Sickle cell mice

Knockout-transgenic sickle cell mice were kindly provided by Drs. Tim Townes and Tom Ryan from the University of Alabama at Birmingham, Birmingham, AL. Sickle mice used in these studies were homozygous null for the murine **α**- and **β**-globin alleles and carried two transgenes encoding the human **α**-, **β**-, and **γ**-globin gene sequences. Specifically, one transgene consisted of a 22-kb DNA fragment of the human **β**-globin locus control region (LCR) linked to a 9.7-kb DNA fragment containing the ^{A}**γ**-globin and **β**^{S}-globin genes. The other transgene linked the LCR to a 3.8-kb DNA fragment containing the human **α**1-globin gene. Adult homozygous sickle mice (HbS m**α**$^{0/0}$m**β**$^{0/0}$ LCR **α**/LCR A**γ**-**β**^{S}) synthesize approximately 94% human sickle (HbS), 6% human fetal hemoglobin (HbF), and no murine hemoglobin. The persistent expression of moderate levels of HbF in adult sickle mice is similar to the average HbF levels observed in adult sickle cell patients, which was an important criterion for selecting this model for our pharmacological studies. Sickle cell mice mice faithfully mimic many of the hematological manifestations of patients with SCD, including a chronic hemolytic anemia, stress reticulocytosis, and red blood cell sickling (Fig. S1).

Drug preparation and dose selection

Pomalidomide (Celgene) was prepared at a concentration of 1 mg/ml in sterile saline and injected at a volume of 10 µl/g bw for a final injection dose of 10 mg/kg bw. Hydroxyurea (Sigma) was prepared at a concentration of 10 mg/ml in sterile saline and injected at a volume of 10 µl/g bw for a final injection dose of 100 mg/kg bw. Vehicle (sterile saline) was injected at a volume of 10 **µ**l/g bw. Mice were weighed each week during treatment

and the volume of pomalidomide, hydroxyurea, and vehicle was adjusted accordingly. To determine an effective and non-toxic dose of pomalidomide for our studies, initial experiments investigated the activity of 100 mg/kg of pomalidomide administered daily by i.p. injection for four weeks. This trial produced adverse effects as evidenced by a 20% death rate (2 out of ten mice) in the experimental group with no deaths in the control group, a significantly lower weight gain, a significant reduction of the reticulocyte count to ~50% of control, and a wide range of HbF values reflecting different degrees of drug toxicity. Based on these results, we selected a ten-fold lower dose of pomalidomide (10 mg/kg), increased the treatment period to eight weeks, and introduced a treatment holiday on weekends.

Pharmacokinetic analysis

Pomalidomide and its internal standard ([$^{13}C_5$]-pomalidomide) were extracted from 0.2 mL of mouse samples (1:1, K_3EDTA plasma/25 mM citrate buffer pH 1.5) using a liquid-liquid extraction method with methyl t-butyl ether. The sample extracts were loaded onto a Phenomenex Luna column (5 µm, 2.0 x 50 mm) for separation and the HPLC effluent was introduced into a Sciex API 4000 equipped with an electrospray source for detection. The standard curve, which ranged from 0.5 to 500 ng/mL, was fitted to a weighted ($1/x^2$) quadratic regression model. Pharmacokinetic parameters were calculated using WinNonlin™ (version 5.1.1). The maximum plasma concentration (C_{max}) and the corresponding T_{max} were determined from actual data. The area under the plasma concentration-time curve for pomalidomide from time 0 to 24 hours (AUC_{24h}) was determined by the non-compartmental model using the linear/log trapezoidal rule. Nominal times were used for pharmacokinetic parameter calculations. Plasma concentrations below the limit of quantitation (BLOQ) were treated as zero for calculation purpose.

Figure S1. HPLC hemoglobin profile and RBC morphology in sickle cell mice

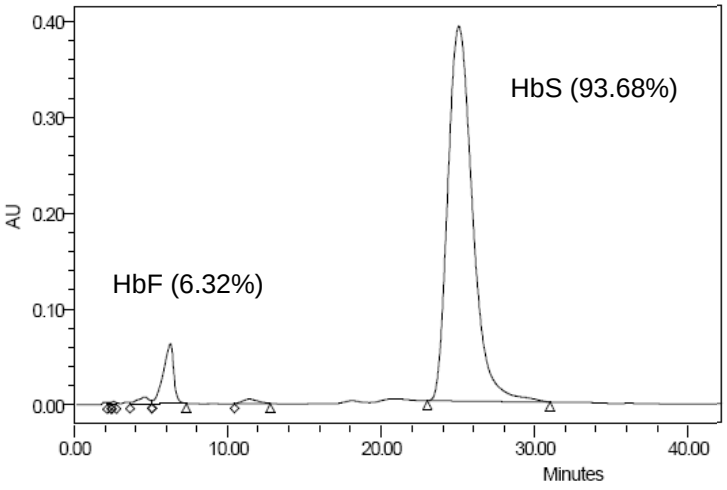
(A) Analytic HPLC of adult hemolysate from a vehicle treated sickle mouse showing typical percentages of fetal and sickle hemoglobin. (B) Sickle mouse red blood cells after 30 min exposure to a hypoxic gas mixture (97% N2, 3% O2). Images were taken with a Zeiss Axiovert 200 and a Plan-Neofluar 40x/1.3 oil objective.

Figure S2. Pharmacokinetics of pomalidomide in transgenic sickle cell mice

(A) Plasma concentration-time profiles from wildtype and sickle cell mice after a single i.p. dose of pomalidomide (10 mg/kg). (B) Pharmacokinetic variables corresponding to plasma concentration – time profiles. Plasma drug concentrations were measured with a high performance triple quadrupole mass spectrometer system (Sciex API 4000) and represent the mean of three samples per time point in both groups. C_{max}, maximum plasma concentration; T_{max}, peak concentration time; $T_{1/2}$, terminal half-life; AUC_{24h}, area under the concentration versus time curve; AUC_{inf}, area under the concentration-time curve extrapolated to infinity; CL/F, apparent clearance.

Figure S1

A


0.40
0.30
0.20
0.10
0.00
AU
HbS (93.68%)
HbF (6.32%)
0.00
10.00
20.00
30.00
40.00
Minutes

B

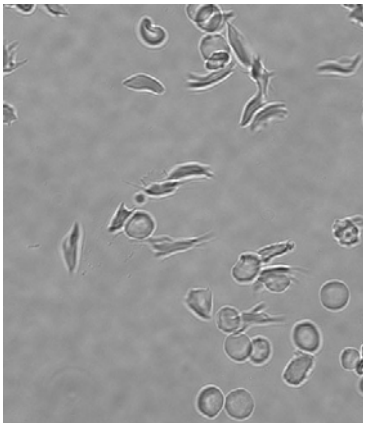
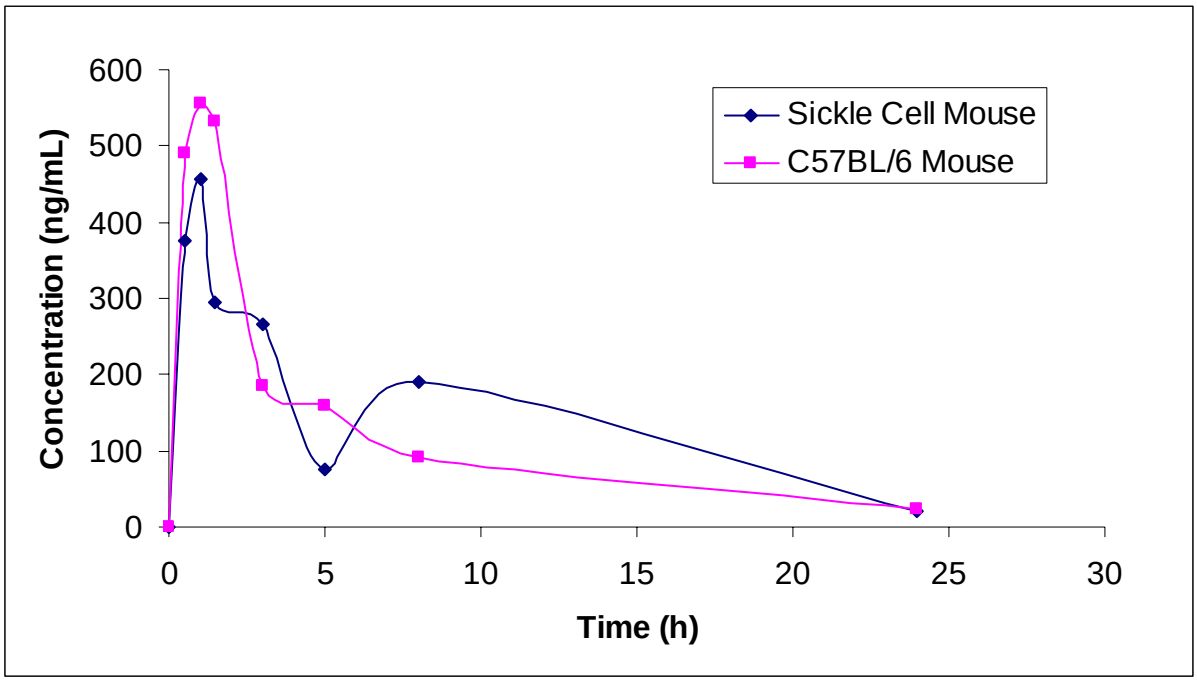


Figure S2

A



B

Animal Groups	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{24h} (ng*h/mL)	AUC_{inf} (ng*h/mL)	CL/F (ml/h/kg)
C57BL/6	555.7	1.0	4.8	2829.0	2997.6	3336.0
Sickle	456.0	1.0	5.9	3354.4	3535.5	2828.5