaffected by pomalidomide, but were significantly reduced by hydroxyurea. Compared to hydroxyurea, pomalidomide had no statistically significant effect on the total WBC count, but caused a significant reduction in the monocyte fraction which could have additional beneficial treatment effects because of the proinflammatory role of sickle monocytes in SCD.

A potential limitation of this study in mice is the difficulty of extrapolating an equivalent HbF-inducing dose of pomalidomide in humans because of the large interspecies differences in drug metabolism. Studies in rats with [14C]-Pomalidomide demonstrated that metabolism makes only a minor contribution to drug clearance whereas monkeys and humans metabolized the compound
extensively. Although further experiments are required to identify pomalidomide's mechanism of HbF induction, our study revealed that pomalidomide is a safe and effective HbF inducing agent unaccompanied by the cytotoxic effects of hydroxyurea in mice with SCD. These results and pomalidomide's immunomodulatory properties, which are the subject of ongoing research in our laboratory, warrant further exploration of this compound as a novel therapy for patients with SCD and other β-hemoglobinopathies.
Acknowledgments

We are grateful to Drs. Tim Townes and Tom Ryan for the gift of knockout-transgenic sickle cell mice. We thank Kimberly Smith and Doris Cawley for their technical assistance. S.E.M. was supported by grants from the Celgene Corporation and National Institutes of Health Roadmap Initiative in Nanomedicine through a Nanomedicine Development Center award (1PN2EY018244).

Authorship

Contributions: S.E.M. was responsible for the overall study, designed the research, analyzed the data, and wrote the manuscript; M.W. performed the research, analyzed the data, and assembled the figures; F.K. performed the fetal hemoglobin analysis; S.Y. analyzed data; Y.J.X. conducted the pharmacokinetic analysis; L.A.M. and L.C. discussed the study and provided vital reagents; P.S.S. contributed to the writing of the manuscript; A.K. conceived the idea and contributed to the writing of the manuscript.

Conflict-of-interest disclosure: S.E.M., M.W., S.Y., P.S.S. and A.K. received funding from Celgene; Y.J.X., and L.C. are employees of Celgene which has a financial interest in pomalidomide. L.A.M. was an employee of Celgene at the time of this study.
References


Table 1. Hematological parameters after eight weeks of treatment with pomalidomide and/or hydroxyurea in transgenic sickle cell mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT (C57BL/6)</th>
<th>Vehicle</th>
<th>Pomalidomide</th>
<th>Hydroxyurea HD</th>
<th>Pomalidomide + Hydroxyurea (C/HD)</th>
<th>Pomalidomide + Hydroxyurea (C/LD)</th>
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<tr>
<td><strong>Peripheral blood</strong></td>
<td></td>
<td></td>
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<tr>
<td>RBC (x10⁶/μl)</td>
<td>8.9 ± 0.1^**</td>
<td>4.8 ± 0.1</td>
<td>5.5 ± 0.2*</td>
<td>4.5 ± 0.5</td>
<td>4.3 ± 0.4^*</td>
<td>5.5 ± 0.2*</td>
</tr>
<tr>
<td>Total Hb (g/dL)</td>
<td>13.7 ± 0.2^**</td>
<td>7.2 ± 0.4</td>
<td>7.7 ± 0.3</td>
<td>6.1 ± 0.5</td>
<td>5.7 ± 0.5^*</td>
<td>7.7 ± 0.3</td>
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<td>Hct (%)</td>
<td>46.6 ± 1.58^**</td>
<td>28.2 ± 1.1</td>
<td>28.9 ± 1.2</td>
<td>23.6 ± 3.0</td>
<td>23.9 ± 2.0</td>
<td>31.7 ± 1.0</td>
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<td>Reticulocytes (%)</td>
<td>3.5 ± 0.2^**</td>
<td>36.8 ± 5.8</td>
<td>40.5 ± 4.7</td>
<td>16.4 ± 3.9^** ***</td>
<td>14.7 ± 2.4^** ***</td>
<td>38.5 ± 2.3</td>
</tr>
<tr>
<td>WBC (x10³/μl)</td>
<td>7.1 ± 0.2^*</td>
<td>14.4 ± 2.7</td>
<td>14.2 ± 3.5</td>
<td>6.9 ± 0.8^*</td>
<td>10.0 ± 1.9</td>
<td>19.1 ± 4.2</td>
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<td>Neutrophils (%WBC)</td>
<td>15.0 ± 2.8</td>
<td>13.1 ± 2.6</td>
<td>19.0 ± 3.4</td>
<td>12.1 ± 1.6</td>
<td>11.0 ± 1.6</td>
<td>33.9 ± 4.4^*</td>
</tr>
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<td>Lymphocytes (%WBC)</td>
<td>78.3 ± 3.7</td>
<td>80.1 ± 3.7</td>
<td>76.3 ± 3.7</td>
<td>84.3 ± 1.8</td>
<td>87.5 ± 1.5</td>
<td>60.7 ± 4.5^**                   ***</td>
</tr>
<tr>
<td>Monocytes (%WBC)</td>
<td>5.0 ± 1.4</td>
<td>7.1 ± 1.0^**</td>
<td>4.1 ± 0.4^**</td>
<td>3.0 ± 0.5^**</td>
<td>1.7 ± 0.3^**</td>
<td>3.7 ± 1.1^*</td>
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<tr>
<td>Platelets (x10³/μl)</td>
<td>1023 ± 126^**</td>
<td>523.3 ± 83</td>
<td>652.6 ± 166</td>
<td>430.4 ± 73</td>
<td>384.3 ± 24</td>
<td>612.0 ± 99</td>
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<td><strong>RBC indices</strong></td>
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<td>MCV (fl)</td>
<td>52.2 ± 1.0</td>
<td>58.1 ± 1.9^**</td>
<td>52.8 ± 1.8^*</td>
<td>52.3 ± 1.6^*</td>
<td>55.9 ± 1.8</td>
<td>57.7 ± 2.1</td>
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<tr>
<td>MCH (pg)</td>
<td>15.4 ± 0.1^**</td>
<td>14.9 ± 1.0</td>
<td>14.0 ± 0.6</td>
<td>13.5 ± 0.8</td>
<td>13.5 ± 0.7</td>
<td>14.1 ± 0.7</td>
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<tr>
<td>MCHC (g/dL)</td>
<td>29.6 ± 0.7</td>
<td>25.9 ± 2.2</td>
<td>27.0 ± 1.8</td>
<td>24.8 ± 0.8</td>
<td>24.0 ± 0.5</td>
<td>24.3 ± 0.5</td>
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<td><strong>Plasma</strong></td>
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<tr>
<td>Free hemoglobin (mg/dL)</td>
<td>26.6 ± 0.2^**</td>
<td>82.4 ± 11.4</td>
<td>81.0 ± 11.3</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td><strong>Extramedullary organs</strong></td>
<td></td>
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<tr>
<td>Spleen weight (% bw)</td>
<td>0.34 ± 0.04^**</td>
<td>4.1 ± 0.3^*</td>
<td>4.9 ± 0.2^*</td>
<td>2.8 ± 0.3^**</td>
<td>2.0 ± 0.1^*</td>
<td>4.1 ± 0.2^**</td>
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</table>

Complete blood counts were measured from whole blood of 14-week-old mice after treatment with the indicated agent(s). RBC indicates red blood cell count; Hb, hemoglobin; Hct, hematocrit; WBC, white blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; C/HD, combination high dose treatment (pomalidomide 10 mg/kg, hydroxyurea 100 mg/kg); C/LD, combination low dose treatment (pomalidomide 10 mg/kg, hydroxyurea 10 mg/kg); NT, not tested. Data are presented as mean ± SEM. The symbol ^ = vs. vehicle; # = vs. pomalidomide; * = p<0.05; ** = p<0.01.
Figure 1. Pomalidomide regulates fetal hemoglobin synthesis and erythropoiesis in transgenic sickle cell mice. Homozygous sickle cell mice were treated with vehicle, pomalidomide, or hydroxyurea for eight weeks by intraperitoneal injection and sacrificed for analysis. (A) HbF protein levels as a percentage of total hemoglobin were determined by HPLC at the end of the study to avoid artifactual increases of HbF from repeated blood draws. F-cells were analyzed by flow cytometry after immunolabeling formalin-fixed RBCs with anti-human FITC-conjugated HbF antibody. (B) Representative images depicting the myeloid to erythroid (M:E) ratio in H&E stained bone marrow sections of the proximal femur. Erythroid cells are recognized by their round, dense, and deeply basophilic nuclei. All images were collected using a Zeiss Axioplan 2 microscope and a Plan-Apochromat 63X/1.4 oil objective. (C) Analysis of bone marrow cellularity, M:E ratio, and megakaryocyte counts. Cellularity in the active treatment groups was determined in reference to the 100% cellular marrow in vehicle treated sickle cell mice. Megakaryocytes were counted in five low magnification optical fields per animal and converted to megakaryocytes/mm². All sections were analyzed by two blinded investigators. a*: significantly different from Veh and HU; p<0.05; b**: significantly different from Veh and PL; p<0.01; Veh, vehicle; PL, pomalidomide; HU, hydroxyurea; C/HD, combination high dose treatment (pomalidomide 10 mg/kg, hydroxyurea 100 mg/kg); C/LD, combination low dose treatment (pomalidomide 10 mg/kg, hydroxyurea 10 mg/kg).
Figure 1

A.

![Graphs showing Hemoglobin F (peak %), Hemoglobin F Cell (pg/cell), and F Cells (%)]

B. Vehicle Pomalidomide Hydroxyurea

![Bone marrow analysis images with labels: Cellularity (%), Myeloid:erythroid (M:E) ratio, Megakaryocytes (cells/mm²)]

C.

<table>
<thead>
<tr>
<th>Bone Marrow Analysis</th>
<th>Vehicle</th>
<th>Pomalidomide</th>
<th>Hydroxyurea</th>
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</thead>
<tbody>
<tr>
<td>Cellularity (%)</td>
<td>100 ± 0.00</td>
<td>97.86 ± 2.14</td>
<td>73.75 ± 3.75; b**</td>
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<tr>
<td>Myeloid:erythroid (M:E) ratio</td>
<td>2.27 ± 0.43</td>
<td>1.20 ± 0.22; a*</td>
<td>3.98 ± 1.81</td>
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<tr>
<td>Megakaryocytes (cells/mm²)</td>
<td>52.5 ± 4.6</td>
<td>51.6 ± 3.2</td>
<td>25.4 ± 4.8; b**</td>
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</table>
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