LETTER TO EDITOR

Rifaximin on intestinally-related pathologic changes in sickle cell disease

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Running title: Rifaximin on intestine in SCD

Source of support: Bausch Health Companies, Inc.

Conflict of Interest: All authors declare no conflict of interest
To the Editor:

Sickle cell disease (SCD) is characterized by episodes of painful vaso-occlusive crises (VOC) and progressive end-organ damage, resulting in poor quality of life and a shortened lifespan. Although sickling is a prerequisite for the development of VOC, the dissociation between the number of sickled erythrocytes observed on the peripheral blood smear and symptoms of VOC suggests that, in addition to sickled erythrocytes, certain co-factors are needed for the pathogenesis of VOC. Many clinical and laboratory observations have implicated neutrophils as one of the co-factors. These observations include higher baseline white cell counts (WBCs), and higher risks for stroke, acute chest syndrome, and premature death in those with a WBC >15 x 10^9/L. In addition, the neutrophils in SCD demonstrated qualitative abnormalities. They have higher levels of activation molecules, e.g. CD64 and CD11b/CD18, and the sera elevated soluble CD62L. To explain the baseline neutrophilia and increased neutrophil activation, we previously hypothesized that changes in the intestinal microbial composition and/or permeability in SCD may be responsible. They provide the portal of entry for the translocation of intestinal bacteria/bacterial products across the intestinal barrier into the systemic circulating, at an inoculum sufficient to stimulate the neutrophils but insufficient to cause infection, causing the higher baseline neutrophil counts and increased neutrophil activation.
Various studies have supported our hypothesis that SCD patients exhibited pathophysiologic changes in the intestine. We previously reported the occurrence of intestinal dysbiosis, and increased intestinal injury and bacterial translocation in SCD patients. Others have found that sickle cell mice treated with the antibiotic cocktail of oral vancomycin, ampicillin, metronidazole, and streptomycin were protected from tumor necrosis factor α (TNFα)-induced fatal VOC. These findings suggest that the intestine might be a therapeutic target for SCD.

Based on these backgrounds, we have carried out a Phase 2 clinical study of oral rifaximin in subjects with SCD (Clinicaltrials.gov: NCT03719729) after Institutional Review Board approval. SCD subjects with at least two painful crises needing inpatient or outpatient intravenous opioid analgesia (IOA) during the preceding 12 months were accrued to the study. Each subject received rifaximin for six months and the subjects were followed for the development of VOC, compared to those expected for a six-month period calculated from the average of the previous 12 months. We found in the study that the frequency of painful VOC and the number of days needing IOA were significantly reduced during the 6-month study period. Since rifaximin is a minimally absorbed oral antibiotics, we carried out an exploratory study to investigate the local effects of rifaximin in modulating the intestinal pathophysiologic changes.
and circulating neutrophils to gain insight into the possible mechanisms of action of rifaximin in mediating the clinical benefits we observed in the clinical study.

To examine changes in neutrophil activation in SCD subjects treated with rifaximin in 10 of the 12 subjects accrued to the study with sufficient serum specimens, we measured the serum CD62L serially. Before being started on rifaximin, serum CD62L levels were elevated in 9 of the ten subjects. Serum CD62L declined consistently following the start of rifaximin ($p = 0.0002$) (Figure 1a(i)), to levels comparable to those reported in non-SCD controls. The levels, however, increased subsequently in two subjects, both coincided with episodes of painful crisis, precipitated by presumed infections, despite continued to have low levels of CANs.

In addition to activated neutrophils, SCD also have increased CANs. Since CANs are involved in the pathogenesis of VOC, we next determined whether the clinical benefits of rifaximin in SCD subjects were associated with changes in CANs. We observed that nine of 12 (75%) subjects exhibited more than 10% CANs in their venous blood before starting rifaximin (median 12.6, range 6-40). In keeping with our previous study, CANs dropped significantly in all 12 subjects after starting rifaximin ($p = 0.005$) (Figure 1a(ii)). Drops in CANs occurred as early as two weeks after rifaximin and remained low throughout the six-month study period. In all cases, the CANs dropped to below 5%. Taking into consideration rifaximin is a minimally absorbed antibiotic, these results not only indicate that rifaximin use resulted in reduced CANs.
but also support the notion that, not unlike in mice\textsuperscript{4}, CANs in human are regulated by intestinal microbiota.

CANs are regulated by intestinal microbiota via Myd88 and toll-like receptor (TLR) 2/4\textsuperscript{4}. Both Myd88 and TLR 2/4 are receptors for pathogen-associated molecular patterns (PAMPs) such as LPS\textsuperscript{7,8}. Therefore, we determined the serum baseline LPS in subjects treated with rifaximin. In keeping with what we reported previously\textsuperscript{3}, the baseline levels of serum LPS were elevated before rifaximin in 12 subjects accrued to the study. The median level of serum LPS was 2.27 µg/ml (range 1-4.56). Serum LPS, however, came down significantly ($p = 0.01$) (Figure 1a(\textit{iii})) in all 12 subjects, to levels comparable to those in non-SCD controls\textsuperscript{3}. The levels remained low throughout the six-month study period.

Serum LPS reflects increased translocation of intestinal bacteria/bacterial products, caused either by increased intestinal permeability from enterocyte damage or by increased intestinal microbial load, across the intestinal barrier into the systemic circulation. We previously reported that SCD subjects showed evidence of enterocyte injury\textsuperscript{3}. Having demonstrated drops in the serum LPS following rifaximin therapy, we next measured changes in the serum iFABP to determine whether the drops could be related to changes in the degrees of enterocyte injury. iFABP is a protein produced by enterocytes and is released into the systemic circulation when there is enterocyte damage. The baseline serum iFABP were elevated in all 12 subjects. The
median serum iFABP was 1.05 ng/ml (range 0.4-3.17). iFABP decreased significantly \( (p = 0.008) \) (Figure 1a(iv)) in all 12 subjects after being started on rifaximin, to levels comparable to those we previously reported in non-SCD controls\(^3\). The reduction in enterocyte injury may, therefore, play a part in reducing the serum LPS. The mechanisms for the reduction in enterocyte injury, however, remain speculative but may be related to the local anti-inflammatory property of rifaximin previously reported\(^9\). Alternatively it may be the end result of a decrease in the frequency and severity of VOCs when the subjects were taking rifaximin.

Urinary 3-IS is a metabolite of dietary L-tryptophan by intestinal flora, particularly, the microbes that produce tryptophanase. Elevated urinary 3-IS reflects either an increase in the abundance of intestinal *Clostridiales* without changes in the total microbial density\(^10\), or an increase in the total microbial density without changes in the relative abundance of *Clostridiales*. *Clostridiales* are major producers of short-chain fatty acids (SCFAs) such as butyrate. To gain insight into the intestinal metabolomic changes, we next measured the urinary 3-IS levels in these subjects. The median baseline urinary 3-IS was high at 152 \( \mu g/ml \) (range 104.8-299.6). Urinary 3-IS dropped significantly following the start of rifaximin \( (p = 0.01) \) (Figure 1b). The drops occurred as early as 4 weeks after starting rifaximin and the levels remained low throughout the study period, at levels comparable to those we reported in non-SCD controls\(^3\). The results we have observed in the urinary 3-IS suggest the need in future study to examine in
detail changes in the metabolomics if the mechanisms of action of rifaximin in SCD patients are to be determined.

Having demonstrated changes in CANs and neutrophil activation, and associated decrease in the serum LPS and enterocyte injury, following use of rifaximin, changes in intestinal microbiome were determined. In keeping with our previous findings\(^2\), SCD subjects in this study did not show any restriction in the \(\alpha\)-diversity of their baseline intestinal microbiome. Following therapy with rifaximin, there was a decrease in \(\alpha\)-diversity but the effect was not statistically significant (Shannon \(p = 0.625\) and tail \(p = 0.695\)\(^{38}\)). We next examined changes in specific genera by first transforming the abundance to additive log ratio (alr)\(^{39,40}\) to mitigate spurious correlation of relative abundances due to the compositional nature of the profiles. Only the top 15 most abundant taxa were considered, with the remainder accumulated in the denominator of the alr ratio. Rifaximin treatment resulted in trend towards increase in the taxonomic abundance of the genera *Bacterioides*, *Akkermansia*, and *Escherichia-Shigella* (\(p = 0.0645, 0.322\), and 0.4316, respectively) (Figure 1c), although they did not reach statistical significance. Based on the Wilcoxon signed-rank test, additional studies with a larger cohort may confirm the trend. Such findings in our study are not surprising in view of the small sample size and heterogeneity of the cohort of subjects. The lack of statistical significance associated with the changes in the microbiome post-treatment does not rule out the hypothesis that rifaximin’s clinical efficacy operates through its modulation of the intestinal microbiome. It is possible that
the clinical benefits from rifaximin are not influenced by any consistent changes in the microbial composition, but rather to changes in the metabolomics as a result of subtle changes in the compositional activity of the same intestinal microbiota.

In summary, rifaximin therapy influenced the intestinal pathophysiologic changes in SCD. It reduced the intestinal injury and increased the intestinal barrier. Associated with these changes, CANs and activated neutrophils were reduced. Although there was no significant change in the intestinal microbiome, rifaximin obviously altered the intestinal metabolomics, as shown by changes in the urinary 3-IS. These results may explain the clinical benefits of rifaximin in reducing VOC in SCD.
Figure legends

**Figure 1**: Effects of rifaximin on subjects with SCD. **a)** Changes in the intestinal pathophysiology in SCD subjects treated with rifaximin. (i). Serum CD62L, a marker of neutrophil activation, declined consistently and occurred in all ten subjects with serum samples for study. (ii). Circulating aged neutrophils (CANs), regulated by intestinal microbiota, were grossly elevated in SCD subjects but decreased dramatically after being started on the antibiotic. Circulating aged neutrophils remained low throughout the study period. (iii). Serum lipopolysaccharides (LPS) dropped after the subjects were treated with rifaximin, suggesting a decrease in the translocation of bacteria/bacterial products across the intestinal barrier into the systemic circulation. (iv). Reductions in the serum LPS were due, at least in part, to improvement in the integrity of the intestinal barrier, as shown by decreases in the intestinal fatty acid binding protein (iFABP). **b)** Changes in the urinary 3-indoxyl sulfate (3-IS) levels following treatment with rifaximin, suggesting that rifaximin alters the intestinal metabolomics. **c)** Changes in taxonomic abundance in intestinal microbiome after being started on rifaximin. Relative abundances were transformed with the additive log ratio (alr), prior to calculating the difference, to reduce the spurious correlations associated with compositional data. Positive differences signify increased abundance post treatment. Histograms represent the frequency of
differences between during and pre-rifaximin treatment alr values, where an increase of abundance during treatment is indicated by a shift of the distribution towards the right. The dashed blue vertical reference line at $x = 0$, indicates no difference due to rifaximin treatment.

References


Supplemental References


S10.Gloor GB, Macklaim JM, Pawlowsky-Glahn V, and Egozcue JJ. Microbiome datasets are compositional: And this is not optional. Front Microbiol. 2017; 8: 2224.
Figure 1a

(i) Serum soluble CD62L (meg/ml)

(ii) % circulating aged neutrophils

(iii) LPS (mg/mL)

(iv) ifABP (ng/mL)

$p = 0.0002$

$p = 0.005$

$p = 0.01$

$p = 0.008$
Figure 1b

$p = 0.01$

[Graph showing urinary 3-4S (mcg/ml) over time (month) for different patients labeled Pt_1 to Pt_12.]