Study of the antimalarial properties of hydroxyethylamine derivatives using green fluorescent protein transformed *Plasmodium berghei*

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A rapid decrease in parasitaemia remains the major goal for new antimalarial drugs and thus, in vivo models must provide precise results concerning parasitaemia modulation. Hydroxyethylamine comprise an important group of alkanolamine compounds that exhibit pharmacological properties as proteases inhibitors that has already been proposed as a new class of antimalarial drugs. Herein, it was tested the antimalarial property of new nine different hydroxyethylamine derivatives using the green fluorescent protein (GFP)-expressing Plasmodium berghei *strain. By comparing flow cytometry and microscopic analysis to evaluate parasitaemia recrudescence, it was observed that flow cytometry was a more sensitive methodology. The nine hydroxyethylamine derivatives were obtained by inserting one of the following radical in the* para *position: H, 4Cl, 4-Br, 4-F, 4-CH₃, 4-OCH₃, 4-NO₂, 4-NH₂ and 3-Br. The antimalarial test showed that the compound that received the methyl group (4-CH₃) inhibited 70% of parasite growth. Our results suggest that GFP-transfected P. berghei is a useful tool to study the recrudescence of novel antimalarial drugs through parasitaemia examination by flow cytometry. Furthermore, it was demonstrated that the insertion of a methyl group at the* para *position of the sulfonamide ring appears to be critical for the antimalarial activity of this class of compounds.*

Key words: experimental malaria - novel antimalarial drugs - hydroxyethylamine

Malaria is the most relevant parasitic disease and, despite the many efforts made to eradicate malaria, the disease still accounts for 0.5 million deaths per year globally (WHO 2015). In Brazil, despite the number of cases has been decreasing, it still accounts for 177,767 cases in 2013 (de Pina-Costa et al. 2014, WHO 2015). The current antimalarial treatment recommended by World Health Organization (WHO) is artemisinin-based combination therapy because of artemisinin's efficacy and ability to lower the rate at which resistance emerges (WHO 2010). However, several cases of resistance to artemisinin derivatives have been observed, first at the Cambodia-Thailand border (Dondorp et al. 2010) and now spread across Southeast Asia (Ashley et al. 2014). Such a scenario compels the discovery of novel antimalarial drugs. Several approaches have been used in antimalarial drug discovery, including the use of drugs that prevent transmission or new infection, stop relapse or can be used in cases of uncomplicated and severe malaria (Aguiar et al. 2012a, Anthony et al. 2012). However, a rapid decrease in parasitaemia remains the major goal for new drugs (Burrows et al. 2013).

The biological activities of hydroxyethylamine core have been extensively studied. Hydroxyethylamines have been described as human immunodeficiency virus (HIV) protease inhibitors (Ghosh et al. 2014) and, over the last several years, this class have been studied for their antimalarial activity (de Souza et al. 2012). The antimalarial mechanism of action of hydroxyethylamines comprises the selective inhibition of plasmodium proteases such as falcipain and plasmepsin without interfering with human proteases (Muthas et al. 2005, Rathi et al. 2013). Indeed, the study of hydroxyethylamine derivatives as a new class of antimalarial drugs could represent a safe antimalarial drug. Recently, it was demonstrated that the insertion of a ciclohexyl group in hydroxyethylamine core synthesised from alkylamines increase the antimalarial of such molecule (de Souza et al. 2012).

Herein, it was tested newly synthesised nine different hydroxyethylamine derived from ring-opening of the (2S,3S)-Boc-phenylalanine epoxide with benzylamine in refluxing isopropanol, according its antimalarial activity using the mouse in vivo model of infection with green fluorescent protein-expressing *Plasmodium berghei* (PbGFP).

MATERIALS AND METHODS

Ethics statement - This work was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on Ethical Use of Laboratory Animals of the Oswaldo Cruz Foundation (Fiocruz) (Rio de Janeiro, Brazil) (permit LW52/12).

Mice and the model of infection - C57BL/6 mice (4-5 weeks old) were provided by the Fiocruz breeding unit and caged with free access to food and fresh water in a room at the Farmanguinhos experimental

facility, with a temperature ranging from 22-24ºC and a 12 h light/dark cycle, until use.

For the nontransfected and PbGFP ANKA infection [GFPcon 259cl2 was kindly provided by Dr L Carvalho (Fiocruz) and is a donations from the Malaria Research and Reference Reagent Resource Center - MR4, deposited by CJ Janse and AP Waters (MRA-865)], the mice were intraperitoneally (i.p.) inoculated with 5 x 10^6 *P. berghei*-parasitised red blood cells withdrawn from a previously infected mouse. Artesunate, chloroquine or primaquine was orally administered to mice on the third day of infection (100 mg/kg, diluted in 10% ethanol and 90% propylene glycol; Farmanguinhos). For the evaluation of survival rate, lethality was registered every day until day 14 post-infection. Mice were euthanised by an i.p. injection with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) prior to pentobarbital (150 mg/kg).

Parasitaemia evaluation - At the indicated time points after infection, a thin blood smear was performed for parasitaemia determination by Diff-Quick staining. The determination of parasitaemia by microscopy was performed by counting five fields of approximately 200 erythrocytes per field. To evaluate parasitaemia by flow cytometry, 4 µL of blood was resuspended in 500 µL of phosphate buffered saline/0.1% azide and the cell suspension was immediately submitted to flow cytometry (FACSCalibur, BD Biosciences), as described (Franke-Fayard et al. 2004). Forward scatter and side scatter were set to gate the total erythrocytes and the percentage of PbGFP-infected erythrocytes was determined by fluorescence intensity. At least 10,000 events were acquired in the gate. The data analyses were performed using Cell-Quest software (BD Immunocytometry Systems, USA).

Antimalarial activity of hydroxyethylamine derivatives - The target compounds 5a-i were obtained as previously described (Facchinetti et al. 2014, Moreth et al. 2014). To evaluate the in vivo antimalarial efficacy of hydroxyethylamine derivatives, the PbGFP four-day suppressive test was used (Fidock et al. 2004). Two hours after infection with PbGFP, mice were randomly assigned to 11 groups: nontreated (vehicle, 200 µL i.p.), artesunate treated [10 mg/kg/day diluted in 5% dimethyl sulfoxide (DMSO)] and a group for each hydroxyethylamine derivatives (5a-i; 10 mg/kg/day diluted in 5% DMSO). Mice were treated daily up to day 4 after infection when parasitaemia determination was performed by
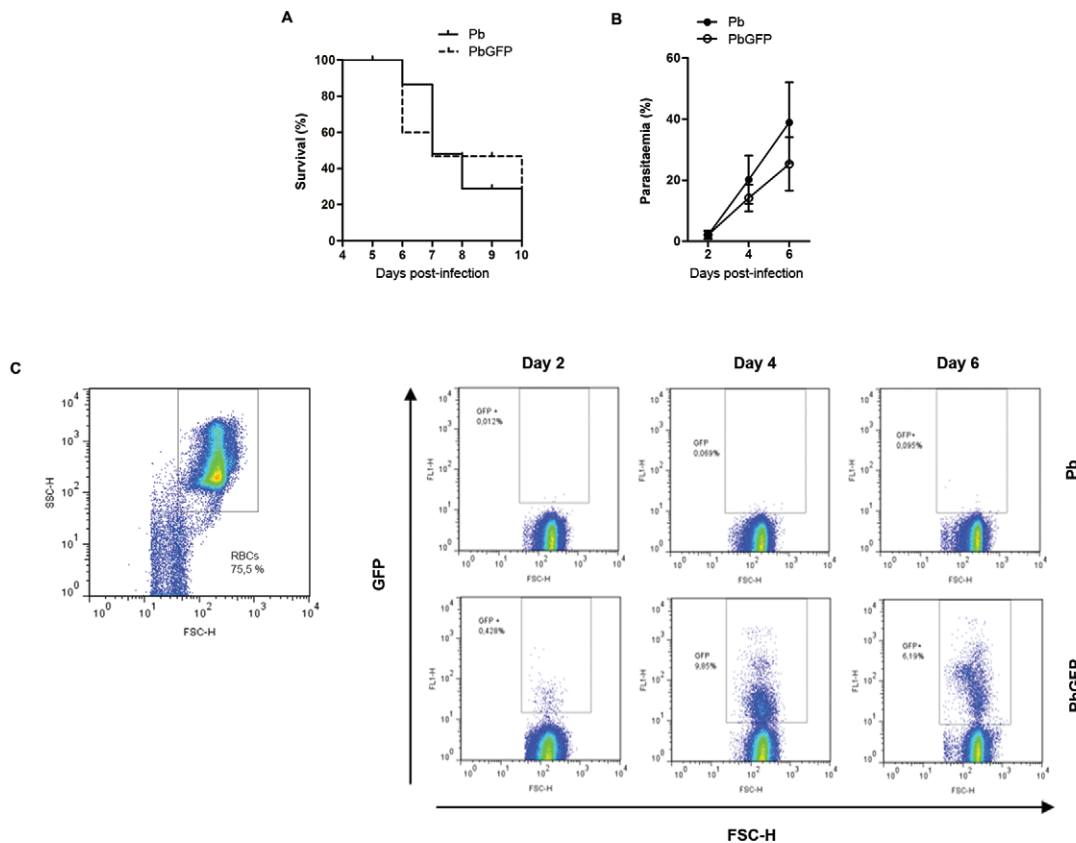

Fig. 1A: survival rates for C57BL/6 mice infected with *Plasmodium berghei* (Pb) (solid line) or green fluorescent protein-expressing Pb (PbGFP) (dashed line). The log-rank test revealed no differences in the survival curves when the Pb-infected (n = 10) and PbGFP-infected C57BL/6 mice (n = 10) were compared. Evolution of parasitaemia in Pb (black symbols) or PbGFP-infected (white symbols) mice measured by microscopy (B) or cytometry (C). The results are expressed as the mean ± standard deviation from at least six animals per group in two different experiments. Gating strategy used to isolate total red blood cells (RBCs) based on forward scatter (FSC) and side scatter (SSC), and representative dot-plots demonstrate the increase in fluorescence, as indicated by an increase in GFP expression in the RBCs is shown in C.

flow cytometry. The results are expressed as drug activity as described previously (Fidock et al. 2004). The difference between the mean value of the control group (taken as 100%) and that of the experimental groups was calculated and expressed as percent reduction (= activity) using the following equation: activity = 100 - [(mean parasitaemia treated/mean parasitaemia control) x 100].
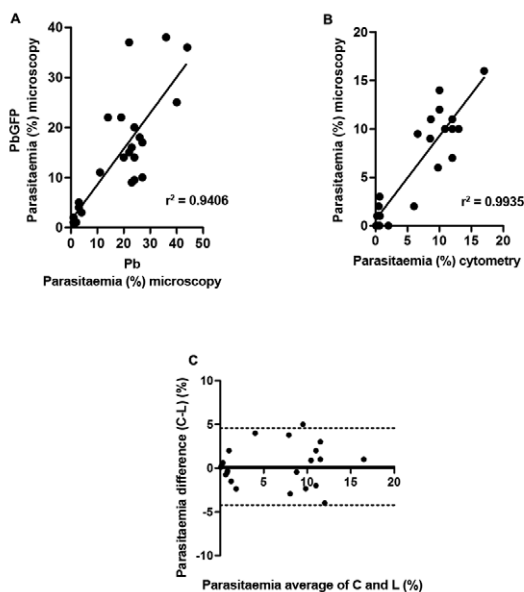

Fig. 2: correlation analyses of parasitaemia estimated by microscopy and cytometry. A: correlation between parasitaemia in mice infected with *Plasmodium berghei* (Pb) or green fluorescent protein-expressing Pb (PbGFP) evaluated by microscopy; B: correlation between parasitaemia in PbGFP-infected mice evaluated by microscopy and cytometry; C: Bland-Altman plot representing the bias (0.1%) and 95% limit of agreement (4.2%) for the parasitaemia evaluation.

Statistical analysis - A log-rank (Mantel-Cox) test was used to compare the percentages of survival and the significance level was set at p < 0.05. The correlation coefficient and Bland-Altman limit were calculated. Additional statistical significance was assessed using ANOVA followed by the Newman-Keuls *t* test. The results are expressed as the mean ± standard error of the means and the significance level in all cases was set at p < 0.05.

RESULTS

Comparison of recrudescence test using Pb and PbGFP-infected mice - In view of the importance to observe the rapid decrease of parasitaemia after antimalarial treatment, it was first compared two methodologies used to the test of new antimalarial drugs (Aguiar et al. 2012b, de Souza et al. 2012). It was observed that Pb and PbGFP-infected mice exhibited similar survival curves (p = 1.00) (Fig. 1A). In addition, the parasitaemia in the Pb and PbGFP groups, as counted by microscopy, was not statistically different and increased up to day 6 post-infection (Fig. 1B). PbGFP-infected erythrocytes were further counted by flow cytometry and it was observed increased levels of parasitaemia up to day 6 post-infection (Fig. 1C). A positive correlation was observed between the parasitaemia counted by microscopy from Pb and PbGFP-infected mice (Fig. 2A). In addition, the evaluation of parasitaemia from PbGFP-infected mice analysed by microscopy or by flow cytometry revealed a significant positive linear correlation (p = 0.006) (Fig. 2B). To confirm that two different methodologies would infer the same result, it was performed a Bland-Altman analysis that also indicated that the evaluation of parasitaemia by cytometry and by microscopy are equivalent (bias = 0.1%; 95% limit of agreement = 4.2%) (Fig. 2C).

Concerning recrudescence studies, up to 48 h after treatment with chloroquine, no infected erythrocytes were found in the blood smears obtained from treated mice (Fig. 3A-C, respectively). However, using flow cy-
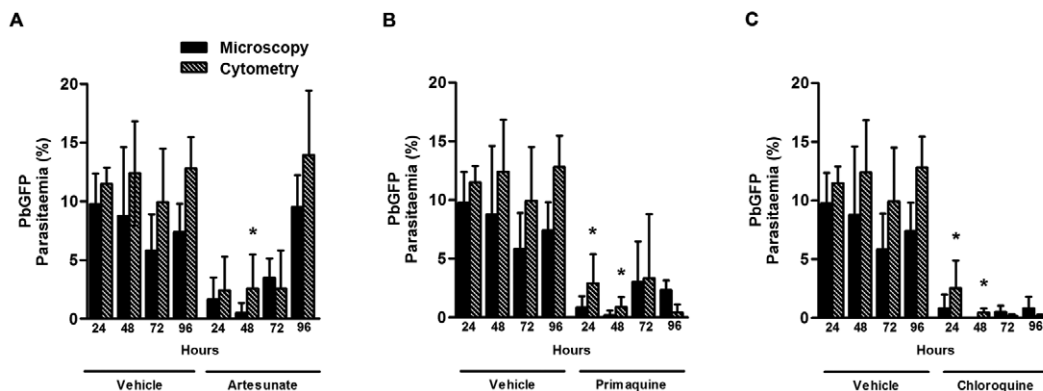

Fig. 3: evaluation of recrudescence after treatment with antimalarial drugs. Mice were infected with green fluorescent protein-expressing *Plasmodium berghei* (PbGFP) and treated with artesunate (A), chloroquine (B) or primaquine (C) at day 3 post-infection and parasitaemia was evaluated up to 96 h after treatment. Parasitaemia was evaluated by microscopy (black bars) and cytometry (hatched bars). The results are expressed as the mean ± standard deviaton from at least six animals per group in two different experiments. Statistically significant differences compared to the group evaluated by microscopy (p < 0.05) are indicated by an asterisk.
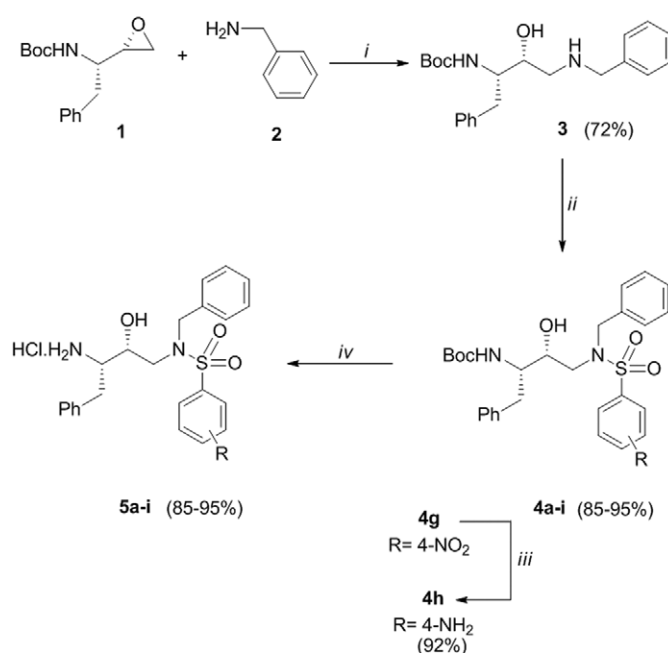
Fig. 4: reaction and conditions (*i*: isopropanol, reflux, 16 h; *ii*: Et$_3$N, DMF, RC$_6$H$_4$SO$_2$Cl, CH$_2$Cl$_2$, r.t., 4 h; *iii*: H$_2$, Pd/C 10%, EtOH, r.t., 16 h; *iv*: HCl gas, EtOH, r.t., 4 h).
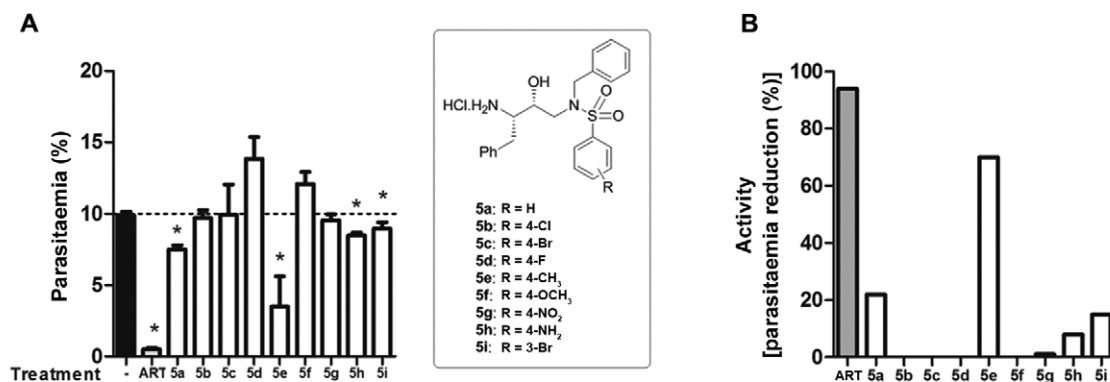

Fig. 5: antimalarial activity of the hydroxyethylamine derivatives. The mice were treated daily with artesunate (ART) or derivatives (10 mg/kg/day; intraperitoneally). Drug activity was evaluated at day 4 after infection and is expressed as (A) parasitaemia levels or as (B) activity, according the following equation: activity = 100 - [(mean parasitaemia treated/mean parasitaemia control) x 100]. It was used at least six animals per group in two different experiments.

tometry, an increase in the parasitaemia of treated mice was observed, especially in mice treated with primaquine or chloroquine. At 72 h and 96 h after treatment, infected erythrocytes were observed in the blood smears at the same extent observed by flow cytometry.

Antimalarial activity of hydroxyethylamine derivatives - Because PbGFP is an effective model to study antimalarial drugs, PbGFP-infected mice were treated with nine hydroxyethylamine derivatives ((2S,3R)-2-(amino)-[4-(N-benzylarenesulfonamido)-3-hydroxy-1-phenylbutane). The preparation of the target compounds 5a-i (Fig. 4) has been previously described (Facchinetti et al. 2014, Moreth et al. 2014).

Of the nine tested compounds, 5a and 5e showed antimalarial activity. Compound 5e was able to reduce 70% of the parasitaemia and was the most active substance of this series (Fig. 5).
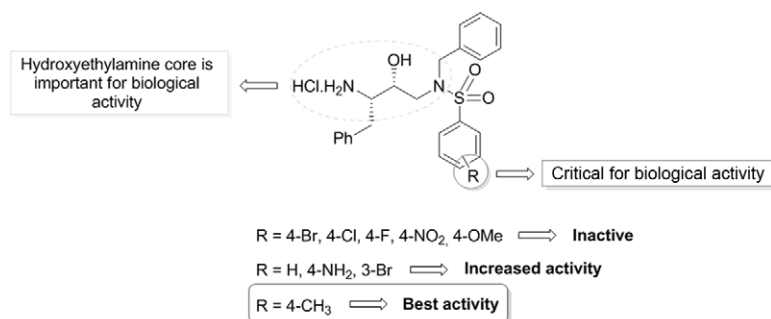
Fig. 6: structure-activity relationship for the studied hydroxyethylamine 5a-i series.

DISCUSSION

Herein, it was proposed the study of newly synthesised hydroxyethylamine derivatives as antimalarial compounds using PbGFP. Furthermore, it was observed that parasitaemia evaluation by flow cytometry reveal low parasitaemia levels, which is not observed by microscopic analysis.

As described before, according the Medicine for Malaria Venture, the "ideal" candidate profile of an antimalarial drug is whom account for fast parasite clearance over 48 h after treatment (Burrows et al. 2013). In such way, it is important to use techniques for parasite evaluation that lead to accurate results. The construction of PbG-FP was performed by Franke-Fayard et al. (2004) and has been used in a wide range of studies (Sultan et al. 1999, Sanchez et al. 2004, Tewari et al. 2010, de Souza et al. 2012), including the screening of novel antimalarial drugs (de Souza et al. 2012, Lam et al. 2013, Wang et al. 2014). It is interesting to note the presence of infected erythrocytes up to 48 h after treatment with chloroquine by flow cytometry that was not detected by microscopic analysis. Flow cytometry allows faster and accurate parasitaemia examination because this technique can identify small amounts of parasites in the blood (Malleret et al. 2011). Although flow cytometry is a costly and complex technology to examine parasitaemia for routine diagnosis purposes, the required instrumentation and materials are widely available in research and development institutions for research purpose (Shapiro et al. 2013). Parasitaemia evaluation by microscopic examination, despite widely used as main test for diagnosis purposes (WHO 2015), is labour and time-consuming, as well as dependent on microscopist training and ability (Payne 1988). Limitations for PbGFP usage as a tool for the discovery of pyrimethamine-based drugs should be addressed, since the construction of Pb-GFP required the insertion of pyrimethamine-resistant gene at the same vector where GFP gene is insert aiming to select the successfully transfected parasites.

Hydroxyethylamine derivative has been used in different biological approaches, as HIV-1 protease inhibitor (Ghosh et al. 2014) and inhibitor of β-secretase 1, an enzyme associated with neurodegeneration (Nordeman et al. 2014). As well, the hydroxyethylamine-based compounds has been tested as antimalarial drugs since this compounds are able to inhibit the activity of plasmepsin (Muthas et al. 2005) and falcipain (Rathi et al. 2013), main enzymes involved in parasite development (Blackman 2008). It was previously showed that hydroxyethylamine derivatives (ciclohexyl group inserted in hydroxyethylamine core) synthesised from alkylamines presented antimalarial activity (de Souza et al. 2012). Herein, it was tested the in vivo activity of new nine different ((2S,3R)-2-(amino)-[4-(N-benzylarenesulfonamido)-3-hydroxy-1-phenylbutane derivatives and observed that the insertion of a methyl group at the *para* position of the sulfonamide ring appears to be critical for the antimalarial activity of this class of compounds (Fig. 6). Interestingly, hydroxyethylamine exhibits no toxic effect on erythrocytes and does not inhibit human proteases (Muthas et al. 2005), suggesting that hydroxyethylamine derivatives would be safe and effective novel antimalarial drugs. In fact, its biological activity may be attributed to a secondary alcohol structural element, which mimics the tetrahedral intermediate during metabolite cleavage by proteases (Cunico et al. 2009). In addition, Jaudzems et al. (2014) showed that the insertion of two methyl group in hydroxyethylamine-based compounds increased compound activity on *Plasmodium faciparum* enzymes when compared to nonmethylated compound.

Together, our results suggest that PbGFP is a useful tool to study the recrudescence of novel antimalarial drugs through parasitaemia examination by flow cytometry. Furthermore, it was demonstrated that the insertion of a methyl group at the *para* position of the sulfonamide ring appears to be critical for the antimalarial activity of this class of compounds.

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