



RESEARCH ARTICLE

REVISED **Testing the effect of PAR1 inhibitors on *Plasmodium falciparum*-induced loss of endothelial cell barrier function**
 [version 3; peer review: 2 approved]

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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 trans endothelial electrical resistance (TEER). A selection of PAR1 inhibitors was tested for their ability to reverse the *P. falciparum* 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, PAR1 inhibitors did not rescue the barrier dysfunction after co-culture with IE lysate.

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.

Open Peer Review

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Any reports and responses or comments on the article can be found at the end of the article.

Keywords

Plasmodium falciparum, cerebral malaria, PAR1, thrombin, endothelium, barrier function



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REVISED Amendments from Version 2

The comment from Prof. Rodriguez on version 2 of the manuscript was noted. In the methods section describing the preparation of IE and IE lysates, a few additional sentences were included to describe the amount of IE suspension or lysate added in the experiments.

Any further responses from the reviewers can be found at the end of the article

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)). 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 (PfEMP1) 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 PfEMP1 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

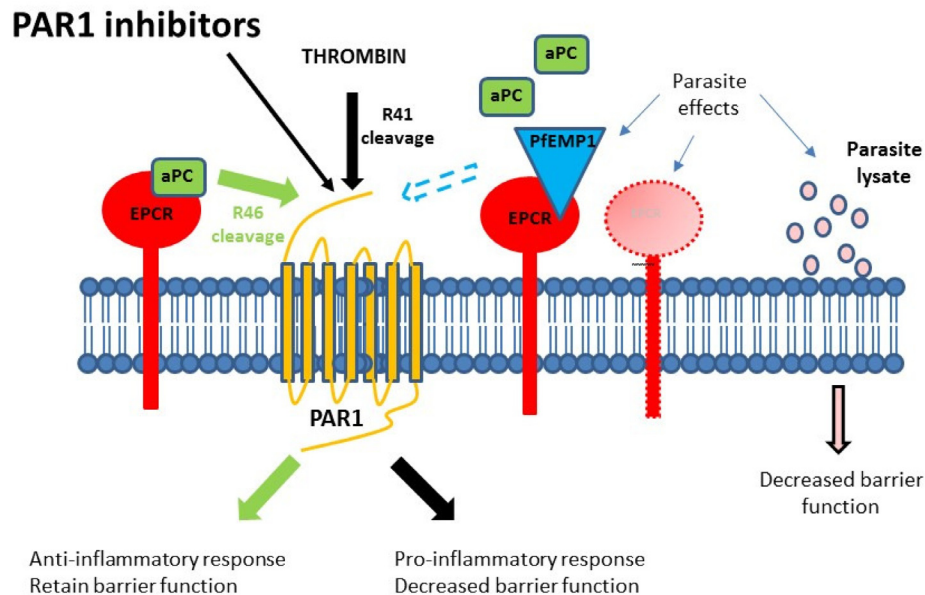


Figure 1. Schematic diagram of the proposed mechanisms of endothelial dysregulation via *Plasmodium falciparum* cytoadherence. Under normal circumstances, the presence of endothelial protein C receptor (EPCR)-activated protein C (aPC) on the endothelial surface is able to modulate the protease-activated 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* erythrocyte membrane protein 1 (PfEMP1) 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.

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 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).

On embarking on this study, our hypothesis was 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

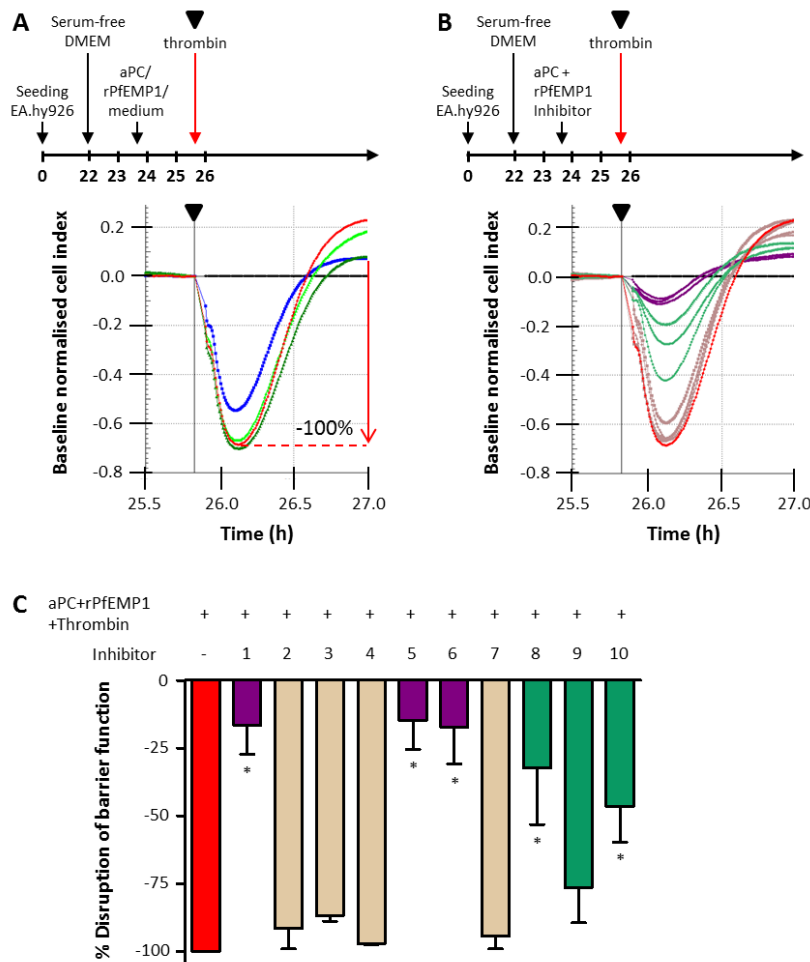


Figure 2. Measuring effect of protease-activated receptor 1 (PAR1) inhibitors on endothelial barrier function of EA.hy926 cells using trans endothelial electrical resistance. (A) Experimental timeline and cell index traces for thrombin induced decrease in barrier function in EA.hy926 cells. Schematic of the experimental timeline (not to scale) indicating the addition of medium and modulators to the cells. Cell index was normalised at the time point immediately prior to the addition of 2 nM thrombin, indicated by the black triangle in the timeline and cell index trace, and medium (black line) was set as baseline. The decrease in normalised cell index by thrombin (red) was set at 100% and the effect of 57 nM aPC (blue), 25 nM recombinant *P. falciparum* erythrocyte membrane protein 1 (rPfEMP1) (bright green) and 57 nM activated protein C (aPC) and 25 nM rPfEMP1 combined (dark green) on thrombin induced decrease in barrier function was determined. (B) The effect of inhibitors on thrombin induced decrease in barrier function. Inhibitors (concentrations in Table 1) were tested in the presence of 57 nM aPC and 25 nM rPfEMP1. The inhibitors in light brown do not have an effect, the inhibitors in light green have an intermediate effect and the inhibitors in purple reverse the effect of thrombin. (C) Graph of the data depicted in B with corresponding colours. Thrombin induced decrease in barrier function was set as 100% (red) and shown are the mean \pm SD of 3 independent experiments for the 10 inhibitors. *denotes a P-value <0.05 .

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 (ItG), 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 (CIDRα1) 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 in aliquots at -20°C.

Preparation of IE and IE lysates

Mid-late trophozoite stage IE were enriched by gelatin flotation and a suspension of 50% parasitaemia at 1% haematocrit

in EGM2-min medium (EGM2 without hydrocortisone and heparin) was prepared. RBC were cultured overnight in complete RPMI medium and subjected to the same procedure as IE to obtain a suspension of 1% haematocrit.

Lysates of these IE and RBC suspensions were prepared by freeze-thawing three times. In TEER experiments, 300 µl IE suspension or lysate (1.5 × 10⁷ IE) was added per well (0.64 cm²) in the E plate. In experiments to determine EPCR expression, 350 µl IE suspension or lysate (1.75 × 10⁷ IE) was added per well (1.9 cm²) in a 24 well plate.

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 at 50,000 cells/cm² in 8-well E plates (L8, surface area 0.64 cm²) 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 for 3 – 6 hours and then with EGM2-min medium 2 hours prior to the addition of 300 µl IE suspension (1.5 × 10⁷ IE per well) or 300 µl 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:

$$\Delta\text{NCI (thrombin + inhibitor)} / \Delta\text{NCI (thrombin - inhibitor)} \times 100.$$

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:

$$\Delta\text{NCI (lysate + inhibitor)} / \Delta\text{NCI (lysate - inhibitor)} \times 100.$$

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

Table 1. Names of protease-activated receptor 1 inhibitors supplied by Eisai and concentrations used in the initial barrier disruption screen.

Inhibitor	Compound name	Conc. used
1	#91	0.1 µM
2	#33	2.5 µM
3	#01	2.0 µM
4	#62	5.0 µM
5	#04	0.1 µM
6	#39	0.5 µM
7	#38	1.0 µM
8	#11	0.1 µM
9	#96	1.0 µM
10	#90	0.5 µM

suspension lysate was also made, as described above. RBC and RBC lysate were used as control and 10 ng/ml TNF was used as a positive control, since it decreases EPCR expression. 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.

Statistical analysis

Statistical significance was calculated by ANOVA with Dunnett's post-test comparing the mean value (\pm SD) for each individual modulator addition (inhibitor, IE, lysate or TNF) with the mean value (\pm 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; note that neither aPC nor rPfEMP1 treatment alone had significant effects on EC barrier func-

tion) 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 RBC, RBC lysate, IE or IE-lysate (Figure 3). RBC and RBC lysate did not signifi-

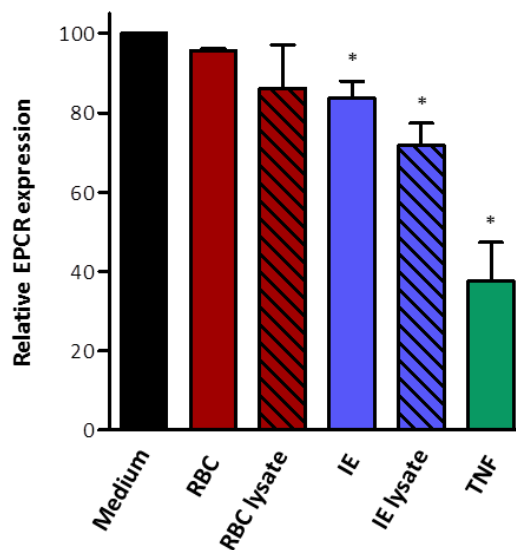


Figure 3. Endothelial protein C receptor (EPCR) expression on human brain microvascular endothelial cells after overnight culturing with *P. falciparum*-infected erythrocytes (IE) or IE lysate. EPCR expression was determined by flow cytometry after overnight culturing with IT4var14 or IT4var37 IE or IE lysate ($n=2$). Red blood cells (RBC), RBC lysate and tumour necrosis factor (TNF) were included as control. EPCR expression on cells with medium only was set at 100%. Shown is mean \pm SD of the combined IT4var14 and IT4var37 data ($n=4$), RBC data ($n=2$) and the TNF data ($n=2$) with * indicating a P-value < 0.05 .

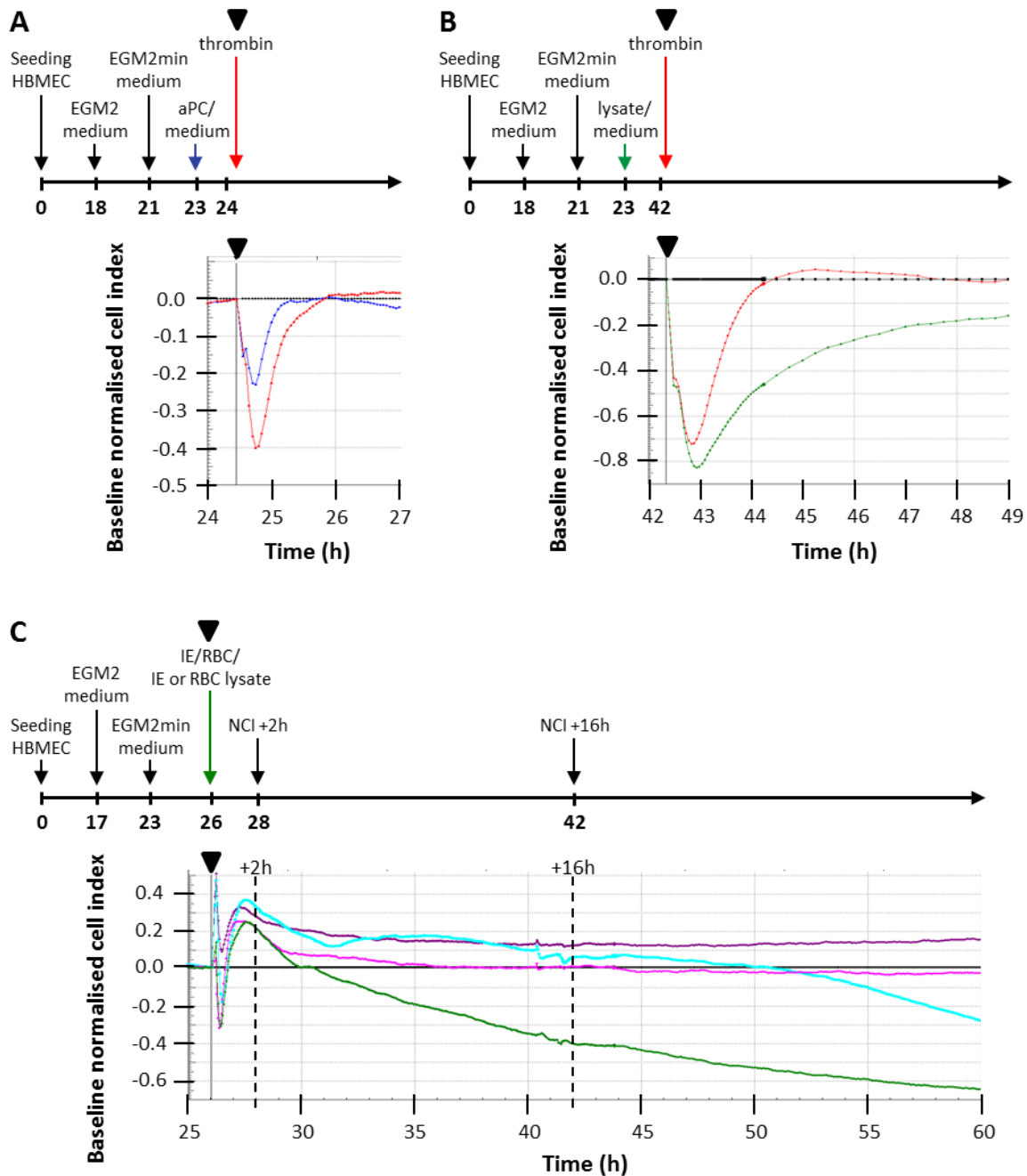


Figure 4. Measuring endothelial barrier function of human brain microvascular endothelial cells (HBMEC) using trans endothelial electrical resistance. (A) Experimental timeline and cell index traces for thrombin induced decrease in barrier function in HBMEC. Schematic of the experimental timeline (not to scale) indicating the addition of medium and modulators to HBMEC. Cell index was normalised at the time point immediately prior to the addition of 2 nM thrombin, indicated by the black triangle in the timeline and cell index trace, 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) Schematic of the experimental timeline and cell index traces for thrombin induced decrease of barrier function after lysate exposure and its recovery over 7 hours in HBMEC. Cell index was normalised at the time point immediately prior to the addition of 2 nM thrombin, indicated by the black triangle in the timeline and cell index trace, and medium (black line) was set as baseline. Shown are the effects of 2 nM thrombin on barrier function of cells in medium (red) and cells exposed to *P. falciparum* infected erythrocytes (IE) lysate for 18.5 hours (green). (C) 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 in the timeline and cell index trace. Medium only was set as baseline (black line). Normalised cell index is shown for 35 hours for red blood cells (RBC) (purple), RBC lysate (magenta), IE (cyan) and IE lysate (green). The decrease in normalised cell index was measured between 2 and 16 hours after addition of cells or lysate.

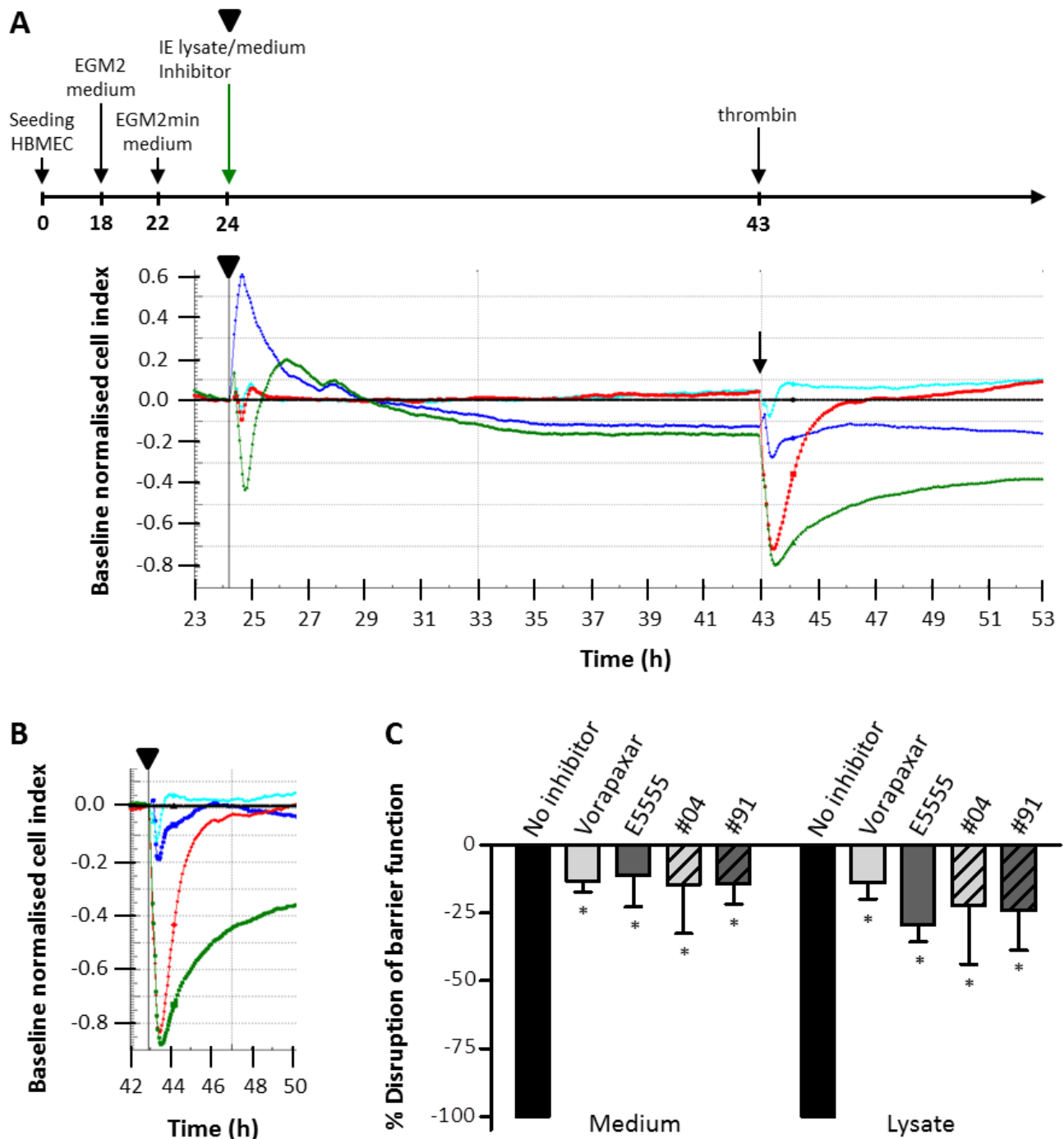


Figure 5. Effect of inhibitors on thrombin-induced decrease in barrier function of human brain microvascular endothelial cells (HBMEC). (A) Experimental timeline and representative cell index traces for *P. falciparum* infected erythrocyte (IE) lysate induced decrease in barrier function in HBMEC and the effect of 0.3 μ M Vorapaxar. Schematic of the experimental timeline (not to scale) indicating the addition of IE lysate and Vorapaxar. Cell index traces are shown for IE lysate in the absence (green) and presence of Vorapaxar (blue) and medium in the absence (red) and presence of Vorapaxar (cyan). Cell index was normalised at the time point immediately prior to the addition of IE lysate, indicated by the black triangle in the timeline and cell index trace, and medium (black line) was set as baseline. After 19 hours 2 nM thrombin was added as indicated by the arrow. (B) The same cell index traces as in A, but normalised at the time point prior to the addition of thrombin (black triangle). Vorapaxar (blue and cyan) reverses the effect of thrombin. (C) Graph of the inhibitor data analysed as depicted in B. The maximum decrease in normalised cell index by thrombin was determined for medium or lysate in the absence of inhibitor and set at 100%. Shown are the mean \pm SD of 2 independent experiments, with * indicating a P-value <0.05 compared to no inhibitor.

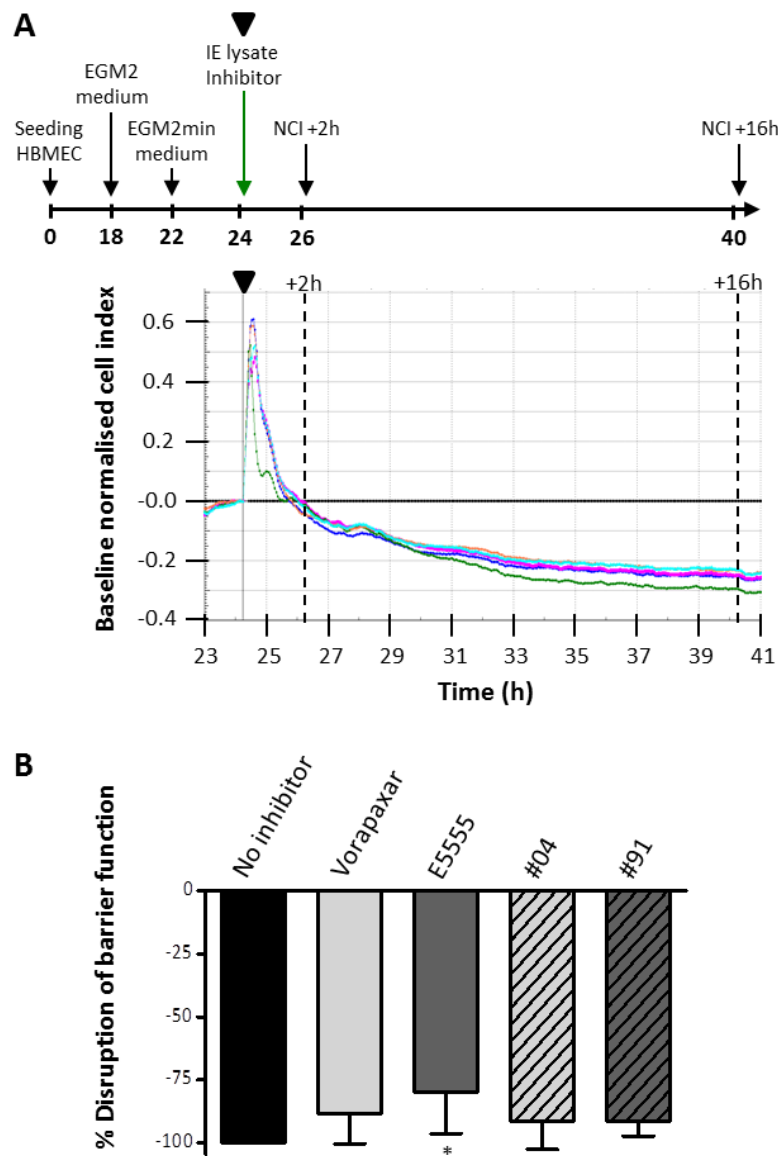


Figure 6. Effect of inhibitors on lysate-induced decrease in barrier function of human brain microvascular endothelial cells (HBMEC).

(A) Experimental timeline and cell index traces for *P. falciparum* infected erythrocyte (IE) lysate induced decrease in barrier function in HBMEC and the effect of the four inhibitors (0.3 μ M). Schematic of the experimental timeline (not to scale) indicating the addition of IE lysate in the absence and presence of inhibitor to the cells. Cell index was normalised at the time point immediately prior to the addition of IE lysate, indicated by the black triangle in the timeline and cell index trace, and medium (black line) was set as baseline. The normalised cell index was determined at 2 and 16 hours after adding the lysate in the absence of inhibitor (green) or in the presence of Vorapaxar (blue), E5555 (magenta), #04 (cyan) or #91 (coral). (B) Graph of the data depicted in A. The decrease in normalised cell index between 2 and 16 hours was calculated and this Δ normalised cell index in absence of inhibitor was set at 100%. Shown is the mean \pm SD of 5 independent experiments with * indicating a P-value <0.05 compared to no inhibitor.

cantly reduce the EPCR expression and IE lysates had a more pronounced effect on EPCR expression compared to whole IE. Lysates 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 4C) 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. After exposure to IE lysate, barrier function could still be further decreased by thrombin, but recovery was slow and in some cases barrier integrity was not restored to the level prior to thrombin addition (Figure 4B).

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) (Avril *et al.*, 2019), with either IE or parasite material affecting barrier integrity. The delayed recovery after thrombin addition was also reported by Avril *et al.* when using schizont-stage IE in their TEER experiments (Avril *et al.*, 2019). 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 ($-79.9 \pm 16.5\%$, $P = 0.026$), 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 reduce 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). Despite the lysate induced decrease in barrier function, thrombin could still reduce the barrier function even further (Figure 4B and Figure 5A). However, the recovery was delayed, an observation that was also reported in TEER experiments with HBMEC and schizont-stage IE, but not with trophozoite-stage IE (Avril *et al.*, 2019). It is likely that that schizont-stage IE rupture during co-culture and release mediators, causing similar effects to IE lysate. We do not know whether the concentrations of parasite factors used in our model to cause endothelial barrier breakdown are the same as those seen during a malaria infection, and measurements from patient plasma may only partially reflect the local concentrations of these in vessels with high levels of cytoadherent IE. New dynamic 3D models of cytoadherence may be able to resolve this issue.

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 nor-malisation.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_E5555-1+#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 \(CC-BY 4.0\)](https://creativecommons.org/licenses/by/4.0/).

Author information

Janet Storm and Yang Wu are joint first authors; Christopher A. Moxon and Alister G. Craig are joint last authors.

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The authors would like to thank our collaborators at Eisai Co. Ltd. in Japan, in particular Drs. Makoto Asada and Kentaro Yoshimatsu, for their generosity in providing access to the PAR1 inhibitors and their engagement throughout the project. The collaboration with Eisai was instigated by the World Intellectual Property Organization (WIPO) and BIO Ventures for Global Health (BVGH), and our thanks to them for their support and encouragement. We would also like to thank Dr Louise Turner, University of Copenhagen for supplying the EPCR-binding rPEMP1.

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Reviewer Report 15 July 2020

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Ana Rodriguez

Department of Microbiology, New York University, New York, NY, USA

I have revised their response and everything is fine.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 05 June 2020

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Ana Rodriguez

Department of Microbiology, New York University, New York, NY, USA

In the methods, Preparation of IE and IE lysates, the concentration of lysate that is added to the endothelial cells is still missing. This can be expressed as number of lysed IE per surface area of the well. This measurement is important since it provides an idea of the likelihood that this phenomenon could be taking place *in vivo*.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Malaria, cerebral malaria, malaria anemia, malaria pathogenesis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 10 Jun 2020

Janet Storm, Liverpool School of Tropical Medicine, Liverpool, UK

The concentration of IE added to HBMEC was added to the methods describing the TEER experiments in version 2:

“For subsequent experiments with HBMEC, the overnight culture medium was first replaced with EGM2 medium for 3 – 6 hours and then with EGM2-min medium 2 hours prior to the addition of 300 μ l IE suspension (1.5×10^7 IE per well) or 300 μ l lysates”

The surface area of the wells and seeding density of HBMEC is also provided in the same methods section. Lysates were prepared from these IE suspensions, so would be an equivalent amount.

Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 20 April 2020

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Ana Rodriguez

Department of Microbiology, New York University, New York, NY, USA

This work explores the possible mechanisms underlying cerebral malaria pathogenesis. It is therefore very relevant. The authors develop an *in vitro* model of the BBB and test the effect of *Plasmodium falciparum* and its effect on the ePCR/thrombin/PAR1 axis in barrier function of brain endothelial cells. The findings implicate that the parasite causes a more potent delayed barrier disruption that is not related to the classical ePCR/PAR1 model.

Minor points:

- Include the number of IE per surface area added in every experiment or the equivalent for IE lysates.
- Include the number of cells seeded per well (or surface area) used in xCelligence experiments.

- Include the Cell index of cultures after the overnight growth before starting the experiments.
- Indicate the stage of IE used in the experiments or if asynchronous cultures were used. If a single stage was used, explain how was it isolated.
- "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)." please indicate the time of incubation with EGM2 medium.
- In Fig. 2, the effect of aPC and recombinant PfEMP1 alone on cells should be included as controls.
- In Fig. 3, a control of uninfected RBC and their lysate should be included.
- In Fig.4, it is not clear which are the lines corresponding to lighter and darker shades of green. Please use different colors.
- In Figure 5, it would be interesting to see the original cell index graph showing the effect of thrombin on cells already incubated with the parasite lysate for 20 h.
- In Figure 6 it is stated that "the normalised cell index was determined at 2 and 16 hours", but it is not clear which time point is shown in the graph or if it is the difference between the values at these two time points. It would be informative to see the original cell index graphs as in figure 4 or to show the results at 2 h and 16 h separately in two independent graphs.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Malaria, cerebral malaria, malaria anemia, malaria pathogenesis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 19 May 2020

Janet Storm, Liverpool School of Tropical Medicine, Liverpool, UK

We thank the reviewer for her constructive comments and have addressed these in our reply and in the manuscript.

Include the number of IE per surface area added in every experiment or the equivalent for IE lysates.

For IE and IE lysates, 300 μ l was added per well and always at 50% parasitaemia at 1% haematocrit. This is 1.5×10^7 IE per well (0.64 cm^2 surface area). This is now added to the methods. In addition, related to comment 4, the preparation of IE suspension has also been added to the methods section.

Include the number of cells seeded per well (or surface area) used in xCelligence experiments.

Cells were seeded at 50,000 cells/ cm^2 , equal to 32,000 cells/well (surface area per well is 0.64 cm^2). This is added to the methods.

Include the Cell index of cultures after the overnight growth before starting the experiments.

On average the cell index, after refreshing the medium and before adding lysates and/or inhibitors, is 6.9. This information is available in full in the raw data (<https://doi.org/10.6084/m9.figshare.11558889>).

Indicate the stage of IE used in the experiments or if asynchronous cultures were used. If a single stage was used, explain how was it isolated.

The section in the methods describing the preparation of lysate has been altered and includes the preparation of IE suspension at 50% parasitaemia at 1% haematocrit. Lysates were made from these IE suspensions.

"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)." please indicate the time of incubation with EGM2 medium.

Fresh EGM2 was normally added in the morning, resulting in 3-6 hrs incubation. This has been added to the methods.

In Fig. 2, the effect of aPC and recombinant PfEMP1 alone on cells should be included as controls.

The TEER traces in figure 2A are a representative and are derived from an experiment that did not include aPC and rPfEMP1 on their own. We choose this representative experiment as it included the inhibitor data shown in figure 2B. From other experiments it was clear that neither aPC nor rPfEMP1 affected the normalised cell index more than 5%, this has been added to the results section.

In Fig. 3, a control of uninfected RBC and their lysate should be included.

These controls are now added to figure 3, its legend and the text describing the result.

In Fig.4, it is not clear which are the lines corresponding to lighter and darker shades of green. Please use different colors.

The colours in new figure 4C (was 4B) have been changed. IE lysate is now green and IE is cyan.

In Figure 5, it would be interesting to see the original cell index graph showing the effect of thrombin on cells already incubated with the parasite lysate for 20 h.

Cell index traces have been added to figure 5, as requested by the other reviewer too. It shows the slow recovery from thrombin after co-culture with IE lysate. For clarity, a similar cell index trace has been added to figure 4. See also our reply to the other reviewer.

In Figure 6 it is stated that "the normalised cell index was determined at 2 and 16 hours", but it is not clear which time point is shown in the graph or if it is the difference between the values at these two time points. It would be informative to see the original cell index graphs as in figure 4 or to show the results at 2 h and 16 h separately in two independent graphs.

Representative cell index traces have also been added to figure 6 and indicate the 2 and 16 hour time points. The difference between the normalised cell index at these time points was calculated and used to make the graph. These calculations are also provided in the raw data file "Combined analysis data for fig6_TEER_includes normalisation_2020-02-04.csv".

Competing Interests: No competing interests were disclosed.

Reviewer Report 06 March 2020

<https://doi.org/10.21956/wellcomeopenres.17089.r37975>

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Maria Bernabeu 

European Molecular Biology Laboratory, Barcelona, Spain

The study from Storm *et al.*, describe how PAR1 inhibitors could counteract the loss of endothelial barrier that occurs in cerebral malaria. The authors show that PAR1 inhibitors revert barrier disruption mediated by thrombin, but are not effective against long-term barrier breakdown caused by parasite lysates. The finding that PAR1 inhibitors are, even more effective than aPC, at rescuing thrombin-induced barrier opening in the presence of PfEMP1 EPCR binders is important for the field. The finding that other therapeutic approaches will be needed for barrier caused by parasitic molecules/toxins is also relevant. Overall, the study is well-designed and the conclusions are supported by the results. However, the study might benefit from additional improvements.

Major comments:

Methods:

- The authors used the same parasitemia and hematocrit for long-term culture or to prepare the IEs lysate and they obtained significant differences in barrier dysfunction. Did the authors observe merozoite egress after long term IE incubation? Are the molecules released on a mid-trophozoite lysate a good proxy of schizont molecules released after rupture? Is the concentration used “physiologically” present in the cerebral malaria microvasculature. Although I agree that the concentration used in this study might be necessary to observe a major barrier breakdown in *in vitro* cultures, it might not accurately mimic the pathogenic mechanisms on patients. A sentence in the discussion reflecting the limitations of the model might improve the interpretation of the paper by readers outside the field.

Results:

- Long term incubation with both IE or IE lysate showed 1) reduced surface expression of EPCR and reduced HBMEC cell index. However, the reduced cell index might not be a direct consequence of loss of EPCR in the surface. As the authors accurately describe in the discussion, loss of barrier in their experiments is more likely a consequence of the presence of parasite histones, HRP2 or other soluble mediators. However, the hypothesis exposed in the introduction might be misleading for the average reader. *“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.”* Since we don’t understand quite well the molecular mechanisms that induce loss of endothelial integrity and surface EPCR, the authors should be more careful while establishing associations.
- I would recommend to include both 1) representative traces of cell index and bar plots in figure 3, 4, 5 and 6 in a similar way than in figure 2. Or include it in supplementary data. Was the experiment shown in figure 4 done on independent days? Although TEER measurements are highly variable depending on the day of the experiment, box plots and dot plots might better represent data distribution than bar graphs.

Discussion:

- Avril *et al.*, 2019 showed using the same methodology that schizont-stage parasites (EPCR binding strains) prolonged thrombin-induced barrier disruption in resting and activated HBMEC, and HBMEC do not get back to baseline after IE incubation. Thrombin and IE colocalization is frequent in the microvasculature in cerebral malaria autopsies. As the effects seen in Avril *et al.* are likely to occur in patients this experiment is highly relevant. Is the experiment in Figure 5 equivalent to Avril *et al.*, 2019 but with trophozoites lysates expressing CD36-binding PfEMP1? At which time-scale was the CI measured in figure 5? A representative cell index trace in Figure 5 will clarify that.

Minor comments:**Abstract:**

- “However, in the IE lysate co-culture system we identified a mechanism that slowly reduces barrier function and which is insensitive to PAR1 inhibitors.”
Please, rephrase to “However, PAR1 inhibitors did not rescue the barrier dysfunction after long-term co-culture with IE lysate”. The molecular mechanisms has not been described in this

manuscript.

Reproducibility of Methods:

- Which amount of IE is used in the long-term co-culture in Figures 3, 4 and 5? The authors give the parasitemia, the hematocrit but as the volume is missing, the quantity or concentration of IE cannot be calculated.

Results:

- Why did the authors test the rescue after short-term EPCR-PfEMP1 steric inhibition on EA.hy.926 cells (HUVEC) (behavior A) and the long-term parasite effects on HBMEC (behavior B)? Is the rescue seen on Figure 2 also reproducible on HBMEC?

Figures and figure legends:

- A visual timeline of the experiments in each figure might improve the clarity. The experiments performed are quite complex and difficult to interpret and to compare with the current figure legends.

References:

- Kessler *et al.*, 2017 is not well cited in the first paragraph as a review of aPC-PAR1 axis. Is this a typo?
- In the current manuscript Avril *et al.*, 2019 is only being cited in the introduction along with 5 other papers. The paper should be introduced in the result section “*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.*” Given the similarities and complementarity between Storm *et al.*, 2020 and Avril *et al.*, 2019, it might be beneficial to discuss it more extensively in the discussion.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Malaria pathogenesis, host-parasite interactions, vascular biology and engineering.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 19 May 2020

Janet Storm, Liverpool School of Tropical Medicine, Liverpool, UK

We thank the reviewer for her constructive comments and have addressed these in our reply and in the manuscript.

The authors used the same parasitemia and hematocrit for long-term culture or to prepare the IEs lysate and they obtained significant differences in barrier dysfunction. Did the authors observe merozoite egress after long term IE incubation?

In similar co-culture experiments (in EC culture medium) the parasites are still healthy looking after 6-7 hours. However, after overnight co-culture they do not develop properly and we hardly ever saw newly formed rings. In using IE, we are specifically looking at the effect of adhesion rather than the release of parasite material.

Are the molecules released on a mid-trophozoite lysate a good proxy of schizont molecules released after rupture?

To mimic the release of schizont molecules, we wanted to use lysate of schizonts and we spent considerable time optimising culture methods to obtain parasites in schizogony. This proved to be rather difficult and therefore we made lysates of trophozoites in a late as possible stage. The cultures were regularly synchronised with sorbitol to keep them synchronous. In the methods section, preparation of IE and lysates has been altered to mid-late trophozoite stage. We did notice that lysates with a higher proportion of schizonts had a greater effect on the cell index, but for the experiments described in this manuscript it was not investigated further.

Is the concentration used “physiologically” present in the cerebral malaria microvasculature. Although I agree that the concentration used in this study might be necessary to observe a major barrier breakdown in in vitro cultures, it might not be accurately mimic the pathogenic mechanisms on patients. A sentence in the discussion reflecting the limitations of the model might improve the interpretation of the paper by readers outside the field.

The reviewer is correct in pointing out that the *in vitro* conditions might not mimic the *in vivo* situation accurately, but the release of parasite molecules at sequestration sites could result in high local concentrations of these parasite factors. Measurements from patient plasma may only partially reflect these local concentrations, but hopefully new 3D models of cytoadherence may be able to resolve this issue. A comment has been added to the discussion to reflect this.

Long term incubation with both IE or IE lysate showed 1) reduced surface expression of EPCR and reduced HBMEC cell index. However, the reduced cell index might not be a direct consequence of loss of EPCR in the surface. As the authors accurately describe in the discussion, loss of barrier in their experiments is more likely a consequence of the presence of parasite histones, HRP2 or other soluble mediators. However, the hypothesis exposed in the introduction might be misleading for the average reader. “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.” Since we don’t understand quite well the molecular mechanisms that induce loss of endothelial integrity and surface EPCR, the authors should be more careful while establishing associations.

The reason to test the PAR1 inhibitors was to test our initial hypothesis that the Thrombin-PAR1 axis plays a significant role in barrier loss in CM. If true, the PAR1 inhibitors should affect the parasite-induced disruption of barrier function. Our results show that this is not the case and the implications of these data are discussed. We have changed the wording at the start of this paragraph to indicate the context of the hypothesis as a starting point. We agree with the reviewer that we do not understand all the mechanisms that contribute to endothelial dysfunction in severe malaria.

I would recommend to include both 1) representative traces of cell index and bar plots in figure 3, 4, 5 and 6 in a similar way than in figure 2. Or include it in supplementary data.

Cell index traces have been added to figure 5 and 6. In figure 5B, the plot shows the effect of Vorapaxar on thrombin induced disruption of barrier function in the absence and presence of lysate. Figure 5A depicts the same traces but over a longer time frame, also showing the effect of Vorapaxar on IE lysate induced decrease in cell index. Figure 6A then shows the effect of all four inhibitors on the lysate induced decrease in cell index.

Note that the calculations are also provided in the raw data files “Combined analysis data for fig5_TEER_includes normalisation_2020-02-04.csv” and “Combined analysis data for fig6_TEER_includes normalisation_2020-02-04.csv”.

Figure 4 already depicts TEER traces, but a panel (4B) has been added on request from both reviewers. It shows that HBMEC exposed to IE lysate recovers slowly from thrombin induced barrier disruption.

Figure 3 shows the EPCR expression data, derived from flow cytometry.

Was the experiment shown in figure 4 done on independent days?

Yes, the data shown in figure 4 A, B and C (note that there is an additional panel) are from three different experiments.

Although TEER measurements are highly variable depending on the day of the experiment, box plots and dot plots might better represent data distribution than bar graphs.

The bar graphs in figure 5 and 6 are showing mean \pm SD, with relatively low SD values. We do not think depicting it as box plots or dot plots will change the interpretation of the data.

Avril *et al.*, 2019 showed using the same methodology that schizont-stage parasites (EPCR binding strains) prolonged thrombin-induced barrier disruption in resting and activated HBMEC, and HBMEC do not get back to baseline after IE incubation. Thrombin and IE colocalization is frequent in the microvasculature in cerebral malaria autopsies. As the effects seen in Avril *et al.* are likely to occur in patients this experiment is highly relevant. Is the experiment in Figure 5 equivalent to Avril *et al.*, 2019 but with trophozoites lysates expressing CD36-binding PfEMP1? At which time-scale was the CI measured in figure 5? A representative cell index trace in Figure 5 will clarify that.

We thank the reviewer for their comment and we observed the same delayed recovery from thrombin treatment after co-culture with IE lysate. We have added a representative cell index trace to show this in figure 5 (A and B) and added text to the results and discussion sections to acknowledge this.

A similar cell index trace was also added to figure 4 to show all three observations with HBMEC in one figure:

A) Barrier function disruption by thrombin with a fast recovery. This can partly be rescued by the addition of aPC.

B) Slow recovery from thrombin treatment after co-culture with IE lysate.

C) Disruption of barrier function by IE lysate.

“However, in the IE lysate co-culture system we identified a mechanism that slowly reduces barrier function and which is insensitive to PAR1 inhibitors.”

Please, rephrase to “However, PAR1 inhibitors did not rescued the barrier dysfunction after long-term co-culture with IE lysate”. The molecular mechanisms has not been described in this manuscript.

The reviewer is correct and we have changed this in the abstract.

Which amount of IE is used in the long-term co-culture in Figures 3, 4 and 5? The authors give the parasitemia, the hematocrit but as the volume is missing, the quantity or concentration of IE cannot be calculated.

This has been added in the methods, it is 300 μ l. The number of IE (or equivalent for lysates) has also been added, as requested by the second reviewer.

Why did the authors test the rescue after short-term EPCR-PfEMP1 steric inhibition on EA.hy.926 cells (HUVEC) (behavior A) and the long-term parasite effects on HBMEC (behavior B)? Is the rescue seen on Figure 2 also reproducible on HBMEC?

The initial screening experiments were done with a cell line to simplify the approach, and compounds that were identified from this screen were entered into more complex investigations using primary human brain endothelial cells. We recognise that the screen may miss some details but we used this as a cost-effective way to down-select the number of experiments needed. As others have reported on the steric effects of PfEMP1, we did not replicate this work on HBMEC.

A visual timeline of the experiments in each figure might improve the clarity. The experiments performed are quite complex and difficult to interpret and to compare with the current figure legends.

A timeline has been added to figure 2, 4, 5 and 6.

Kessler et al., 2017 is not well cited in the first paragraph as a review of aPC-PAR1 axis. Is this a typo?

We had included it to give full coverage but the reviewer is correct and we have removed the reference at this point – it is cited later.

In the current manuscript Avril et al., 2019 is only being cited in the introduction along with 5 other papers. The paper should be introduced in the result section “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.” Given the similarities and complementarity between Storm et

al., 2020 and Avril et al., 2019, it might be beneficial to discuss it more extensively in the discussion.

The reference to *Avril et al* has been added to the results and discussion section, specifically after adding TEER traces to figure 4 and 5 describing a similar observation with delayed thrombin recovery after IE lysate exposure.

Competing Interests: No competing interests were disclosed.
