# The novel ETA receptor antagonist HJP-272 prevents cerebral microvascular hemorrhage in cerebral malaria and synergistically improves survival in combination with an artemisinin derivative

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## ARTICLE INFO

## ABSTRACT

*Aim:* To investigate the association between vasculopathy and survival during experimental cerebral malaria (ECM), and to determine whether targeting the endothelin-1 (ET-1) pathway alone or in combination with the anti-malaria drug artemether (a semi-synthetic derivative of artemisinin) will improve microvascular hemorrhage and survival.

*Main methods:* C57BL/6 mice infected with *Plasmodium berghei* ANKA (PbA) were randomly assigned to four groups: no treatment, artemether treated, $ET_A$ receptor antagonist (HJP-272) treated, or HJP-272 and artemether treated. The uninfected control mice were treated with HJP-272 and artemether. We analyzed survival, cerebral hemorrhage, weight change, blood glucose levels and parasitemia.

*Key findings:* Our studies demonstrated decreased brain hemorrhage in PbA-infected (ECM) mice treated when HJP-272, a 1,3,6-trisubstituted-2-carboxy-quinol-4-one novel $ET_A$ receptor antagonist synthesized by our group, is used in conjunction with artemether, an anti-malarial agent. In addition, despite adversely affecting parasitemia and weight in non-artemether treated infected mice, HJP-272, seemed to confer some survival benefit when used as adjunctive therapy, though this did not reach significance.

*Significance:* Previous studies demonstrate that the endothelin pathway is associated with vasculopathy, neuronal injury and inflammation in ECM. As demonstrated here, components of the ET-1 pathway may be important targets for adjunctive therapy in ECM, and may help in preventing hemorrhage and in improving survival when used as adjunctive therapy during malaria infection. The data presented suggest that our novel agent, HJP-272, may ameliorate alterations in the vasculature which can potentially lead to inflammation, neurological dysfunction, and subsequent death in mice with ECM.

## Introduction

Cerebral malaria (CM) in humans is the deadliest complication of *Plasmodium falciparum* infection, leading to encephalopathy and high rates of mortality in developing countries. Numerous studies indicate that alteration of the cerebral vasculature are crucial in the pathogenesis of CM. Magnetic resonance studies of murine CM *in vivo* demonstrated vascular damage, including edema, a reduction in brain perfusion, breakdown of the blood brain barrier (BBB) and hemorrhage due to inflammatory factors (Penet et al., 2005). Previously, we demonstrated

that experimental CM (ECM) was associated with cerebral vasculopathy with an increase in endothelin level, which resulted in a reduction in cerebral blood flow and neuronal dysfunction (Kennan et al., 2005; Machado et al., 2006). These abnormalities resulted in cognitive deficits in ECM during acute infection and after successful antimalarial therapy (Desruisseaux et al., 2008; Dai et al., 2010).

Endothelin-1 (ET-1) in the brain is increasingly recognized as a contributor in the pathogenesis of CM. It is a very potent vasoconstrictor, which can be produced in vascular endothelial cells as well as neurons and astrocytes (Kedzierski and Yanagisawa, 2001; Schinelli, 2002). ET-1 has several pharmacological effects on vasculature mediated through the ET receptors ($ET_A$ or $ET_B$) (Arai et al., 1990). The predominant biological effects produced by ET-1 are vasodilation through activation of the $ET_B$ receptor and vasoconstriction and smooth muscle cell proliferation through the activation of the $ET_A$ receptor (Douglas and Ohlstein, 1997). Elevated concentrations of ET-1 lead to increased

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synthesis of inflammatory mediators and leukocyte adhesion molecules mediating inflammation-induced vasoconstriction, resulting in reduction of cerebral blood flow. Moreover, ET-1's participation in the inflammatory process is believed to contribute to vascular dysfunction and disruption of the integrity of the BBB (Teder and Noble, 2000; Matsuo et al., 2001).

Current antimalarial drugs such as artemether have been used as therapeutic treatments in the prevention of severe malaria in most sub-Saharan African countries (Frosch et al., 2011). Although artemether is a particularly useful antimalarial treatment, cognitive deficits of CM persist after successful cure of the infection as antimalarials only eradicate the parasites, but do not provide neuroprotection (Sinclair et al., 2009). Consequently, achieving a parasitological cure is insufficient in the amelioration and/or protection of the neurological dysfunction caused by malaria. Therefore, it is necessary to develop adjunctive therapies directed at the prevention of neuronal dysfunction.

ET-1's facilitation in inflammation-induced vasoconstriction plays a key role in the pathogenesis of CM, perhaps serving as an important target for prevention of neuronal damage. To determine whether targeting the $ET_A$ receptor has the potential to attenuate neurological deficits in ECM, a series of novel $ET_A$ receptor antagonists was designed and synthesized. Comparative molecular field analysis was used to identify regions of the molecule that could accommodate various substituents and a structure activity relationship was performed. The most potent analog in the series reduces ET-1 binding to the receptor by 50% at nano-molar concentrations. Use of this novel $ET_A$ receptor blocker, 1,3,6-trisubstituted-2-carboxy-quinol-4-one (HJP-272), resulted in attenuation of hemorrhage in the brain of mice infected with PbA, which may provide the neural protection necessary in the treatment of CM.

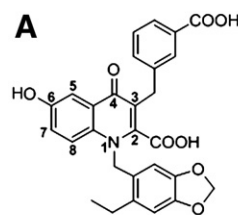## Materials and methods

### Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experiments were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine. All efforts were made to minimize suffering.

### Endothelin receptor antagonist

A novel $ET_A$ receptor antagonist, 1,3,6-trisubstituted-2-carboxy-quinol-4-one (HJP-272; Fig. 1A), was synthesized by our group using comparative molecular field analysis (CoMFA) as previously reported (Patet et al., 2010). It was used as one of the treatments to examine the role of the $ET_A$ receptor in neuroprotection in combination with artemether, a semi-synthetic ether derivative of artemisinin used in the treatment of malaria. Artemisinin derivatives have been shown to be particularly effective in the treatment of cerebral malaria [Klayman, 1985; Barnes et al., 2009].

### Animal infection and study design

Six-week-old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were infected via intraperitoneal (IP) injection with $10^5$ *Plasmodium berghei* ANKA (PbA) parasitized RBCs. The infected mice were randomly assigned to four groups: no treatment, artemether treated (50 mg/kg; IP injection; Cabrales et al., 2010), the novel $ET_A$ receptor antagonist (HJP-272) treated at a dose of 50 mg/kg via IP injection, or HJP-272 and artemether treated (Fig. 1B; $n = 7$ in each group). The HJP-272 dose chosen was previously established to have the most reliable biological effect without evidence of toxicity (Olgun et al., 2010). The uninfected control mice were treated with HJP-272 and artemether (Fig. 1B; $n = 7$ in each group). Tail blood

**Fig. 1.** HJP-272 structure and Experimental Design: (A) Chemical structure of selective $ET_A$ receptor antagonist (HJP-272) 1,3,6-trisubstituted-2-carboxy-quinol-4-one. (B) PbA-infected mice were randomly assigned to four groups with different treatments. Uninfected control mice were treated with HJP-272 ($ET_A$RB) 50 mg/kg via intraperitoneal (IP) injection and artemether 50 mg/kg IP. $n = 7$ mice per group. $ET_A$RB = HJP-272.

| Experiment Group | Sample Size | Infection Condition | Treatment Condition |
|---|---|---|---|
| group 1 | 7 | PbA | $ET_A$RB |
| group 2 | 7 | PbA | $ET_A$RB+Artemether |
| group 3 | 7 | PbA | Artemether |
| group 4 | 7 | PbA | No treatment |
| group 5 | 7 | Con | $ET_A$RB+Artemether |

smears of infected mice were stained with modified Giemsa stain (Sigma-Aldrich, St Louis, MO) and examined daily under a light microscope from day 4 post-infection (PI) for parasitemia (defined as percentage of parasitized RBCs). Weight and blood glucose were measured at days 0, 2, 4 and every day from day 6 PI. Mice were examined daily for locomotor activity and coat condition.

Treatment with either HJP-272, 50 mg/kg via intraperitoneal (IP) injection, or vehicle was initiated at day 3 PI for all mice and continued for 7 days. For treatment with artemether, infected mice were allowed to manifest features of ECM, then treated with either artemether at a dose of 50 mg/kg via IP injection for 5 days in order to eradicate parasites or were given no antimalarial therapy (injected with vehicle). Artemether treatment was initiated when infected mice reached 3 pre-determined criteria as previously reported (Dai et al., 2010) briefly, weight loss, significant change in glucose from baseline (hypo or hyperglycemia) and decreased activity. Artemether therapy was initiated at day 7 PI in all uninfected control mice, the earliest day in which infected mice received treatment with artemether. Mortality was recorded and mice without artemether therapy were euthanized using carbon dioxide at day 8 or day 9 PI. Mice treated with artemether were euthanized 5 days after cessation of artemether. Brains were harvested and divided down the midline. For histology one half of the brain was fixed in 10% normal buffered formalin (NBF) and stored at room temperature.

### Histology

Brain tissue was fixed, embedded in paraffin then sectioned into 4 μm sagittal slices at the midline. Brain sections were stained with hematoxylin–eosin. Two separate sagittal sections were examined for each animal ($n = 5$ PbA + HJP-272; 4 PbA + HJP-272 + Artemether; 3 PbA + Artemether; 9 PbA and 6 uninfected controls). For the purposes of histological analysis, brain sections of 7 untreated PbA mice from parallel replicate experiments using the same parameters as those utilized in this experiment were used in order to increase the number of mice analyzed ($n = 9$ for PbA mice). Photographs were taken using a Nikon Microphot-FXA microscope system and a Nikon digital sight DS-5 M camera (Nikon Corporation, Japan). Examiners blinded to the conditions of the specimens reviewed the stained sagittal sections using a semi-quantitative method for hemorrhage analysis based on a scale of 0–4 as previously reported (Dai et al., 2010) Briefly 0 = red blood cells (RBCs) are confined within the blood vessels; 1 = one vessel with minimal extravasation of RBCs in one field at 10× magnification;

2 = greater than 1 vessel with minimal extravasation; 3 = 1 vessel with extensive extravasation; 4 = greater than 1 vessel with extensive extravasation.

### Statistical analysis

Hemorrhage scores, percentage of weight change, percentage of glucose change and parasitemia were statistically analyzed by *t*-tests, one-way ANOVA with post-hoc Tukey's multiples comparison analysis and 2-way ANOVA with post-hoc Bonferroni analysis using GraphPad Prism v5.04 (GraphPad Software, La Jolla, California). Survival data was analyzed by the Kaplan–Meier and logrank test with GraphPad Prism.

### Results

#### Effects of HJP-272 on parasitemia, activity, weight and glucose

Infected mice receiving no anti-parasitic treatment experienced a gradual increase in parasitemia, with greater than 7% infected red blood cells (iRBCs) at 6 dpi and a peak at 9 dpi (PbA + HJP-272 $20.5 \pm 5.1\%$ v. PbA $18.2 \pm 1.8\%$, $p =$ NS; Fig. 2A). Although parasitemia in the infected mice treated with artemether was greater than 7% iRBCs at the beginning of artemether treatment (PbA + HJP-272 + Artemether $12.8 \pm 1.3\%$ v. PbA + Artemether $14.1 \pm 2.1\%$, $p =$ NS; Fig. 2A), 3 days after initiation of artemether treatment parasitemia returned to 0% (Fig. 2A). There was a significant effect of HJP-272 therapy on



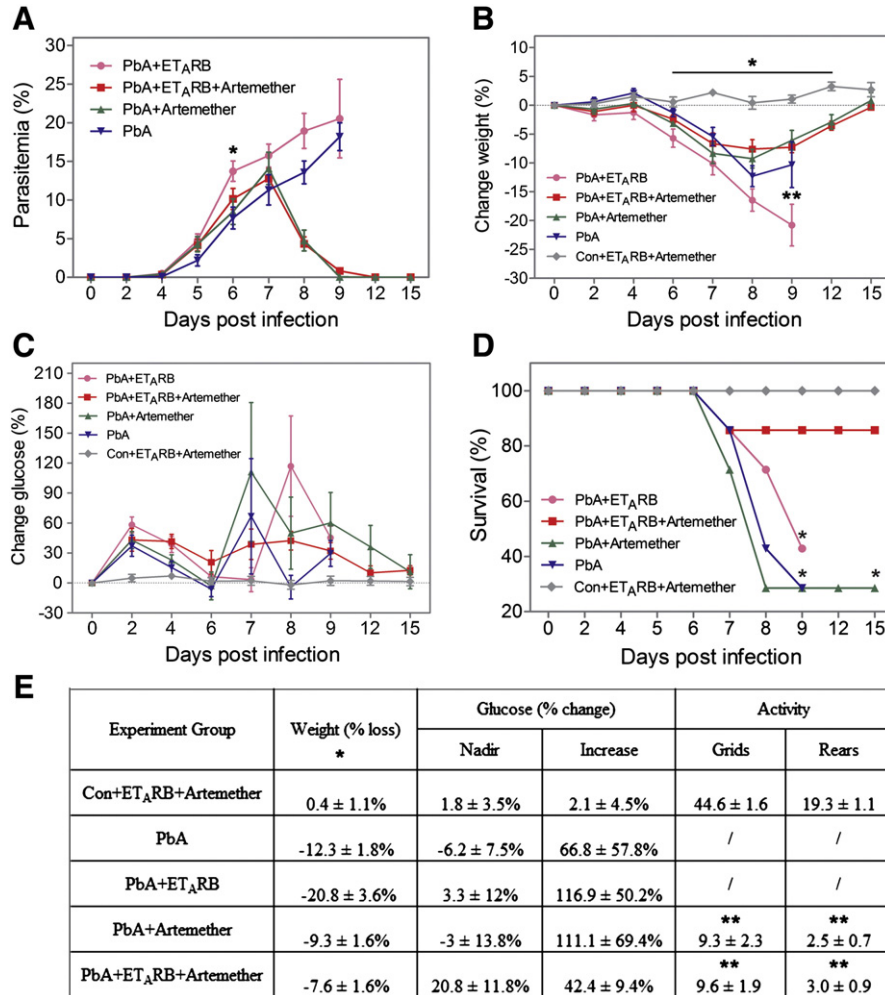| Experiment Group | Weight (% loss) * | Glucose (% change) | | Activity | |
|---|---|---|---|---|---|
| | | Nadir | Increase | Grids | Rears |
| Con+ET$_A$RB+Artemether | $0.4 \pm 1.1\%$ | $1.8 \pm 3.5\%$ | $2.1 \pm 4.5\%$ | $44.6 \pm 1.6$ | $19.3 \pm 1.1$ |
| PbA | $-12.3 \pm 1.8\%$ | $-6.2 \pm 7.5\%$ | $66.8 \pm 57.8\%$ | / | / |
| PbA+ET$_A$RB | $-20.8 \pm 3.6\%$ | $3.3 \pm 12\%$ | $116.9 \pm 50.2\%$ | / | / |
| PbA+Artemether | $-9.3 \pm 1.6\%$ | $-3 \pm 13.8\%$ | $111.1 \pm 69.4\%$ | ** $9.3 \pm 2.3$ | ** $2.5 \pm 0.7$ |
| PbA+ET$_A$RB+Artemether | $-7.6 \pm 1.6\%$ | $20.8 \pm 11.8\%$ | $42.4 \pm 9.4\%$ | ** $9.6 \pm 1.9$ | ** $3.0 \pm 0.9$ |

Fig. 2. Parasitemia, weight loss, glucose change, survival and activity levels: (A) By 6 dpi, parasitemia was greater than 7% in all PbA-infected mice. Treatment with HJP-272 (ET$_A$RB) resulted in higher parasitemia than vehicle at 6 days post-infection (dpi) ($F_{1,83} = 7.72$; $p < 0.01$). However, this effect was only transient as there were no further effects on parasitemia on subsequent days. All mice treated with artemether had resolution of their parasitemia by 9 dpi. (B) Infected animals demonstrated a gradual decrease in weight after infection with significant decrease occurring after 6 dpi ($F_{4,30} = 6.07$; $p < 0.01$), and a nadir occurring after 7 dpi ($F_{4,26} = 10.07$; $p < 0.0001$). HJP-272 therapy resulted in significantly greater weight loss than vehicle in non-artemether treated infected mice ($F_{1,31} = 17.02$; $p < 0.001$), with this effect being evident at 9 dpi. Weights of infected animals increased to original levels after artemether treatment (15 dpi). (C) Infection with *P. berghei* ANKA (PbA) significantly affected serum glucose when compared to uninfected controls in all treatment groups with infection conferring higher glucose levels at terminal stages of disease (7 and 8 dpi in non-artemether treated mice). Though therapy with HJP-272 did not independently affect glucose, it resulted in a significant delay in the time taken to develop hyperglycemia in infected mice without artemether treatment (day 8 in HJP-272 treated mice v. Day 7 in vehicle treated mice; $F_{5,107} = 3.78$; $p < 0.01$). Likewise, in infected mice treated with artemether, though HJP-272 did not independently affect glucose measurements, it contributed to attenuation of the hyperglycemia observed at day 7 ($F_{7,123} = 2.12$; $p < 0.05$). All surviving infected mice recovered stabilization of blood glucose by 15 dpi. (D) Kaplan–Meier survival curves in PbA-infected mice are significantly different when compared to controls. Treatment with artemether alone had no effect on the mortality of infected mice. Likewise, treatment with HJP-272 alone did not result in improved survival. When used in combination with artemether, HJP-272 seemed to result in improved survival when compared to PbA-infected mice with and without artemether that did not receive therapy; however, this did not quite reach statistical significance ($p = 0.054$ and 0.057 respectively). There was no difference in survival between infected mice with combined treatment and uninfected control mice. (E) Infection with PbA conferred significant decreases in weight compared to uninfected control groups regardless of treatment. Likewise, infection with PbA resulted in wide fluctuations in glucose starting at 7 dpi. PbA-infected mice had a significantly lower activity levels than uninfected control mice at 7 dpi as demonstrated by the number of grids crossed ($F_{2,17} = 113.3$; $p < 0.0001$) and the number of rears ($F_{2,17} = 106.7$; $p < 0.0001$) in 1 minute (Fig. 2E). There were no effects of HJP-272 therapy on the activity levels of infected mice. Initial $n = 7$ per group; Values plotted as mean ± standard error (SEM). ET$_A$RB = HJP-272. *$p < 0.05$, **$p < 0.01$.

parasitemia levels in all infected mice prior to institution of artemether treatment, with HJP-272 resulting in higher parasitemia than vehicle ($F_{1,83} = 7.72$; $p<0.01$). However, this effect was only transient and occurred at 6 days post-infection (dpi) in non-artemether treated mice. As previously stated, mice treated with artemether had resolution of parasitemia regardless of HJP-272 administration.

PbA-infected mice had a significantly lower activity levels than uninfected control mice at 7 dpi as demonstrated by the number of grids crossed ($F_{2,17} = 113.3$; $p<0.0001$) and the number of rears ($F_{2,17} = 106.7$; $p<0.0001$) in 1 minute (Fig. 2E). There were no effects of HJP-272 therapy on the activity levels of infected mice.

All infected mice showed fluctuations in weight over time (illustrated as the percent change from original weight) during active infection, with significant decreases of original body weight, compared to uninfected control mice, starting at 6 dpi ($F_{4,30} = 6.07$; $p<0.01$; Fig. 2B) and a nadir occurring after 7 dpi ($F_{4,26} = 10.07$; $p<0.0001$; Fig. 2B, E). HJP-272 therapy resulted in significantly greater weight loss than vehicle in non-artemether treated infected mice ($F_{1,31} = 17.02$; $p<0.001$), with this effect being evident at 9 dpi. All artemether-treated infected mice recovered their baseline weight by 15 dpi.

Infection with *P. berghei* ANKA (PbA) significantly affected serum glucose when compared to uninfected controls in all treatment groups (with and without artemether and with and without HJP-272) with infection conferring higher glucose levels at terminal stages of disease (Fig. 2C). Though therapy with HJP-272 did not independently affect glucose, it resulted in a significant delay in the time taken to develop hyperglycemia in infected mice without artemether treatment ($F_{5,107} = 3.78$; $p<0.01$). Likewise, in infected mice treated with artemether, though HJP-272 did not independently affect glucose measurements, it contributed to attenuation of the hyperglycemia observed at day 7 ($F_{7,123} = 2.12$; $p<0.05$). All surviving infected mice recovered stabilization of blood glucose by 15 dpi (Fig. 2C).

*Effects of HJP-272 on survival*

Mice receiving artemether treatment were sacrificed 5 days after the cessation of artemether. Mice without artemether treatment were sacrificed at 9 dpi. At 9 dpi, Kaplan–Meier survival curves were plotted and differences in rates of survival in the various groups were observed: PbA + HJP-272 + Artemether 85.7%; PbA + Artemether 28.6%; PbA + HJP-272 42.8%; and PbA 28.6%. Significant effect of PbA-infection on survival was demonstrated by the Logrank test with infected mice having significantly higher mortality than uninfected controls (Fig. 2D; $p<0.05$). Treatment with artemether alone had no effect on the mortality of infected mice. Likewise, treatment with HJP-272 alone did not result in improved survival. When used in combination with artemether, HJP-272 seemed to result in improved survival when compared to PbA-infected mice with and without artemether that did not receive therapy; however, this did not quite reach statistical significance ($p = 0.054$ and $0.057$ respectively). There was no difference in survival between infected mice with combined treatment and uninfected control mice (Fig. 2D). Furthermore, there was no mortality observed in the uninfected control group treated with HJP-272 and artemether, indicating that combined treatment with these two drugs had no potential toxicity (Fig. 2D).

*Effect of HJP-272 on brain hemorrhage*

Hematoxylin and eosin (H&E) staining of sagittal brain sections demonstrated significant group effects on microvascular hemorrhage (Fig. 3), with PbA infection with or without artemether therapy resulting in higher hemorrhage scores in the olfactory bulb ($F_{4,17} = 4.74$; $p<0.01$), the thalamus ($F_{4,21} = 4.64$; $p<0.01$), the hypothalamus ($F_{4,23} = 4.29$; $p<0.01$) and the brainstem ($F_{4,23} = 8.65$; $p<0.001$) than in uninfected controls. Interestingly, the degree of microvascular hemorrhage induced by PbA infection continued to progress in the thalamus, the

hypothalamus and in the brainstem despite treatment with artemether, indicating that during acute disease, anti-malarial treatment alone was not sufficient to halt damage to cerebral microvessels despite effective clearance of parasites from the blood. Adjunctive therapy with HJP-272, in combination with artemether, significantly lessened hemorrhage in the olfactory bulb, the thalamus and the brainstem (Fig. 3).

## Discussion

In the current study, we employed HJP-272, a novel $ET_A$ receptor antagonist synthesized by our group to determine the role of ET-1 during ECM and to ascertain the antagonist's ability to ameliorate brain microvascular damage and survival during severe disease. A significant attenuation of hemorrhage was observed in PbA-infected mice treated with a combination of HJP-272 and artemether in the regions of the brainstem, the thalamus and the olfactory bulb. In addition, therapy with HJP-272 helped ameliorate or helped delay, in artemether-treated and non-artemether-treated infected mice respectively, wide fluctuations in blood glucose induced by infection. Furthermore, HJP-272 seemed to confer a synergistic survival benefit when combined with artemether which was independent of its effect on hemorrhage and which was not due to any unintended intrinsic antimalarial effect as PbA-infected mice treated with HJP-272 developed parasitemias that were either comparable or higher than the untreated infected mice. Interestingly, treatment with HJP-272 in fact resulted in an initial worsening in parasitemia which resolved as disease progressed, suggesting that endothelin may play a protective role in the control of parasitism during early infection. This phenomenon has also been observed during treatment with other parasitic diseases (Camargos et al., 2002).

We and others have previously demonstrated persistent working and spatial memory deficits with ECM which mimics the process seen in survivors of pediatric CM (Dai et al., 2010; Reis et al., 2010). Subcortical regions of the brain, including the thalamus, are intricately involved in spatial learning and memory (Davoodi et al., 2009; Lopez et al., 2009). The lesions in the thalamus observed in out ECM mice, both prior to anti-malarial treatment and after artemether therapy, may be involved in the memory deficits previously recorded in our mouse model. ET-induced vasculopathy may play an important role in the decline in memory in ECM as adjunctive therapy with an $ET_A$ receptor antagonist ameliorated hemorrhage in that region of the brain. These findings underscore the need for future studies correlating improvement in microvascular hemorrhage to cognitive dysfunction both during acute infection and after treatment with an anti-malarial agent.

Endothelial inflammation and a hyper-active immune response, including elevated levels of the pro-inflammatory cytokines TNF-α, IFN-γ and IL-1, are hallmarks of severe malaria (Akanmori et al., 2000; Wenisch et al., 1996). Both human and experimental studies indicate that encephalitis resulting from CM is related to the production of inflammatory mediators, leading to an activation of cerebral vascular endothelium ( Akanmori et al., 2000; Hunt and Grau, 2003). We have previously demonstrated that vasculopathy and cognitive dysfunction in ECM highly correlate with inflammation throughout the brain (Kennan et al., 2005; Desruisseaux et al., 2008). In this regard, it has been reported that abnormal levels of ET-1 result from damaged or ischemic endothelial and epithelial cells and have been linked to the induction of this dysregulated inflammation (Lou et al., 2001; Sibson et al., 2002). Overexpression of ET-1 has been demonstrated to activate macrophages via stimulation of pro-inflammatory transcription factor NF-κB, leading to production of pro-inflammatory cytokines, such as TNF-α and IL-1 (Bohm and Pernow, 2007; Shao et al., 2011). Moreover, ET-1 induces the release of monocyte chemoattractant protein-1 (MCP-1), which promotes an inflammatory response (Sutcliffe et al., 2009). These immune responses likely contribute to vascular dysfunction
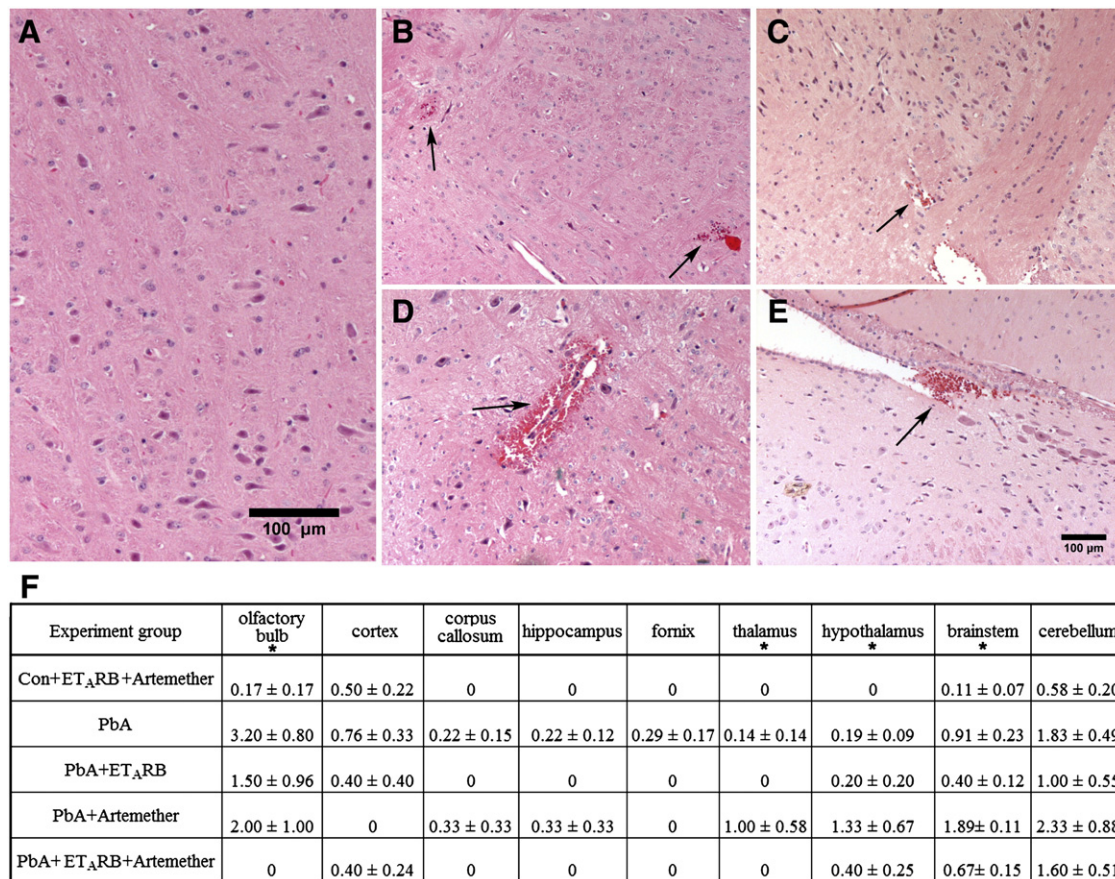
**Fig. 3.** Pathology of ECM with different treatments: While control mice treated with HJP-272 ($ET_A RB$) and artemether demonstrated no hemorrhage (A), PbA infected mice displayed a significant degree of hemorrhage (D — arrow; F) in several regions of the brain, particularly in the brainstem ($F_{4,23} = 8.65$; $p < 0.001$), and the olfactory bulb ($F_{4,17} = 4.74$; $p < 0.01$). (B — arrows; F) HJP-272 treatment alone improved hemorrhage in the brainstem. (E; F) Even with artemether treatment, PbA infected mice still have significant hemorrhage which worsened despite treatment in the brainstem (E- arrow), the thalamus (F; $F_{4,21} = 4.64$; $p < 0.01$), and the hypothalamus (F; $F_{4,23} = 4.29$; $p < 0.01$). (C; F) Combined treatment with HJP-272 and artemether improved hemorrhage in the brainstem (C- arrow), the thalamus and the olfactory bulb (F). $n = 9$ PbA-infected mice with no treatment; 6 uninfected controls; 6 PbA-infected mice with both artemether and HJP-272; 5 PbA-infected mice with HJP-272 alone; 3 PbA-infected mice with artemether alone. $ET_A RB = $ HJP-272. *$p < 0.01$.

and potential downstream dysregulation with resulting neurological sequelae. Therefore, regulation of ET-1's action via $ET_A$ receptor antagonists may increase the survival in ECM by attenuating the associated inflammation in addition to its protective effects on the vasculature.

The $ET_A$ receptor is found on various neurons and mediates both vasoconstriction and signal transduction in the brain (Yamada and Kurokawa, 1998; Douglas and Ohlstein, 1997). Intraventricular administration of ET-1 in conscious rats resulted in behavioral, physiological and hypermetabolic effects. Most of these effects occurred at the doses that cause no changes in cerebral blood flow (Douglas and Ohlstein, 1997). Injection of ET-1 into dorsolateral periaqueductal gray matter of mice reduced pain response in a dose-dependent manner (D'Amico et al., 1996). These data suggest that $ET_A$ plays a role in neurotransmission that is necessary in CNS function. ET-1 was also found to exacerbate apoptosis and neuronal cell death during ischemic conditions through activation of its receptor (Leung et al., 2004; Taylor et al., 2004).

In an ischemic brain, ET-1 induces a decrease in glutamate uptake by reducing glutamate transporters in astrocytes and enhances glutamate excitotoxicity, resulting in intensifying neuronal damage (Ishibashi et al., 2001; Montgomery et al., 2006). Recent evidence implicated that ET-1 modulates glutamate release onto magnocellular neurons in the supraoptic nucleus (Zampronio et al., 2010). Controlling ET-1's action therefore may also reduce ischemic brain damage and restore proper regulation of glutamate.

Finally, ET-1 may regulate glucose metabolism in ECM. Infected mice treated with both the $ET_A$ receptor antagonist and artemether

had more stable blood levels of glucose than infected mice treated with artemether alone. Furthermore, in infected mice with no anti-malarial treatment, administration of the $ET_A$ receptor antagonist resulted in a delay in the volatile fluctuations in blood glucose. Chronic treatment with ET-1 has been demonstrated to reduce insulin signaling by inducing degradation of the insulin receptor substrate 1 (IRS-1) and inhibition of insulin-stimulated tyrosine phosphorylation of IRS-1 (Ishibashi et al., 2001; Usui et al., 2005). It is possible that the $ET_A$ receptor antagonist may improve both neurological function and survival in ECM by restoring proper insulin signaling and hence proper regulation of glucose metabolism.

It is important to note that though treatment with the anti-malarial agent, artemether alone or with HJP-272 alone did not have any effect on survival, we observed a synergistic improvement in survival in mice treated with HJP-272 combined with artemether. Though this did not quite reach significance due to the initial number of mice in our experiment, it was nonetheless an important observation which suggests that mechanisms beyond either drug's effects on hemorrhage are important in the management of ECM, and which warrants further investigation. In this regard, treatment with HJP-272 ameliorated hemorrhagic damage in the brainstem. Irreversible brainstem glial cell injury and dysfunction have been described in cases of severe human CM (Medana et al., 2001). ET-1 induced increase in hemorrhagic damage in the brainstem observed during infection in our model not only point toward damage to the central respiratory center as a possible explanation for the increased mortality associated with ECM despite treatment with artemether, but it may also be implicated in downstream

neuronal and glial cell damage with ECM. Adjunctive administration of HJP-272 resulted in significant amelioration of this damage to the respiratory center and may be important in future survival studies using this model.

There was a progression of vascular damage in the brain after successful treatment of acute malarial disease with artemether, particularly in the brainstem, the thalamus and the hypothalamus. This is different from our previous observations of resolved hemorrhage with anti-parasitic treatment (Dai et al., 2010); however the mice in our previous studies were examined long after completion of a prolonged chloroquine course, i.e. 21 days after completion of a 10-day course of chloroquine long after the resolution of acute disease, and not during the acute process as with this study where mouse brains were examined 5 days after a 5-day course of artemether.

Cerebral malaria is a potentially fatal disease characterized by irreversible neurological insults. In this study, we have identified a possible therapeutic candidate that regulates the inflammatory ET-1 pathway, hence decreasing the hemorrhage in the brain microvasculature in mice infected with PbA. Whether the improvement in vascular damage results in proper restoration of CNS function and whether the drug reverses the long-term cognitive deficits associated with severe disease still needs to be determined. However, based on these initial tests, this antagonist presents a promising adjunctive agent for the management of cerebral malaria.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Acknowledgments

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2012.07.006.

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