

High-Throughput Assay to Screen Small Molecules for Their Ability to Prevent Sickling of Red Blood Cells

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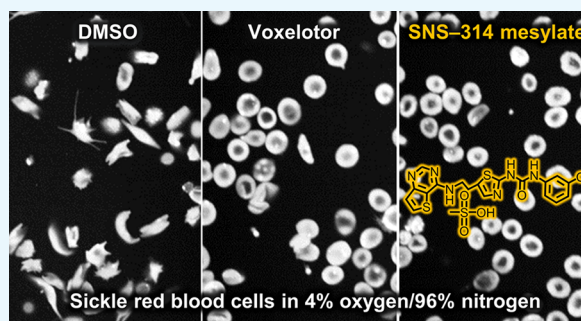
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ABSTRACT: Sickle cell disease (SCD) is an inherited disorder of hemoglobin (Hb); approximately 300,000 babies are born worldwide with SCD each year. In SCD, fibers of polymerized sickle Hb (HbS) form in red blood cells (RBCs), which cause RBCs to develop their characteristic “sickled” shape, resulting in hemolytic anemia and numerous vascular complications including vaso-occlusive crises. The development of novel antisickling compounds will provide new therapeutic options for patients with SCD. We developed a high-throughput “sickling assay” that is based on an automated high-content imaging system to quantify the effects of hypoxia on the shape and size of RBCs from HbSS SCD patients (SS RBCs). We used this assay to screen thousands of compounds for their ability to inhibit sickling. In the assay, voxelotor (an FDA-approved medication used to treat SCD) prevented sickling with a z' -factor > 0.4 , suggesting that the assay is capable of identifying compounds that inhibit sickling. We screened the Broad Repurposing Library of 5393 compounds for their ability to prevent sickling in 4% oxygen/96% nitrogen. We identified two compounds, SNS-314 mesylate and voxelotor itself, that successfully prevented sickling. SNS-314 mesylate prevented sickling in the absence of oxygen, while voxelotor did not, suggesting that SNS-314 mesylate acts by a mechanism that is different from that of voxelotor. The sickling assay described in this study will permit the identification of additional, novel antisickling compounds, which will potentially expand the therapeutic options for SCD.



INTRODUCTION

Sickle cell disease (SCD) is an autosomal recessive inherited disorder of hemoglobin (Hb) in red blood cells (RBCs); there are approximately 100,000 SCD patients in the United States.^{1,2} Patients with SCD have a mutation in the gene encoding the β -globin chain, resulting in sickle Hb (HbS). When the concentration of deoxygenated HbS (deoxyHbS) in RBCs increases, fibers of polymerized HbS form, causing RBCs to develop the characteristic “sickled” shape.^{3,4} The sickling of RBCs reduces their deformability and enhances their adhesion to the vascular endothelium, resulting in anemia and vaso-occlusive crises.^{2,5,6}

Four medications, hydroxyurea, L-glutamine, voxelotor, and crizanlizumab, are currently approved by the FDA for the treatment of SCD. Hydroxyurea increases the concentration of fetal Hb in RBCs, thereby reducing the concentration of HbS in RBCs and inhibiting the polymerization of HbS.^{5,7–9} L-Glutamine increases the concentration of reduced nicotinamide adenine dinucleotide in RBCs, preventing oxidative damage.^{10,11} Voxelotor is an aromatic aldehyde, which allosterically enhances the affinity of HbS for oxygen, decreasing the concentration of deoxyHbS and reducing the sickling.^{12,13} Crizanlizumab is an anti-P selectin antibody,

which prevents sickle cell crises by inhibiting the intracellular adhesions between RBCs, leucocytes, platelets, and the vascular endothelium.^{14,15} Hydroxyurea, L-glutamine, and crizanlizumab decrease the frequency of vaso-occlusive sickle cell crises.^{16–18} However, not all patients respond favorably to hydroxyurea therapy,^{19,20} and L-glutamine and crizanlizumab do not reduce the sickling of RBCs. Voxelotor improves the anemia associated with SCD but does not decrease the frequency of sickle cell crises.²¹

The identification of novel antisickling compounds will provide additional therapeutic options for patients with SCD. To identify such compounds, we sought to develop a high-throughput “sickling assay” that quantifies the ability of compounds to inhibit the sickling of RBCs from patients with HbSS disease (SS RBCs). Because the sickling assay is based on the appearance of RBCs, rather than an effect on

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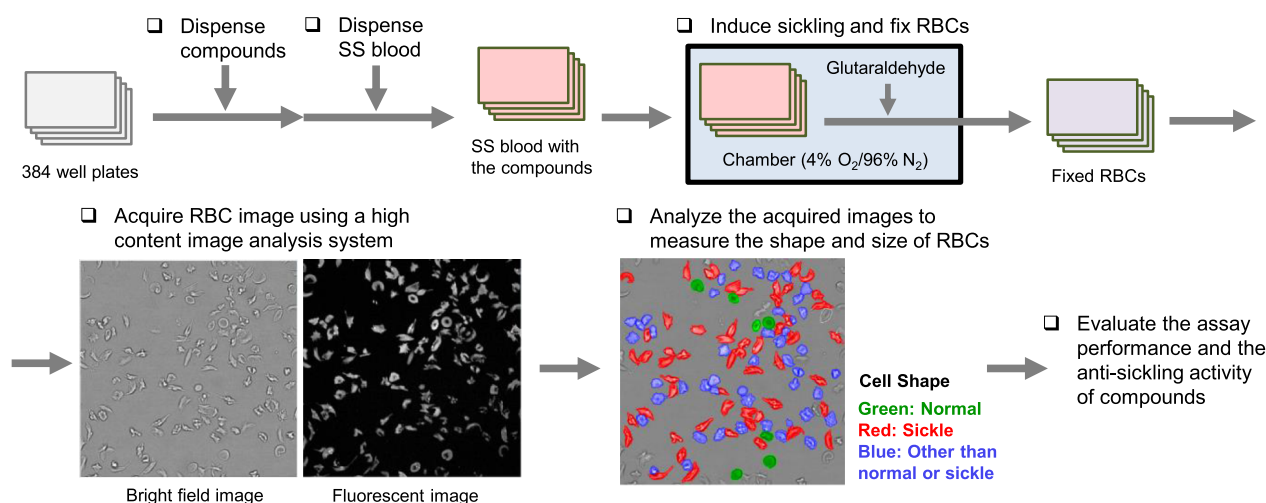


Figure 1. Scheme of the assay used to evaluate the antisickling activity of compounds.

purified Hb, the assay can identify compounds that prevent RBC sickling by a variety of mechanisms, including reducing sickling without increasing the affinity of Hb for oxygen.

In this study, we used a high-content imaging and analysis system to permit computerized acquisition of the appearance of RBCs. Computer acquisition reduced the operator-dependent variability associated with assessing and quantifying the shape of RBCs and permitted the rapid screening of a large number of compounds for their ability to inhibit the sickling of SS RBC. We used the high-throughput sickling assay to screen 5393 compounds in the “Broad Repurposing Library” for their potential ability to prevent sickling of SS RBCs in 4% oxygen. This collection of chemicals includes compounds at different stages of preclinical and clinical development and was developed by the Broad Institute of MIT and Harvard.²² In a blinded screen, we identified two compounds, SNS-314 mesylate (SNS-314M) and voxelotor, that successfully prevent sickling.

METHODS

Sickling Assay. The sickling assay, which can assess the antisickling activity of compounds, consists of the following steps: acquisition of blood samples, induction of hypoxic sickling, preliminary assessment of blood samples, automated quantification of sickling, and evaluation of the sickling assay performance and the ability of compounds to prevent sickling. An overview of the procedure is shown in Figures S1 and 1.

Blood Samples. The Institutional Review Boards of Mass General Brigham, Boston Medical Center and Boston University Medical Campus, and Boston Children’s Hospital approved the use of blood samples from SCD patients, and all participants provided informed consent prior to study procedures. Patients with homozygous HbSS disease provided blood samples (SS blood) in tubes containing ethylenediaminetetraacetic acid, and blood samples were stored at 4 °C. Samples were evaluated within 48 h of blood collection.

Induction of Sickling in 4% Oxygen/96% Nitrogen. SS blood samples were diluted 1:1000 with a modified HEMOX solution, pH 7.4, containing *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (30 mM), sodium chloride (135 mM), potassium chloride (5 mM), calcium chloride (0.9 mM), magnesium chloride (0.5 mM), iron(II) chloride (9 μM), and glucose (5 mM) in water. Diluted SS blood (20 μL/well) was

aliquoted into 384-well plates (CellCarrier 384-well Ultra microplates, PerkinElmer, Waltham, MA).

Assay plates, a plate shaker (Incu-Mixer MP, Benchmark Scientific, Sayreville, NJ), and an oxygen meter (MiniOx 250E, Ohio Medical, Gurnee, IL) were placed in a chamber made of an inflatable polyethylene bag (AtmosBag, MilliporeSigma, St. Louis, MO, Figure S2). The chamber was purged with a mixture of nitrogen (purity 99.9%) and air to maintain a concentration of oxygen gas of 3.9–4.1%, and the plates were shaken (1000 rpm) within the chamber at 37 °C for 1 h. After incubation, samples were fixed with 20 μL/well of 2% glutaraldehyde in phosphate buffer (Electron Microscopy Sciences, Hatfield, PA), sealed, and shaken in the chamber at 37 °C for 15 min. The plates were subsequently removed from the chamber and centrifuged at 1000 rpm for 1 min to sediment RBCs to the bottom of the plates for the subsequent assessment of RBC appearance.

Preliminary Assessment of Blood Samples. An inverted microscope (TS100, Nikon, Melville, NY) was initially used to determine the shape of RBCs and determine whether the assay was capable of detecting inhibition of sickling. The percentage of sickled cells in RBCs treated with voxelotor (MedChemExpress, Monmouth Junction, NJ) 30 μM (as a positive control) or the diluent dimethyl sulfoxide (DMSO) alone (as a negative control) was determined by visual inspection (Figure S3).

Automated Quantification of Sickling. To automatically quantify the antisickling activity of compounds in a high-throughput manner, images of RBC samples were acquired, and RBC appearance of each sample was determined using an automated image analysis system. Images were acquired from fixed blood samples using the Opera Phenix high-content screening system (PerkinElmer). The system was used in the confocal mode with 63× water immersion objective using the brightfield and fluorescence channels. The latter was set with excitation 640 nm and emission 650–760 nm. Four fields per well were acquired, which allowed the analysis of approximately 500 cells per well (Figure S4).

Harmony image analysis software (PerkinElmer) was used for cell segmentation and quantification. Cell segmentation is a task to identify the boundary of each cell in an image. For the image analysis, the cells were segmented using the fluorescence channel. Use of the STAR morphology method for brightfield

and fluorescence channels permitted extraction of a large and diverse set of properties for the quantification of morphological changes. These properties included the outer shape of cells and the distribution of intensity inside the cell, including symmetry and compactness. The linear classifier method, which is a supervised classification task using “PhenoLOGIC machine learning concept”, was applied to train software to recognize different cell populations based on the extracted morphological parameters. The treated SS RBCs were used to “train” the software to identify the different shapes of the cells. Round and concave SS RBCs prepared in room air and elongated SS RBCs prepared in 4% oxygen were defined as normal and sickle cells, respectively. Cells that were neither normal nor sickled were defined as “other” in the software.

Based on these definitions of RBC shape, the number of normal cells, sickle cells, and other cells was counted automatically by the software, and the percentage of cells with each shape feature was determined for each well on the plate. The average cell area ($\mu\text{m}^2/\text{cell}$; total area of the counted cells/number of the cells) in each well was also measured using the Harmony software.

Screen of Compounds to Identify Compounds That Prevent Sickling. The 5393 compounds in the Broad Repurposing Compound Library,²² each dissolved in DMSO, together with voxelotor, and DMSO alone were dispensed into individual wells in 384-well plates using a liquid handling machine (Echo 555 acoustic liquid handlers, Beckman Coulter Life Sciences, Indianapolis, IN) to create preprepared assay ready plates (ARPs). SS blood samples diluted 1:1000 with the modified HEMOX solution (20 $\mu\text{L}/\text{well}$) were dispensed into the ARPs and incubated in 4% oxygen at 37 °C for 1 h as described above in “Induction of Sickling in 4% Oxygen/96% Nitrogen”. The percentage of cells with each shape and the average cell area in each sample were determined automatically as described above.

Elimination of Compounds That Enhance the Oxidation of Hemoglobin. To assess whether screened compounds reduce sickling by enhancing the oxidation of Hb, the absorbance of compound-treated Hb samples was measured at 630 nm (A_{630}). Each compound of interest, dissolved in DMSO (60 μM for each group, DMSO 5 vol %), was incubated with purified HbA in HEMOX solution (20 μM as Hb tetramer prepared as described²³). Each compound alone in HEMOX solution was used as control. The compound-treated and the control samples (100 $\mu\text{L}/\text{well}$) were dispensed onto a 384-well plate (Nunc 262120, Thermo Scientific, MA), and the A_{630} of samples was measured at 37 °C every 10 min for 6 h using a plate reader (Multiskan GO, Thermo Scientific). Each compound was tested three times ($n = 3$ experimental replicates). The values of A_{630} were averaged and then calibrated by subtracting the A_{630} of control from that of the corresponding sample. If the calibrated A_{630} of a compound-treated Hb was greater than that of DMSO-treated Hb, then the compound was considered to increase the oxidation of Hb and was removed from the screening pool.

Effect of Increasing Concentration of Compounds on Sickling. The DMSO solution of the compounds (eight different concentrations, 60 nL/each), together with positive (voxelotor) and negative (DMSO alone) controls, was dispensed into individual wells in 384-well plates using the liquid handling machine to create ARPs. SS blood samples (20 $\mu\text{L}/\text{well}$) were subsequently dispensed into each well of the ARPs and were incubated in 4% oxygen at 37 °C for 1 h. The

percentage of sickled cells in each sample was determined using the automated image analysis system.

The dose–response curves of the compounds and the resulting percentage of sickle cells were obtained, and the EC_{50} of compounds was determined by nonlinear regression (eq 1) using GraphPad Prism 9 (GraphPad Software, La Jolla, CA).

$$Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{(\log\text{EC}_{50} - X) \times \text{HillSlope}}) \quad (1)$$

X is the log of the concentration of a compound. Y is the percentage of sickle cells. “Top and bottom” are plateaus in the same units as Y . $\log\text{EC}_{50}$ has the same log unit as X . HillSlope is a slope factor or Hill slope (unitless).

RESULTS

Sickling Assay. We developed a “sickling assay” to quantify the effects of deoxygenation on the shape of SS RBCs (Figures S1 and 1). Blood samples were obtained from a total of 18 patients with homozygous HbS SCD (SS blood samples). SS blood samples were diluted 1:1000 with buffer (pH 7.4) and 0.3 vol % DMSO and incubated in 4% oxygen for 1 h to induce sickling. Glutaraldehyde was used to fix the RBCs, and the appearance of the cells was initially assessed by light microscopy. Cells were manually “scored” as being “elongated” (sickled), “normal” (round and concave), or “other” than normal or sickled. Each blood sample was tested in quadruplicate. A blood sample was considered adequate for the screening assay if the average percentage of sickled cells was greater than 40% (Figure S3A). Of the 18 blood samples, 11 samples met this criterion. The explanation as to why seven blood samples did not have at least 40% sickled cells is uncertain but might be related to variation in the concentration of fetal Hb. Of the 7 samples that did not have at least 40% sickled cells, 3 were obtained from patients who were treated with hydroxyurea.

Voxelotor was used as a positive control for the inhibition of sickling. In the presence of voxelotor (30 μM), the mean percentage of sickle cells was less than 10% in all 11 blood samples (Figure S3B), confirming that the assay was adequate to identify compounds that inhibit sickling and that voxelotor can be used as a positive control.

Initial Screen to Identify Compounds That Prevent Sickling. The sickling assay was used to screen the 5393 compounds in the “Broad Repurposing Library”²² for their potential ability to prevent sickling of SS RBCs in 384-well plates (Table S1). The positive control, voxelotor, was placed in columns 1 and 23 (32 total wells), and the negative control, DMSO, was placed in columns 2 and 24 (32 total wells). One compound from the library was dispensed into each of the remaining 320 wells. Each compound was tested in two plates.

The final concentration of voxelotor was 30 μM , and each compound was tested at a concentration that was either between 0.3 and 25 μM (1285 compounds) or between 25 and 35 μM (4108 compounds), depending on the solubility of each compound in the diluent (DMSO). Each compound in the plate was incubated with SS RBCs for 15 min at room temperature (in air), prior to exposing the cells to 4% oxygen.

Samples were incubated in 4% oxygen, fixed with glutaraldehyde, and then the percentage of sickle cells was determined using an automated imaging system, which was able to acquire bright-field and fluorescence images of samples (Figure S4) and assess the number, morphology, and apparent

area of RBCs in each well (Figure 1). Fluorescence images of RBCs were successfully obtained without adding a fluorescent dye to samples because treatment with glutaraldehyde generated fluorescent substances in RBCs.^{24,25} These images permitted us to use Harmony software for accurate cell segmentation and extraction of hundreds of morphological parameters, which were utilized by the PhenoLOGIC machine learning method to train software to recognize different cell populations. Data for each plate were acquired over the course of approximately 30 min.

Six Compounds Prevented Sickling without Altering the Cell Area and the Round Shape of RBCs. In cells treated with DMSO alone, $44 \pm 5.3\%$ had a sickled appearance. In cells treated with voxelotor, $4.4 \pm 1.4\%$ of the cells (mean \pm SD) were sickled. To assess the performance of the sickling assay, the z' -factor, a value that quantifies the performance of an assay,²⁶ was determined for each plate using the mean and SD value of the percentage of sickle cells in voxelotor- and DMSO-treated RBCs. The value of the z' -factor was greater than 0.4 in all of the plates, confirming that the assay was adequate²⁷ to screen for compounds that inhibit sickling.

The potential ability of the screened compounds to prevent sickling of SS RBCs was evaluated as indicated in the flow chart (Figure 2). First, the percentage of sickle cells for each sample in each plate was compared with the average percentage in DMSO-treated samples that were tested in the same plate (μ_{DMSO}). Approximately, 99.8% of DMSO samples

did not reduce the percentage of sickle cells by greater than 15% compared to μ_{DMSO} , a result that was considered to “not prevent sickling”. Of the 5393 screened compounds, 183 compounds reduced the percentage of sickle cells by greater than 15% in each of the duplicate wells and were therefore identified as potential candidates that prevented sickling.

To consider the possibility that some of the 183 compounds reduced the percentage of sickle cells by shrinking RBCs, rather than preventing sickling, the automated imaging system was used to measure an “average cell area” of a sample ($\mu\text{m}^2/\text{cell}$). Voxelotor-treated RBCs were considered the model of normal cells, and the average cell area of each sample was compared to the mean average cell area of the voxelotor-treated RBCs that were tested in the same plate ($\mu'_{\text{voxelotor}}$). In all voxelotor-treated samples, the ratio of the average cell area to $\mu'_{\text{voxelotor}}$ was greater than 0.8, which was considered to “not reduce the cell area”. In 149 of the 183 compound-treated samples, the ratio of the average cell area to $\mu'_{\text{voxelotor}}$ was less than 0.8 in either or both duplicate plates, suggesting that these compounds reduced sickling by shrinking RBCs. The remaining 34 compounds were considered to have prevented sickling without reducing the cell area.

To consider the possibility that some of the 34 compounds reduced sickling but still failed to maintain the normal shape of RBCs, the percentage of “other” shaped cells (neither normal nor sickled, Figure 1) for each well was compared with the average percentage of “other” shaped cells in the voxelotor-treated RBCs that were tested in the same plate ($\mu''_{\text{voxelotor}}$). Approximately, 99.6% of voxelotor-treated RBCs did not increase the percentage of “other” cells by greater than 15% compared to $\mu''_{\text{voxelotor}}$. Of the 34 compounds identified as preventing sickling without decreasing the apparent area of the RBCs, 28 compounds increased the percentage of “other” cells by greater than 15% compared to $\mu''_{\text{voxelotor}}$ in either or both plates. The results suggested that these 28 compounds affected the gross appearance of normal RBCs, which may reflect damage to the surface of RBCs. The remaining six compounds (designated compounds 1–6) decreased the percentage of sickle cells without altering the average cell area or increasing the percentage of “other” cells.

Compound 1 Prevented Sickling but Increased the Number of Spherocytes. The acquired images of RBCs treated with compounds 1–6 (Figures S5 and S6) were manually evaluated to confirm that the shape of the RBCs was in fact round and concave. The shape of some of the RBCs in compound 1-treated samples was round but not concave (Figure S5C), indicating that compound 1 may prevent sickling by increasing the percentage of spherocytes, a shape that was included as “normal” in the automated analysis. Because compound 1 caused spherocytosis, in which the deformability of RBCs is reduced,²⁸ this compound was eliminated from further consideration. The shape of RBCs treated with compounds 2–6 was round and concave (Figures S5D and S6C–F), suggesting that these five compounds decreased the percentage of sickle cells without altering the average cell area or the shape of normal RBCs.

Compounds 2–4 Prevented Sickling but Enhanced the Oxidation of Hb. Oxidation of Hb inhibits the formation of sickled cells by decreasing the concentration of deoxy-HbS.^{29,30} However, because the oxidation of Hb impairs the ability to bind and deliver oxygen to tissues, compounds that induce oxidation are not helpful for the treatment of SCD. To evaluate the ability of the compounds to induce the oxidation

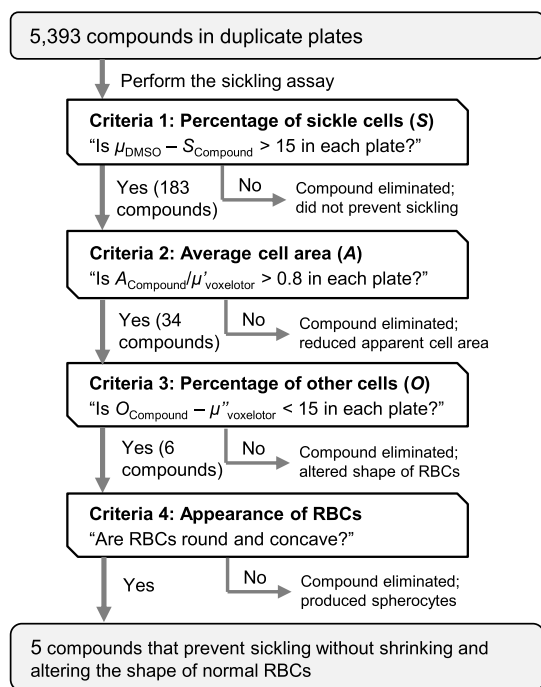


Figure 2. Flow chart to screen 5393 compounds and identify compounds that prevent sickling without shrinking and altering the shape of normal RBCs. S_{compound} : percentage of sickle cells in a compound-treated sample. μ_{DMSO} : mean percentage of sickle cells in the DMSO-treated samples tested in the same plate. A_{compound} : apparent cell area of a compound-treated sample. $\mu'_{\text{voxelotor}}$: mean average cell area in the voxelotor-treated samples tested in the same plate. O_{compound} : percentage of other cells in a compound-treated sample. $\mu''_{\text{voxelotor}}$: mean percentage of other cells in the voxelotor-treated samples tested in the same plate.

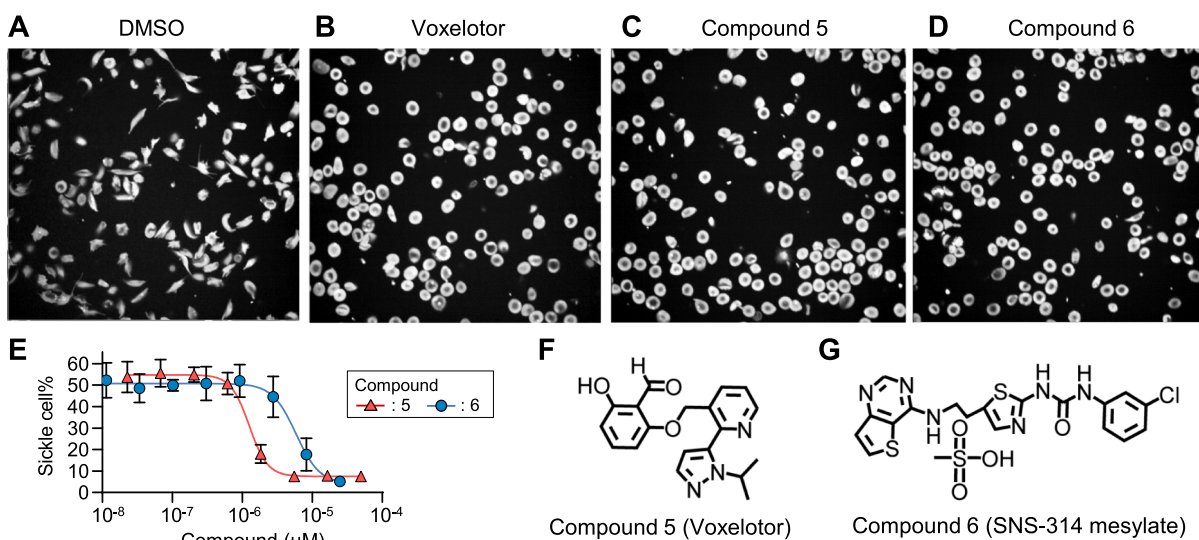


Figure 3. Fluorescence image of SS RBCs in 4% oxygen treated with DMSO (A), voxelotor (B), compound 5 ($50 \mu\text{M}$) (C), and compound 6 ($25 \mu\text{M}$) (D). Dose–response curve of compounds 5 and 6 in terms of percentage of sickle cells (E). Each plot and error bar represent mean and SEM, respectively. The values are derived from two independent biological replicates. The structures of compound 5 [(F) voxelotor] and compound 6 [(G) SNS-314 mesylate].

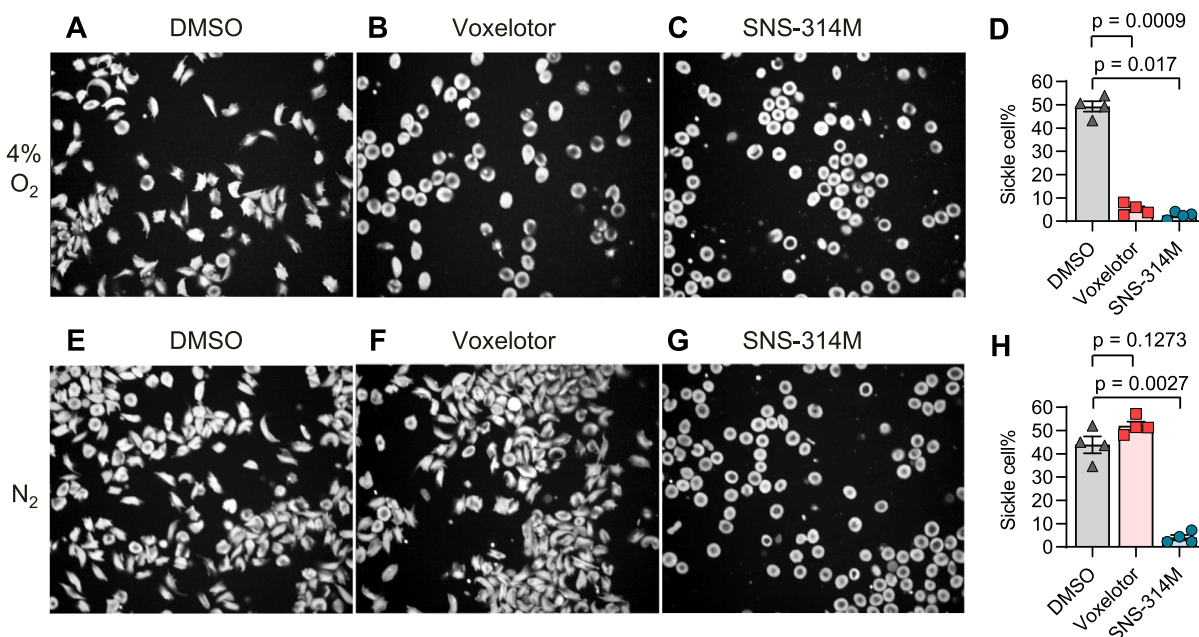


Figure 4. Representative images of SS RBCs treated with DMSO (A), voxelotor $30 \mu\text{M}$ (B), and SNS-314M $30 \mu\text{M}$ (C) in 4% oxygen in nitrogen. The percentage of sickle cells in the SS RBCs treated with compounds in 4% oxygen in nitrogen (D). Representative images of SS RBCs treated with DMSO (E), $30 \mu\text{M}$ voxelotor (F), and $30 \mu\text{M}$ SNS-314M (G) in 99.9% nitrogen. The percentage of sickle cells in SS RBCs treated with compounds in 99.9% nitrogen (H). (A–C) and (E–G) are representative of four replicated samples for each compound. Each bar and error bar, respectively, indicate mean and SEM ($n = 4$ biological replicates). One-way ANOVA with Tukey’s multiple comparison test was used to compare the percentage of sickle cells in samples treated with DMSO vs voxelotor vs SNS-314M. In these comparisons, an adjusted p -value was calculated using GraphPad Prism 9, and a p -value less than 0.05 was considered statistically significant.

of Hb, purified Hb was treated with compounds 2–6 and the absorbance of light at 630 nm (A_{630}) was measured at 37°C . Increased absorption of light at A_{630} is associated with increased metHb concentration (Figure S7A).

Treatment of purified Hb ($20 \mu\text{M}$ tetramer) with DMSO (5 vol %) for 6 h did not alter the A_{630} of Hb ($A_{630} < 0.03$, Figure S7B,C), confirming that DMSO does not oxidize Hb. Treatment of purified Hb with each of the compounds 2–4 ($60 \mu\text{M}$ each in DMSO 5 vol %) increased A_{630} greater than

that of DMSO-treated Hb, indicating that these three compounds induced the formation of metHb. We therefore eliminated compounds 2–4 from further consideration. Compounds 5 and 6 did not alter A_{630} over the course of 6 h ($A_{630} < 0.03$, Figure S7C), indicating that these two compounds inhibit sickle cell formation by a mechanism that is independent of Hb oxidation.

Compounds 5 and 6 Inhibited Sickling in a Concentration-Dependent Manner. To further evaluate

the ability of compounds 5 and 6 to prevent sickling, SS RBCs were treated with each compound at eight different concentrations and were then exposed to 4% oxygen. The percentage of sickle cells was determined using the sickling assay (Figure 3A–D), and the dose–response curves of the compounds, in terms of preventing RBC sickling, were determined (Figure 3E). A linear regression curve was used to determine EC_{50} values for each compound, which is the concentration of a compound that decreases the percentage of sickle cells halfway between the baseline and maximum response. The mean EC_{50} values of compounds 5 and 6 were 1.3 and 5.9 μ M, respectively ($n = 2$ independent biological replicates), indicating that the activity of compound 5 to prevent sickling was greater than that of compound 6. “Unblinding” of the Broad Repurposing Compound Library revealed that compound 5 was voxelotor (Figure 3F) and compound 6 was SNS-314 mesylate (SNS-314M, Figure 3G).

SNS-314M Prevents Sickling in Nitrogen. To assess the ability of SNS-314M and voxelotor to prevent sickling under conditions in which the compounds were unable to alter the concentration of deoxyHbS, the sickling assay was conducted in pure nitrogen (purity: 99.9%).

In pure nitrogen, the percentage of sickle cells in SNS-314M-treated blood samples was significantly lower than that in DMSO-treated blood samples (4.0 ± 1.2 vs 44 ± 3.6 , mean \pm SEM, p -value = 0.0027, $n = 4$ independent biological replicates, Figure 4E,G,H). However, as expected because voxelotor decreases sickling by increasing the affinity of Hb for oxygen, the percentage of sickle cells in voxelotor-treated blood samples in pure nitrogen was similar to that in DMSO-treated SS RBCs (52 ± 1.8 vs 44 ± 3.6 , p -value = 0.127, $n = 4$ independent biological replicates, Figure 4E,F,H).

Taken together, these results indicate that SNS-314M, but not voxelotor, prevented sickling in the absence of oxygen and that SNS-314M prevents sickling by a mechanism that is different from that of voxelotor.

DISCUSSION

We developed a high-throughput sickling assay, which used an automated high-content imaging system to quantify the effects of deoxygenation on the shape and size of SS RBCs. Based on results using an FDA-approved, commercially available inhibitor of RBC sickling (voxelotor), we showed that the assay clearly identified compounds that inhibit sickling. The assay was used to screen the 5393 compounds in the Broad Repurposing Library for their potential ability to prevent sickling of SS RBCs. Of the 5393 screened compounds, two compounds, SNS-314M and voxelotor (a component of the compound library), prevented RBC sickling without altering the average cell area or the shape of normal RBCs. Neither of the compounds increased the oxidation of Hb.

Several assays have been reported to quantify the percentage of sickle cells in a 96- or 384-well plate format, but these assays have limitations that prevent their use for high-throughput screening. One assay is based on laser-induced polymerization of Hb in sickle trait cells (heterozygous for normal HbA and HbS, with a mixed AS RBC phenotype) in nitrogen.³¹ In this assay, the precise time at which fibers of HbS distort the cells can be determined, but specialized equipment is required to induce the polymerization of HbS. In another assay, RBCs in a 384-well plate were incubated in nitrogen and the number of normal and sickle cells was measured continuously for 12 h.³² Although the rate of sickling can be analyzed, this assay is slow,

which reduces speed and throughput of the measurement. One solution to increase the throughput is to assess the percentage of sickle cells at a single time point. In a previously reported sickling assay,³³ RBCs from patients with sickle cell anemia were treated in 2% oxygen for 2 h and then fixed with glutaraldehyde in a 96-well plate. Sickling of samples was assessed using imaging flow cytometry in air.³³ However, glutaraldehyde must be removed from each sample prior to flow cytometry, preventing application of this assay to a high-throughput setting.

The assay described herein is optimal for the screening of a large number of compounds. SS RBCs were fixed with glutaraldehyde in 384-well plates after 1 h of gas exposure and the percentage of sickled cells was measured automatically at a rate of one plate every 30 min. Although the 15 min incubation prior to exposing the cells to 4% oxygen was sufficient to identify voxelotor and SNS-314M in this study, longer incubations might be required to identify compounds that modify sickle cell formation by altering RBC metabolism. An unexpected advantage of using glutaraldehyde to fix cells was that this treatment permitted us to obtain fluorescence images (without staining with a dye), which facilitated the automated measurements of the cell appearance and area. The blinded identification of voxelotor among the 5393 compounds supports the ability of this assay to identify compounds that inhibit sickling.

SNS-314M was developed as an inhibitor of Aurora kinases,³⁴ which are serine/threonine kinases essential for cell proliferation. SNS-314 showed potent antitumor activity in mouse xenograft models of human cancer.³⁵ To our knowledge, SNS-314M has not been studied as a treatment for SCD. The mechanism by which SNS-314M inhibits sickling has not yet been determined.

The automated image analysis allowed us to screen compounds for their antisickling activities. Evaluating the RBC area and the percentage cells with shapes “other” than concave or sickled permitted us to eliminate compounds with potentially hazardous effects. One limitation of the automated analysis is that the software could not detect spherocytes. Manual image analysis was therefore required, for a limited number of compounds, to confirm that the compounds prevented sickling without altering the round and concave shape of RBCs.

CONCLUSIONS

We used an automated acquisition system to develop a high-throughput sickling assay that permits screening of compounds for their effects on sickling of SS RBCs. The sickling assay was used to perform a screen of 5393 compounds for their potential ability to prevent sickling of SS RBCs. Two compounds, SNS-314M and voxelotor, were found to prevent RBC sickling without altering the average cell area or shape of RBCs. The sickling assay described in this study will facilitate the identification of additional, novel antisickling compounds, which will potentially expand the therapeutic options for SCD.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c00541>.

Scheme of the sickling assay; chamber to expose plates to 4% O₂/96% N₂; bright-field images of an SS RBC

sample treated with DMSO and voxelotor (visualized using an inverted microscope), examples of the bright-field and fluorescence images of DMSO-treated SS RBCs; bright-field images of an SS RBC sample treated with DMSO, voxelotor, and compounds 1–6; time course of A_{630} of HbA treated with compounds 2–6; and summary of the method to screen the 5393 compounds (PDF)

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Notes

The authors declare the following competing financial interest(s): Massachusetts General Hospital has filed a patent related to SNS-314 mesylate.

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REFERENCES

- <https://www.cdc.gov/ncbddd/sicklecell/data.html> (accessed Jan 17, 2022).
- Kato, G. J.; Piel, F. B.; Reid, C. D.; Gaston, M. H.; Ohene-Frempong, K.; Krishnamurti, L.; Smith, W. R.; Panepinto, J. A.; Weatherall, D. J.; Costa, F. F.; Vichinsky, E. P. Sickle cell disease. *Nat. Rev. Dis. Prim.* **2018**, *4*, 18010.
- Noguchi, C. T.; Schechter, A. N. Sickle hemoglobin polymerization in solution and in cells. *Annu. Rev. Biophys. Biophys. Chem.* **1985**, *14*, 239–263.
- Eaton, W. A.; Hofrichter, J. Sickle cell hemoglobin polymerization. *Adv. Protein Chem.* **1990**, *40*, 63–279.
- Steinberg, M. H. Pathophysiology of sickle cell disease. *Baillieres Clin. Haematol.* **1998**, *11*, 163–184.
- Kato, G. J.; Steinberg, M. H.; Gladwin, M. T. Intravascular hemolysis and the pathophysiology of sickle cell disease. *J. Clin. Invest.* **2017**, *127*, 750–760.
- Charache, S.; Dover, G.; Moyer, M.; Moore, J. Hydroxyurea-induced augmentation of fetal hemoglobin production in patients with sickle cell anemia. *Blood* **1987**, *69*, 109–116.
- Navenot, J.-M.; Merghoub, T.; Ducrocq, R.; Muller, J.-Y.; Krishnamoorthy, R.; Blanchard, D. New method for quantitative determination of fetal hemoglobin-containing red blood cells by flow cytometry: application to sickle-cell disease. *Cytometry* **1998**, *32*, 186–190.
- Platt, O. S. Hydroxyurea for the treatment of sickle cell anemia. *N. Engl. J. Med.* **2008**, *358*, 1362–1369.
- Niihara, Y.; Zerez, C. R.; Akiyama, D. S.; Tanaka, K. R. Increased red cell glutamine availability in sickle cell anemia: demonstration of increased active transport, affinity, and increased glutamate level in intact red cells. *J. Lab. Clin. Med.* **1997**, *130*, 83–90.
- Quinn, C. T. L-Glutamine for sickle cell anemia: more questions than answers. *Blood* **2018**, *132*, 689–693.
- Howard, J.; Hemmaway, C. J.; Telfer, P.; Layton, D. M.; Porter, J.; Awogbade, M.; Mant, T.; Gretler, D. D.; Dufu, K.; Hutchaleelaha, A.; Patel, M.; Siu, V.; Dixon, S.; Landsman, N.; Tonda, M.; Lehrer-Graiwer, J. A phase 1/2 ascending dose study and open-label extension study of voxelotor in patients with sickle cell disease. *Blood* **2019**, *133*, 1865–1875.
- Oksenberg, D.; Dufu, K.; Patel, M. P.; Chuang, C.; Li, Z.; Xu, Q.; Silva-Garcia, A.; Zhou, C.; Hutchaleelaha, A.; Patskovska, L.;

- Patskovsky, Y.; Almo, S. C.; Sinha, U.; Metcalf, B. W.; Archer, D. R. GBT440 increases haemoglobin oxygen affinity, reduces sickling and prolongs RBC half-life in a murine model of sickle cell disease. *Br. J. Haematol.* **2016**, *175*, 141–153.
- (14) Matsui, N. M.; Borsig, L.; Rosen, S. D.; Yaghai, M.; Varki, A.; Embury, S. H. P-selectin mediates the adhesion of sickle erythrocytes to the endothelium. *Blood* **2001**, *98*, 1955–1962.
- (15) Kutlar, A.; Kanter, J.; Liles, D. K.; Alvarez, O. A.; Cançado, R. D.; Friedrisch, J. R.; Knight-Madden, J. M.; Bruederle, A.; Shi, M.; Zhu, Z.; Ataga, K. I. Effect of crizanlizumab on pain crises in subgroups of patients with sickle cell disease: A SUSTAIN study analysis. *Am. J. Hematol.* **2019**, *94*, 55–61.
- (16) Charache, S.; Terrin, M. L.; Moore, R. D.; Dover, G. J.; Barton, F. B.; Eckert, S. V.; McMahon, R. P.; Bonds, D. R. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. *N. Engl. J. Med.* **1995**, *332*, 1317–1322.
- (17) Niihara, Y.; Miller, S. T.; Kanter, J.; Lanzkron, S.; Smith, W. R.; Hsu, L. L.; Gordeuk, V. R.; Viswanathan, K.; Sarnaik, S.; Osunkwo, I.; Guillaume, E.; Sadanandan, S.; Sieger, L.; Lasky, J. L.; Panosyan, E. H.; Blake, O. A.; New, T. N.; Bellevue, R.; Tran, L. T.; Razon, R. L.; Stark, C. W.; Neumayr, L. D.; Vichinsky, E. P. A Phase 3 Trial of off-Glutamine in Sickle Cell Disease. *N. Engl. J. Med.* **2018**, *379*, 226–235.
- (18) Ataga, K. I.; Kutlar, A.; Kanter, J.; Liles, D.; Cancado, R.; Friedrisch, J.; Guthrie, T. H.; Knight-Madden, J.; Alvarez, O. A.; Gordeuk, V. R.; Gualandro, S.; Colella, M. P.; Smith, W. R.; Rollins, S. A.; Stocker, J. W.; Rother, R. P. Crizanlizumab for the Prevention of Pain Crises in Sickle Cell Disease. *N. Engl. J. Med.* **2017**, *376*, 429–439.
- (19) Steinberg, M. H.; Voskaridou, E.; Kutlar, A.; Loukopoulos, D.; Koshy, M.; Ballas, S. K.; Castro, O.; Barton, F. Concordant fetal hemoglobin response to hydroxyurea in siblings with sickle cell disease. *Am. J. Hematol.* **2003**, *72*, 121–126.
- (20) Yahouédéhou, S. C. M. A.; Adorno, E. V.; da Guarda, C. C.; Ndidi, U. S.; Carvalho, S. P.; Santiago, R. P.; Aleluia, M. M.; de Oliveira, R. M.; Gonçalves, M. d. S. Hydroxyurea in the management of sickle cell disease: pharmacogenomics and enzymatic metabolism. *Pharmacogenomics J.* **2018**, *18*, 730–739.
- (21) Vichinsky, E.; Hoppe, C. C.; Ataga, K. I.; Ware, R. E.; Nduba, V.; El-Beshlawy, A.; Hassab, H.; Achebe, M. M.; Alkindi, S.; Brown, R. C.; Diuguid, D. L.; Telfer, P.; Tsitsikas, D. A.; Elghandour, A.; Gordeuk, V. R.; Kanter, J.; Abboud, M. R.; Lehrer-Graiwer, J.; Tonda, M.; Intondi, A.; Tong, B.; Howard, J.; Investigators, H. T. A Phase 3 Randomized Trial of Voxelotor in Sickle Cell Disease. *N. Engl. J. Med.* **2019**, *381*, 509–519.
- (22) Corsello, S. M.; Bittker, J. A.; Liu, Z.; Gould, J.; McCarren, P.; Hirschman, J. E.; Johnston, S. E.; Vrcic, A.; Wong, B.; Khan, M.; Asiedu, J.; Narayan, R.; Mader, C. C.; Subramanian, A.; Golub, T. R. The Drug Repurposing Hub: a next-generation drug library and information resource. *Nat. Med.* **2017**, *23*, 405–408.
- (23) Nakagawa, A.; Ferrari, M.; Schleifer, G.; Cooper, M. K.; Liu, C.; Yu, B.; Berra, L.; Klings, E. S.; Safo, R. S.; Chen, Q.; Musayev, F. N.; Safo, M. K.; Abdulmalik, O.; Bloch, D. B.; Zapol, W. M. A Triazole Disulfide Compound Increases the Affinity of Hemoglobin for Oxygen and Reduces the Sickling of Human Sickle Cells. *Mol. Pharm.* **2018**, *15*, 1954–1963.
- (24) Lee, K.; Choi, S.; Yang, C.; Wu, H.-C.; Yu, J. Autofluorescence generation and elimination: a lesson from glutaraldehyde. *Chem. Commun.* **2013**, *49*, 3028–3030.
- (25) Abay, A.; Simionato, G.; Chachanidze, R.; Bogdanova, A.; Hertz, L.; Bianchi, P.; van den Akker, E.; von Lindern, M.; Leonetti, M.; Minetti, G.; Wagner, C.; Kaestner, L. Glutaraldehyde—A Subtle Tool in the Investigation of Healthy and Pathologic Red Blood Cells. *Front. Physiol.* **2019**, *10*, 514.
- (26) Zhang, J.-H.; Chung, T. D. Y.; Oldenburg, K. R. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screen* **1999**, *4*, 67–73.
- (27) Iversen, P. W. Assay Guidance Manual [Internet], Assay Guidance Manual; Markossian, S.; Grossman, A.; Brimacombe, K.; Arkin, M.; Auld, D.; Austin, C. P.; Baell, J.; Chung, T. D. Y.; Coussens, N. P.; Dahlin, J. L.; Devanarayan, V.; Foley, T. L.; Glicksman, M.; Hall, M. D.; Haas, J. V.; Hoare, S. R. J.; Inglese, J.; Iversen, P. W.; Kales, S. C.; Lal-Nag, M.; Li, Z.; McGee, J.; McManus, O.; Riss, T.; Saradjian, P.; Sittampalam, G. S.; Tarselli, M.; Trask, O. J., Jr.; Wang, Y.; Weidner, J. R.; Wildey, M. J.; Wilson, K.; Xia, M.; Xu, X., Eds.; Bethesda (MD), 2004. <https://www.ncbi.nlm.nih.gov/books/NBK53196/> (accessed Jan 17, 2022).
- (28) Huisjes, R.; Bogdanova, A.; van Solinge, W. W.; Schifflers, R. M.; Kaestner, L.; van Wijk, R. Squeezing for Life—Properties of Red Blood Cell Deformability. *Front. Physiol.* **2018**, *9*, 656.
- (29) Beutler, E. The effect of methemoglobin formation in sickle cell disease. *J. Clin. Invest.* **1961**, *40*, 1856–1871.
- (30) Franklin, I. M.; Rosemeyer, M. A.; Huehns, E. R. Sickle cell disease: the proportion of liganded haemoglobin needed to prevent crises. *Br. J. Haematol.* **1983**, *54*, 579–587.
- (31) Li, Q.; Henry, E. R.; Hofrichter, J.; Smith, J. F.; Cellmer, T.; Dunkelberger, E. B.; Metaferia, B. B.; Jones-Straehle, S.; Boutom, S.; Christoph, G. W.; Wakefield, T. H.; Link, M. E.; Staton, D.; Vass, E. R.; Miller, J. L.; Hsieh, M. M.; Tisdale, J. F.; Eaton, W. A. Kinetic assay shows that increasing red cell volume could be a treatment for sickle cell disease. *Proc. Natl. Acad. Sci. U.S.A.* **2017**, *114*, E689–E696.
- (32) Dunkelberger, E. B.; Metaferia, B.; Cellmer, T.; Henry, E. R. Theoretical Simulation of Red Cell Sickling Upon Deoxygenation Based on the Physical Chemistry of Sickle Hemoglobin Fiber Formation. *J. Phys. Chem. B* **2018**, *122*, 11579–11590.
- (33) Fertrin, K. Y.; Samsel, L.; van Beers, E. J.; Mendelsohn, L.; Kato, G. J.; McCoy, J. P., Jr. Sickle Cell Imaging Flow Cytometry Assay (SIFCA). *Methods Mol. Biol.* **2016**, *1389*, 279–292.
- (34) Oslob, J. D.; Romanowski, M. J.; Allen, D. A.; Baskaran, S.; Bui, M.; Elling, R. A.; Flanagan, W. M.; Fung, A. D.; Hanan, E. J.; Harris, S.; Heumann, S. A.; Hoch, U.; Jacobs, J. W.; Lam, J.; Lawrence, C. E.; McDowell, R. S.; Nannini, M. A.; Shen, W.; Silverman, J. A.; Sopko, M. M.; Tangonan, B. T.; Teague, J.; Yoburn, J. C.; Yu, C. H.; Zhong, M.; Zimmerman, K. M.; O'Brien, T.; Lew, W. Discovery of a potent and selective aurora kinase inhibitor. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4880–4884.
- (35) Arbitrario, J. P.; Belmont, B. J.; Evanchik, M. J.; Flanagan, W. M.; Fucini, R. V.; Hansen, S. K.; Harris, S. O.; Hashash, A.; Hoch, U.; Hogan, J. N.; Howlett, A. R.; Jacobs, J. W.; Lam, J. W.; Ritchie, S. C.; Romanowski, M. J.; Silverman, J. A.; Stockett, D. E.; Teague, J. N.; Zimmerman, K. M.; Taverna, P. SNS-314, a pan-Aurora kinase inhibitor, shows potent anti-tumor activity and dosing flexibility in vivo. *Cancer Chemother. Pharmacol.* **2010**, *65*, 707–717.