



Compstatin Cp40 blocks hematin-mediated deposition of C3b fragments on erythrocytes: Implications for treatment of malarial anemia



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ABSTRACT

During malarial anemia, 20 uninfected red blood cells (RBCs) are destroyed for every RBC infected by *Plasmodium falciparum* (Pf). Increasing evidence indicates an important role for complement in destruction of uninfected RBCs. Products of RBC lysis induced by Pf, including the digestive vacuole and hematin, activate complement and promote C3 fragment deposition on uninfected RBCs. C3-opsonized cells are then subject to extravascular destruction mediated by fixed tissue macrophages which express receptors for C3 fragments. The Compstatin family of cyclic peptides blocks complement activation at the C3 cleavage step, and is under investigation for treatment of complement-mediated diseases. We demonstrate, that under a variety of stringent conditions, second-generation Compstatin analogue Cp40 completely blocks hematin-mediated deposition of C3 fragments on naïve RBCs. Our findings indicate that prophylactic provision of Compstatin for malaria-infected individuals at increased risk for anemia may provide a safe and inexpensive treatment to prevent or substantially reduce malarial anemia.

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1. Introduction

The pathogenesis of malarial anemia is associated with at least two separate mechanisms of red blood cell (RBC) destruction. RBCs infected with *Plasmodium falciparum* (Pf) are destroyed during growth and replication of the organism; the growing schizont within the RBC lyses, followed by rupture of the RBC membrane and release of the digestive vacuole (DV) as well as merozoites which invade additional RBCs and continue the pattern of RBC infection and destruction [1,2]. Secondly, severe malarial anemia occurs at relatively low parasite burdens, and thus an additional mechanism or mechanisms must operate to account for destruction of large numbers of uninfected RBCs [3–7].

Abbreviations: APC, alternative pathway of complement; Cp40, second generation Compstatin analogue; DV, digestive vacuole; MESF, molecules of equivalent soluble fluorochrome; NHS, normal human serum; PNH, paroxysmal nocturnal hemoglobinuria; RBC, red blood cell.

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Numerous studies have implicated complement in destruction of uninfected RBCs in malaria, but elucidation of pathways whereby complement may mediate this reaction remained elusive [2,8,9]. Now, increasing evidence indicates that in malaria, uninfected RBCs are opsonized with complement C3 fragments (C3b/iC3b/C3dg), and the opsonized RBCs can be destroyed extravascularly by fixed tissue macrophages, in spleen and liver, which express receptors specific for C3 fragments. In vitro experiments have revealed that breakdown products generated during lysis of Pf-infected RBCs, including heme/hematin and the DV, activate the alternative pathway of complement (APC) and promote C3 fragment deposition on uninfected RBCs [1,5,10–13].

These findings provide the key link by which lysis of Pf-infected RBCs leads to C3 fragment-opsonization of uninfected cells followed by their extravascular clearance and destruction. We previously reported that *anti*-C3b mAb 3E7, which blocks the APC, inhibited complement activation mediated by hematin, and prevented C3-fragment deposition on RBCs [10]. In principle mAb 3E7 could be used to treat malarial anemia, but practical/financial obstacles render this approach problematic. An alternative strategy to block the APC is centered on use of Compstatin (Cp40), a 13 amino acid cyclic peptide which binds to C3 and blocks complement activation at the C3 cleavage step in the classical and APC C3 convertases [14]. In this communication we report on a series of in vitro experiments which demonstrate that Cp40 is quite effective at inhibiting hematin-mediated C3b deposition on RBCs. Our

findings suggest that Cp40 might find use in treatment and prevention of severe malarial anemia.

2. Materials and methods

We followed our previously reported procedures to study hematin-mediated C3b deposition on RBCs [10]. Blood was collected in EDTA, RBCs were isolated and washed, and duplicate or triplicate samples were reconstituted in polystyrene tubes (12 × 75 mm) to hematocrits of 5% or 50% in 50% autologous normal human serum (NHS), in the presence or absence of mAb 3E7 (final concentration 200 µg/ml) or Cp40 (final concentration 25 µM unless noted). Alternatively an IgG1 isotype control was used in place of mAb 3E7, or a scrambled peptide (same amino acid composition as Cp40) was used in place of Cp40. Hematin (Sigma-Aldrich, final concentration 400 µg/ml) was added and the samples were incubated for 20 min at 37 °C. The RBCs were washed once and in most experiments reconstituted under the same conditions for a second and third reaction cycle. Subsequently the cells were washed twice and probed with Al488- or Al647-labeled mAbs 7C12 or 1H8, specific for C3b/iC3b or C3b/iC3b/C3dg, respectively, and analyzed by flow cytometry. The intensities of the fluorescent signals are reported in units of molecules of equivalent soluble fluorochrome (MESF) [10], and means and SD are reported. In some experiments, blood was anticoagulated by collection in lepirudin (50 µg/ml final concentration), and either whole blood or RBCs reconstituted in 50% lepirudin plasma (± Cp40) were reacted with hematin and then processed as described above.

Unpaired single tail t tests were conducted to test the hypothesis that addition of Cp40 or mAb 3E7 would reduce C3b deposition on RBCs reacted with hematin in NHS. Significant differences between experimental samples versus controls are noted as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3. Results and discussion

3.1. Promotion and inhibition of C3b deposition mediated by hematin

Washed RBCs from donors 1 and 2 were reacted at 5% hematocrit in autologous 50% NHS with 400 µg/ml hematin, ± mAb 3E7 or varying amounts of Cp40. As previously reported, reaction with hematin for one or more treatment cycles mediates C3b deposition on RBCs, and mAb 3E7 blocks the reaction (Fig. 1A–B). There is some C3b deposition on cells reacted only in NHS, which is reduced to background in the presence of mAb 3E7. We now report that concentrations of Cp40 as low as 3 µM completely block hematin-mediated C3b deposition. Controls with an IgG1 isotype control in place of mAb 3E7, and a scrambled peptide in place of Cp40 (Fig. 1C–D, donors 3 and 2, respectively) additionally demonstrate the specific action of Cp40 and mAb 3E7, although we note that the scrambled peptide modestly reduced C3b deposition for donor 2. We previously reported that reaction of RBCs in NHS with hematin at concentrations as low as 150 µg/ml (corresponding to lysis of approximately 3% of circulating RBCs) promotes C3b deposition on the RBCs [10]. We conducted the present experiments at hematin concentrations of 400 µg/ml, thus setting a greater challenge for testing the capacity of Cp40 to block the hematin-mediated C3b deposition reaction.

Deposition of C3b on RBCs is very rapid, and if the hematin is added to the RBC-NHS mixture *first*, then the inhibitors (Cp40 or mAb 3E7) are only effective if added within the next 2 min (Fig. 1E–F, donors 1 and 2). The results illustrated in Fig. 1E–F are based on only one treatment cycle, indicating that the C3b deposition reaction is indeed quite potent.

3.2. More stringent tests for inhibition of C3b deposition

We next tested the efficacy of Cp40 in blocking hematin-mediated C3b deposition on RBCs under more stringent conditions, including

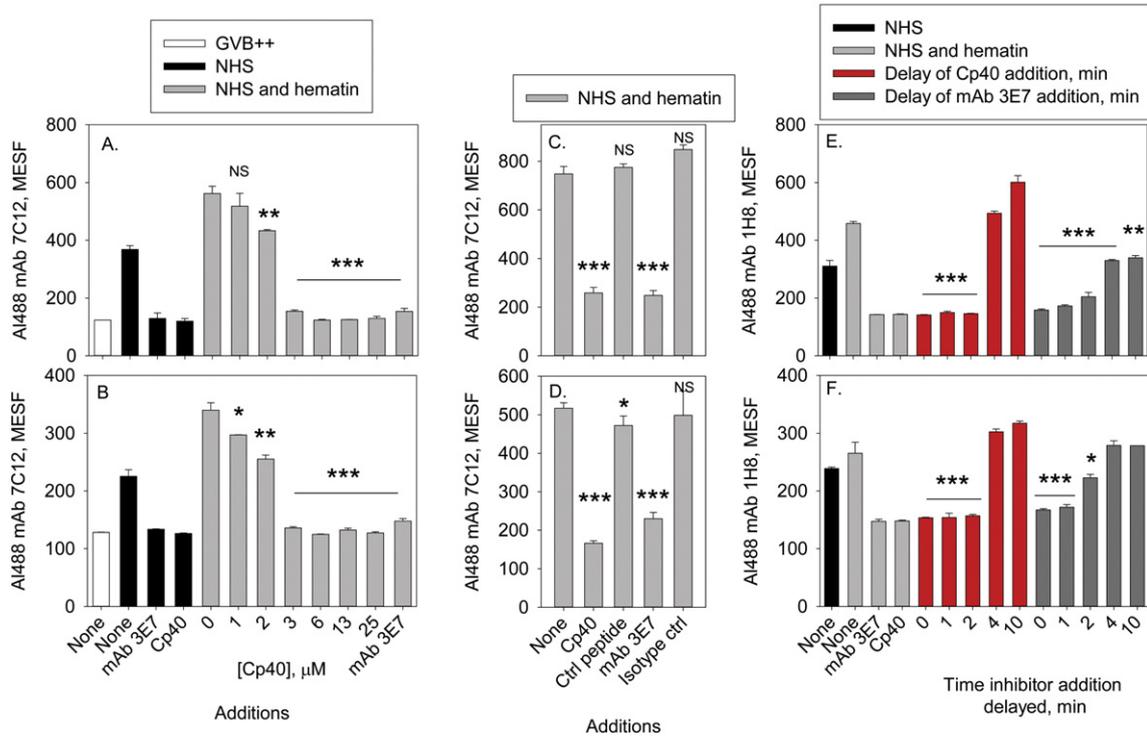


Fig. 1. In 50% NHS, hematin mediates deposition of C3b fragments on RBCs and both mAb 3E7 and Cp40 block this reaction. (A,B) Normal blood donors 1 and 2, 5% hematocrit, three cycles of reaction; cells reacted in gelatin veronal buffer (GVB++) define the background signal. Cp40 concentrations of 3 µM or more completely blocked C3b deposition. At lower concentrations of Cp40, C3b deposition is clearly evident. (C,D) For normal blood donors 3 and 2, controls demonstrate that a scrambled peptide (same amino acid composition) in place of Cp40 does not inhibit hematin-mediated C3b deposition, and similarly, an IgG1 isotype control in place of mAb 3E7 does not block C3b deposition. (E,F) For blood donors 1 and 2, a kinetic experiment indicates that treatment of samples with Cp40 or mAb 3E7 strongly inhibits C3b deposition if the reagents are added within 2 min after the hematin is added; one cycle of reaction, all at 5% hematocrit. Significant differences between treated samples compared to samples reacted with hematin alone are noted.

whole blood. RBCs (final hematocrit 5% or 50%) were reacted with hematin, in 50% autologous NHS, and we also conducted experiments in which hematin was added to complement replete whole blood samples anti-coagulated in lepirudin. The results, for blood donors 3 and 4, (Fig. 2A–D), based on developing with two different mAbs specific for C3 fragments, demonstrate that Cp40 effectively blocks C3b deposition under all three conditions examined.

3.3. Potential of compstatin in the treatment of malaria

Our findings, taken in context with numerous reports [5,6,8,11,12,15–17] may have important implications for possible use of Cp40 in treating malarial anemia. These reports all lead to the following generalized concept: RBC breakdown products, including hematin and DV, produced when Pf-infected RBCs are lysed, activate the APC. Complement activation under these conditions mediates opsonization of uninfected RBCs by C3b, which can decay to iC3b/C3dg. These opsonized RBCs are subject to extravascular clearance and destruction based on their recognition by fixed tissue macrophages which express complement receptors.

This paradigm can explain why ~20 uninfected RBCs are cleared for every Pf-infected lysed RBC [3,4,6,7]. Under normal homeostasis, safeguards neutralize dangerous RBC breakdown products [10]. Haptoglobin chelates and removes hemoglobin, and hemopexin safely disposes of heme. However, the large burden resulting from RBC lysis in malaria can saturate the clearance capacity of these agents, and the scenario we have described will occur. Therefore prophylactic Cp40 therapy, that is early treatment of malaria-infected individuals most likely to develop

severe anemia, could be quite effective because it would prevent deposition of C3 activation fragments on, and destruction of, naïve RBCs.

In a related model, Berg et al. reported that Cp40 abrogated complement activation mediated by both hemin and hemozoin, where the readout was generation of soluble C5b–C9, produced as a consequence of activation of the terminal phase of complement [13]. The investigators did not examine C3b deposition on RBCs, but their clinical observations and laboratory measurements revealed that circulating levels of soluble C5b–C9 were correlated with disease severity in malaria patients, thereby suggesting a role for complement in the pathogenesis of this disease.

3.4. Similarities of C3b deposition in paroxysmal nocturnal hemoglobinuria (PNH)

There is precedence for complement promoting clearance of C3 fragment-opsonized RBCs [18]. Recently Risitano et al. reported compelling data in PNH, indicating that extravascular clearance of C3 fragment-sensitized PNH RBCs plays an important role in RBC destruction in some patients who receive eculizumab treatment [19,20]. Due to low levels of complement control protein CD55, PNH RBCs are opsonized with C3 fragments, even though they are protected against intravascular lysis due to eculizumab inhibition of C5 cleavage. PNH RBCs opsonized with C3 fragments are thus subject to erythrophagocytosis by fixed tissue macrophages [20]. Cp40 blocks C3-fragment deposition on PNH RBCs, and is in clinical development for PNH and other indications [14,21].

If safety and efficacy of Cp40 can be demonstrated in the current trials, then it would be reasonable to investigate use of Cp40 for prevention or treatment of malarial anemia. Currently there is no viable and

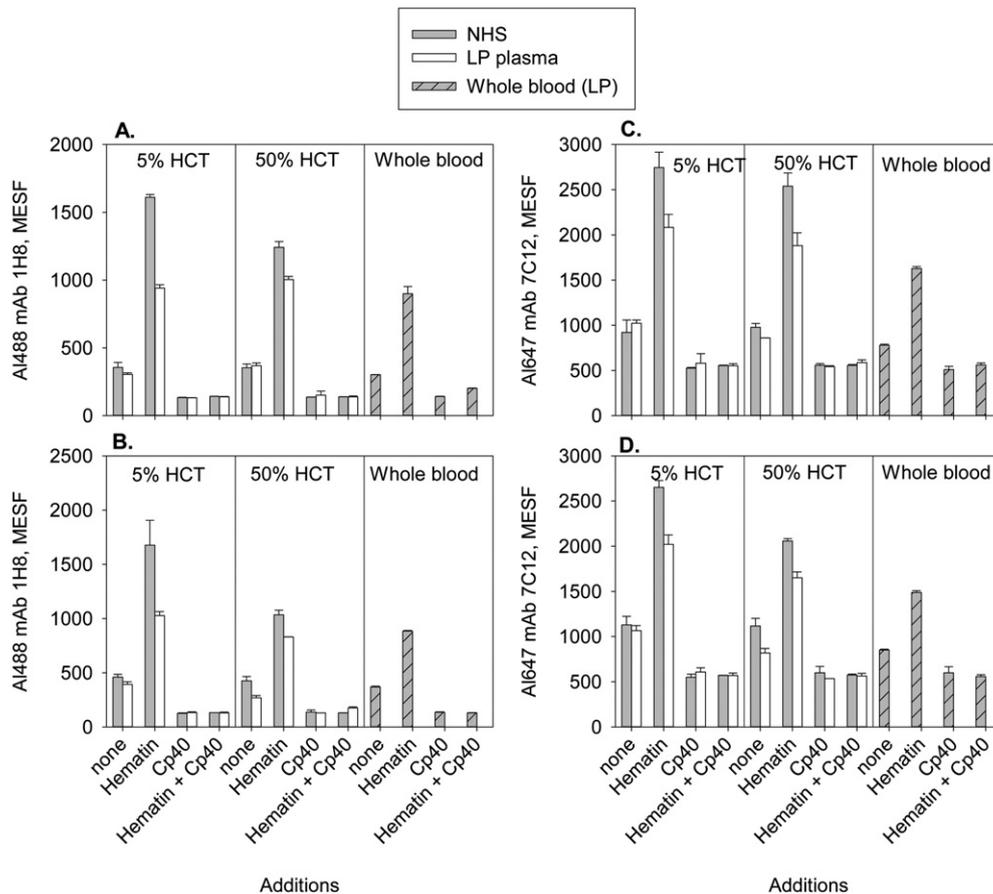


Fig. 2. Cp40 is equally effective in blocking hematin-mediated deposition of C3b on human RBCs in whole blood. RBCs were reacted in 50% NHS or in 50% lepirudin anti-coagulated plasma (LP plasma) at a hematocrit of 5% or 50%. Alternatively, the reaction was examined in whole blood anti-coagulated with lepirudin (whole blood (LP)). Three cycles. (A,C) Donor 3; (B,D) Donor 4. In all cases the differences between inhibitor-treated samples and samples reacted with hematin alone were statistically significant ($p < 0.01$ or $p < 0.001$) but symbols were omitted from the figures for clarity.

specific approach for treatment or prevention of severe malarial anemia. In the present context, malaria-infected individuals would have to receive Cp40 prophylactically before DV and free hematin flood the bloodstream. Therefore, a major challenge will be to identify patients most likely to benefit from Cp40 treatment. The recent study of Goncalves et al. [7] on 882 children in Tanzania delineated several factors (presence of placental malaria, high-transmission season, absence of sickle cell trait) that could be used to define groups of malaria-infected children most likely to benefit from prophylactic Cp40 treatment.

Finally, we speculate that Cp40 might also find use in sickle cell disease, a syndrome that similarly includes intravascular lysis of RBCs and complement activation, as well as chronic ischemia reperfusion injury, a distinct lesion that promotes complement activation [22–25].

Competing interest

J.D.L. and D.R. are inventors of patents and patent application describing complement inhibitors and their clinical use. J.D.L. is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors for therapeutic purposes.

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