HHS Public Access

Author manuscript

Blood Vessel Thromb Hemost. Author manuscript; available in PMC 2025 June 06.

Published in final edited form as:

Blood Vessel Thromb Hemost. 2025 May ; 2(2): . doi:10.1016/j.bvth.2025.100045.

Small-molecule inhibitor screen to identify mechanisms of sickle hemoglobin clearance by liver endothelium

Tomasz W. Kaminski[1], **Hong Zhang**[2], **Omika Katoch**[1,3], **Qizhen Shi**[3,4], **Gregory J. Kato**[5], **Prithu Sundd**[1,4], **Tirthadipa Pradhan-Sundd**[3,4]

[1]Hemostasis and Thrombosis Program, Versiti Blood Research Institute, Milwaukee, WI;

[2]BioMagis Inc, San Diego, CA;

[3]Transfusion Medicine, Vascular Biology and Cell Therapy Program, Versiti Blood Research Institute, Milwaukee, WI;

[4]Departments of Medicine, Biomedical Engineenring and Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, Milwaukee, WI;

[5]Blood Science Consulting, Tilghman, MD

Abstract

Intrahepatic accumulation of cell-free hemoglobin (Hb) is a significant pathology linked with hemolytic disorders such as sickle cell disease (SCD). In addition to hepatic Kupffer cells, liver sinusoidal endothelial cells (LSECs) were recently reported to contribute to Hb clearance in SCD mice and patients via currently unknown endocytic mechanism. Using small-molecule inhibitors of endocytic pathway components in primary human and mouse LSECs, we show that sickle-Hb (HbS) uptake by LSECs occurs predominantly through micropinocytosis or fluid-phase endocytosis. However, inhibiting clathrin-mediated endocytosis, receptor recycling, or drop in pH also significantly attenuated HbS uptake by LSECs. LSEC-driven HbS uptake was independent of haptoglobin. Finally, we found that the presence of lipid droplets promotes endothelial HbS internalization, whereas hypolipidemic condition inhibits it. In conclusion, this study identifies previously unknown alternative mechanism of LSEC-mediated HbS internalization. Our findings also inform the need to evaluate the therapeutic potential of blocking these mechanisms to ameliorate hemolysis-associated liver damage in SCD and other hemolytic disorders.

Correspondence: Tirthadipa Pradhan-Sundd, Transfusion Medicine, Vascular Biology, and Cell Therapy, Versiti Blood Research Institute, 8733 W Watertown Plank Rd, Milwaukee, WI 53226; tpradhan@versiti.org.

Authorship

Contribution: T.P.-S. designed the research; T.W.K. and H.Z. performed the research; O.K., G.J.K., Q.S., and P.S. contributed vital new reagents or analytical tools; H.Z., T.W.K., and T.P.-S. analyzed data; and T.P.-S. wrote the manuscript.

The current affiliation for G.J.K. is CSL Behring, King of Prussia, PA.

Conflict-of-interest disclosure: G.J.K. currently works at CSL Behring. The remaining authors declare no competing financial interests.

Data are available on request from the corresponding author, Tirthadipa Pradhan-Sundd (tpradhan@versiti.org).

The full-text version of this article contains a data supplement.

Introduction

Hemolysis is a prevalent pathophysiological phenomenon in both hematologic and nonhematologic disorders. The accumulation of extracellular hemoglobin (Hb) has been linked to hepatic injury. Sickle cell disease (SCD) is one such hemolytic disorder characterized by sickling of red blood cells, leading to hemolysis and the release of cell-free Hb into the blood circulation.[1,2] Hb binds to plasma haptoglobin (Hp), and the Hb-Hp complex travels to the liver, where it gets cleared up by the scavenger receptor CD163 expressed on hepatic Kupffer cells[3–5] for degradation to heme, followed by heme oxygenase-1–dependent metabolism to generate iron, carbon monoxide, and bilirubin.[4,6–9] Hp-Hb binding and the enzymatic degradation of heme by heme oxygenase-1 are the protective mechanisms that prevent Hb or heme accumulation under hemolytic condition.[6,10–12] However, chronic hemolysis associated with SCD overwhelms these protective mechanisms and depletes Hp,[13,14] leading to elevated levels of erythrocyte-derived damage-associated molecular patterns (eDAMPs), causing damage to organs such as liver and kidney.[15–17]

Recently, it was shown that the liver plays a predominant role in red blood cell, Hb, and iron clearance.[18,19] Accumulating evidence highlights the pivotal role of the hepatic Kupffer cells and monocyte-derived macrophages, among other phagocytic cells including blood monocytes and spleen red pulp macrophages, in the clearance of Hb and other eDAMPs (heme and iron).[20,21] Our recent findings demonstrated, to our knowledge, for the first time that along with hepatic Kupffer cells and monocyte-derived macrophages, liver sinusoidal endothelial cells (LSECs) also participate in Hb clearance.[19] However, the exact mechanisms underlying LSEC-mediated Hb clearance are not completely understood.

Using small-molecule inhibitor screen of various endocytic pathways and LSEC receptor blockers in in vitro cultured primary LSECs, we show that fluid-phase endocytosis is the main endocytic route of LSEC-mediated Hb internalization. Although fluid-phase endocytosis was the main endocytic route, CME was also found to contribute to LSEC-mediated Hb internalization. Changes in pH and impaired lysosomal degradation inhibited sickle-Hb (HbS) clearance in LSECs. We also found that LSEC-mediated Hb internalization was not dependent on Hp but adversely affected by heme and iron. Finally, we show that Hb internalization is lipid dependent and that the lipid-depleting agents simvastatin, imanixil, and nystatin significantly impede endothelial Hb internalization. In conclusion, we identified various alternative methods of LSEC-mediated Hb internalization, which might provide useful therapeutic strategies to combat hemolysis-related organ damage in SCD.

Methods

Primary liver endothelial cell culture

Mouse sinusoidal endothelial cells (catalog no. ABC-TC3208) were procured from AcceGen and cultured in complete RPMI medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin for a maximum of 5 passages to preserve their LSEC identity. Human hepatic sinusoidal endothelial cells (catalog no. 10HU-021) were obtained from iXCells and cultured in endothelial cell growth medium (iXCells; catalog no. MD-0010) for

a maximum of 4 passages to maintain their hepatic sinusoidal endothelial cell identity. All experiments involving various chemical treatments were conducted when the cells reached 70% to 80% confluence. LSECs were passaged up to 2 times to maintain their structural and functional integrity (characterization is shown in supplemental Figure 1)

Design of the small-molecule inhibitor of endocytosis

We conducted a small-molecule inhibitor screen (as shown in supplemental Figure 2A) by selecting a number of endocytosis blockers and LSEC-specific endocytosis inhibitors. Inhibitor concentration and administration duration are listed in supplemental Table 1. Broadly, the blockers were divided into the following groups (Table 1).

Compounds that were able to reduce/increase Hb internalization ≥50% were grouped into strong regulators (listed in supplemental Tables 2 and 3), whereas compounds that were able to affect Hb internalization by <50% were grouped into intermediate regulators (listed in supplemental Table 4). Compounds that did not show any change in Hb internalization are listed in supplemental Table 5. Only the strong regulators of Hb internalization are discussed in this study.

Flow cytometric analysis

Mouse LSECs were incubated with the specified stimuli at the indicated time point, followed by the addition of 2 μM Alexa 488 Hb for an additional 1 hour. After incubation, the cells were washed, detached with 0.25% trypsin with EDTA, and subjected to a single wash. Subsequently, the cells were stained with 5 μL of calcium-free apoptotic-dead cell tag 647 for 5 minutes without. The cells were analyzed by gating on live cells. The flow cytometry gating strategy is depicted in supplemental Figure 3.

Immunofluorescence and confocal imaging

Mouse LSECs were seeded in 96-well plates overnight. The next day, the cells were cultured with RPMI without serum for 1 hour before being treated with indicated chemicals for the indicated time. The cells were continuously treated with 2 μM Alexa 488–conjugated Hb for an additional 1 hour. After incubation, the cells were washed, detached with trypsin, and subjected to washes 5 times. The cells were incubated with DRAQ5 for 10 minutes, followed by 1 wash. The cells were imaged with Leica fluorescent microscope under a 20× objective.

Statistical analysis

The unpaired 2-tailed Student *t* test was used to determine statistical significance between the 2 groups (*$P < .05$ or **$P < .01$). When >2 groups were compared, statistical analysis was performed using 1- and 2-way analysis of variance with the Bonferroni correction. The calculations were done with Prism version 7.0a (GraphPad Software). All in vitro studies are either a collection of 3 independent experiments or reflect at least 3 independent experiments. Error bars indicate standard deviation.

Results

Hb internalization to LSECs is a slow process and happens within hours

To determine the time taken by LSECs to internalize normal-Hb (HbA) and HbS, we administered HbA or HbS tagged with fluorescein isothiocyanate to human and mice primary LSEC culture and performed flow cytometric analysis at 0 minute and 1 hour after HbA/HbS administration (Figure 1A). When compared, HbA internalization (as measured by overall Hb intensity across the cells) appeared faster than HbS (Figure 1B).

To verify that HbA/HbS is taken up by LSECs and not bound to the cell surface of LSECs, we performed an immunofluorescence assay. Figure 1C demonstrates that the administration of HbA/HbS resulted in the gradual buildup of Hb inside LSECs over time, as indicated by the presence of small, distinct puncta of Hb. Interestingly, as shown in Figure 1C, the internalization of HbA/HbS appeared to be a slow process, and all LSECs were found to be positive for Hb staining by 1 hour. Finally, we compared the fluorescence intensity of HbA and HbS inside LSECs 1 hour after administration. Interestingly, HbA exhibited a stronger fluorescence intensity than HbS, suggestive of a slower uptake or an increased degradation of HbS compared with HbA (Figure 1C). Together, these observations indicate that Hb internalization in LSECs happens overtime, and HbA exhibits a faster internalization rate than HbS.

HbA/HbS internalization to LSECs is pH dependent

Next, as shown in supplemental Figure 2A and Table 1, we used a subset of small-molecule inhibitors that are known to affect various endocytic pathways to identify regulators of LSEC-mediated Hb internalization. A critical aspect of endocytosis is the formation of early endosomes, which requires the maintenance of an acidic internal pH, typically ranging from 6.0 to 6.2. The low pH in the early endosome compartment facilitates the dissociation of ligands from their receptors.[22–24] To determine the effect of pH on HbA/HbS internalization, we exposed in vitro cultured LSECs to increasing doses of ammonium chloride, chloroquine, concanamycin A, or monensin for the stipulated time point mentioned in supplemental Table 1, followed by incubation with fluorescein isothiocyanate–tagged HbA/HbS for 1 hour. Chloroquine phosphate (a lysosomotropic weak base) and monensin (a carbocyclic polyether Na^+ ionophore), which prevents endosomal acidification by serving as an Na^+/H^+ antiporter,[25] significantly reduced HbA/HbS internalization to LSECs, as seen by flow cytometric analysis and confocal microscopy image analysis (Figure 1E–G; data not shown).

Concanamycin A, a selective inhibitor of vacuolar-type H^+-adenosine triphosphatases and hence a potent inhibitor of endosomal acidification,[26] induced a significant reduction in HbA/HbS internalization (by 5000-fold at a concentration of only 20 nM; Figure 1F–G). Altogether, these data show that HbA/HbS use a pH-dependent internalization route, possibly an endosomal cell entry pathway.

HbS/HbA enters liver endothelial cells predominantly through alternative endocytic routes

Proteins use a variety of pH-dependent endocytic pathways, including clathrin-mediated endocytosis (CME), dynamin-mediated (usually clathrin mediated but can be clathrin independent) endocytosis, clathrin-independent endocytosis, macropinocytosis, and caveola-mediated endocytosis.[22,27–29] We have recently shown that blocking micropinocytosis can mildly affect Hb internalization to LSECs.[19] Because micropinocytosis is a type of endocytosis and several proteins are known to use various pathways simultaneously for internalization, we further characterized the internalization route of Hb to LSECs. To assess Hb internalization mechanism, we first treated primary LSECs with certain doses of inhibitors that are known to block different endocytic pathways, followed by analyses of Hb trafficking by flow cytometry and imaging analysis. The list of inhibitors used is shown in supplemental Table 1. Phenylarsine oxide, which is reported to inhibit fluid-phase endocytosis as well as abolish the development of clathrin-coated endocytic vesicles by interfering with the interaction between the adapter protein AP-2 and the clathrin-coated pit lattice,[30,31] almost completely inhibited Hb internalization at a concentration of 20 μM, as seen by flow cytometry analysis (Figure 2B) and confocal imaging (Figure 2C). A similar effect was observed with Pitstop 2, which inhibits CME,[32] at concentrations of 20 μM, suggesting that both fluid-phase endocytosis as well as CME can regulate Hb internalization in LSECs (Figure 2D–F). To further confirm the effect of CME, we next used various dynamin blockers in in vitro cultured LSECs, namely dynamin inhibitors, dynasore, and dynamin inhibitor 1. Dynamin functions as a mechanochemical enzyme that drives membrane fission and as a regulatory guanosine triphosphatase in CME.[33] Dynamin inhibitor blocks membrane fusion in CME. Dynasore is an inhibitor of dynamin and inhibits CME.[34] We treated LSECs with dynamin inhibitor as well as dynasore for up to 30 minutes before Hb administration to the culture media, followed by the measurement of internalized HbS intensity by flow cytometry and confocal imaging. As shown in Figure 2G–I, both these treatments did not significantly reduce the internalization of HbS to LSECs, suggesting that LSEC-mediated HbS internalization is dependent on clathrin coat formation but not on dynamin-induced membrane fusion. Because CME is also involved in receptor recycling,[35] we further asked whether HbS internalization is impaired by blocking receptor recycling pathway. To inhibit receptor recycling, we used dansylcadaverine, which is known to block CME by inhibiting receptor recycling.[36] Remarkably, dansylcadaverine treatment showed a stronger reduction in Hb internalization. As shown in Figure 2K–L, HbS internalization was significantly reduced, suggesting that either receptor recycling or CME vessel formation promotes HbS internalization in LSECs (Figure 2J). Taken together, the data presented in Figure 2 suggest that HbA/HbS enters LSECs by several endocytic routes, including fluid-phase endocytosis/micropinocytosis (predominant route) as well as CME (alternative route).

Cathepsin B–mediated lysosomal degradation promotes LSEC-mediated HbA/HbS clearance

To further evaluate the possible involvement of other routes of endocytosis, we blocked caveolin-mediated endocytosis using filipin III (Figure 3A).[37] Interestingly, filipin III did not have a significant effect on HbS internalization by LSECs (Figure 3B–C). Similar effect was seen using nocodazole, which is also known to block caveola-dependent

endocytosis (supplemental Figure 4A–B).[38,39] We next examined the effect of blocking macropinocytosis (Figure 3D) using wortmannin, blebbistatin, and 5-[N-ethyl-N-isopropyl] amiloride.[40,41] 5-[N-ethyl-N-isopropyl] amiloride administration caused significant cell death in LSECs, and thus, its potential role in Hb internalization was not tested. Blebbistatin inhibits myosin II light chain formation and abrogates macropinocytosis. As shown in Figure 3E,G, blebbistatin treatment did not significantly affect Hb internalization. We could find HbS-positive green dots inside LSECs after treatment. However, the overall pattern of HbS distribution inside LSECs appeared different after blebbistatin treatment. Similar to blebbistatin, wortmannin selectively inhibits macropinocytosis (Figure 3D) by blocking PI3K.[42] Wortmannin treatment did not have a significant effect in HbS internalization (Figure 3F–G).

To finally test whether cathepsin-mediated processing within the endosomes is required for HbS internalization, we blocked cathepsin B and cathepsin L (Figure 3H).[43,44] Cathepsin B is an intercellular cysteine protease, which promotes proteolysis of endocytosed proteins in the lysosome. When we blocked cathepsin B activity using a specific inhibitor, CA047, it significantly inhibited HbS internalization to LSECs. As shown in Figure 3I–J, both imaging and flow cytometric analysis revealed reduced HbS staining and colocalization in LSECS. However, blocking cathepsin L using an inhibitor did not significantly affect Hb internalization.

The proteolytic cleavage of proteins by cathepsin B is reliant on the acidic pH of the surrounding environment, but cathepsin L does not require this acidic condition. Thus, the difference seen in cathepsin L– and cathepsin B–mediated proteolytic cleavage could be due to pH changes. Altogether, our data suggest that Hb internalization is independent of caveola-mediated endocytosis or macropinocytosis. Moreover, we show that cathepsin B–mediated (and not cathepsin L–mediated) lysosomal degradation of protein promotes Hb internalization and degradation in LSECs.

Effects of SCD-related pathophysiology on endothelial Hb internalization

Hemolysis and accumulation of cell-free HbS in SCD are associated with increased inflammation and elevated levels of heme and iron in the hepatic sinusoidal endothelial area.[2,45–49] Therefore, to further understand HbS internalization mechanism in SCD disease context, we next examined whether Hb, heme, and iron accumulation as well as lipopolysaccharide (LPS) treatment can affect HbS internalization to LSECs (Figure 4A). Moreover, because Hb is known to bind to Hp and Hb-Hp complex formation accelerates Hb internalization,[4] we also tested whether the presence of Hp promotes LSEC-induced Hb internalization (Figure 4A). Administration of oxy-Hb or iron to the culture media did not significantly affect HbS internalization by LSECs (supplemental Figure 4A–B). However, blocking iron trafficking using a small-molecule iron transport inhibitor, NSC306711[50]/ferristatin (transferrin [Tf] receptor blocker), led to significant reduction in HbS internalization by LSECs (Figure 4B–C). Similar effect was seen after blocking toll-like receptor 4 (TLR4) using TAK242[51,52] (Figure 4A–C), suggesting that heme and iron trafficking promotes LSEC-mediated Hb internalization.

Next, we added LPS to the culture media to mimic inflammation in cultured LSECs. Hb internalization was not affected after LPS treatment compared with dimethyl sulfoxide–treated cells, based on flow cytometric analysis and confocal imaging (Figure 4B–C). Increasing the concentration of LPS (200 ng, 600 ng, and up to 1200 ng) added to the culture media did not significantly affect LSEC-mediated Hb internalization (supplemental Figure 4C–D). Similarly, adding increasing concentrations of Hp to the media did not affect Hb internalization (Figure 4B–C; supplemental Figure 4E–F). Taken together, our results suggested that although TAK242 and NSC306711 treatment can significantly impede Hb internalization, administration of Hp, LPS, oxy-Hb, and iron does not have a clear effect on endothelial Hb internalization.

HbS/HbA internalization to liver endothelial cells is accelerated by the presence of cellular lipids

Because we observed that both receptor-driven CME and fluid-phase endocytosis, which are vesicle-driven endocytic pathways, can affect Hb internalization, we next examined the impact of blocking endocytic vesicle formation on HbS internalization. Because vesicles consist of lipid bilayers,[53] we investigated how the presence or absence of lipids might affect HbS internalization. To examine the effect of lipids, we increased lipid concentration using a lipid cocktail, and conversely, we reduced lipid levels using a lipid blocker, simvastatin.[54] Remarkably, administration of the lipid cocktail significantly increased Hb internalization (Figure 5A,C). Both flow cytometric analysis and confocal imaging showed significant increase in HbS internalization (Figure 5A,C). Blocking lipid in the cell culture media using simvastatin led to reduced internalization of Hb. As shown in Figure 5B–C, both flow cytometric analysis and confocal imaging showed significant decrease in Hb internalization. Interestingly, HbS internalization was more affected by the presence or absence of lipids than HbA. Subsequently, we examined the cellular architecture after treatment with lipids and simvastatin. The cell-cell adhesion seen in dimethyl sulfoxide– and lipid-treated cells was lost in simvastatin-treated LSECs (Figure 5D). Moreover, because nystatin is known to block lipid-mediated endocytosis (along with blocking micropinocytosis),[55] we administered nystatin and measured HbA/HbS internalization 30 minutes after treatment. Nystatin treatment showed a stronger reduction in HbS internalization, as shown by both imaging and flow cytometric analysis (Figure 5E). Finally, we blocked lipid internalization using imanixil, which is a low-density lipoprotein receptor inducer as well as a cholesterol reducing agent.[56,57] Imanixil treatment caused significant impairment in liver endothelial Hb internalization, as shown by flow cytometric analysis and confocal imaging (Figure 5E–F). These combined results suggest that the presence of lipids can significantly enhance Hb internalization to LSECs, whereas hypolipidemic condition inhibits it.

Discussion

Here, we demonstrate that Hb clearance by LSECs occurs through >1 endocytic pathway. Although fluid-phase endocytosis/micropinocytosis was seen as the predominant route for Hb internalization, CME, lysosomal degradation, and receptor recycling were also shown to affect HbS internalization to LSECs. Among the factors affecting HbS internalization, pH and heme-iron content were prominent. Finally, we show that LSEC-mediated Hb

internalization is dependent on the presence of lipids. Altogether, these results uncover the mechanism of Hb entry to LSECs. Targeting LSEC-mediated Hb internalization by either overexpressing the receptors or manipulating the conditions for internalization may serve as a novel therapeutic target in treating SCD and other pathological states involving persistent hemolysis.

Pharmacological interventions that alter cellular pH impeded Hb entry, indicating a pH-dependent mechanism for Hb uptake. As observed for many other proteins,[58] a low pH may be required to cause conformational changes in surface glycoproteins required to initiate fusion of the protein membrane with the target cell membrane. However, it is important to note that pH alteration may have unintended consequences, such as disrupting receptor recycling, inhibiting endosome maturation, or neutralizing the trans-Golgi network.[59,60] Consequently, additional tests must be conducted to validate the pH dependency of Hb internalization.

Endocytosis can be differentiated into phagocytosis and pinocytosis. Phagocytosis has been observed only in specialized cells engulfing large particles. Because the vast majority of known small proteins use pinocytic pathways, we hypothesized that Hb protein (~5 nm in diameter) enters LSECs by fluid-phase endocytosis/micropinocytosis. Indeed, blocking fluid-phase endocytosis/micropinocytosis significantly inhibited Hb internalization, confirming our hypothesis. Pinocytosis can be categorized into dynamin-dependent subpathways, including CME, caveola-mediated endocytosis, and poorly characterized clathrin-independent dynamin-mediated endocytosis, as well as dynamin-independent pathways, commonly referred to as macropinocytosis, lipid raft-mediated endocytosis, and nonclathrin/non-caveolar endocytosis. Our experiments here show that Hb primarily uses a lipid-mediated endocytosis pathway. Moreover, the Hp-independent internalization of Hb also hints at a non–receptor-mediated fluid-phase trafficking of Hb to endothelial cells. We observed a mild reduction in Hb internalization upon blocking CME-mediated pathway.

CME usually happens quickly, with proteins attached to the exterior of target cells getting inside them within minutes. However, we found Hb internalization by LSECs to be a slow process, taking almost an hour to complete, suggesting that CME is most likely an alternative pathway used by Hb to enter LSECs or it indirectly regulates HbS internalization. Several proteins are known to use multiple pathways to enter their target cells, or they use specific yet distinct pathways in different cell types. It would be intriguing to investigate whether LSECs possess the distinctive ability for Hb internalization via many concurrent pathways. One of the key findings of our study is that lipid promotes Hb internalization by LSECs, and blocking lipid/cholesterol synthesis by simvastatin, imanixil, or nystatin treatment blocked Hb internalization by LSECs. Along with that, our screen revealed a negative role of NSC306711/ferristatin in endothelial Hb internalization. The small molecule NSC306711 decreases the uptake of Tf-bound iron by internalizing and degrading the unoccupied Tf receptors.[50] Interestingly, prior studies have demonstrated that NSC306711-driven internalization of Tf is independent of clathrin or dynamin but is sensitive to the lipid-depleting agents filipin and nystatin, implying a function of lipid raft in ferristatin-induced Tf receptor internalization and degradation.[50] We propose that the NSC306711-driven impairment of Hb internalization could also be due to blockage of lipid-assisted endocytic

trafficking. We propose several mechanisms that may contribute to lipid-mediated Hb internalization. First, the positive role of lipids in Hb internalization could be by promoting the formation of lipid-rich vesicles/microparticles that transport Hb to the LSECs. Second, Hb and lipids might travel together to LSECs, and blocking lipid receptors might also affect Hb internalization. Finally, the presence of lipids in the culture media might signal (eat me signal) endothelial cells to engulf the Hb proteins. Future experiments would be needed to determine the mechanisms contributing to the need for lipids in Hb internalization by LSECs.

A predominant pathophysiology associated with SCD is intravascular and extravascular hemolysis. Previous studies have shown that products of hemolysis (ie, heme/decompartmentalized Hb) inflame the endothelial-vascular system.[3,48,49,61] These byproducts are also known to cause sterile inflammation and activation of the leukocytes, platelets, and endothelial cells leading to vaso-occlusion.[2] Here, we are showing that, along with several other known deleterious effects, hemolysis (increased accumulation of heme and iron) can also impair Hb clearance. Understanding how each of these components influence the internalization/clearance of Hb would be useful in deciphering the complex heterogeneity associated with SCD organ damage.

Interestingly, we found that LPS did not influence HbS transport; however, TLR4, which is involved in LPS-induced endothelium activation, affected HbS transport. The observed disparity between LPS- and TLR4-mediated HbS transport may be attributable to dosage, including both the concentration of LPS and the duration of administration. Alternatively, LPS may induce a slight elevation in TLR4, insufficient to affect a notable alteration in HbS internalization.

Our experiments are limited by the fact that some of the inhibitors used in the primary screen might have off-target effects and thus can lead to false positivity or negativity. Several exotic endocytic pathways, including the interleukin 2, glycosylphosphotidylinositol-anchored protein enriched compartments, flotillin, and ARF6 pathways, were not examined in this study. Although it is unlikely, Hb could possibly enter different cell types by means of different pathways based on the presence or absence of external stimuli. The differences in HbS and HbA internalization could have therapeutic implications in SCD.

In summary, in this study, we identified alternative mechanisms of LSEC-mediated HbS transport. Because increased uptake of HbS by LSECs impedes HbS clearance and facilitates LSEC senescence, which subsequently exacerbates hepatobiliary damage,[19] these findings on alternative methods of HbS trafficking may inform the development of new therapies to ameliorate liver damage associated with SCD as well as other hemolytic disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the Versiti Blood Research Institute Shared Resources (core grant: SCR_025503) for its services, instrumentation, and specialist support.

This work was supported by the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases grant 1K01DK125617-01 (T.P.-S.), an American Society of Hematology junior faculty scholar award (T.P.-S.), and Versiti startup funds (T.P.-S.); National Heart, Lung, and Blood Institute grants R01HL128297 (P.S.), R01HL141080 (P.S.), and R01HL166345 (P.S.); and American Heart Association grants 18TPA34170588 (P.S.) and 23TPA1074022 (T.W.K.). T.W.K. was supported by American Heart Association postdoctoral fellowship AHA828786.

References

1. Kato GJ, Piel FB, Reid CD, et al. Sickle cell disease. Nat Rev Dis Primers. 2018;4:18010. [PubMed: 29542687]
2. Sundd P, Gladwin MT, Novelli EM. Pathophysiology of sickle cell disease. Annu Rev Pathol. 2019;14(1):263–292. [PubMed: 30332562]
3. Schaer DJ, Buehler PW, Alayash AI, Belcher JD, Vercellotti GM. Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins. Blood. 2013;121(8):1276–1284. [PubMed: 23264591]
4. Thomsen JH, Etzerodt A, Svendsen P, Moestrup SK. The haptoglobin-cd163-heme oxygenase-1 pathway for hemoglobin scavenging. Oxid Med Cell Longev. 2013;2013:523652. [PubMed: 23781295]
5. Nielsen MJ, Andersen CBF, Moestrup SK. CD163 binding to haptoglobin-hemoglobin complexes involves a dual-point electrostatic receptor-ligand pairing. J Biol Chem. 2013;288(26):18834–18841. [PubMed: 23671278]
6. Gbotosho OT, Kapetanaki MG, Kato GJ. The worst things in life are free: the role of free heme in sickle cell disease. Front Immunol. 2020;11:561917. [PubMed: 33584641]
7. Liu Y, Jing F, Yi W, et al. HO-1hi patrolling monocytes protect against vaso-occlusion in sickle cell disease. Blood. 2018;131(14):1600–1610. [PubMed: 29437594]
8. Nakamura K, Zhang M, Kageyama S, et al. Macrophage heme oxygenase-1-SIRT1-p53 axis regulates sterile inflammation in liver ischemia-reperfusion injury. J Hepatol. 2017;67(6):1232–1242. [PubMed: 28842295]
9. Belcher JD, Mahaseth H, Welch TE, Otterbein LE, Hebbel RP, Vercellotti GM. Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice. J Clin Invest. 2006;116(3):808–816. [PubMed: 16485041]
10. Ofori-Acquah SF, Hazra R, Orikogbo OO, et al. Hemopexin deficiency promotes acute kidney injury in sickle cell disease. Blood. 2020;135(13): 1044–1048. [PubMed: 32043112]
11. Graw JA, Mayeur C, Rosales I, et al. Haptoglobin or hemopexin therapy prevents acute adverse effects of resuscitation after prolonged storage of red cells. Circulation. 2016;134(13):945–960. [PubMed: 27515135]
12. Belcher JD, Chen C, Nguyen J, et al. Haptoglobin and hemopexin inhibit vaso-occlusion and inflammation in murine sickle cell disease: role of heme oxygenase-1 induction. PLoS One. 2018;13(4):e0196455. [PubMed: 29694434]
13. Santiago RP, Guarda CC, Figueiredo CVB, et al. Serum haptoglobin and hemopexin levels are depleted in pediatric sickle cell disease patients. Blood Cell Mol Dis. 2018;72:34–36.
14. Yalamanoglu A, Deuel JW, Hunt RC, et al. Depletion of haptoglobin and hemopexin promote hemoglobin-mediated lipoprotein oxidation in sickle cell disease. Am J Physiol Lung Cell Mol Physiol. 2018;315(5):L765–L774. [PubMed: 30047285]
15. Lacaille F, Allali S, De Montalembert M. The liver in sickle cell disease. J Pediatr Gastroenterol Nutr. 2021;72(1):5–10. [PubMed: 32740518]
16. Feld JJ, Kato GJ, Koh C, et al. Liver injury is associated with mortality in sickle cell disease. Aliment Pharmacol Ther. 2015;42(7):912–921. [PubMed: 26235444]

17. Pradhan-Sundd T, Kato GJ, Novelli EM. Molecular mechanisms of hepatic dysfunction in sickle cell disease: lessons from Townes mouse model. Am J Physiol Cell Physiol. 2022;323(2):C494–C504. [PubMed: 35759437]
18. Theurl I, Hilgendorf I, Nairz M, et al. On-demand erythrocyte disposal and iron recycling require transient macrophages in the liver. Nat Med. 2016;22(8): 945–951. [PubMed: 27428900]
19. Kaminski TW, Katoch O, Li Z, et al. Impaired hemoglobin clearance by sinusoidal endothelium promotes vaso-occlusion and liver injury in sickle cell disease. Haematologica. 2024;109(5):1535–1550. [PubMed: 37941440]
20. Willekens FLA, Werre JM, Kruijt JK, et al. Liver Kupffer cells rapidly remove red blood cell-derived vesicles from the circulation by scavenger receptors. Blood. 2005;105(5):2141–2145. [PubMed: 15550489]
21. Terpstra V, Van Berkel TJC. Scavenger receptors on liver Kupffer cells mediate the in vivo uptake of oxidatively damaged red blood cells in mice. Blood. 2000;95(6):2157–2163. [PubMed: 10706889]
22. Cosson P, De Curtis I, Pouyssegur J, Griffiths G, Davoust J. Low cytoplasmic pH inhibits endocytosis and transport from the trans-Golgi network to the cell surface. J Cell Biol. 1989;108(2):377–387. [PubMed: 2918022]
23. Freedman SD, Kern HF, Scheele GA. Acinar lumen pH regulates endocytosis, but not exocytosis, at the apical plasma membrane of pancreatic acinar cells. Eur J Cell Biol. 1998;75(2):163–173. [PubMed: 9548373]
24. Davoust J, Gruenberg J, Howell KE. Two threshold values of low pH block endocytosis at different stages. EMBO J. 1987;6(12):3601–3609. [PubMed: 3428267]
25. Caì Y, Postnikova EN, Bernbaum JG, et al. Simian hemorrhagic fever virus cell entry is dependent on CD163 and uses a clathrin-mediated endocytosis-like pathway. J Virol. 2015;89(1):844–856. [PubMed: 25355889]
26. Dröse S, Altendorf K. Bafilomycins and concanamycins as inhibitors of V-ATPases and P-ATPases. J Exp Biol. 1997;200(pt 1):1–8. [PubMed: 9023991]
27. Sun X, Yau VK, Briggs BJ, Whittaker GR. Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells. Virology. 2005; 338(1):53–60. [PubMed: 15936793]
28. Dollery SJ, Delboy MG, Nicola AV. Low pH-induced conformational change in herpes simplex virus glycoprotein B. J Virol. 2010;84(8):3759–3766. [PubMed: 20147407]
29. Long G, Pan X, Kormelink R, Vlak JM. Functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis. J Virol. 2006;80(17):8830–8833. [PubMed: 16912330]
30. Wang LH, Rothberg KG, Anderson RGW. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. J Cell Biol. 1993;123(5):1107–1117. [PubMed: 8245121]
31. Gibson AE, Noel RJ, Herlihy JT, Ward WF. Phenylarsine oxide inhibition of endocytosis: effects on asialofetuin internalization. Am J Physiol. 1989;257(2 pt 1):C182–C184. [PubMed: 2475026]
32. Dutta D, Williamson CD, Cole NB, Donaldson JG. Pitstop 2 is a potent inhibitor of clathrin-independent endocytosis. PLoS One. 2012;7(9):e45799. [PubMed: 23029248]
33. Mettlen M, Pucadyil T, Ramachandran R, Schmid SL. Dissecting dynamin's role in clathrin-mediated endocytosis. Biochem Soc Trans. 2009;37(pt 5): 1022–1026. [PubMed: 19754444]
34. Harper CB, Popoff MR, McCluskey A, Robinson PJ, Meunier FA. Targeting membrane trafficking in infection prophylaxis: dynamin inhibitors. Trends Cell Biol. 2013;23(2):90–101. [PubMed: 23164733]
35. Grant BD, Donaldson JG. Pathways and mechanisms of endocytic recycling. Nat Rev Mol Cell Biol. 2009;10(9):597–608. [PubMed: 19696797]
36. Van Leuven F, Cassiman JJ, Van Den Berghe H. Primary amines inhibit recycling of α2M receptors in fibroblasts. Cell. 1980;20(1):37–43. [PubMed: 6156003]
37. Schnitzer JE, Oh P, Pinney E, Allard J. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. J Cell Biol. 1994;127(5):1217–1232. [PubMed: 7525606]

38. Le PU, Nabi IR. Distinct caveolae-mediated endocytic pathways target the Golgi apparatus and the endoplasmic reticulum. J Cell Sci. 2003;116(pt 6): 1059–1071. [PubMed: 12584249]
39. Hamm-Alvarez SF, Sonee M, Loran-Goss K, Shen WC. Paclitaxel and nocodazole differentially alter endocytosis in cultured cells. Pharm Res (N Y). 1996;13(11):1647–1656.
40. Straight AF, Cheung A, Limouze J, et al. Dissecting temporal and spatial control of cytokinesis with a myosin II inhibitor. Science. 2003;299(5613): 1743–1747. [PubMed: 12637748]
41. Kovács M, Tóth J, Hetényi C, Málnási-Csizmadia A, Sellers JR. Mechanism of blebbistatin inhibition of myosin II. J Biol Chem. 2004;279(34):35557–35563. [PubMed: 15205456]
42. Pundir P, Catalli A, Leggiadro C, Douglas SE, Kulka M. Pleurocidin, a novel antimicrobial peptide, induces human mast cell activation through the FPRL1 receptor. Mucosal Immunol. 2014;7(1):177–187. [PubMed: 23839065]
43. Scarcella M, d'Angelo D, Ciampa M, et al. The key role of lysosomal protease cathepsins in viral infections. Int J Mol Sci. 2022;23(16):9089. [PubMed: 36012353]
44. Xie Z, Zhao M, Yan C, et al. Cathepsin B in programmed cell death machinery: mechanisms of execution and regulatory pathways. Cell Death Dis. 2023; 14(4):255. [PubMed: 37031185]
45. Belcher JD, Beckman JD, Balla G, Balla J, Vercellotti G. Heme degradation and vascular injury. Antioxid Redox Signal. 2010;12(2):233–248. [PubMed: 19697995]
46. Belcher JD, Bryant CJ, Nguyen J, et al. Transgenic sickle mice have vascular inflammation. Blood. 2003;101(10):3953–3959. [PubMed: 12543857]
47. Belcher JD, Chen C, Nguyen J, et al. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. Blood. 2014;123(3):377–390. [PubMed: 24277079]
48. Kato GJ, Steinberg MH, Gladwin MT. Intravascular hemolysis and the pathophysiology of sickle cell disease. J Clin Invest. 2017;127(3):750–760. [PubMed: 28248201]
49. Gladwin MT, Kanias T, Kim-shapiro DB. Hemolysis and cell-free haemoglobin drive an intrinsic mechanism for human disease. J Clin Invest. 2012; 122(4):1205–1208. [PubMed: 22446184]
50. Horonchik L, Wessling-Resnick M. The small-molecule iron transport inhibitor ferristatin/ NSC306711 promotes degradation of the transferrin receptor. Chem Biol. 2008;15(7):647–653. [PubMed: 18635001]
51. Bennewitz MF, Jimenez MA, Vats R, et al. Lung vaso-occlusion in sickle cell disease mediated by arteriolar neutrophil-platelet microemboli. JCI Insight. 2017;2(1):e89761. [PubMed: 28097236]
52. Bhattacharyya S, Wang W, Tamaki Z, et al. Pharmacological inhibition of toll-like receptor-4 signaling by TAK242 prevents and induces regression of experimental organ fibrosis. Front Immunol. 2018;9:2434. [PubMed: 30405628]
53. Subtil A, Gaidarov I, Kobylarz K, Lampson MA, Keen JH, Mcgraw TE. Acute cholesterol depletion inhibits clathrin-coated pit budding. Proc Natl Acad Sci U S A. 1999;96(12):6775–6780. [PubMed: 10359788]
54. Adams SP, Alaeiilkhchi N, Wright JM. Simvastatin for lowering lipids. Cochrane Database Syst Rev. 2023;2023(2):CD014857.
55. Fadeyibi O, Rybalchenko N, Mabry S, Nguyen DH, Cunningham RL. The role of lipid rafts and membrane androgen receptors in androgen's neurotoxic effects. J Endocr Soc. 2022;6(5):bvac030.
56. Huettinger M, Hermann M, Goldenberg H, Granzer E, Leineweber M. Hypolipidemic activity of HOE-402 is mediated by stimulation of the LDL receptor pathway. Arterioscler Thromb. 1993;13(7):1005–1012. [PubMed: 8318502]
57. Hoffmann A, Schmalz M, Leineweber M. Cholesterol lowering action of HOE 402 in the normolipidemic and hypercholesterolemic Golden Syrian hamster. Biochim Biophys Acta. 1996;1299(1):95–102. [PubMed: 8555258]
58. Vázquez-Calvo Á, Saiz JC, McCullough KC, Sobrino F, Martín-Acebes MA. Acid-dependent viral entry. Virus Res. 2012;167(2):125–137. [PubMed: 22683298]
59. Mercer J, Schelhaas M, Helenius A. Virus entry by endocytosis. Annu Rev Biochem. 2010;79:803–833. [PubMed: 20196649]
60. Clague MJ, Urbé S, Aniento F, Gruenberg J. Vacuolar ATPase activity is required for endosomal carrier vesicle formation. J Biol Chem. 1994;269(1): 21–24. [PubMed: 8276796]

61. Vinchi F, Tolosano E. Therapeutic approaches to limit hemolysis-driven endothelial dysfunction: scavenging free heme to preserve vasculature homeostasis. Oxid Med Cell Longev. 2013;2013:396527. [PubMed: 23781294]

Key Points

- Liver endothelial cell–mediated Hb internalization predominantly occurs via fluid-phase endocytosis.
- Presence of lipid droplets promotes endothelial HbS internalization, whereas hypolipidemic condition inhibits it.

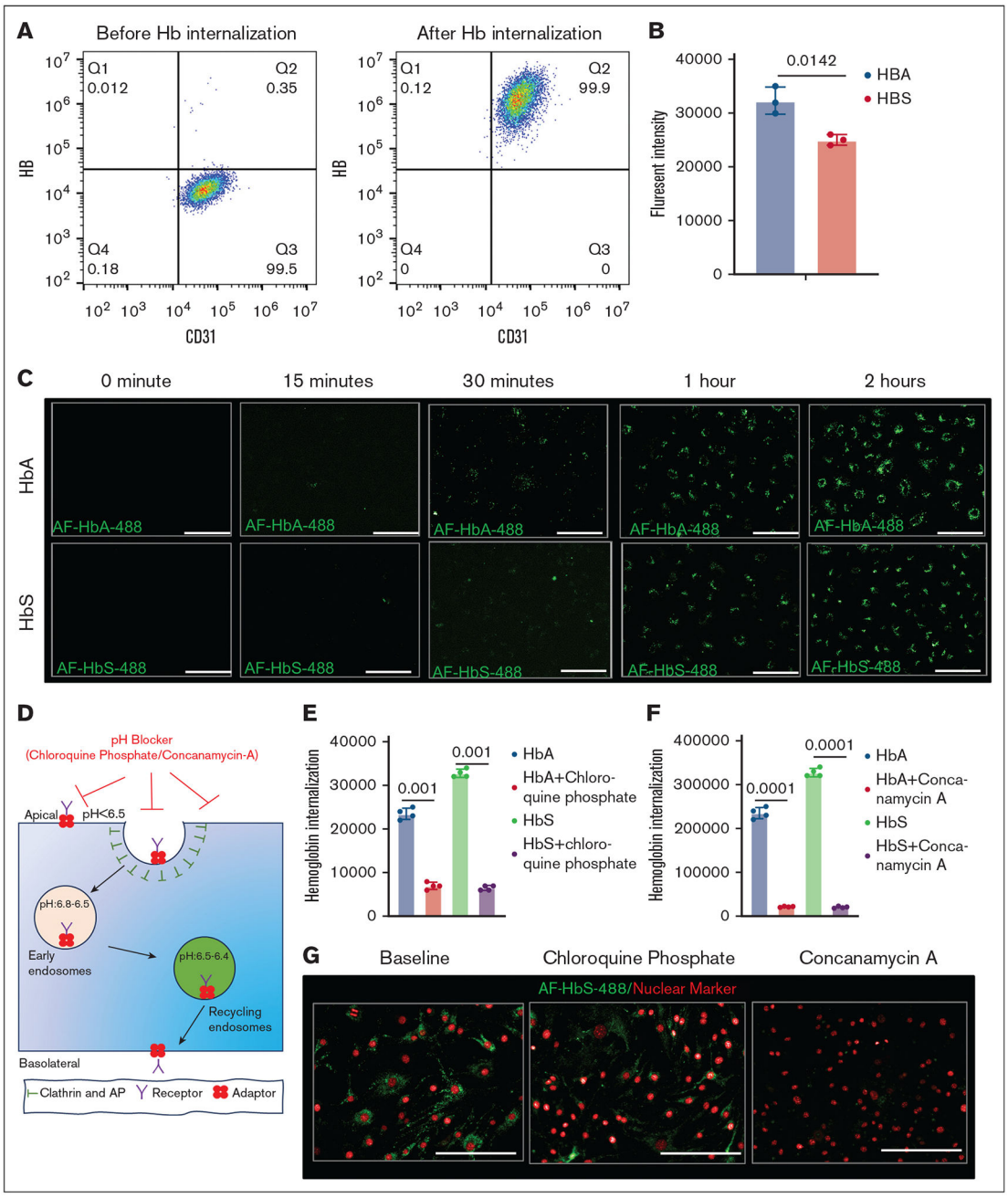

Figure 1. Endothelial Hb internalization is a slow process and depends on cellular pH.

(A) Flow cytometry analysis of Hb internalization in cells; distribution of CD31 (liver endothelial cell marker) and Hb before internalization (left), and the distribution after internalization (right). (B) Quantification of fluorescence intensity of internalized HbA and HbS, indicating a significant difference in internalization efficiency. (C) Time course study of HbA and HbS internalization in cells at 0, 15, and 30 minutes, 1 hour, and 2 hours. Images show the fluorescence of AF-HbA-488 and AF-HbS-488, demonstrating the time-dependent internalization of Hb. Scale bars, 20 μm. (D) Schematic representation of the internalization and trafficking pathway of Hb in cells, highlighting the role of pH

blockers (chloroquine phosphate and concanamycin A) in altering Hb internalization. (E) Quantification of Hb internalization in the presence of chloroquine phosphate. Results indicate a significant reduction in Hb internalization when treated with chloroquine phosphate compared with baseline ($P < .05$). (F) Quantification of Hb internalization in the presence of concanamycin A. Results indicate a significant reduction in Hb internalization when treated with concanamycin A compared with baseline (*$P < .05$). (G) Confocal microscopy images showing the internalization of AF-HbS-488 in the presence of chloroquine phosphate and concanamycin A. Nuclear marker is shown in red, indicating the distribution of internalized Hb within the cells. Scale bars, 20 μm. $P < .05$. HBB, hemoglobin beta.

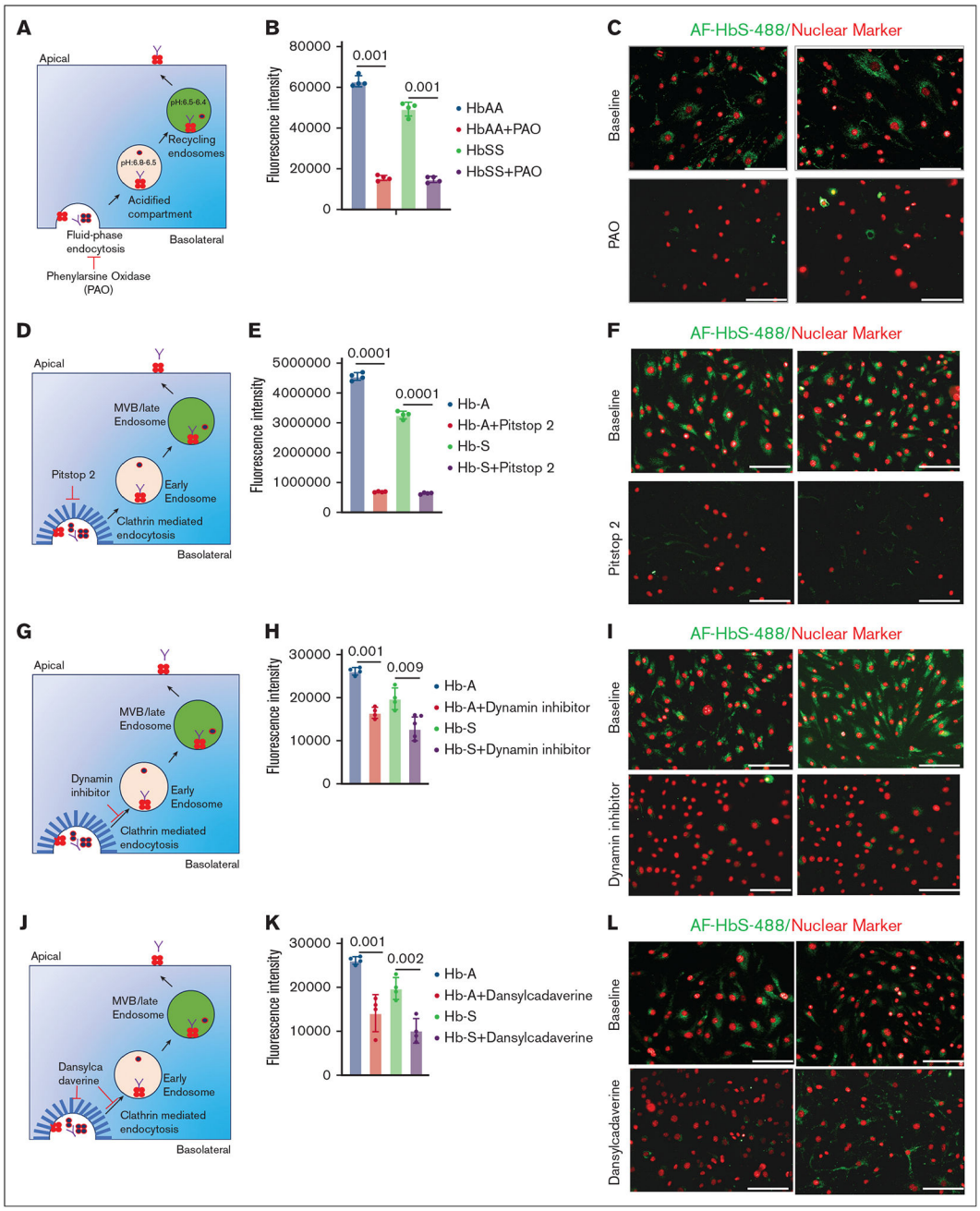

Figure 2. Liver endothelial cell–driven Hb internalization depends on alternative endocytic routes.

(A) Schematic representation of the internalization and trafficking pathway of Hb in cells, highlighting the role of phenylarsine oxide (PAO) in altering Hb internalization through fluid-phase endocytosis. (B) Quantification of fluorescence intensity of internalized HbA and HbS in the presence of PAO, indicating a significant reduction in internalization efficiency when treated with PAO. (C) Confocal microscopy images showing the internalization of AF-HbS-488 in the presence of PAO. Nuclear marker is shown in red, indicating the distribution of internalized Hb within the cells. Scale bars, 20 μm. (D) Schematic representation of the internalization and trafficking pathway of Hb in cells,

highlighting the role of Pitstop 2 in altering Hb internalization through the inhibition of CME. (E) Quantification of fluorescence intensity of internalized HbA and HbS in the presence of Pitstop 2, indicating a significant reduction in internalization efficiency when treated with Pitstop 2. (F) Confocal microscopy images showing the internalization of AF-HbS-488 in the presence of Pitstop 2. Nuclear marker is shown in red, indicating the distribution of internalized Hb within the cells. (G) Schematic representation of the internalization and trafficking pathway of Hb in cells, highlighting the role of dynamin inhibitor in altering Hb internalization through the inhibition of CME. (H) Quantification of fluorescence intensity of internalized HbA and HbS in the presence of dynamin inhibitor, indicating a significant reduction in internalization efficiency when treated with dynamin inhibitor. (I) Confocal microscopy images showing the internalization of AF-HbS-488 in the presence of dynamin inhibitor. Nuclear marker is shown in red, indicating the distribution of internalized Hb within the cells. (J) Schematic representation of the internalization and trafficking pathway of Hb in cells, highlighting the role of dansylcadaverine in altering Hb internalization through inhibition of CME. (K) Quantification of fluorescence intensity of internalized HbA and HbS in the presence of dansylcadaverine, indicating a significant reduction in internalization efficiency when treated with dansylcadaverine ($P < .05$). (L) Confocal microscopy images showing the internalization of AF-HbS-488 in the presence of dansylcadaverine. Nuclear marker is shown in red, indicating the distribution of internalized Hb within the cells. Scale bars, 20 μm. *$P < .05$. MVB, multivesicular bodies.

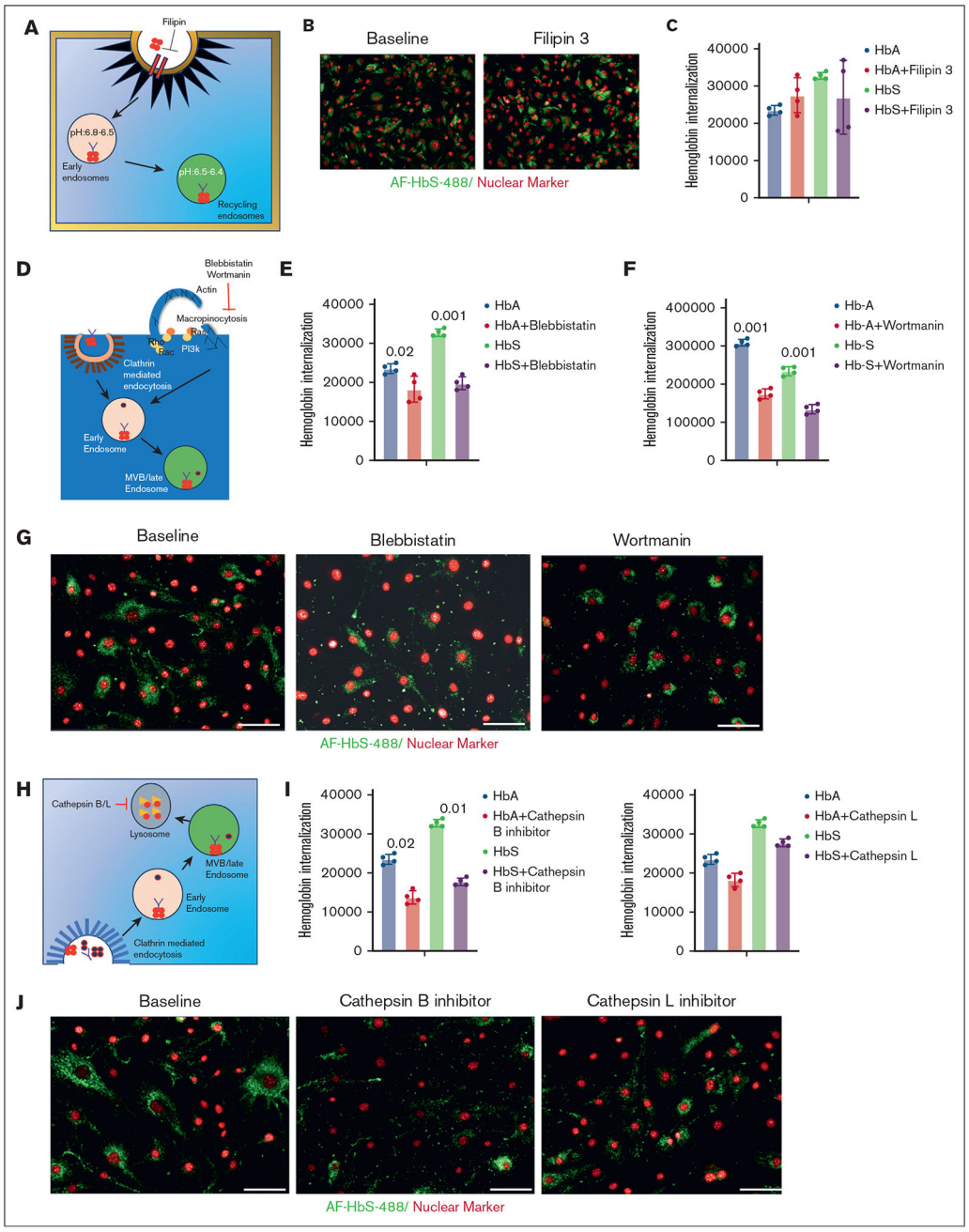

Figure 3. The effect of blocking caveola-mediated endocytosis and lysosomal degradation in LSEC-mediated Hb internalization.

(A) Schematic representation of the internalization and trafficking pathway of Hb in cells, highlighting the role of filipin III in altering Hb internalization through inhibition of caveola-mediated endocytosis. (B) Confocal microscopy images showing the internalization of AF-HbS-488 in the presence of filipin III. Nuclear marker is shown in red, indicating the distribution of internalized Hb within the cells. (C) Quantification of fluorescence intensity of internalized HbA and HbS in the presence of filipin III, indicating a significant reduction in internalization efficiency when treated with filipin III. (D) Schematic representation of the internalization and trafficking pathway of Hb in cells, highlighting the role of blebbistatin

and wortmannin in altering Hb internalization through inhibition of macropinocytosis. (E) Quantification of fluorescence intensity of internalized HbA and HbS in the presence of blebbistatin, indicating a significant reduction in internalization efficiency when treated with blebbistatin. (F) Quantification of fluorescence intensity of internalized HbA and HbS in the presence of wortmannin, indicating a significant reduction in internalization efficiency when treated with wortmannin. (G) Confocal microscopy images showing the internalization of AF-HbS-488 in the presence of blebbistatin and wortmannin. Nuclear marker is shown in red, indicating the distribution of internalized Hb within the cells. (H) Schematic representation of the internalization and trafficking pathway of Hb in cells, highlighting the role of cathepsin B and cathepsin L inhibitors in altering Hb internalization through inhibition of lysosomal degradation. (I) Quantification of fluorescence intensity of internalized HbA and HbS in the presence of cathepsin B and cathepsin L inhibitors, indicating a significant reduction in internalization efficiency when treated with cathepsin B and cathepsin L inhibitors. (J) Confocal microscopy images showing the internalization of AF-HbS-488 in the presence of cathepsin B and cathepsin L inhibitors. Nuclear marker is shown in red, indicating the distribution of internalized Hb within the cells. Scale bars, 20 μm. $*P < .05$. MVB, multivesicular bodies.

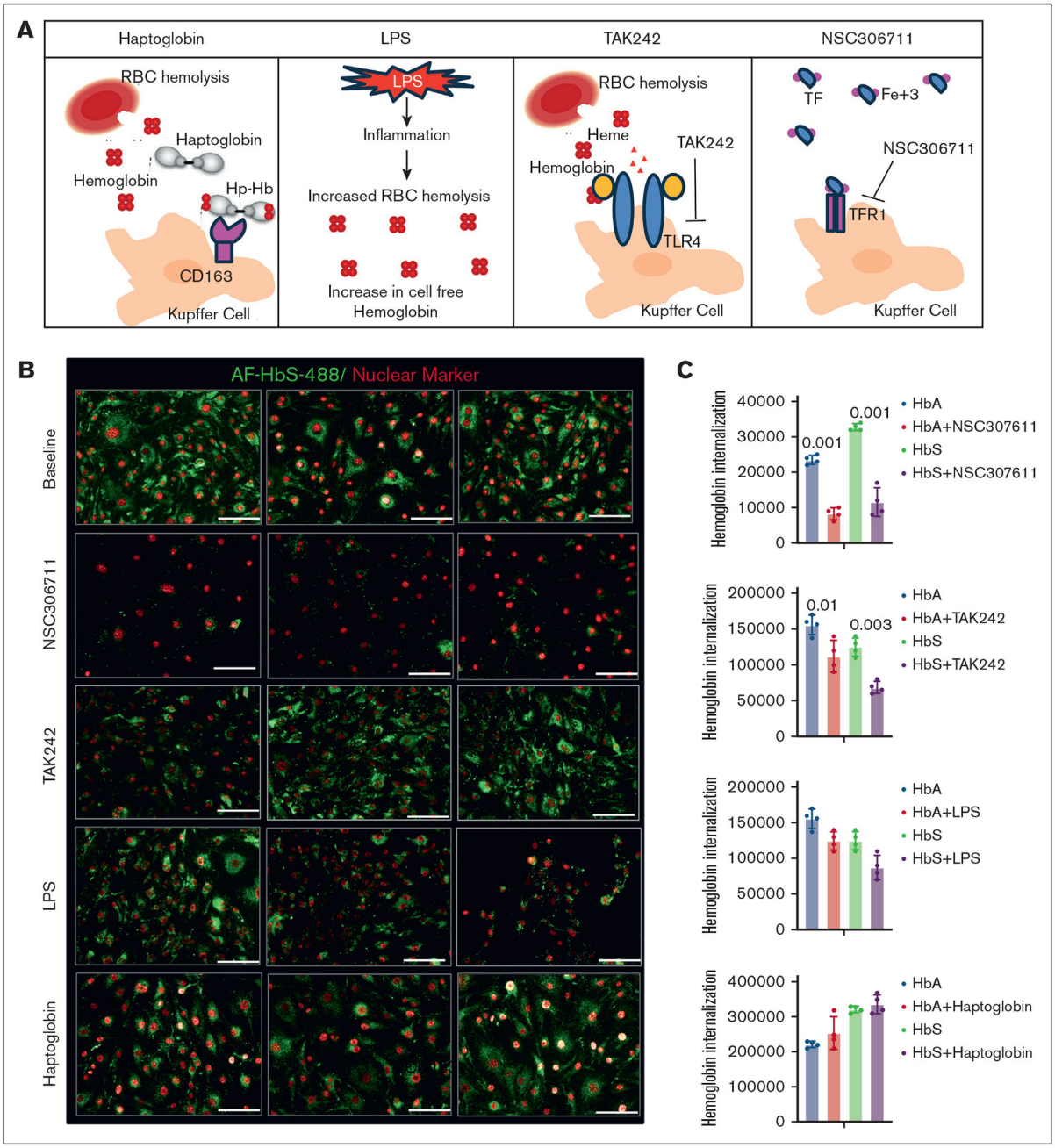

Figure 4. The effect of SCD pathophysiology on LSEC-mediated Hb internalization.

(A) Schematic representation of the different pathways and inhibitors affecting Hb internalization in Kupffer cells. The pathways include the following: Hp binding to Hb and interacting with CD163; LPS inducing inflammation, leading to increased red blood cell hemolysis and free Hb; TAK242 inhibiting TLR4 signaling, affecting Hb internalization; and NSC306711 interfering with TFR1-mediated iron uptake. (B) Confocal microscopy images showing the internalization of AF-HbS-488 under baseline conditions and in the presence of Hp, LPS, TAK242, and NSC306711. Nuclear marker is shown in red, indicating the distribution of internalized Hb within the cells. Scale bars, 20 μm. (C) Quantification of

Hb internalization under various treatments: Hb internalization with Hp, LPS, TAK242, and NSC306711; # and * indicating significant changes compared with its respective baseline ($P < .05$). RBC, red blood cell; TFR1, transferrin receptor 1.

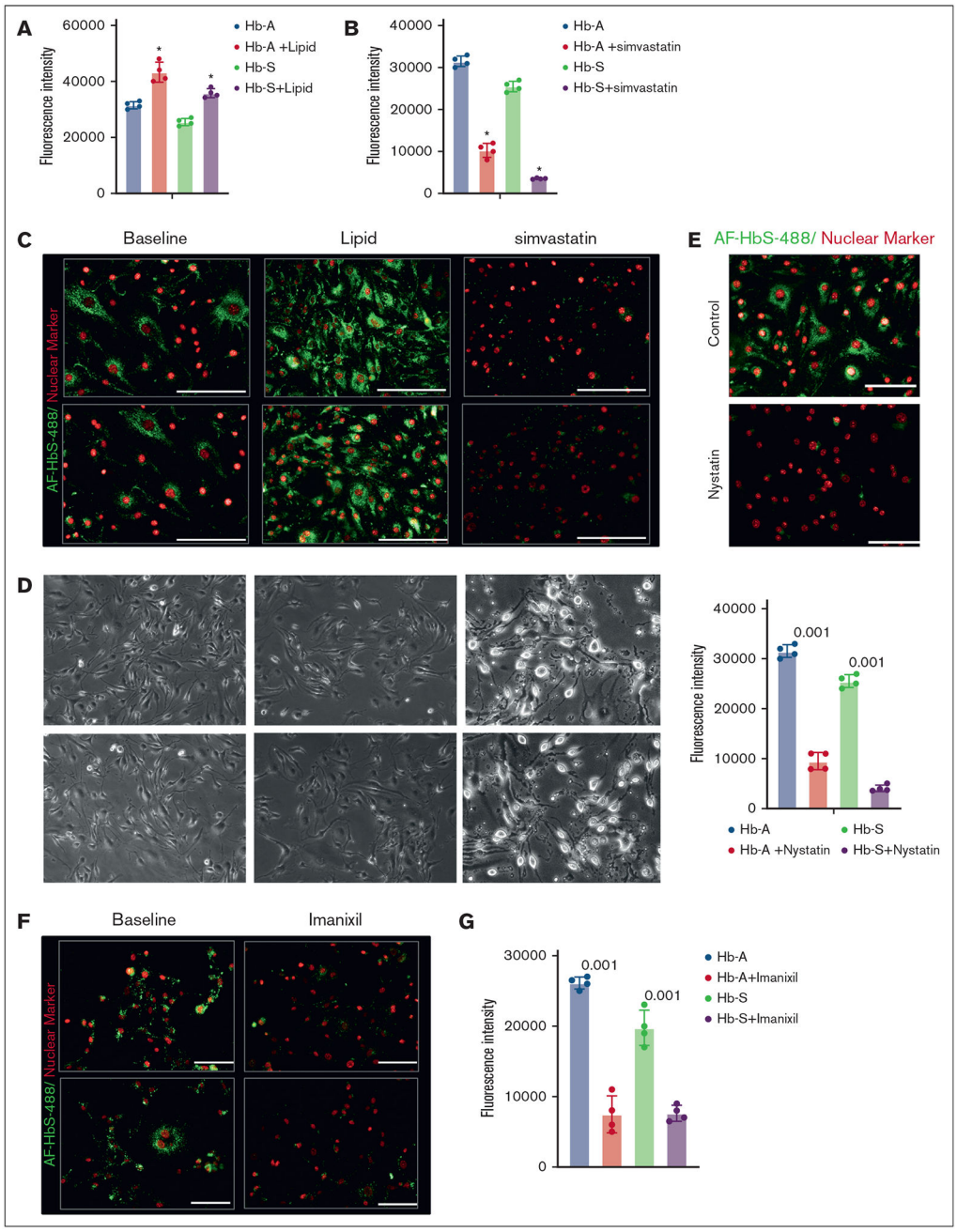

Figure 5. Presence of lipid promotes Hb internalization in LSECs.

(A) Quantification of fluorescence intensity of internalized HbA and HbS in the presence of lipid solution, indicating a significant reduction in internalization efficiency when treated with lipid solution. (B) Quantification of fluorescence intensity of internalized HbA and HbS in the presence of simvastatin, indicating a significant reduction in internalization efficiency when treated with simvastatin. (C) Confocal microscopy images showing the internalization of AF-HbS-488 at baseline, with lipid solution and with simvastatin. Nuclear marker is shown in red, indicating the distribution of internalized Hb within the cells. (D) Phase-contrast microscopy images showing the morphological changes in

cells at baseline, with lipid solution, and with simvastatin. (E) Confocal microscopy images showing the internalization of AF-HbS-488 with and without nystatin treatment. Nuclear marker is shown in red, indicating the distribution of internalized Hb within the cells. The quantification graph indicates a significant reduction in internalization efficiency when treated with nystatin. (F) Representative immunohistochemistry images showing the internalization of AF-HbS-488 with and without imanixil treatment. Nuclear marker is shown in red, indicating the distribution of internalized Hb within the cells. The quantification graph indicates a significant reduction in internalization efficiency when treated with imanixil. Scale bars, 20 μm. $*P < .05$.

Table 1.

List of all chemicals/small-molecule inhibitors used in the screen

Category of blocker	Inhibitors used
pH regulators	Concanamycin A, chloroquine, and NH4Cl
General endocytosis blockers	Pitstop 2, dynasore, and dynamin hydrate
Micropinocytosis	Phenylarsine oxidase and nystatin
Macropinocytosis	Wortmannin, blebbistatin, and EIPA
Actin cytoskeleton related	Cytochalasin, latrunculin A, and paclitaxel
Lipid related	Lipid cocktail, simvastatin, imanixil, nystatin, and NSC306711 (ferristatin)
CME	Dynamin inhibitors, dynasore hydrate, and dansylcadaverine,
Caveola-mediated endocytosis	Filipin III and nocodazole
Lysosomal degradation blocker	Cathepsin L inhibitor, cathepsin B inhibitor, amantadine, and Z-Phe-Tyr(tBu)-diazomethylketone
Receptor recycling blockers	Dansylcadaverine
SCD related	Oxy-Hb, iron dextran, Hp, LPS, TAK242, ferristatin (NSC306711), IL-4, and TNFα
ATP dependence/P2X receptors	NF279, NF499, and dipyridamole
LSEC specific receptors/blockers	Methyl-β-cyclodextrin, amiloride hydrochloride hydrate, infliximab, GW4869, KF38739, SBI115, AZD4547, and H2L5186303

ATP, adenosine triphosphate; EIPA, 5-[N-ethyl-N-isopropyl] amiloride; IL-4, interleukin-4; TNFα, tumor necrosis factor α.