RESEARCH ARTICLE

Testing the effect of PAR1 inhibitors on *Plasmodium falciparum*-induced loss of endothelial cell barrier function [version 1; peer review: awaiting peer review]

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Abstract

Background: Sequestration and cytoadherence of *Plasmodium falciparum*-infected erythrocytes (IE) to microvascular endothelium alters endothelial barrier function and plays a role in the pathogenesis of severe malaria. Binding of IE is mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) and the PfEMP1 variants that binds to endothelial protein C receptor (EPCR) have, in particular, been associated with the dysregulation of the coagulation/inflammation pathways in endothelial cells. This has prompted speculation about the role of protease-activated receptor-1 (PAR1) activation and signalling in causing endothelial activation and loss of barrier function in cerebral malaria.

Methods: We used a co-culture of primary human brain microvascular endothelial cells (HBMEC) with *P. falciparum* material, recombinant PfEMP1 or lysates from IE, and measured barrier function by transendothelial electrical resistance (TEER). A selection of PAR1 inhibitors was tested for their ability to reverse the and thrombin induced decrease in barrier function.

Results: An initial screen in the presence of recombinant PfEMP1 identified a few inhibitors that were able to reduce the rapid thrombin-induced barrier disruption even when activated protein C (aPC) was unable to do so. However, in the IE lysate co-culture system we identified a mechanism that slowly reduces barrier function and which is insensitive to PAR1 inhibitors.

Conclusions: The selected PAR1 inhibitors were able to reverse the disruption of barrier function by thrombin but did not reverse the IE lysate induced disruption of barrier function, implicating a different PAR1-independent mechanism. These findings have implications for the design of adjunct therapies to reduce brain swelling in cerebral malaria.
Keywords
Plasmodium falciparum, cerebral malaria, PAR1, thrombin, endothelium, barrier function

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Introduction

The pathology of cerebral malaria (CM) is not fully understood but is associated with sequestration of *P. falciparum*-infected erythrocytes (IE) and involves interactions between IE and host endothelial cells (EC) as well as host inflammation (for a review see (Wassmer & Grau, 2017)). In a clinical study in Malawi, brain swelling was found to be strongly associated with fatal outcome in CM (Seydel et al., 2015), which has subsequently been confirmed by studies in India (Mohanty et al., 2017). The aetiology of this swelling is probably multifactorial, involving cytopathic and vasogenic mechanisms (e.g. local and systemic inflammation; endothelial cell death; IE and erythrocyte accumulation). Multiple lines of work indicate involvement of the protein C (PC)–protease-activated receptor-1 (PAR1) axis and the consequences of the loss or inactivation of endothelial protein C receptor (EPCR) (for a review see (Mohan Rao et al., 2014)) (Kessler et al., 2017). PAR1 is a seven-transmembrane G protein-coupled receptor and is the prototypical thrombin receptor. It contains its own tethered ligand and exhibits biased antagonism. Cleavage events at two different sites lead to opposing effects: when cleaved at amino acid R41 by thrombin, PAR1 is pro-inflammatory and barrier disrupting whereas non-canonical cleavage of PAR1 at amino acid R46 by activated PC (aPC) has a barrier stabilising and anti-inflammatory effect, significantly reducing the inflammatory and barrier disruptive effect of thrombin on endothelial cells. Non-canonical cleavage of PAR1 by aPC is dependent on aPC binding to the active site of EPCR (Figure 1). This dysregulation of the coagulation system does not often result in clinically evident thrombosis or bleeding in children and some researchers in this field have stressed the distinction between cytopathic and coagulant effects in this pathway (Mosnier et al., 2007). However, laboratory measurements of coagulation factors do show coagulation activation that is associated with fatal outcome in cerebral malaria (CM) (Moxon et al., 2015).

Two mechanisms for the action of cytoadherence on coagulation/inflammation dysregulation have been proposed (Figure 1). The direct binding of the *P. falciparum* variant surface protein *P. falciparum* erythrocyte membrane protein 1 (PIEMP1) to EPCR blocks the conversion of PC to aPC, affecting the ability of this host control system to control PAR1 cleavage by thrombin and altering the PAR1 signalling pathway (Avril et al., 2019; Gillrie et al., 2015; Kessler et al., 2017; Mosnier & Lavstsen, 2016; Sampath et al., 2015). This mechanism is dependent on the presence of EPCR-binding parasite variants, and these PIEMP1 types are strongly associated with severe malaria (Mkumbaye et al., 2017; Storm et al., 2019). However, the same effect can also be achieved through the reduction of expression of EPCR on the endothelial surface, which is thought to be expressed at lower levels in the brain microvasculature. We (Moxon et al., 2013) have shown that this does occur and is associated with CM, and while linked to IE sequestration, this appears to be due to receptor shedding rather than steric inhibition and does not require cytoadherence via EPCR (Moxon et al., 2013). Either mechanism would disrupt EPCR function and its capacity to form a complex with aPC and modify PAR1 signalling; thus leading to an unmodified effect of

Figure 1. Schematic diagram of the proposed mechanisms of endothelial dysregulation via *Plasmodium falciparum* cytoadherence.

Under normal circumstances, the presence of endothelial protein C receptor (EPCR)-activated protein C (aPC) on the endothelial surface is able to modulate the protease-activated receptor 1 (PAR1) response (by cleavage of R46) to thrombin along a cytoprotective pathway. In the presence of IE, the binding site for aPC can be occupied by *P. falciparum*-infected erythrocyte membrane protein 1 (PIEMP1) or EPCR expression can be reduced, both limiting the availability of EPCR-aPC and resulting in cytopathic responses due to unmodified thrombin cleavage (R41) of PAR1. *P. falciparum*-infected erythrocytes (IE) are also able to release soluble factors that can influence endothelial integrity directly.

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Figure 1: Schematic diagram of the proposed mechanisms of endothelial dysregulation via *Plasmodium falciparum* cytoadherence.
thrombin with pro-inflammatory changes and loss of EC barrier function. Because the dysfunction of EPCR is at the level of the receptor, these cannot be mitigated through the addition of aPC (Figure 2A).

Our hypothesis is that, through both of these mechanisms, the Thrombin-PAR1 coagulation/inflammation axis plays a significant role in barrier loss in CM, and that adjunct treatments targeting this might alleviate mortality or post-CM neurological sequelae in CM. Since the activation of PC may be prevented by either of these mechanisms of EPCR disruption (steric inhibition or receptor cleavage) it is important to identify treatments that could be barrier stabilising to EPCR abrogation by either mechanism. We therefore investigated a range of PAR1 antagonists that are able to directly inhibit thrombin cleavage of the PAR1 extracellular domain without affecting other thrombin-dependent pathways. We tested their ability in preventing loss of barrier function in human endothelial cells in response to treatment with parasite material.

Methods

Culture of endothelial cells and Plasmodium falciparum
The human umbilical vein endothelial cell line EA.hy926 (ATCC) was cultured in DMEM medium supplemented with 10%
foetal calf serum. Primary human brain microvascular endothelial cells (HBMEC, Cell Systems) were cultured in Endothelial Cell Growth Medium 2 containing 2% foetal calf serum, 5 ng/ml epidermal growth factor, 10 ng/ml basic fibroblast growth factor, 20 ng/ml insulin-like growth factor, 0.5 ng/ml vascular endothelial growth factor 165, 1 µg/ml ascorbic acid, 0.2 µg/ml hydrocortisone and 22.5 µg/ml heparin (EGM2, Promocell). The Plasmodium falciparum IT4 lab strains IT4var16 (I6G), IT4var14 (A4) and IT4var37 (4E12) were cultured according to our standard laboratory methods (Wu et al., 2018) in complete RPMI medium (RPMI 1640 with 25 mM HEPES, 11 mM glucose, 2 mM glutamine, 0.2% NaHCO₃, 25 mg/l gentamicin and 10% Human Serum, pH 7.4) in normal group O red blood cells (RBC) at 3% haematocrit.

rPfEMP1 preparation and PAR1 antagonists

Recombinant EPCR-binding PfEMP1 (rPfEMP1) was produced as previously described (Turner et al., 2015). In short, HIS-tagged cysteine-rich interdomain region domain α1 (CDRα1) protein derived from the IT4var20 PfEMP1 sequence was produced in the baculo-virus expression system, purified by nickel affinity chromatography and validated for binding to EPCR in ELISA. PAR1 antagonists, supplied by Eisai Co. Ltd. (Japan), and listed in Table 1, or commercially sourced from Sigma (Varopaxar), were dissolved at 10 mM in DMSO and stored at -20°C.

Preparation of parasite lysates

Lysates of RBC and mid-trophozoite stage IE were prepared by freeze-thawing three times a suspension of 1% haematocrit or a suspension of 50% parasitaemia at 1% haematocrit, respectively, in EGM2-min medium (EGM2 without hydrocortisone and heparin). Enrichment of the IE by gelatine flotation was used to obtain 50% parasitaemia.

Trans endothelial electrical resistance (TEER) analysis

Barrier function was measured by TEER with the iCELLigence™ system (ACEA Biosciences). EA.hy926 cells and HBMEC (up to passage 9) were seeded in 8-well E plates (L8) and TEER was recorded at specific time intervals and expressed as cell index. For the initial inhibitor screen, EAhy926 cells were grown overnight and when confluent, the culture medium was exchanged for serum-free DMEM medium and combinations of 25 nM rPfEMP1, 57 nM aPC or PAR1 inhibitors (see Table 1 for concentrations) or vehicle were added 2 hours prior to treatment with 2 nM thrombin (see Figure 2 for details, n=3). For subsequent experiments with HBMEC, the overnight culture medium was first replaced with EGM2 medium and then with EGM2-min medium 2 hours prior to the addition of lysates in the absence or presence of selected PAR1 inhibitors (n=5). After ~20 hours, the additional effect of 2 nM thrombin was determined (n=2). Control experiments were performed with medium or inhibitor only. The cell index of medium only was set as baseline to correct for non-specific fluctuations of cell index during the experiment.

The decrease in barrier function by modulators was determined by normalising the cell index at the time point immediately prior to modulator addition. The maximum decrease in normalised cell index (NCI) by thrombin was determined for medium or lysate in the absence of inhibitor and set at 100%. The protective effect of inhibitor on the thrombin-induced disruption of barrier function calculated as follows:

\[ \text{ΔNCI (thrombin + inhibitor)} / \text{ΔNCI (thrombin – inhibitor)} \times 100. \]

For lysates, the normalised cell index was determined at 2 and 16 hours after adding the IT4var16 lysate. The decrease in normalised cell index was calculated and ΔNCI in absence of inhibitor was set at 100%. The effect of inhibitor on the lysate-induced decrease in barrier function was calculated as follows:

\[ \text{ΔNCI (lysat + inhibitor)} / \text{ΔNCI (lysat – inhibitor)} \times 100. \]

Measuring EPCR expression on endothelial cells

The effect of IT4var14 and IT4var37 IE or IE lysate on the EPCR expression on HBMEC was determined after 16 - 20 hours of co-culture. A suspension of 50% parasitaemia at 1% haematocrit in EGM2-min medium was prepared and from this suspension lysate was also made, as described above. HBMEC were detached with Accutase®, washed with cold PBS/1% BSA/2 mM EDTA, labelled with phycoerythrin conjugated rat anti-human EPCR antibody (Biolegend, 351904), washed and stained for cell viability with live/dead fixable yellow stain (Invitrogen). EPCR expression levels of viable cells were detected by flow cytometry; 10 ng/ml TNF was used as a positive control, since it decreases EPCR expression.

Statistical analysis

Statistical significance was calculated by ANOVA with Dunnett’s post-test comparing the mean value (± SD) for each individual modulator addition (inhibitor, IE, lysate or TNF) with the mean value (± SD) of the condition without modulator (GraphPad Prism version 5). A P-value < 0.05 was deemed significant.
Results
Thrombin-induced barrier disruption and the effect of prior treatment with either PAR1 inhibitors or aPC in the presence of rPfEMP1
In the initial screen we investigated whether the effect of EPCR binding by PfEMP1 on thrombin-mediated barrier disruption of the human umbilical vein endothelial cell line EA.hy926 could be modified by PAR1 inhibitors. The loss of barrier function caused by the addition of thrombin was restored by the prior addition of aPC in the absence of rPfEMP1. However, aPC had no effect on preventing loss of barrier function when rPfEMP1 was bound to EPCR (see condition rPfEMP1-aPC-thrombin in Figure 2) blocking aPC-mediated barrier strengthening. Thus, aPC might not be a useful treatment when EPCR-binding IE are present, such as in severe malaria. However, normal barrier function was obtained with the addition of inhibitors 1, 5 and 6 (Figure 2), suggesting that they are able to act to stabilise EC barrier function despite the loss of protective aPC-mediated PAR1 cleavage. Raw data for Figure 2, in addition to that for Figure 3–Figure 6, are available as Underlying data (Storm et al., 2020).

Developing an endothelial cell barrier model using IE lysates
Based on these findings we identified a subset of the Eisai compounds that were able to inhibit the action of thrombin on barrier function in the presence of rPfEMP1 and aPC. Next we used a co-culture system with primary HBMEC and IE. The work focussed on three Eisai compounds: E5555 (Atopaxar (Serebruany et al., 2009)), #04 (inhibitor 5) and #91 (inhibitor 1). E5555 was included as it has been through Phase 1 and Phase 2 clinical testing. In addition, we used the clinically licenced PAR1 inhibitor Vorapaxar (brand name Zontivity (Aralez Pharmaceuticals)), obtained as a non-clinical compound from Sigma-Aldrich.

The effect of PfEMP1 ligation to EPCR on barrier function has been described elsewhere (Gillrie et al., 2015; Kessler et al., 2017), but there has been little work on the impact of EPCR removal from the endothelial surface during cytoadherence. To address this, we focussed on EPCR abrogation in our co-culture model and first measured the EPCR expression on HBMEC after overnight co-culture with IE or IE-lysate (Figure 3). Lysates had a more pronounced effect on EPCR expression to whole IE and were easier to prepare and more reproducible in TEER experiments. We therefore switched to a HBMEC/IE lysate co-culture system for subsequent experiments.

We observed that there were two ‘behaviours’ taking place in the TEER system (Figure 4):

A. A rapid response to thrombin in reducing EC barrier function transiently, which could be controlled under normal conditions using aPC (Figure 4A).

B. A slower reduction in barrier function, activated by treatment with IE lysate and producing a cumulative effect over 30 hours (Figure 4B) that was not seen in control wells with RBC lysate where barrier function remained constant throughout the incubation period. IE had less of an effect on barrier function than IE lysate. The fluctuations in cell index in the first 2 hours are consistent in each experiment, probably due to the addition of cells or cellular material.

Figure 3. Endothelial protein C receptor (EPCR) expression on human brain microvascular endothelial cells after overnight culturing with P. falciparum-infected erythrocytes (IE) or IE lysate. EPCR expression was determined by flow cytometry after overnight culturing with IT4var14 or IT4var37 IE or IE lysate (n=2). EPCR expression on cells with medium only was set at 100%. Shown is mean ± SD of the combined IT4var14 and IT4var37 data (n=4) and the tumour necrosis factor (TNF) data (n=2) with * indicating a P-value <0.05.
Figure 4. Measuring endothelial barrier function of human brain microvascular endothelial cells (HBMEC) using trans endothelial electrical resistance. (A) Cell Index traces for thrombin induced decrease in barrier function in HBMEC. Cell index was normalised at the time point immediately prior to the addition of 2 nM thrombin, indicated by the black triangle, and medium (black line) was set as baseline. Shown are the effect of 2 nM thrombin (red) on barrier function and the protection by 10 nM activated protein C (aPC) (blue) added 1 hour prior to thrombin. (B) Decrease in barrier function by IE, RBC and their lysates. Cell index of HBMEC was monitored and normalised at the time point immediately prior to the addition of cells or lysates, indicated by the black triangle at 26 hours. Medium only was set as baseline (black line). Normalised cell index is shown for 30 hours for red blood cells (RBC) (purple), RBC lysate (magenta), P. falciparum-infected erythrocytes (IE) (dark green) and IE lysate (light green). The decrease in normalised cell index was measured between 2 and 16 hours after addition of cells or lysate.
Behaviour A was as expected and confirms that aPC rescues thrombin induced barrier disruption in HBMEC as seen above in EAhy926 cells. Behaviour B has been reported previously by several investigators using a range of co-culture systems (Tripathi et al., 2007) (Gillrie et al., 2012), with either IE or parasite material affecting barrier integrity. Based on these findings we have tested the compounds for their ability to block both behaviours.

Effect of PAR1 inhibitors on thrombin-induced and lysate-induced reduction in EC barrier function

Compounds were tested for their ability to inhibit disruption of barrier function by thrombin and the slower effect caused by the IE lysate directly. All tested PAR1 inhibitors were able to significantly reverse thrombin-induced barrier disruption even in the presence of IE lysate (Figure 5). However, three of the four inhibitors had no significant effect on the lysate-induced decrease in barrier function when used at 0.3 µM (Figure 6) or at 0.6 µM (see Underlying data; Storm et al., 2020). Only E5555 significantly reverses the decrease in barrier function by IE lysate (−79.9 ± 16.5%, P = 0.026), albeit only by 20%. This still leaves a substantial EC barrier disruption by IE lysates, indicating that this phenomenon is independent of PAR1.

Discussion

There is considerable interest in developing adjunct therapies for CM based on controlling brain swelling, following the MRI finding of a strong association between this phenotype
and death from CM (Mohanty et al., 2017; Seydel et al., 2015) and that brain swelling has also been associated with EPCR-binding IE phenotype (Kessler et al., 2017). As expected, the PAR1 inhibitors (including Vorapaxar) were able to block the effect of thrombin on transient reduction in barrier function, including in the presence of IE lysate. However, they had no significant effect on the slower reduction in barrier function caused by the lysate itself (thrombin independent).

Blocking of the thrombin-mediated pathway with the PAR1 antagonists might reduce brain swelling sufficiently to relieve mortality/morbidity in CM, but whether these types of inhibitors could be used safely in children/adults with multiple ring haemorrhages in their brains is a concern given the potential bleeding risk, owing to their inhibition of PAR1 in platelets (https://www.rxlist.com/zontivity-side-effects-drug-center.htm). Further consideration of risk/benefit calculations will be required to answer this.

The slower effect on brain barrier function by IE lysate requires further investigation. Other work has implicated a role for β-catenin in regulating brain barrier integrity (Gallego-Delgado et al., 2016), showing that IE-derived soluble mediators are able to cause a reduction in TEER of brain endothelial cells over several hours. Similarly, parasite kinins were also shown to reduced brain endothelial cell barrier function, as well as enhancing IE cytoadherence (Silva et al., 2019), and HRP2 has been implicated in modifying barrier function (Pal et al., 2016). Parasite histones have also been shown to cause barrier disruption on lung and brain endothelial cells and to be released in culture by IE and in vivo in patients with CM (Gillrie et al., 2012) and are increased in children with CM and associated with blood brain barrier breakdown and brain swelling (Moxon et al., 2019). For histones, there are some therapeutic avenues that could be explored. Non-anticoagulant heparins prevent histone-induced lethality in bacterial sepsis models in vivo (Wildhagen et al., 2014) and block the toxic effects of plasmodial histones in vitro. A non-anticoagulant heparin, Sevuparin, is being used in phase II trials for malaria for its potential to block parasite binding (Leitgeb et al., 2017; Saiwaew et al., 2017). Based on the work in this paper, an option would be to consider a combination therapy, based on stopping the thrombin-mediated effect using a PAR1 inhibitor combined with a non-anti-coagulant heparin to mitigate the histone effect. Interestingly, the two pathways are not completely separate, as aPC cleaves histones to regulate histone concentration in the vascular compartment (Gillrie et al., 2012; Xu et al., 2009) and histones can reduce aPC production (Kowalska et al., 2014).

In summary, we have shown that PAR1 inhibitors are able to reduce thrombin-induced barrier disruption even when the normal aPC control pathway has been disabled due to direct EPCR engagement with PfEMP1 or by reduced EPCR expression on EC induced by parasite lysates. The identification of a second pathway for barrier disruption induced by parasite lysate that is insensitive to PAR1 inhibitor treatment suggests that a more complex approach to adjunct therapy may be needed.

Data availability

Underlying data

Figshare: Data acquired from testing the effect of PAR1 inhibitors on Plasmodium falciparum-induced loss of endothelial cell barrier function. https://doi.org/10.6084/m9.figshare.11558889 (Storm et al., 2020).

This project contains the following underlying data:

- Combined analysis data for fig 2_TEER_includes normalisation.csv (normalised data used to generate Figure 2).
- Combined analysis data for fig5_TEER_includes normalisation.csv (normalised data used to generate Figure 5).
- Combined analysis data for fig6_TEER_includes normalisation.csv (normalised data used to generate Figure 6).
- Data for fig2A+B+C_TEER_EA cells+inhibitors+thrombin_3.csv.
- Data for fig2C_TEER_EA cells+inhibitors+thrombin_1.csv.
- Data for fig2C_TEER_EA cells+inhibitors+thrombin_2.csv.
- Data for fig3_flow cytometry HBMEC.csv.
- Data for fig4A_TEER_aPC+thrombin_all time points shown.csv.
- Data for fig4B_TEER_RBC+IE+lysate_all time points shown.csv.
- Data for fig5+6_TEER_lysate+thrombin_#04-2+#91-2.csv.
- Data for fig5+6_TEER_lysate+thrombin_#91-1.csv.
- Data for fig5+6_TEER_lysate+thrombin_E5555-14#04-1.csv.
- Data for fig5+6_TEER_lysate+thrombin_E5555-2.csv
- Data for fig5+6_TEER_lysate+thrombin_Vorapaxar-1.csv.
- Data for fig5+6_TEER_lysate+thrombin_Vorapaxar-2.csv.
- Data for fig6_TEER_lysate_Vorapaxar+E5555+04+#91-1.csv.
- Data for fig6_TEER_lysate_Vorapaxar+E5555+04+#91-2.csv.
- Data for fig6_TEER_lysate_Vorapaxar+E5555+04+#91-3.csv.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).
References


