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Screen of the ReFRAME Compound Library for Therapeutic Agents to Prevent Red Blood Cell Sickling Using an Improved High Throughput Sickling Assay

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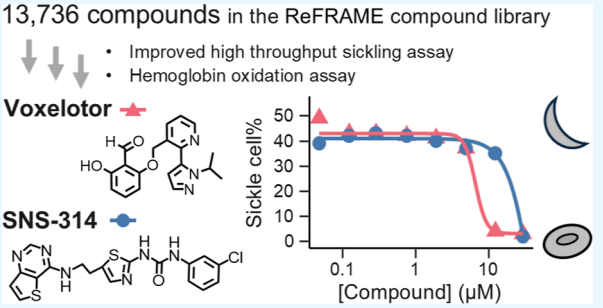
ABSTRACT: Sickle cell disease (SCD) is an autosomal recessive disorder of blood characterized by a mutation in the β chain of hemoglobin (Hb), leading to the production of sickle Hb (HbS). In SCD, under low oxygen conditions, red blood cells (RBCs) containing HbS form a characteristic "sickle" shape, resulting in chronic hemolytic anemia and acute vaso-occlusive crises. Current therapies for SCD have limitations in efficacy or availability, highlighting the need for new anti-sickling drugs. To facilitate the discovery of new anti-sickling compounds, we previously developed a high throughput sickling assay, which permits rapid screening of thousands of compounds for the ability to inhibit RBC sickling. In this study, we improved the sickling assay by optimizing the assay condition and expanded our screening efforts by evaluating the Repurposing, Focused Rescue, and Accelerated Medchem (ReFRAME) compound library, which contains approximately 2.5 times more compounds than previously screened. We were able to increase the number of blood samples that were adequate for identifying anti-sickling compounds in the improved sickling assay and identified voxelotor and SNS-314 as compounds that successfully prevented sickling. The improved sickling assay will increase access to valuable blood samples from SCD volunteers, providing more opportunities to develop anti-sickling compounds for treating SCD.



INTRODUCTION

Sickle cell disease (SCD) is an autosomal recessive disorder of blood characterized by a mutation in the gene encoding the β-globin chain of hemoglobin (Hb), resulting in the production of sickle hemoglobin (HbS).[1,2] In SCD, as the concentration of oxygen in blood decreases, the concentration of deoxygenated HbS increases, forming fibers of polymerized HbS in red blood cells (RBCs), which develop the characteristic "sickle" shape.[3,4] The sickling of RBCs damages their membrane resulting in hemolytic anemia, reduces their deformability, and enhances their adhesion to the vascular endothelium, leading to microvascular occlusion, which causes pain, inflammation, damage to nearly every vital organ, and premature death.[1,5,6] In the US, the estimated average life expectancy for patients with SCD is 20 years less than the general population,[7,8] in African countries, 50−90% of children born with SCD die before their fifth birthday.[9]

Three disease-modifying therapies—hydroxyurea,[6,10,11] L-glutamine,[12,13] and crizanlizumab[14,15]—have been approved by the FDA for the treatment of SCD. These therapies provide several benefits, including reducing the frequency of pain crises, increasing Hb concentrations, and lowering the rate of hospitalizations.[16−18] However, each has limitations: a subset of patients does not respond to hydroxyurea, although the underlying reasons why these individuals fail to respond remain unclear.[19] While both L-glutamine and crizanlizumab reduce vaso-occlusive crises,[16,17] they do not prevent RBC sickling. Emerging treatments for SCD include allogenic hematopoietic stem cell transplantation[20,21] and autologous hematopoietic stem cell transplantation including novel gene therapies[22] that either correct the genetic mutation (Lyfgenia)[23] or enhance fetal Hb production (Casgevy).[24] Lyfgenia and Casgevy have been approved by the FDA and currently available to patients for the treatment of SCD. Although these cell and gene therapies are curative, their application is restricted by limited donor availability (for allogenic stem cell transplantation), the requirement for specialized facilities and clinical expertise,[25] manufacturing bottlenecks and inefficiencies, and extremely high costs. Furthermore, the conditioning

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regimens used in these therapies can increase the risk of long-term complications.[26]

Considering the limitations of the current therapies, the development of widely available and affordable anti-sickling drugs is required to address the needs of the vast majority of patients with SCD. In addition, the availability of affordable and accessible small molecules could circumvent the challenges for those unable or unwilling to get gene therapies or transplants. The discovery of new anti-sickling small molecules would significantly expand the available therapeutic options. To facilitate this discovery, we developed a high throughput "sickling assay" that quantifies the ability of compounds to inhibit sickling of RBCs in the setting of low partial pressure of oxygen, which induces RBC sickling.[27] Using the sickling assay, we screened 5393 compounds in the Broad repurposing compound library and identified voxelotor (an anti-sickling compound[28]) as well as SNS-314 mesylate as a novel small molecule that successfully inhibited RBC sickling while maintaining the round and concave shape of RBCs without enhancing oxidation of Hb.[27] These results highlight the capability of the sickling assay to identify anti-sickling compounds in large compound libraries.

In this study, we expanded our previous screening efforts by evaluating the Repurposing, Focused Rescue, and Accelerated Medchem (ReFRAME) compound library, which contains approximately 2.5 times more compounds than previously screened, with the goal of identifying additional anti-sickling agents. The ReFRAME compound library[29] is developed by Calibr-Skaggs Institute for Innovative Medicine funded generously by the Gates Foundation and the Wellcome Trust Foundation. The library comprises compounds that have either reached clinical development, entered into investigational new drug (IND)-enabling studies, or undergone extensive preclinical evaluation. We optimized the sickling assay to increase the number of blood samples which are suitable for screening and screened 13,736 compounds from the ReFRAME library and identified four compounds, including voxelotor and SNS-314, that inhibited sickling without enhancing oxidation of Hb.

METHODS

Screen of Compounds for their Ability to Inhibit Sickling Using the Sickling Assay. Compounds were evaluated for their anti-sickling activity using the sickling assay by the following steps: (1) acquisition of blood samples, (2) induction of sickling in 4% oxygen/96% nitrogen, (3) preliminary assessment of blood samples, (4) automatic quantification of sickling, and (5) evaluation of the sickling assay performance and the ability of compounds to prevent sickling.

Acquisition of Blood Samples. The Institutional Review Boards of Mass General Brigham and Boston Children's Hospital approved the collection and the use of blood samples from patients with homozygous HbS (SS blood samples), and all participants provided informed consent prior to blood collection. Blood samples were collected from the participants in tubes containing EDTA and were stored at 4 °C. The samples were used within 48 h of blood collection.

Induction of Sickling in 4% Oxygen/96% Nitrogen. The procedure for inducing sickling followed the previously reported method,[27] with modifications to the buffer components and oxygen concentration to increase the number of sickled cells in samples for assessment of anti-sickling activity. The specific changes are as follows:

A buffer (pH 7.4) was prepared as previously reported,[27] which contains *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. Each SS whole blood sample was diluted in the buffer and water in a ratio of 1:900:100. The diluted SS blood (20 μL/well) was aliquoted onto 384-well plates (CellCarrier 384-well Ultra or Phenoplate microplates, Revvity, Waltham, MA). The samples were incubated as previously reported[27] in 4% (3.6−4.0%) oxygen in nitrogen at 37 °C for 1 h. After the incubation, the blood samples were treated with glutaraldehyde as previously reported[27] to assess RBC appearance in air.

Preliminary Assessment of Blood Samples. An inverted microscope was used to determine whether the RBCs were sufficiently sickled to permit their use for the screen, as previously reported.[27] A blood sample was considered adequate for the screen if the average percentage of sickled cells (n = 4 wells) was greater than 40%.

Screen of Compounds to Identify Compounds that Prevent Sickling by Automatic Quantification of Sickling. The DMSO solution of compounds in the ReFRAME compound library, together with voxelotor (the positive control that inhibits sickling), and DMSO (diluent for the compounds) alone were dispensed into individual wells in 384-well plates using an acoustic liquid handler by Calibr-Skaggs Institute for Innovative Medicine (San Diego, CA). SS blood samples were diluted in a 1:900:100 ratio using the buffer and water, and diluted blood samples (20 μL/well) were dispensed onto the plates. The samples were incubated in 4% oxygen at 37 °C for 1 h and then treated with glutaraldehyde as described in "Induction of sickling in 4% oxygen/96% nitrogen" for the subsequent assessment of RBC appearance in air.

To assess RBC appearance, the percentage of cells with "round" (normal), "elongated" (sickle), and "other" (neither normal nor sickle) shape, and the cell area (μm^2/cell; total area of the counted cells/number of the cells) in each sample were determined automatically using the Opera Phenix high-content imaging system (Revvity, Waltham, MA) as previously reported.[27]

Identification of Compounds that Reduce Sickling While Maintaining the Normal Shape and Size of RBCs. The compounds that inhibit RBC sickling in 4% oxygen, while maintaining the normal size and shape of RBCs, were identified using the four criteria (Criteria 1−4) as previously reported.[27]

Elimination of Compounds that Enhance Oxidation of Hb. To evaluate whether the screened compounds reduce sickling by enhancing oxidation of Hb, human adult Hb (HbA, 20 μM tetramer) was treated with each compound (60 μM) in HEMOX solution and dispensed into a 384-well plate, as previously reported.[27] The plate was placed in a plate reader (Multiskan GO, Thermo Scientific, MA), and the absorption spectra of the samples were measured from 500 to 700 nm in air. The samples were incubated at 37 °C in air for 8 h, after which the absorption spectra were measured again. Each compound was tested in triplicate (n = 3). The spectra were averaged, and the control spectra were subtracted from the corresponding sample spectra for calibration. If the calibrated absorbance at 630 nm (A_{630}) of a compound-treated Hb

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exceeded that of DMSO-treated Hb, the compound was considered to enhance oxidation of Hb and was eliminated from further screening.

Assessing the Effect of Compound Concentration on Sickling. To further evaluate the ten compounds that were identified to inhibit sickling at a single concentration, we repeated the sickling assay using eight different concentrations (30, 12, 5.0, 1.9, 0.76, 0.29, 0.12, and 0.048 μM) of each compound and determined the EC_{50} values for each compound from their respective dose−response curves using four-parameter logistic regression (eq 1) with R version 4.4.1[30] and the nplr package (version 0.1-7).[31]

$$y = \text{bottom} + \frac{\text{top} - \text{bottom}}{1 + 10^{\{(\text{Xmid} - X) \times \text{HillSlope}\}}} \quad (1)$$

In eq 1, X is the log of the concentration of a compound. Y is a percentage of sickle cells. "top and bottom" are plateaus in the same units as Y. The value of 10^{Xmid} is an EC_{50}, a concentration of a compound that decreases the percentage of sickle cells halfway between the baseline and maximum response. HillSlope is a slope factor or Hill slope (unitless).

RESULTS

An Improved Sickling Assay Method Increased the Percent of Sickled Cells and Increased the Number of SS Blood Samples that could be Used in the Assay. The sickling assay[27] was used to screen the 13,736 compounds in the ReFRAME library for their ability to prevent sickling of SS RBCs. In this assay, RBCs were exposed to 4% oxygen in 384-well plates to induce sickling, and images were acquired using an automated image analysis system, as previously described.[27] The analysis system determines the percentages of "elongated" (sickled), "round" (normal), and "other" (neither normal nor sickled) RBCs, along with the apparent area of the RBCs, which are used to evaluate the ability of the compounds in inhibiting sickling while maintaining the normal shape and size of the RBCs.

A limitation of our screening process was that some blood samples exhibited insufficient sickling with sickle cell percent less than 40%, which hindered the evaluation of the anti-sickling activity of compounds and slowed the screening process. The low sickle cell percent was likely due to a high proportion of RBCs that were "other" (neither round nor elongated) cells. A report indicated that increased salt concentrations in samples can lead to the formation of "granular" or "mosaic"-shaped RBCs, instead of the typical elongated sickle cells.[32] Additionally, we found a reduction in sample volume of approximately 10% after the sickling assay. These findings suggest that fewer sickled cells would be formed during the assay, potentially due to slight condensation of the samples in the 384-well plate.

To enhance the formation of sickle cells in the sickling assay, the buffer (used to dilute SS blood; see Methods) was diluted with water at a ratio of 9:1. This diluted solution was then used to prepare blood samples for the assay, which evaluated the effect of dilution on the percentage of sickled cells. A total of 20 SS blood samples were evaluated, all collected from donors undergoing hydroxyurea treatment for SCD. We found that the sickle cell percent in the diluted solution was 39 ± 2.0 (mean ± standard error of the mean, SEM), significantly higher than that in the undiluted solution (30 ± 2.5, $p = 2.1 \times 10^{-4}$, $n = 20$ biological replicates; Figure 1). Furthermore, the number of

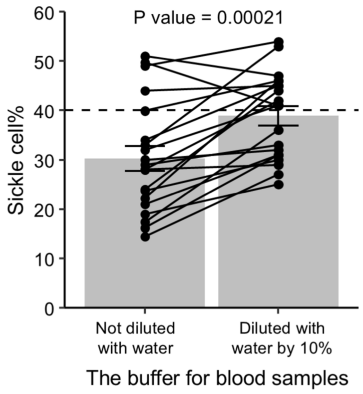

Figure 1. Sickle cell percent of 20 SS blood samples in the buffer and the one diluted with water at 9:1 volume ratio in the sickling assay. The bar graphs represent the mean percentage of sickle cells, with error bars showing the mean ± SEM (n = 20 biological replicates). A two-sided paired t-test was performed using R 4.4.1 to compare the sickle cell percents between the two groups, and differences were considered statistically significant if the p-value was less than 0.05. The dashed line indicates 40% of sickle cells, above which a blood sample was considered adequate for screening compounds for their anti-sickling activity.

blood samples with a sickle cell percent greater than 40% increased from four to 11 out of 20 by the dilution of the buffer. These results suggest that diluting the buffer by 10% increases the number of sickled cells, thereby improving the suitability of blood samples for identifying anti-sickling compounds. Consequently, in the screen of the ReFRAME library in 384-well plates, whole SS blood samples were diluted at a 1:900:100 ratio using the buffer and water.

Screen of the ReFRAME Compound Library Using the Improved Sickling Assay. The improved sickling assay was used to screen 13,736 ReFRAME compounds in a total of eighty-seven 384 well plates (24 columns and 16 rows) for their ability to inhibit sickling. For each plate, voxelotor was placed in columns 1 and 23 (32 total wells) as the positive control (30 μM as the final concentration) to inhibit sickling (Figure S1). DMSO (the diluent of the compounds) was placed in columns 2 and 24 (32 total wells) as the negative control (Figure S2) and the compounds were dispensed onto the remaining 320 wells. The final concentration of each compound was between 1.5 and 25 μM (137 compounds), 25 and 35 μM (13,590 compounds), or 35 and 145 μM (nine compounds). Out of the 13,736 screened compounds, 1241 compounds were tested only once, and the remaining 12,495 compounds were tested twice based on the availability of the compounds.

To assess the quality of the sickling assay for identifying anti-sickling compounds, the z'-factor[33] for sickle cell percentage was calculated for each plate using the mean and standard deviation of the sickle cell percentages in voxelotor- and DMSO-treated RBCs. Across all 87 plates, the z'-factor value exceeded 0.4 (0.68 ± 0.10, mean ± SEM), confirming that the improved sickling assay remains suitable for screening compounds for their ability to inhibit sickling.[34]

Ten of the 13,736 Compounds Prevented Sickling at a Single Concentration While Maintaining the Normal Shape and Size of RBCs. The ability of the screened 13,736 compounds to prevent RBC sickling while maintaining normal cell size and shape was assessed using four criteria (Criteria 1−4; Figure 2, Table S1) as previously reported.[27] Among the

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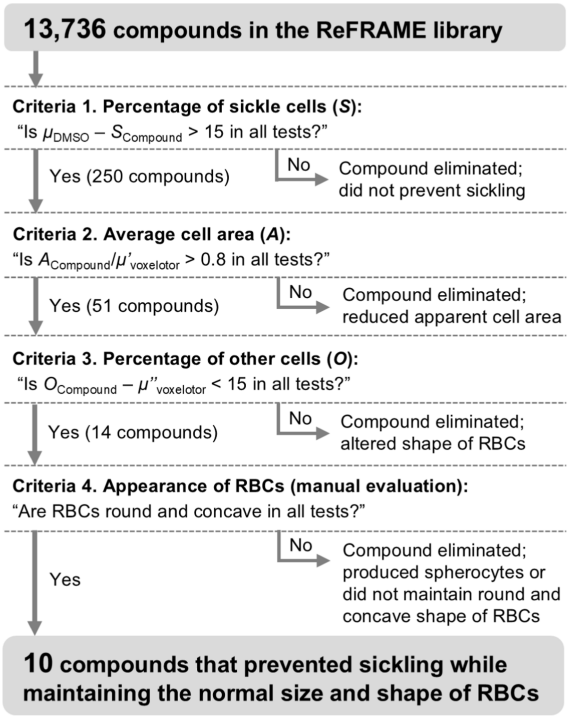

Figure 2. A flowchart to screen 13,736 compounds for the ability of the compounds to prevent sickling while maintaining the normal size and shape of normal RBCs. $S_{compound}$: percentage of sickle cells in a compound-treated sample. μ_{DMSO}: mean percentage of sickle cells in the DMSO-treated samples tested in the same plate. $A_{compound}$: apparent cell area of a compound-treated sample. $\mu_{voxelotor}'$: 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. $\mu_{voxelotor}''$: mean percentage of other cells in the voxelotor-treated samples tested in the same plate.

13,736 compounds, 250 compounds met Criteria 1 for sickle cell percent in all tests, indicating a reduction in sickling of over 15% compared to DMSO controls. Additionally, in 51 of the 250 compounds, the ratio of cell area to the average cell area of voxelotor-treated samples (representing normal RBCs) exceeded 0.8 across all tests (Criteria 2), indicating that these 51 compounds preserved the normal size of RBCs. Furthermore, 14 of the 51 compounds showed the percentage of "other" cells (neither normal nor sickled) at least 15% lower than the average in the voxelotor-treated samples in all tests (Criteria 3), suggesting that these 14 compounds (compounds **1**−**14**) maintained the normal shape of RBCs.

Next, the acquired images of RBCs treated with compounds **1**−**14** were manually assessed to verify that RBCs maintained a round and concave shape (Criteria 4; Figure 2). We found that ten out of the 14 compounds (compounds **1**−**10**) maintained the round and concave shape of RBCs (Figures S3−S12). In contrast, RBCs treated with compounds **11**−**14** did not retain the round and concave shape. Images of RBCs treated with compound **11** were blurred during the initial acquisition (Figure S13), and no cells were detected 1 week later, indicating potential lysis caused by compound **11**, which is unfavorable for SCD treatment. Consequently, compound **11** (aescin) was eliminated from further investigation. Additionally, spherocytes were observed in all samples treated with compounds **12**−**14** (Figures S14−S16), which would be detrimental for treating SCD as spherocyte formation is associated with the reduced deformability of RBCs.[35] Thus, compound **12** (an analog of AD 0261), **13** (amiodarone), and **14** (isopropyl alcohol) were also eliminated from further consideration.

In summary, compounds **1**−**10** were identified in the 13,736 ReFRAME compounds as potential anti-sickling compounds that prevent sickling while maintaining the normal shape and size of RBCs.

Compounds 1−6 Prevented Sickling but Enhanced Oxidation of Hb. Compounds that enhance oxidation of Hb are unsuitable for treating SCD, as oxidized Hb (metHb) cannot deliver oxygen to tissues. To assess the tendency of the ten compounds to increase metHb formation, purified normal Hb (HbA) was treated with each of compounds **1**−**10**, and absorbance spectra of the Hb samples were recorded from 500 to 700 nm (n = 3 experimental replicates) before and 8 h after incubating the samples at 37 °C. The formation of metHb was evaluated by measuring the increase in absorbance at 630 nm (A_{630}), which correlates with elevated metHb concentration (Figure S17).[36]

After the 8 h-incubation, the absorbance spectrum pattern of Hb treated with compounds **1**−**6** were changed and A_{630} was increased greater than the control (Figure S18), while compounds **7**−**10** did not increase A_{630} (Figure S19). These results indicate that compounds **1**−**6** enhanced oxidation of Hb and likely inhibited sickling by decreasing the concentration of deoxyHbS.[37,38] Compounds **1**−**6** were ethopropazine hydrochloride, phenothiazine, AD 0261, fluorapacin, CI-201678, and Δ^9-tetrahydrocannabivarin respectively, and all these compounds were eliminated from further investigation.

Voxelotor and SNS-314 in the ReFRAME Library Inhibited Sickling in a Concentration-Dependent Manner. To further evaluate the efficacy of compounds **7**−**10** in inhibiting sickling, the sickling assay was conducted to measure the dose−response curves of these compounds using three blood samples. When SS blood samples were treated with compounds **7** and **8** at a concentration of 30 μM, both compounds were reaffirmed as anti-sickling compounds (Figure 3C,D,G,H). The dose−response curves for compounds **7** and **8** (Figure 3G,H) demonstrated a reduction in the percentage of sickled cells as the concentration of the compounds increased. Compounds **7** and **8** were voxelotor (Figure 3K) and SNS-314 respectively (Figure 3L), both of which we previously identified as the anti-sickling compounds in the Broad Repurposing library.[27] The mean EC_{50} value for voxelotor was determined to be 7 μM (n = 3 biological replicates), whereas the EC_{50} value for SNS-314 could not be determined due to the failure of the dose−response curves to fit the four-parameter logistic regression model that was used to determine EC_{50} (eq 1, see Methods). Based on the observed trends in the dose−response curves (Figure 3H), the EC_{50} value for SNS-314 is estimated to be between 10 and 30 μM.

SNS-314 was developed as an inhibitor of Aurora kinases;[39] these kinases are involved in cell division. In our screening of the 13,736 compounds, we identified 33 compounds (Table S2), in addition to SNS-314, that are reported to be Aurora kinase inhibitors.[40] However, none of these compounds met Criteria 1 (Figure 2), indicating that the 33 Aurora kinase inhibitors did not inhibit sickling in our screen.

Bismuth Ethenedithiol and Cetrorelix Acetate Would Not be Useful for the Treatment of SCD. Compounds **9** and **10** did not fully reduce the percentage of sickled cells (Figure 3I,J), while voxelotor and SNS-314 did (Figure 3G,H).

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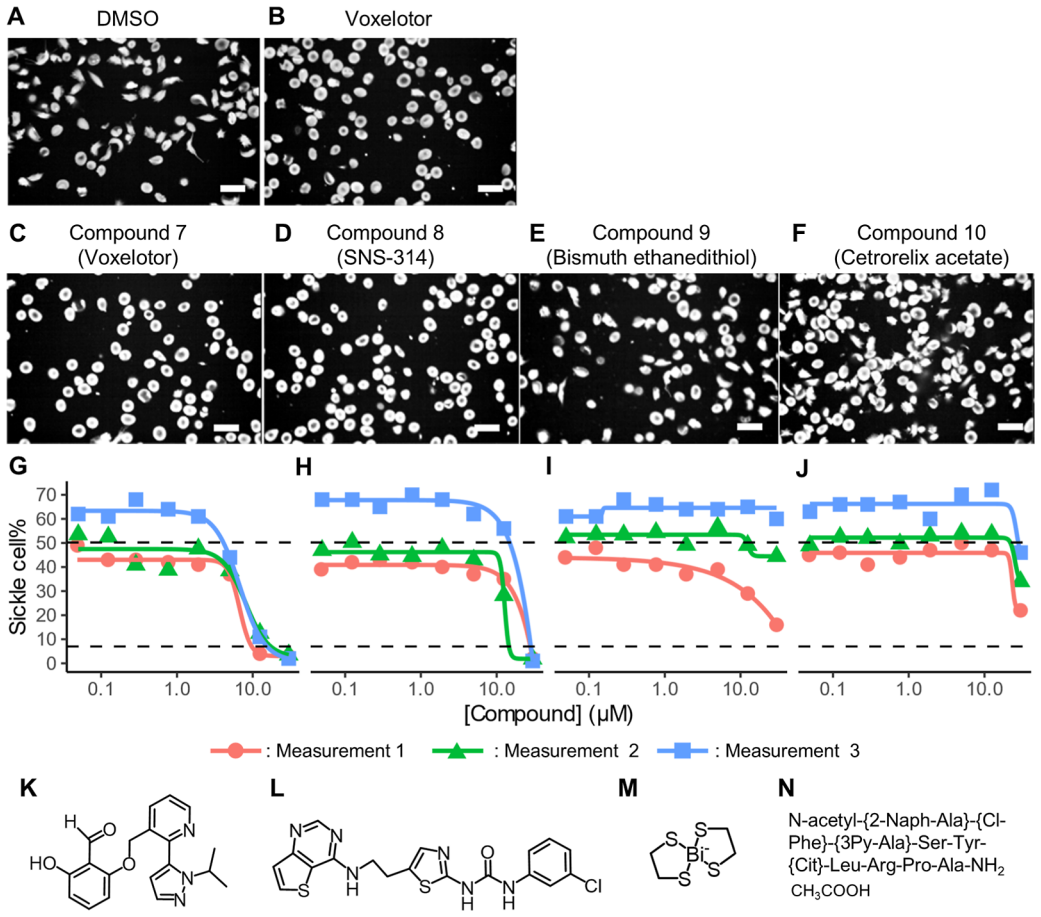

Figure 3. Representative fluorescent image of SS RBCs treated with DMSO (A), voxelotor 30 μM as the positive control of the assay (B), compound **7** 30 μM (C), compound **8** 30 μM (D), compound **9** 30 μM (E), and compound **10** 30 μM (F) in 4% oxygen. The bars in the images indicate 20 μm. Dose−response curves of compounds **7** (G), **8** (H), **9** (I), and **10** (J) on the sickle cell percentage in three measurements (measurements 1−3). The upper and lower dotted lines in panels (G−J) indicate the average sickle cell percent of DMSO-treated samples and voxelotor-treated samples in all the tests, respectively. The structure of compounds **7** (voxelotor, K), **8** (SNS-314, L), **9** (bismuth ethanedithiol, M), and **10** (cetrorelix acetate, N).

Applying the criteria outlined in Figure 2 to the dose−response data for compounds **9** and **10** at a concentration of 30 μM revealed that these compounds were not classified as compounds that inhibit sickling while maintaining the normal shape and size of RBCs in any of the tests. These findings suggest that the EC_{50} values for compounds **9** and **10** are likely greater than 30 μM, and their anti-sickling activity is lower than that of voxelotor or SNS-314. Compound **9** was bismuth ethanedithiol (BiEDT, Figure 3M), an antimicrobial agent,[41] and compound **10** was cetrorelix acetate (Figure 3N), a synthetic decapeptide with a gonadotropin-releasing hormone (GnRH) antagonistic activity.[42]

We assessed whether analogs of BiEDT inhibit sickling. The ReFRAME library included bismuth-containing compounds[41] including bismuth subsalicylate, gastrodenol (bismuth tripotassium dicitrate), bismuth subgallate, bismuth oxychloride, bismuth oxide, and bismuth subnitrate. None of these compounds satisfied Criteria 1 (Figure 2), indicating that the compounds did not reduce the sickle cell percent compared to DMSO in our screen. In addition, it has been reported that bismuth induces cell shrinkage of RBCs.[43] Considering these findings, BiEDT was eliminated from further investigation.

We also assessed whether analogs of cetrorelix inhibit sickling. The ReFRAME library included abarelix, degarelix, ganirelix acetate, goserelin acetate, histrelin acetate, and NBI-42902, which are GnRH antagonists or agonists[44,45] and none of these compounds satisfied Criteria 1 (Figure 2) in our screen. The dose response curves of cetrorelix acetate (Figure 3J) suggest that greater than 30 mM of cetrorelix acetate would be required to inhibit sickling of whole blood because 1000-fold diluted blood was used in the sickling assay. However, cetrorelix acetate was not soluble at 30 mM in whole blood. Given the low anti-sickling activity and low aqueous solubility, cetrorelix acetate was eliminated for further investigation.

In summary, voxelotor and SNS-314 have been identified in the ten ReFRAME compounds that prevent sickling in a dose response manner while maintaining the normal shape and size of RBCs. The anti-sickling activity of BiEDT and cetrorelix will be lower than voxelotor and SNS-314.

Comparison of Newly Identified Anti-Sickling Compounds with Previously Reported Compounds from the Same ReFRAME Compound Library. In our study, we identified ten anti-sickling compounds (compounds **1**−**10**) from the ReFRAME compound library (13,736 compounds at single concentration), while a previous study reported the names of 99 anti-sickling compounds from the same library (12,657 compounds at multiple concentrations).[46] To compare these findings, we re-evaluated the results of the 99 compounds in our screen (Figure S20).

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Out of the 99 compounds (Table S3−5), two were the same compound (exifone), and 20 (including exifone) were not found in the list of our screened compounds (Table S3). Out of the remaining 78 compounds, only ethopropazine and voxelotor were identified as anti-sickling compounds in our screen (Figure S20). Ethopropazine was eliminated from our further study because the compound enhanced oxidation of Hb (Figure S18B). SNS-314, which we identified to inhibit sickling, was not included in the 78 compounds. Conversely, mitapivat in the 78 compounds was not identified as an anti-sickling compound in our screen (Table S4). Mitapivat is used for the treatment of hemolytic anemia in adults with pyruvate kinase deficiency[47] and is currently being studied for the treatment of SCD.

DISCUSSION

We conducted a high throughput sickling assay with an improved method to screen 13,736 compounds in the ReFRAME compound library for their ability to prevent RBC sickling in 4% oxygen. Out of the 13,736 screened compounds, ten compounds were identified to prevent sickling while maintaining the normal shape and size of RBCs. Six of the ten compounds were eliminated from further investigation because these compounds enhanced oxidation of Hb. Out of the remaining four compounds, voxelotor and SNS-314 demonstrated a dose-dependent inhibition of sickling, verifying our previous findings in the screen of the Broad compound library.[27]

The improved sickling assay, in which the buffer for blood samples was diluted with water by 10%, increased the percentage of sickled cells and thereby increased the number of blood samples that were adequate for identifying anti-sickling compounds. Based on the components of the buffer, it is estimated that the osmolarity of the buffer is similar to a 0.8−0.9% sodium chloride solution, as sodium chloride at 135 mM is the major component of the buffer (without the addition of 10% excess water). The good value of z'-factor (>0.4) and the successful identification of voxelotor and SNS-314 in screening the ReFRAME library confirm that the improved assay remains effective in detecting anti-sickling compounds. The improved assay will increase the utility of valuable SS blood samples, providing more opportunities to develop anti-sickling compounds for treating SCD.

SNS-314, identified in this study, as well as in our previous screen, presents an interesting candidate for SCD treatment due to its ability to prevent sickling in the complete absence of oxygen, whereas voxelotor is ineffective under these conditions.[27] This suggests that voxelotor and SNS-314 may inhibit sickling through distinct mechanisms. Unfortunately, the relatively low solubility of SNS-314 in water limits its clinical applicability. The precise mechanism by which SNS-314 inhibits sickling remains unclear, but it is unlikely that Aurora kinases are involved in this process, as more than 30 Aurora kinase inhibitors did not exhibit any inhibitory effect on sickling in our screening. It will be worth exploring analogs of SNS-314 to investigate which structural aspects of the compound mediate the anti-sickling activity. We anticipate that these studies will lead to the identification of novel compounds that will have greater anti-sickling activity.

The ten compounds we identified as anti-sickling compounds differ from the 99 compounds reported by Metaferia and colleagues using the same library.[46] This discrepancy is likely due to differences in assay methods, including the type of blood used (homozygous or heterozygous for HbS), the conditions under which samples were incubated (such as oxygen concentration, incubation time, and the type of blood diluent), the concentration of the tested compounds, and the criteria employed to identify compounds that inhibit sickling. Metaferia and colleagues incubated sickle cell *trait* RBCs (as opposed to the homozygous sickle cells RBCs used in our assay) with 12,657 ReFRAME compounds at multiple concentrations up to 10 μM under nitrogen conditions, collecting images of the samples over time to analyze the sickled cells. In contrast, we incubated SS RBCs at a single concentration ($\leq$30 μM) in 4% oxygen only for 1 h. The 98 compounds that exhibited statistically significant anti-sickling[46] included voxelotor and proprapazine but not the other compounds that we identified to inhibit sickling. Proprapazine enhanced oxidation of Hb, which will not be beneficial for the treatment of SCD; thus, incorporation of an assay to evaluate metHb will be a crucial component of assays to develop anti-sickling compounds for the treatment of SCD.

One limitation of our sickling assay is its inability to detect compounds that exhibit their anti-sickling effects slowly. For example, mitapivat was not identified in our screening, although it was previously reported to inhibit sickling by Metaferia and colleagues.[46] This discrepancy may arise from the longer incubation time required in a 4% oxygen environment for mitapivat to effectively inhibit sickling in our assay. Mitapivat is a pyruvate kinase activator, which reduces the concentration of 2,3-DPG and increases the concentration of ATP in RBCs.[48] The reduction in 2,3-DPG enhances the affinity of RBCs for oxygen, thereby preventing sickling by decreasing the concentration of deoxygenated HbS. It has been reported that when blood samples from 15 pyruvate kinase-deficient patients were incubated with mitapivat at 37 °C for 24 h, enzymatic activity of pyruvate kinase increased in all samples.[49] This report suggests that an extended incubation period may be necessary for our sickling assay to demonstrate a significant reduction in sickling for compounds such as mitapivat.

A recent report indicates that the global prevalence of SCD increased from 5.5 million in 2000 to 7.7 million in 2021.[50] Additionally, voxelotor was withdrawn from the market in September 2024 due to safety concerns, including a high mortality rate, although the underlying cause of increased mortality rate is unclear.[51] Given the rising number of SCD patients and the lack of key therapeutic options, there is a critical need to develop novel anti-sickling compounds to expand treatment options. The sickling assay described in this study will be useful for facilitating the development of additional therapeutics for SCD.

CONCLUSIONS

We improved the high throughput sickling assay, thereby increasing the utility of valuable blood samples from sickle cell disease volunteers for the development of anti-sickling compounds to treat sickle cell disease. Utilizing this improved assay, we screened 13,736 compounds from the ReFRAME library for their ability to prevent red blood cell sickling in 4% oxygen. We identified voxelotor and SNS-314, which inhibited sickling in a dose-dependent manner while maintaining the normal size and shape of RBCs without enhancing oxidation of hemoglobin.

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■ ASSOCIATED CONTENT

Data Availability Statement

The results of screening the 13,736 ReFRAME compounds are available at https://reframedb.org.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c11077.

Summary of screening 13,736 ReFRAME compounds. Bright field images of a SS blood sample treated with the controls (DMSO and voxelotor) and compounds **1**−**14**. Absorption spectra of oxyHb and metHb. Absorption spectra of HbA treated with compounds **1**−**10** before incubation and 8 h after the incubation. List of Aurora kinase inhibitors that were tested in the ReFRAME library. The comparison of our identified anti-sickling compounds and the ones reported by another group (PDF)

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Notes

The authors declare no competing financial interest.

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