Universidade Federal de Minas Gerais
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BUSCA DE NOVOS FÁRMACOS PARA O TRATAMENTO DA MALÁRIA HUMANA ATRAVÉS DE DIFERENTES ABORDAGENS

Belo Horizonte
Janeiro 2015

Julia Penna Coutinho

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Tese apresentada ao Programa de Pós-Graduação em Medicina Molecular da Faculdade de Medicina da Universidade Federal de Minas Gerais como requisito parcial para a obtenção do grau de Doutor em Medicina Molecular.

Orientadora:
Prof(a) Dra. Antoniana Ursine Krettli

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UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA MOLECULAR

FOLHA DE APROVAÇÃO

BUSCA DE NOVOS FÁRMACOS PARA O TRATAMENTO DA MALÁRIA HUMANA ATRAVÉS DE DIFERENTES ABORDAGENS

JULIA PENNA COUTINHO

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em MEDICINA MOLECULAR, como requisito para obtenção do grau de Doutor em MEDICINA MOLECULAR, área de concentração MEDICINA MOLECULAR.

Aprovada em 27 de janeiro de 2015, pela banca constituída pelos membros:

Prof(a). Antoniana Ursine Krettli - Orientador
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Belo Horizonte, 27 de janeiro de 2015.

UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA MOLECULAR

ATA DA DEFESA DE TESE DA ALUNA
JULIA PENNA COUTINHO

Realizou-se, no dia 27 de janeiro de 2015, às 14:00 horas, Auditório do Centro de Pesquisas René Rachou - Fiocruz/MG, da Universidade Federal de Minas Gerais, a defesa de tese, intitulada *BUSCA DE NOVOS FÁRMACOS PARA O TRATAMENTO DA MALÁRIA HUMANA ATRAVÉS DE DIFERENTES ABORDAGENS*, apresentada por JULIA PENNA COUTINHO, número de registro 2011656766, graduada no curso de FARMÁCIA, como requisito parcial para a obtenção do grau de Doutora em MEDICINA MOLECULAR, à seguinte Comissão Examinadora: Prof(a). Antoniana Ursine Krettli - Orientadora (FIOCRUZ), Prof. Alexandre Guimaraes de Almeida Barros (UFMG), Prof(a). Silvane Maria Fonseca Murta (FIOCRUZ), Prof(a). Caroline Junqueira Giusta (FIOCRUZ), Prof. Rubens Lima do Monte Neto (FIOCRUZ).

A Comissão considerou a tese:

(X) Aprovada

( ) Reprovada

Finalizados os trabalhos, lavrei a presente ata que, lida e aprovada, vai assinada por mim e pelos membros da Comissão.
Belo Horizonte, 27 de janeiro de 2015.

Prof(a). Antoniana Ursine Krettli

Prof. Alexandre Guimaraes de Almeida Barros

Prof(a). Silvane Maria Fonseca Murta

Prof(a). Caroline Junqueira Giusta

Prof. Rubens Lima do Monte Neto

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SUMÁRIO

RESUMO

A malária humana permanece como uma das principais doenças parasitárias do mundo, afetando milhões de pessoas ao ano e causando intensa morbidade e mortalidade, sobretudo em crianças da África. A doença causa ainda pobreza em países com alta prevalência, incluindo o Brasil, na Região Amazônica. Devido à resistência disseminada do *Plasmodium falciparum* aos antimaláricos disponíveis e do *P. vivax* à cloroquina, inclusive no Brasil, novas terapias são necessárias, já que o tratamento medicamentoso permanece como a principal estratégia no controle da doença. A busca de novos fármacos tem como alvo principal os estágios eritrocíticos do *Plasmodium*, mas o interesse em reduzir a transmissão e erradicar a malária inclui combater as formas sexuadas, tornando necessário o desenvolvimento de fármacos contra os gametócitos. No presente trabalho a proposta principal foi a busca por novos compostos com ação dupla, sendo avaliada a atividade esquizonticida sanguínea, *in vitro* e *in vivo*, e bloqueadora da transmissão, *in vitro*. Os compostos foram selecionados por diferentes abordagens: (i) estudos computacionais de modelagem molecular, tendo como alvos específicos as enzimas lactato desidrogenase (atorvastatina, itraconazol e posaconazol) e topoisomerase I (pterocarpanquinona e derivados) do *P. falciparum*; (ii) o conhecimento popular do uso de infusões da planta medicinal *Aspidosperma nitidum* contra a malária na Amazônia Brasileira, e, (iii) o desenvolvimento de compostos híbridos, derivados da ligação do artesunato com mefloquina (MEFAS) ou com primaquina (PRIMAS). Os resultados demonstraram que: (i) atorvastatina, itraconazol e posaconazol, comercialmente disponíveis, e extratos e frações da casca do caule de *A. nitidum*, foram ativos *in vitro* contra *P. falciparum*; (ii) atorvastatina, posaconazol e compostos isolados de *A. nitidum*, inibiram ativamente a parasitemia de camundongos experimentalmente infectados com *P. berghei*, e, (iii) MEFAS e PRIMAS apresentaram atividade dupla, sendo ativos contra as formas assexuadas e contra os gametócitos do *P. falciparum*. Conclui-se que o conhecimento científico gerado neste trabalho poderá contribuir para a formulação de novos fármacos com ação antimalárica dupla e seletiva.

ABSTRACT

The human malaria remains one of the major parasitic diseases in the world, affecting millions of people each year and causing severe morbidity and mortality, especially in children in Africa. The disease causes poverty in countries with high prevalence, including Brazil, in the Amazon Region. Due to the spread of *Plasmodium falciparum* resistance to available antimalarial drugs and of *P. vivax* to chloroquine, including in Brazil, new therapies are needed, as drug treatment remains the main strategy to control the disease. The search for new drugs has the erythrocytic stages of *Plasmodium* as the main target, but the interest in reducing malaria transmission and eradicating the disease includes eliminating sexual forms, which requires the development of drugs against gametocytes. In this study the main proposal was to search for new compounds with dual activity of blood schizonticidal activity evaluated *in vitro* and *in vivo*, as well as transmission blocking activity, *in vitro*. The compound selection for screening was performed by different approaches: (i) *docking* studies having the enzymes as specific targets lactate dehydrogenase (atorvastatin, itraconazole, posaconazole) and topoisomerase I (pterocarpanquinones and derivatives) of *P. falciparum*; (ii) the popular knowledge about the use of infusions of the medicinal plant *Aspidosperma nitidum* against malaria in the Brazilian Amazon, and, (iii) the development of hybrid compounds, derived from artesunate with mefloquine (MEFAS) or primaquine (PRIMAS). The results showed that: (i) the commercially available drugs atorvastatin, itraconazole, posaconazole, and the extracts and fractions of *A. nitidum* stem bark were active *in vitro* against *P. falciparum*; (ii) atorvastatin, posaconazole and the compounds isolated from *A. nitidum*, also inhibited the parasitemia of mice experimentally infected with *P. berghei*, and, (iii) MEFAS and PRIMAS showed a dual activity against *P. falciparum* asexual forms and gametocytes. It can be concluded that scientific knowledge generated in this study may contribute to the development of new drugs with dual and selective antimalarial activity.

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ACT	*Artemisinin Combined Therapy* - Terapia combinada de artemisinina
ART	Artemisinina
AS	Artesunato
ATP	Trifosfato de adenosina
BGM	Linhagem celular de rim de macaco verde
BSA	*Bovine Serum Albumin* - Soro Albumina Bovina
CPqRR	Centro de Pesquisas René Rachou
CQ	Cloroquina
DHA	Diidroartemisinina
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
DND*i*	*Drugs for Neglected Diseases initiative*
EDTA	*Ethylenediaminetetraacetic acid* - Ácido etilenodiamino tetra-acético
ELISA	*Enzyme Linked Immuno Sorbent Assay* - Ensaio imunoenzimático
FIOCRUZ	Fundação Oswaldo Cruz
G6PDd	Glicose-6-fosfato desidrogenase deficiente
HepG2 A16	Linhagem celular de hepatoma humano
HRPII	*Histidine rich protein II* - Proteína rica em Histidina II
*Hss*TopoI	Topoisomerase I humana
IC_{50}	Concentração inibitória de 50% do crescimento do parasito
IS	Índice de seletividade
LDH	Lactato desidrogenase
MDL_{50}	Concentração letal mínima para 50% das células
MEFAS	Artesunato de Mefloquina
MR4	*Methods in Malaria Research*
MTT	Sal tetrazólico 3-(4,5-dimetiltiazol-2-yl)-2,5 difenitetrazol
MQ	Mefloquina
MS	Ministério da Saúde
NADH	Nicotinamida adenina dinucleotídeo
OMS	Organização Mundial da Saúde
*Pf*s25	*Sexual stage antigen of Plasmodium falciparum*

PIB	Produto Interno Bruto
PBS	*Phosphate buffered saline* - Salina tamponada com fosfato
PBS-T	Salina tamponada com fosfato acrescentada de 0,05% de Tween 20
*Pf*LDH	Lactato desidrogenase de *Plasmodium falciparum*
*Pf*TopoI	Topoisomerase I de *Plasmodium falciparum*
PQ	Primaquina
PRIMAS	Artesunato de Primaquina
QN	Quinina
RPMI	*Roswell Park Memorial Institute* - Meio de cultura
SBF	Soro bovino fetal
SVS	Secretaria de Vigilância Sanitária
TMB	3,3′,5,5′-Tetramethylbenzidine
Topo	Topoisomerase
Tween 20	Polioxietileno-sorbitano-monolaurato
UFAM	Universidade Federal do Amazonas
WHO	*World Health Organization*

1. Introdução

A malária humana é uma das doenças parasitárias mais graves e um dos maiores problemas de saúde pública do mundo, sendo endêmica em 97 países nas regiões tropicais e subtropicais. Em 2013 aproximadamente 198 milhões de casos e 584 mil mortes foram relatadas no mundo; cerca de 3,2 bilhões de pessoas estavam sob o risco de infecção, especialmente na África subsaariana (**Figura 1**), onde crianças menores que cinco anos e gestantes são mais gravemente afetadas. Os parasitos causadores da doença pertencem ao gênero *Plasmodium*, sendo cinco espécies responsáveis pela infecção humana: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* e *P. knowlesi*, todas transmitidas pela picada da fêmea do mosquito anofelino infectado (WHO 2014).

A infecção por malária é em geral sintomática, aguda sistêmica febril, mas nas áreas hiperendêmicas, tanto de alta como de média transmissão, ocorre a doença crônica assintomática após anos de exposição (Bloland *et al*., 1999). A gravidade dos casos clínicos na fase aguda é influenciada por vários fatores, sobretudo a espécie do parasito. O *P. falciparum* é responsável pela forma mais grave da doença e pela quase totalidade dos óbitos; o *P. vivax* causa alta morbidade, representando um reservatório de infecção latente, que dificulta o atual controle da malária (Anstey *et al*., 2009).

O *P. vivax*, espécie mais amplamente distribuída no mundo, é responsável por quase 90% dos casos de malária no Brasil e ameaça 40% da população exposta em áreas de risco (Hay *et al*., 2004). Essa espécie é responsável por padrões incomuns de complicações clínicas e casos fatais (Lacerda *et al*., 2012a; WHO 2014). Casos dessa malária grave foram registrados na Índia (Kochar *et al*., 2009), na Indonésia (Tjitra *et al*., 2008) e também no Brasil (Alexandre *et al*., 2010; Lacerda *et al*., 2012b).

O controle da malária é realizado utilizando diferentes medidas: (i) diagnóstico específico e imediato do parasito; (ii) tratamento adequado espécie-específico e imediato com antimaláricos, e, (iii) proteção individual contra a picada de mosquitos, realizada com repelentes e uso de mosquiteiros impregnados com inseticidas (WHO 2014).

O principal método de diagnóstico laboratorial é o exame de microscopia da gota espessa, confeccionado com sangue da polpa digital do paciente e corado com Giemsa, o mais utilizado pelo Ministério da Saúde (MS) (WHO 2012). Existem testes rápidos para diagnóstico do *P. falciparum*, baseados na pesquisa indireta de proteínas específicas do parasito, com a utilização de anticorpos monoclonais contra uma proteína rica em histidina (*Histidine Rich Protein* II ou HRPII), ou contra a enzima lactato desidrogenase (LDH) (WHO

1996). A identificação do DNA do parasito, a partir da reação da cadeia em polimerase, possibilita o diagnóstico dos pacientes assintomáticos, ou com baixas parasitemias (Erdman & Kain, 2008).

Embora diversas vacinas estejam em fase de testes clínicos nas áreas endêmicas (Alonso *et al*., 2005; Herrera *et al*., 2007; Schwartz *et al*., 2012; RTS,S Clinical Trials *et al*., 2012; Reyes-Sandoval *et al*., 2014; Crompton *et al*., 2014), nenhuma delas está disponível. O tratamento medicamentoso permanece como a ferramenta mais importante para impedir a morbidade, o óbito e controlar a malária humana. O surgimento e a disseminação de parasitos resistentes a múltiplos antimaláricos, representam desafios adicionais no controle da doença, estando associados ao aumento da morbidade e da mortalidade nas áreas endêmicas (WHO 2014; Sonko *et al*., 2014).

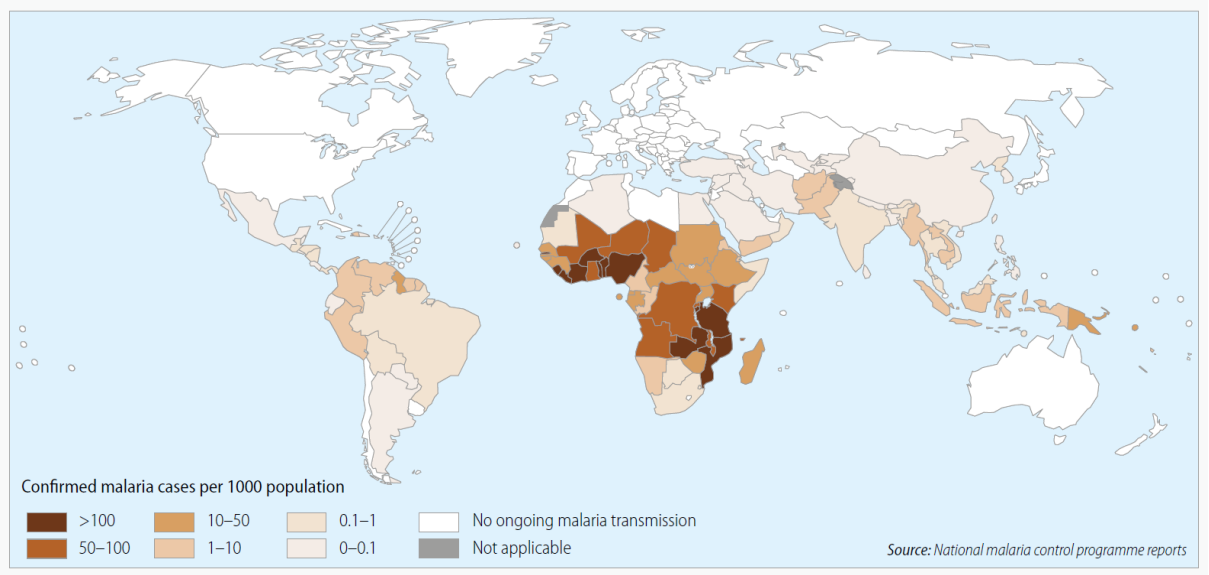


Figura 1- Países com transmissão contínua de malária, 2013. Fonte: *World Malaria Report* 2014. (Organização Mundial da Saúde - OMS).

1.1. A malária no Brasil

No Brasil, três espécies de plasmódio são responsáveis pela malária humana: *P. falciparum*, *P. vivax* e *P. malariae* (Secretaria de Vigilância Sanitária - SVS 2013); 99% dos casos ocorrem na Amazônia Legal (Acre, Amapá, Amazonas, Mato Grosso, Pará, Rondônia, Roraima e Tocantins) (**Figura 2**), onde as condições socioeconômicas e ambientais favorecem a proliferação dos mosquitos anofelinos, os vetores da doença, sendo *Anopheles*

darlingi o principal (WHO 2013a). Em 2013 foram registrados 178 mil casos de malária, sendo aproximadamente 80% causados pelo *P. vivax* (MS 2014).

Na região extra-Amazônica ocorre 1% dos outros casos de malária, importados de áreas endêmicas do Brasil, de outros países das Américas do Sul e Central, da África e da Ásia. Esses casos refletem problemas relacinados à falta de diagnóstico rápido e atraso no tratamento, especialmente dos pacientes com *P. falciparum* (36% dos casos registrados em 2013); consequência do atendimento por profissionais de saúde pouco experientes com os sintomas clínicos da doença, levando ao óbito dos pacientes muitas vezes (Krettli *et al*., 2013).

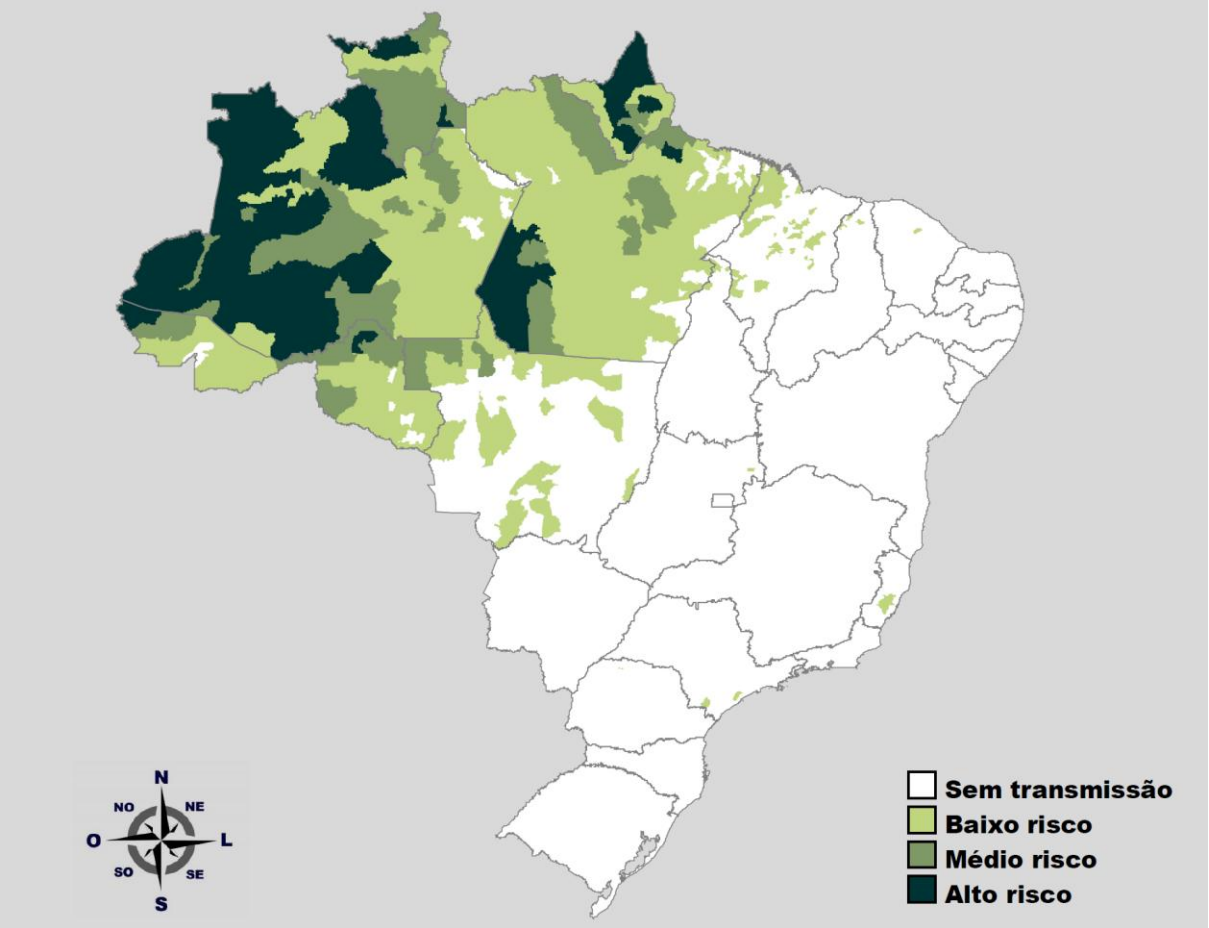


Figura 2- Risco da malária por município de infecção, Brasil, 2013. Fonte: Sinan/SVS/MS e Sivep-Malária/SVS/MS.

1.2. Ciclo de vida do *Plasmodium*

O ciclo biológico dos plasmódios envolve dois hospedeiros: o vertebrado, no qual ocorre a multiplicação assexuada ou esquizogônica, e o inseto vetor, no qual se desenvolve a fase sexuada ou esporogônica (**Figura 3**). A infecção humana inicia quando a fêmea infectada do mosquito *Anopheles* spp., no momento do repasto sanguíneo, inocula esporozoítos sob a epiderme do vertebrado.

Em modelo experimental da malária em camundongos com *P. berghei*, cerca de 10% dos esporozoítos inoculados permanecem na pele, no sítio de inoculação, onde podem se desenvolver e originar merozoítos infectantes (Gueirard *et al*., 2010); a maioria dos esporozoítos evolui no fígado, após migrar e alcançar os hepatócitos e se desenvolverem no vacúolo parasitóforo (Mota *et al*., 2001). Não está claro o mecanismo pelo qual os esporozoítos passam dos capilares sinusoidais do fígado até os hepatócitos, que pode ser através das células de Kupffer ou do endotélio dos vasos sanguíneos (Pradel & Frevert, 2001; Mota *et al*., 2002; Frevert 2004). Utilizando o modelo de malária murina, foi demonstrado que parte dos esporozoítos pode atingir o fígado sem passar pelas células de Kupffer (Gueirard *et al*., 2010).

A primeira demonstração do desenvolvimento dos esporozoítos foi relatada em um voluntário humano inoculado, no qual as formas exoeritrocíticas ou teciduais foram detectadas por microscopia nos hepatócitos, em biópsias obtidas após laparoscopia (Shortt & Garnham, 1948). Posteriormente, nas infecções por *P. vivax* e *P. ovale*, foram ainda evidenciadas formas latentes nos hepatócitos, denominadas hipnozoítos (esporozoítos "dormentes"), aos quais foram atribuídas as recaídas tardias da doença (Krotoski *et al*., 1980).

Nos hepatócitos, após um período de incubação assintomática de cerca de uma a duas semanas, os esporozoítos se diferenciam por esquizogonia e formam os esquizontes maduros, resultando em milhares de merozoítos liberados dos tecidos através de um processo de brotamento de vesículas, os merosomas (Sturm *et al*., 2006). Os merozoítos liberados do fígado invadem as hemácias através do reconhecimento de receptores específicos, ou proteínas de superfície do parasito, como: *Merozoite Surface Protein* ou MSP-1, *Apical Membrane Antigen* 1 ou AMA-1, *Duffy-Binding Protein* ou DBP, entre outras (Wertheimer & Barnwell, 1989; Triglia *et al*., 2000; Goel *et al*., 2003; Deepak *et al*., 2004; Weiss *et al*., 2015).

Os parasitos da fase eritrocítica são os responsáveis pelos sintomas da malária, associados ao momento da liberação dos novos merozoítos das hemácias parasitadas pelos

esquizontes maduros (Meis & Verhave, 1988). O principal sintoma da infecção é a febre periódica elevada, geralmente em múltiplos de 24 horas, variando entre as espécies causadoras da doença, num processo sincronizado iniciado após a invasão de novas células, aparentemente mediado pela melatonina (Hotta *et al*., 2000). No caso do *P. falciparum*, o amadurecimento das formas sanguíneas ocorre nos capilares profundos, em concentrações mais baixas de oxigênio. Esse conhecimento possibilitou o cultivo contínuo do ciclo eritrocítico dos parasitos, descrito por Trager & Jensen, (1976).

Alguns merozoítos sanguíneos se diferenciam em gametócitos masculinos e femininos, as formas sexuadas, essenciais para a infecção do mosquito vetor e da transmissão ao hospedeiro vertebrado. Os mecanismos envolvidos na produção dos gametócitos não estão bem descritos, mas recentemente foi demonstrada no *P. berghei* uma cascata de proteínas, ApiP2, que se ligam ao DNA, como essenciais à produção e maturação de gametócitos (Sinha *et al*., 2014).

Os gametócitos maduros (estágio V) são as formas evolutivas responsáveis pela produção dos parasitos do ciclo sexuado ou esporogônico, quando ingeridos pelo mosquito suscetível (Sinden 1983; Baton & Ranford-Cartwright, 2004). Minutos após o repasto sanguíneo pelo inseto vetor, no lúmen do intestino médio, ocorre a exflagelação do gametócito masculino (ou microgametócito) com a liberação de oito microgametas, e a diferenciação do gametócito feminino em macrogameta. Esses eventos são regulados por fatores como diminuição da temperatura do sangue ingerido pelo mosquito, aumento do pH e exposição dos parasitos ao ácido xanturênico, um subproduto do metabolismo do triptofano (Billker *et al*., 1998). A fusão dos gametas masculino e feminino forma o zigoto, que se torna uma estrutura móvel denominada oocineto, o qual posteriormente atravessa a membrana peritrófica e se aloja entre o epitélio do intestino médio e a lâmina basal, onde evolui (Meis *et al*., 1989). Cada oocineto forma um oocisto e o parasito se multiplica por esporogonia formando milhares de esporozoítos, que migram para a hemolinfa (Meis *et al*., 1992), invadem as glândulas salivares do mosquito através de interações específicas entre receptores do parasito e ligantes presentes na superfície da glândula salivar, como a *Circumsporozoite Protein* ou CSP (Touray *et al*., 1992) e a *Thrombospondin-Related Anonymous Protein* ou TRAP (Vlachou *et al*., 2006). Quando inoculados no hospedeiro vertebrado, os esporozoítas iniciam um novo ciclo evolutivo.

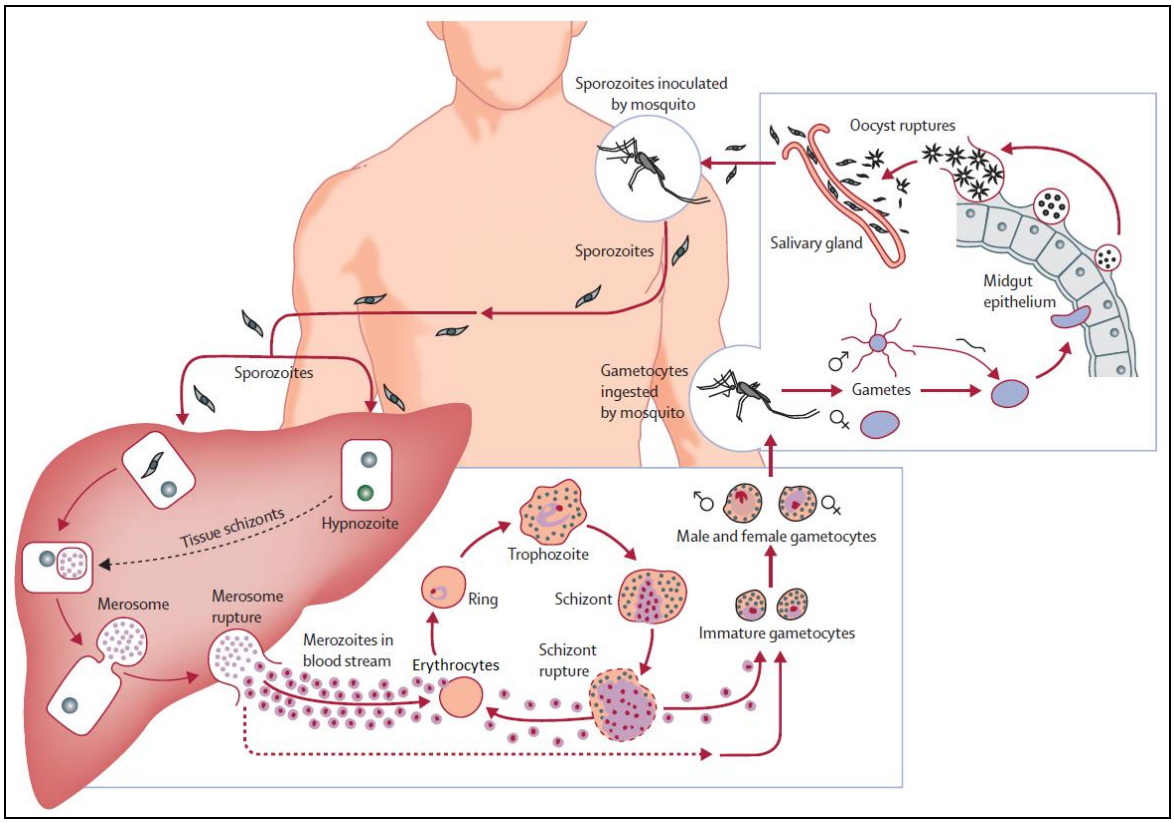


Figura 3- Esquema do ciclo biológico dos plasmódios. Adaptada de Mueller *et al*., 2009.

1.3. Tratamento farmacológico da malária

O tratamento medicamentoso da malária é espécie-específico e tem como principais objetivos: (i) reduzir a morbidade e a mortalidade, devendo ser realizado rapidamente após o diagnóstico; (ii) garantir a cura completa da infecção, prevenindo a progressão da doença não complicada para forma grave e potencialmente fatal, no caso do *P. falciparum*; (iii) reduzir o reservatório do parasito humano, diminuindo a transmissão da doença; e, (iv) evitar o surgimento e a disseminação da resistência aos antimaláricos (WHO 2013b).

Os antimaláricos disponíveis compreendem quinolinas, aminoálcoois, sulfonamidas e sulfonas, biguanidas, diaminopirimidinas, lactonas sesquiterpênicas (artemisinina e seus derivados semissintéticos), naftoquinonas e antibióticos (WHO 2010a). Os principais antimaláricos estão listados na **Tabela 1**.

Tabela 1- Principais antimaláricos disponíveis e seus estágios-alvo no parasito.

Antimalárico	Classificação química	Estágios-alvo no parasito			
		FEE[a]	HP[b]	RBC[c]	GM[d]
Amodiaquina	4-aminoquinolina			*	
Arteméter	Endoperóxido semissintético			*	*
Artesunato	Endoperóxido semissintético			*	*
Atovaquona	Naftoquinona	*		*	*
Azitromicina	Antibiótico macrocíclico			*	
Bulaquina[e]	8-aminoquinolina				*
Cloroquina	4-aminoquinolina			*	
Clorproguanil	Biguanidas	*		*	*
Clindamicina	Antibiótico derivado das lincosamidas	*		*	
Dihidroartemisinina	Endoperóxido semissintético			*	*
Doxiciclina	Antibiótico derivado da tetraciclina	*		*	
Lumefantrina	Aminoálcool			*	
Mefloquina	Aminoálcool			*	*
Piperaquina	Bisquinolina			*	
Pirimetamina	Diaminopirimidina	*		*	*
Primaquina	8-aminoquinolina	*	*	*	*
Proguanil	Biguanidas	*		*	*
Quinina	Aminoálcool			*	
Sulfadoxina	Sulfonamida			*	*
Tafenoquina[f]	8-aminoquinolina		*		
Tetraciclina	Antibiótico	*		*	

[a]FEE: formas exoeritrocíticas; [b]HP: hipnozoítos; [c]RBC: formas eritrocíticas; [d]GM: gametócitos, formas sexuadas; [e]Licenciada apenas na Índia; [f]Em fase de ensaios clínicos.
Tabela adaptada de Aguiar *et al*., 2012a.

A descoberta do primeiro tratamento da malária foi feita há quase 400 anos, portanto, muito antes de ser conhecido o seu agente etiológico. A observação de que pacientes com uma doença febril aguda podiam ser curados após ingestão de infusões de cascas de plantas da Amazônia Peruana (Garnham 1966), rapidamente levou ao uso das espécies *Cinchona calisaya* e *C. succirubra*. Anos depois, em 1820, foi caracterizado por químicos franceses, o

alcaloide responsável pela cura dos pacientes, denominado quinina (QN). Apesar da toxicidade quando utilizada por longos períodos de tempo, a QN permanece sendo utilizada no tratamento da malária complicada pelo *P. falciparum* (WHO 2010a, b) e, mais recentemente, em pacientes com *P. falciparum* resistente aos derivados de ART (Van Hong *et al*., 2014).

A partir do anel quinolínico da QN, diversos antimaláricos sintéticos foram desenvolvidos, as 4- e 8-aminoquinolinas. Dentre eles, a cloroquina (CQ), antimalárico de baixa toxicidade, reduzido custo de produção e bem tolerado entre adultos e crianças, foi utilizada mundialmente durante décadas e motivou a campanha de erradicação da malária, iniciada em 1955. A CQ foi utilizada como monoterapia no tratamento de pacientes e na profilaxia da doença em áreas endêmicas na forma de sal cloroquinado na Região Amazônica Brasileira até a década de 1960 (Sweeney 2000), quando surgiu a resistência do *P. falciparum* à CQ. A campanha de erradicação foi interrompida, mas o uso desse fármaco reduziu drasticamente a doença (Rosenthal & Miller, 2001), sendo ainda hoje utilizada em combinações com outros antimaláricos. A CQ ainda é utilizada como monoterapia no tratamento da doença causada pelo *P. vivax* em áreas onde não ocorre resistência.

A mefloquina (MQ), um 4-quinolinometanol de efeito esquizonticida sanguíneo, foi sintetizada também a partir do anel quinolínico, desenvolvida por pesquisadores americanos. Muito empregada no tratamento da malária a partir dos anos 80 (Palmer *et al*., 1993), a MQ era mais ativa que a CQ e utilizada em dose única, mas se mostrou tóxica, ocasionando efeitos colaterais graves, inclusive sintomas neurológicos (Patchen *et al*., 1989). Por ter meia-vida prolongada, o uso da MQ em larga escala potencializa a seleção rápida de parasitos resistentes. Atualmente, a MQ é recomendada somente em combinação com outros compostos, sobretudo com derivados de artemisinina, no tratamento do *P. falciparum* multi-resistente (WHO 2006).

A primaquina (PQ), uma 8-aminoquinolina, por ser ativa contra os hipnozoítos hepáticos, é utilizada na prevenção de recaídas tardias, frequentes na malária causada pelo *P. vivax* (Wells *et al*., 2010). Apesar de não afetar os esporozoítos e possuir baixa atividade contra o estágio eritrocítico do parasito, a PQ possui também ação gametocitocida, sendo utilizada como bloqueadora da transmissão da doença humana (Brueckner *et al*., 2001).

A ART, uma lactona sesquiterpênica, é um produto natural isolado da planta *Artemisia annua*, caracterizada e utilizada contra a malária humana por pesquisadores chineses, sendo dotada de intensa e rápida atividade (WHO 2006). Modificada quimicamente por ser pouco

solúvel, originou diversos fármacos, como o arteméter, arteéter e artesunato (AS), seus derivados semi-sintéticos (Li & Wu, 2003).

Atualmente, a OMS recomenda a *Artemisin-Combined Therapy* (ACT) no tratamento do *P. falciparum* resistente, uma combinação de derivados da ART com outro antimalárico (WHO 2010b). A escolha do esquema de tratamento é baseada na eficácia terapêutica da combinação na área onde se pretende utilizá-la. O tratamento baseado em associações medicamentosas visa dificultar o aparecimento de cepas de plasmódios resistentes aos antimaláricos, reduzir o tempo de tratamento do paciente, aumentando sua adesão e promovendo a cura da infecção de maneira eficiente (WHO 2008). Mesmo a malária não complicada, causada pelo *P. falciparum*, deve ser tratada com uma das ACTs recomendadas pela OMS. Os fármacos comumente utilizados em associações com derivados de ART são lumefantrina, amodiaquina, MQ, sulfadoxina-pirimetamina e antibióticos. Malária grave por *P. falciparum* deve ser tratada com AS injetável, seguido pelo tratamento completo com uma efetiva ACT, assim que o paciente puder tomar medicamentos por via oral (WHO 2013b).

A malária causada pelo *P. vivax* deve ser tratada com CQ em áreas onde o fármaco ainda é eficaz. Em áreas onde a resistência do *P. vivax* à CQ já foi relatada, uma apropriada ACT deve ser utilizada. Para evitar recaídas tardias e garantir a cura radical da malária por *P. vivax*, a CQ e as ACTs devem ser combinadas à PQ, administrada durante 14 dias (WHO 2013b). Entretanto, os metabólitos da PQ podem causar anemia hemolítica grave em pacientes geneticamente deficientes de glicose-6-fosfato desidrogenase (G6PDd) (Carmona-Fonseca *et al*., 2009; Ashley *et al*., 2014a). Este efeito secundário exige uma pré-triagem para G6PDd em pacientes com malária por *P. vivax*, limitando assim o uso da PQ (SVS 2010; Baird & Surjadjaja, 2011). Em áreas visando a eliminação do *P. falciparum* e onde a PQ ainda não está implantada como gametocitocida, é recomendada uma única dose de 0,25mg/kg de PQ no primeiro dia de tratamento com ACT, sem a necessidade de teste para pacientes G6PDd (WHO 2006).

No Brasil, a primeira opção de tratamento para a malária falciparum é a combinação de arteméter (480mg/dia) e lumefantrina (2880mg/dia) por quatro dias, associada a PQ em dose única (45mg) para evitar a transmissão. No caso da malária vivax não complicada, a CQ é ainda o medicamento de escolha (1500mg/dia, durante três dias), em associação com a PQ (210mg/dia, durante sete dias), para evitar as recaídas tardias da doença (MS 2010).

1.4. Resistência aos antimaláricos

A resistência aos fármacos ocorre como consequência de fatores como más práticas de tratamento, inadequada adesão do paciente aos tratamentos prescritos, má qualidade dos medicamentos, entre outros (WHO 2013b). Apesar dos antimaláricos disponíveis, os índices de morbidade e mortalidade da malária estão aumentando, principalmente devido à disseminada resistência dos parasitos aos mesmos.

O primeiro relato do *P. falciparum* resistente à CQ ocorreu nos anos 60, na Colômbia, no vale do Rio Magdalena, seguido de outros no sudeste da Ásia. Os casos de parasitos resistentes aos antimaláricos continuaram crescendo e se disseminaram na maioria dos países endêmicos (Ridley 2002; Wongsrichanalai *et al*., 2002). Marcadores moleculares associados a resistência à CQ estão relacionados ao gene transportador *multi-drug resistance* 1 ou *mdr*1, codificador de uma proteína expressa na membrana do vacúolo digestivo do parasito ao longo do seu desenvolvimento intraeritrocítico (Cowman *et al*., 1991). Outro gene relacionado a resistência à CQ, *chloroquine resistance transporter* ou *crt*, codifica uma proteína de transporte também expressa na membrana do vacúolo digestivo, relacionada com o acúmulo de CQ e com a conversão da hematina solúvel em hemozoína (Bray *et al*., 1998).

Em 2008 foram relatados os primeiros casos de falha terapêutica às ACTs em casos de malária causada pelo *P. falciparum* (Noedl *et al*., 2008). Essa resistência pode ser definida como: (i) aumento no tempo da eliminação do parasito, evidenciado em ≥10% de casos com parasitos detectáveis no terceiro dia após o tratamento com ACT, e, (ii) insucesso do tratamento, evidenciado pela persistência de parasitos por sete dias e recrudescência dentro de 28-42 dias (WHO 2013b). Essa resistência foi recentemente associada a mutações pontuais na proteína "kelch" (kelch13) (Ariey *et al*., 2014; Miotto *et al*., 2015; Taylor *et al*., 2015).

A resistência do *P. falciparum* à ART encontra-se disseminada no sudeste e sudoeste Asiático (Miotto *et al*., 2013) e foi detectada no oeste da África (Ashley *et al*., 2014b). Recentemente, casos graves de malária refratários ao tratamento com os derivados de ART foram relatados em pacientes procedentes de Angola (Van Hong *et al*., 2014). Caso a resistência à ART se espalhe para a Índia ou na África subsaariana, as consequências globais podem ser desastrosas. Nenhum medicamento antimalárico alternativo com o mesmo nível de eficácia e tolerabilidade como ACTs encontra-se disponível (Ashley *et al*., 2014b). Novos antimaláricos tem sido desenvolvidos, mas o tempo gasto para isso pode ser mais longo do que aquele necessário à seleção de parasitos resistentes aos compostos disponíveis (Bloland 2001).

Casos de malária pelo *P. vivax* com reduzida sensibilidade à CQ, documentados inicialmente na Papua Nova Guiné em 1989 (Rieckmann *et al*., 1989), estão distribuídos no mundo, em regiões da Indonésia (Ratcliff *et al*., 2007; Sutanto *et al*., 2009), Myanmar, Corea do Sul, Vietinan, Índia, Turquia, Etiópia e regiões do sudoeste da África e América do Sul (Suwanarusk *et al*., 2007; Price *et al*., 2009; Gonçalves *et al*., 2014), inclusive no Brasil (Baird 2004; de Santana Filho *et al*., 2007; Marques *et al*., 2013).

1.5. Busca de antimaláricos esquizonticidas sanguíneos

1.5.1. Reposicionamento de fármacos

A estratégia do reposicionamento de fármacos, designado pela utilização daqueles já aprovados para uso humano e empregados no tratamento de outras doenças, pode levar à identificação de novos alvos farmacológicos com novas indicações terapêuticas, acelerando a descoberta de novos antimaláricos. Nessa busca, o sinergismo entre fármacos pode ser utilizado através de combinações terapêuticas (Borisy *et al*., 2003). Como exemplo, a atorvastatina, testada e ativa *in vitro* contra *P. falciparum* em associação com outros antimaláricos, apresentou efeitos sinérgicos (Parquet *et al*., 2010; Dormoi *et al*., 2014).

O reposicionamento de fármacos é uma estratégia financeiramente interessante, inclusive para a indústria farmacêutica. Várias etapas do desenvolvimento e lançamento de novos fármacos foram cumpridas, tornando esse tipo de pesquisa significativamente mais eficaz em termos de custos, tempo e esforços. Abordagens *in silico* podem ser utilizadas, juntamente com métodos *in vitro*, para o reposicionamento de fármacos (Ekins *et al*., 2011). Estudos computacionais tem sido utilizados para avaliar a interação entre fármacos já comercializados e novos alvos antiplasmodiais, com base na estrutura e na energia de ligação entre eles (Cockell *et al*., 2010).

1.5.2. Modelagem molecular e novos alvos farmacológicos

Programas computacionais de modelagem molecular são ferramentas atuais utilizadas na descoberta e no planejamento de novos fármacos, pois permitem predizer o modo de ação, a afinidade da ligação entre molécula e o sítio ativo (proteínas e/ou enzimas), gerando informações teóricas que sugerem possíveis mecanismos de ação (Huang & Zou, 2010).

A busca de novos alvos moleculares para o desenvolvimento de fármacos tem sido conduzida como estratégia para combater a resistência dos parasitos da malária humana aos antimaláricos atualmente disponíveis. Mecanismos envolvendo a inibição de enzimas específicas do parasito podem contribuir para a potencialização do efeito dos fármacos. A interação dos fármacos com receptores com atividade enzimática leva à inibição da ação da enzima, seja pela ligação direta do fármaco no sítio ativo onde uma transformação enzimática é catalisada, ou pelo impedimento da ligação de substâncias endógenas nos seus receptores (Golan *et al*., 2008). Neste trabalho, as enzimas alvo do *P. falciparum* exploradas foram a lactato desidrogenase e a topoisomerase I.

1.5.2.1. Lactato desidrogenase do *P. falciparum* (*Pf*LDH)

A CQ, fármaco muito utilizado no tratamento da malária no século XX, interage com o sítio ativo da LDH, enzima fundamental para a sobrevivência do parasito (Cameron *et al*., 2004). Embora o principal mecanismo de ação da CQ proposto na literatura seja a inibição da polimerização da hemozoína, foi sugerido que a sua atividade é potencializada pela inibição da *Pf*LDH (Egan & Ncokazi, 2005). Na fase eritrocítica do ciclo, o plasmódio necessita de constante suprimento de energia e, para isso, degrada a hemoglobina do hospedeiro em aminoácidos essenciais. A hematina, produzida nesse processo, é um forte inibidor da *Pf*LDH (Read *et al*., 1999). Além disso, a enzima LDH atua na fase final do processo glicolítico, convertendo piruvato em lactato, sendo o NADH, cofator da enzima, essencial para essa conversão.

A *Pf*LDH tem um sítio ativo bastante distinto ao da LDH humana, sugerindo que fármacos inibidores da enzima plasmodial sejam inibidores seletivos do parasito. Estudos de ancoramento molecular de potenciais inibidores da *Pf*LDH foram realizados em compostos estruturalmente semelhantes ao NADH, propondo sua inibição competitiva. Um análogo ao NADH poderia ter uma conformação energética estável no sítio ativo da enzima, competindo pelo sítio de interação do cofator e, portanto, inibindo a ação da LDH.

1.5.2.2. Topoisomerase I do *P. falciparum* (*Pf*TopoI)

A replicação exata e a segregação do DNA envolvem numerosas etapas, muitas das quais podem constituir alvos apropriados para os fármacos. Até o momento, as enzimas

utilizadas como alvo com maior sucesso nesse processo são as topoisomerases, enzimas envolvidas na replicação, transcrição, recombinação e reparo do DNA. Existem dois tipos de topoisomerases: as do tipo I (TopoI) formam e reúnem quebras de fita simples no DNA e as do tipo II (TopoII) executam funções de nuclease e ligase em ambas as fitas do DNA (Golan *et al*., 2008).

Diversos agentes quimioterápicos provocam lesão do DNA ao explorar a função natural das topoisomerases; esses fármacos interferem na função da enzima, induzindo a destruição do DNA (Golan *et al*., 2008). Considerando-se que a enzima TopoI humana (*Hss*TopoI) apresenta 42% de similaridade com a enzima *Pf*TopoI (Reguera *et al*., 2006), a enzima plasmodial representa um alvo molecular potencial e seletivo contra a malária no desenvolvimento de novos fármacos.

1.5.3. Etnofarmacologia e plantas medicinais

Os produtos naturais são utilizados há milênios na busca pelo alívio e cura de doenças. A ingestão de ervas e folhas com esse objetivo talvez tenha sido uma das primeiras formas de utilização de plantas, especialmente na medicina tradicional chinesa. Até hoje muitas espécies e preparados vegetais medicinais são estudados com esse fim e na busca pelo entendimento de seu mecanismo de ação, visando o isolamento dos princípios ativos (Viegas *et al*., 2006). Dois compostos ativos derivados de plantas medicinais foram descobertos para o tratamento da malária humana: (i) QN, alcaloide isolado das espécies *C. calisaya* e *C. succirubra*, utilizada há mais de 350 anos no tratamento da malária (Garnham 1966; Meshnick 1997; Meshnick & Dobson, 2001), muito ativa mesmo contra os parasitos resistentes aos antimaláricos sintéticos derivados desse alcaloide (Ridley 2002), e, (ii) ART, uma lactona sesquiterpênica isolada da planta medicinal *A. annua*, de uso milenar (Klayman 1985). Ambas foram utilizadas no desenvolvimento de novos fármacos, sintéticos e semi-sintéticos, utilizados nas combinações terapêuticas preconizadas pela OMS (WHO 2013b).

Grande parte da população dos países em desenvolvimento ainda depende da medicina tradicional para sua atenção primária, especialmente no continente Africano (WHO 2002). Mais de 80% dessa população utiliza práticas tradicionais nos seus cuidados básicos de saúde, sendo muitas espécies vegetais reconhecidamente utilizadas contra a malária (Adebayo & Krettli, 2010). No Brasil, na Região Amazônica, a dificuldade de acesso aos fármacos antimaláricos leva ao uso de recursos naturais e plantas medicinais no tratamento de muitas

doenças (Oliveira *et al*., 2003). Além disso, nas últimas décadas, o interesse populacional pelas terapias naturais tem aumentado significativamente, mesmo nos países industrializados, onde os produtos naturais recuperam espaço e importância no desenvolvimento de novos fármacos, *per-se* ou como fonte de novos padrões moleculares bioativos (Viegas *et al*., 2006).

A busca por novos fármacos derivados de plantas medicinais é uma estratégia considerada promissora, menos dispendiosa e muito importante no controle da malária (Krettli *et al*., 2001; Krettli 2009). Responsável por perdas econômicas e sociais das sociedades mais acometidas pela doença (Sachs & Malaney, 2002), a malária continua causando empobrecimento na África, na Ásia e em algumas regiões da Amazônia, além de dezenas de óbitos (Junior *et al*., 2014; Sonko *et al*., 2014).

Recentes programas multidisciplinares visando a descoberta de novos antimaláricos a partir de plantas medicinais representam uma importante alternativa a ser melhor explorada, especialmente na Amazônia Brasileira, onde existe uma exuberante flora (Krettli *et al*., 2001) e onde diversas espécies de plantas são utilizadas no tratamento de enfermidades (Oliveira *et al*., 2003). Através da coleta de informações dessas populações, residentes nativos ou migrantes da Amazônia Brasileira, estudos etnofarmacológicos permitiram a seleção de dezenas de plantas utilizadas popularmente contra a malária humana, com comprovada atividade antimalárica experimental (Brandão *et al*., 1992; Carvalho *et al*., 1992; Krettli *et al*., 2001).

O gênero *Aspidosperma* (Apocynaceae) é comumente utilizado na Amazônia por suas diferentes propriedades medicinais (Weniger *et al*., 2001; Ferreira *et al*., 2004), sendo a espécie *A. nitidum* Benth., "carapanaúba", a mais citada nas entrevistas realizadas com mais de oito mil habitantes, para o tratamento da malária. Nesses inquéritos conduzidos pelo nosso grupo e por outros, além de *A. nitidum*, foram citadas as espécies *A. album*, *A. discolor*, *A. excelsum* e *A. polineuron*, também utilizadas como antimaláricas (Brandão *et al*., 1992; de Oliveira *et al*., 2011).

1.6. Busca de antimaláricos bloqueadores da transmissão

Um fármaco ideal para o tratamento da malária deve ser ativo contra todas as formas evolutivas do parasito, sendo capaz de eliminar a infecção aguda sintomática causada pelas formas sanguíneas, bloquear a transmissão da doença e evitar as recaídas tardias causadas pelo *P. vivax*, eliminando os hipnozoítos. Reduzir a transmissão e impedir a propagação dos parasitos resistentes é atualmente um dos principais objetivos para o controle da malária,

sendo a eliminação dos gametócitos, as formas sexuadas do parasito, uma estratégia essencial (Abdul-Ghani *et al*., 2014).

A descoberta de novos fármacos efetivos no tratamento da malária envolve esforços concentrados nas formas assexuadas do parasito, responsáveis pelos sintomas agudos da doença (Rosenthal 2003; Roll Back Malaria 2008; Gamo *et al*., 2010), inclusive nosso grupo (Penna-Coutinho *et al*., 2011; Aguiar *et al*., 2012b; Penna-Coutinho *et al*., 2013; de Souza *et al*, 2014a, de Souza *et al*., 2014b). As possibilidades de controle terapêutico visam também o estágio sexuado do plasmódio (gametócito), responsável pela transmissão da malária do hospedeiro vertebrado para o mosquito vetor. Os gametócitos maduros (estágio V), sendo os únicos estágios do parasito infectantes para os mosquitos, representam um elo fundamental no bloqueio da transmissão do parasito pelo vetor. Eliminar ou comprometer sua viabilidade irá impedir o ciclo do parasito e a infecção de novos indivíduos e, consequentemente, reduzir a prevalência da malária (Delves *et al*., 2013).

1.6.1. Gametócitos

Os parasitos assexuados dos plasmódios originam gametócitos em baixa frequência (0,2 a 1%), e merozoítos sexualmente comprometidos provenientes de um esquizonte pré-comprometido, formam gametócitos do mesmo sexo (Sinden 1983). Os gametócitos do *P. falciparum*, desenvolvem-se lentamente ao longo de um período de 10-14 dias, ao contrário dos do *P. vivax*, se diferenciam em cinco estágios morfológicos de maturação (I-V) (Hawking *et al*., 1971) (**Figura 4**). Tanto os eritrócitos invadidos pelas formas maduras assexuadas, como aqueles invadidos pelos gametócitos imaturos (estágios I-III), são sequestrados no baço e na medula óssea, devido à expressão de novas proteínas na superfície das hemácias e à rigidez da membrana do eritrócito parasitado (Tiburcio *et al*., 2012). A única forma circulante infectante para os mosquitos vetores são os gametócitos maduros (estágio V) (Sinden 1983).

A susceptibilidade dos gametócitos aos antimaláricos varia durante o processo de desenvolvimento. Os gametócitos jovens (estágios I-III) são susceptíveis aos antimaláricos esquizonticidas sanguíneos, como a CQ, pirimetamina, ART e seus derivados (Chutmongkonkul *et al*., 1992; Smalley 1977; Delves *et al*., 2012). No entanto, nos quatro últimos dias do processo de gametocitogênese, os gametócitos do *P. falciparum* entram em um processo de repouso (parada do ciclo celular) por um período máximo de 22 dias. Ainda que infectantes para o mosquito vetor, os gametócitos maduros (estágios IV-V) são insensíveis à maioria dos esquizonticidas (Chutmongkonkul *et al*., 1992, Smalley 1977),

exceto à PQ, um esquizonticida tecidual (Burgess & Bray, 1961; Smalley 1977; Butcher 1997; Adjalley *et al*., 2011).

Algumas diferenças ainda podem ser observadas entre as espécies: no *P. falciparum* os gametócitos podem sobreviver mais tempo do que as fases assexuadas (Butcher 1997), tornando mais difícil sua eliminação. No *P. vivax* os gametócitos aparecem na corrente sanguínea simultaneamente com as formas assexuadas, sugerindo que os esquizontes hepáticos podem estar envolvidos nesse processo (Baker 2010).

Os gametócitos do *P. falciparum* são o único estágio de transmissão produzido em grande escala *in vitro* (D'Alessandro *et al*., 2013; Lelièvre *et al*., 2012; Tanaka & Williamson, 2011). Por isso, mesmo que a eliminação farmacológica desses parasitos seja complicada, é possível se estudar o bloqueio da transmissão da malária utilizando os gametócitos como alvo.

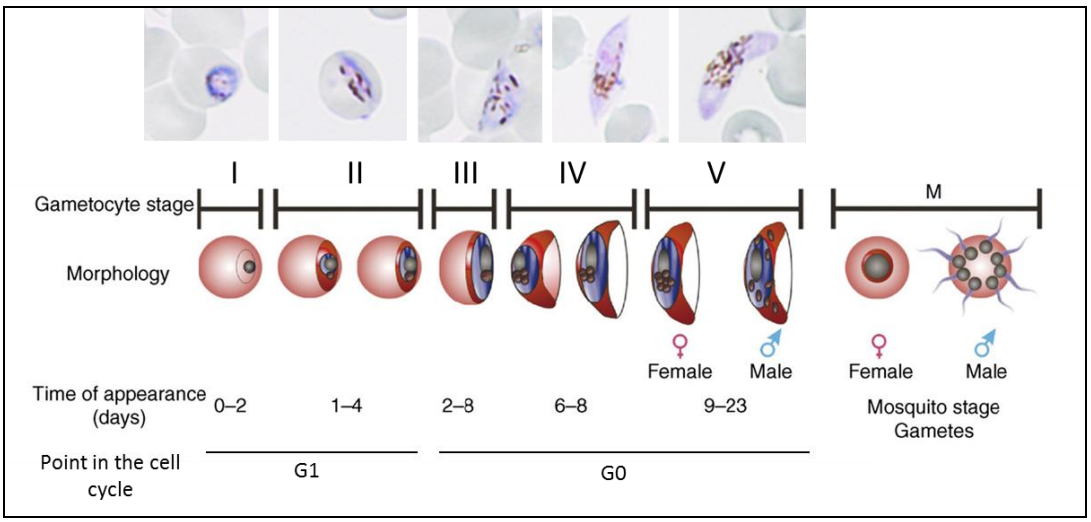


Figura 4- Processo de gametocitogênese do *Plasmodium falciparum*. Adaptada de Dixon *et al*., 2008.

1.6.2. Moléculas híbridas

Os compostos multicompetentes, híbridos provenientes da ligação entre duas ou mais substâncias, resultando em uma única molécula, podem apresentar características favoráveis ao tratamento da malária, como por exemplo: (i) sinergismo entre os componentes; (ii) reduzir o risco de interações medicamentosas; (iii) tornar o esquema terapêutico mais simples, melhorando a adesão ao tratamento; (iv) melhorar a farmacocinética individual e a

estabilidade, e, (v) reduzir a toxicidade dos fármacos de origem (Morphy & Rankovic, 2005; Walsh & Bell, 2009).

Os derivados da ART apresentam ação rápida contra todos os estágios sanguíneos do parasito, assexuados e também os gametócitos. No entanto, por terem meia-vida curta, um tratamento longo (sete dias) com esses fármacos é necessário para sua ação e, por isso, são indicados apenas em associações medicamentosas (ACTs) com fármacos de meia-vida longa, especialmente a MQ. Além de diminuir o tempo de tratamento e aumentar a adesão individual, as ACTs reduzem as chances de seleção da resistência medicamentosa (WHO 2010a).

A combinação de AS com MQ é a mais empregada no combate do *P. falciparum* resistente à CQ (Meshnick 2002); entretanto, o alto custo dessa combinação representa um problema para muitos países onde a malária é endêmica (Meunier 2008). A combinação em dose fixa de AS e MQ (1:2) é produzida no Brasil, por Farmanguinhos (Instituto de Tecnologia em Fármacos, Fundação Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brasil), e disponibilizada no Brasil e na África a baixo custo, em um projeto liderado pelos "médicos sem fronteira" (DNDi 2007). Uma nova molécula, híbrida do AS e da MQ (MEFAS), foi sintetizada e patenteada em Farmanguinhos (N. Boechat *et al*., *international patent application* WO 2005/100370 A1) após ser testada pelo nosso grupo. Nos ensaios, MEFAS se mostrou mais ativa que a combinação entre a MQ e o AS (1:1) *in vitro* contra *P. falciparum* resistente à CQ (W2) e sensível à CQ (3D7), e apresentou atividade curativa da malária em camundongos infectados com *P. berghei* (de Pilla Varotti *et al*., 2008).

A PQ é único fármaco utilizado no Brasil para bloquear a transmissão da malária (SVS 2010), mas como seus metabólitos podem causar anemia hemolítica grave em pacientes G6PDd (Carmona-Fonseca *et al*., 2009), seu uso requer supervisão médica (SVS 2010), sendo limitado em áreas onde existe elevada prevalência de G6PDd (Baird & Surjadjaja, 2011). Alguns fármacos bloqueadores da transmissão da malária tem sido desenvolvidos, alguns em fase de ensaios clínicos, como a Bulaquina, menos tóxica do que a PQ em pacientes G6PDd (Krudsood *et al*., 2008), e com atividade gametocitocida mais rápida que a PQ (Puri & Dutta, 2005).

PRIMAS, sal híbrido derivado da ligação entre o AS e a PQ, foi também sintetizado pelo grupo da Dra. Núbia Boechat, Farmanguinhos, com o objetivo de se reduzir a toxicidade e de melhorar a atividade inibidora da transmissão da malária pela PQ.

2. Justificativa

O controle da malária depende essencialmente do tratamento medicamentoso dos pacientes sintomáticos na fase aguda. Um dos maiores obstáculos ainda é a resistência do *P. falciparum* aos antimaláricos disponíveis, inclusive aos derivados de ART (Noedl *et al*., 2008; Dondorp *et al*., 2009; Phyo *et al*., 2012; Miotto *et al*., 2013; Ashley *et al*., 2014b). Outro problema é o *P. vivax* resistente à CQ, inclusive no Brasil (Baird 2004; de Santana Filho *et al*., 2007; Price *et al*., 2009; Graf *et al*., 2012; Marques *et al*., 2013). Descobrir e desenvolver novos fármacos, ativos contra as diversas formas evolutivas do parasito, além de aumentar as opções de antimaláricos disponíveis, ajudaria a controlar melhor a doença, hoje com poucas alternativas terapêuticas, além das ACTs.

Várias abordagens tem sido propostas visando melhorar o tratamento da malária, sendo as mais importantes: (i) otimizar terapias com fármacos existentes, com novas dosagens e/ou em combinações medicamentosas; (ii) desenvolver novos análogos de fármacos existentes; (iii) estudar a atividade antimalárica de compostos ativos contra outras doenças; (iv) buscar compostos ativos contra alvos específicos do parasito; (v) identificar compostos derivados de produtos naturais ativos contra malária, e, (vi) buscar fármacos ativos contra as diversas formas evolutivas do parasito (Rosenthal & Miller, 2001).

Dada a experiência do nosso grupo de pesquisa em estudar a atividade antimalárica de novos fármacos (de Pilla Varotti *et al*., 2008; Aguiar *et al*., 2012b; de Souza *et al*., 2014a; de Souza *et al*., 2014b), mais recentemente propusemos buscar um antimalárico ideal, de produção mais fácil e a custos baixos em comparação com os derivados de ART (revisto em Krettli 2009; Krettli *et al*., 2009; Aguiar *et al*., 2012a). Poucas classes de antimaláricos foram introduzidas na prática clínica nas últimas décadas (Ekland & Fidock, 2008) pelas dificuldades intrínsecas na descoberta e no desenvolvimento de novos fármacos. A estratégia de analisar medicamentos disponíveis no mercado farmacêutico aprovados para uso humano no tratamento de outras doenças teria uma aplicabilidade imediata se também fosse útil no controle da malária.

A proposta deste projeto foi identificar novos compostos esquizonticidas sanguíneos e avaliar a atividade gametocitocida de moléculas ativas contra os estágios sanguíneos assexuados do *P. falciparum*, o caso do híbrido MEFAS (de Pilla Varotti *et al*., 2008). Considerando que a luta contra a malária tem focalizado na sua erradicação, buscou-se identificar fármacos capazes de interromper a transmissão para os mosquitos vetores, com atividade gametocitocida secundária à atividade esquizonticida sanguínea.

3. Objetivos

3.1. Objetivo geral

Identificar novos antimaláricos utilizando estratégias de reposicionamento de fármacos e prospecção de moléculas ativas de origem natural e sintética.

3.2. Objetivos específicos

- ✓ Avaliar a atividade esquizonticida sanguínea *in vitro* contra *P. falciparum* (cepas resistentes e sensíveis à CQ) de atorvastatina, itraconazol, posaconazol, novas moléculas de síntese (pterocarpanquinonas e derivados), e de extratos e frações purificadas de *A. nitidum*;

- ✓ Avaliar a atividade citotóxica das pterocarpanquinonas e derivados e de extratos de *A. nitidum* contra linhagens celulares neoplásica (HepG2) ou normal (BGM);

- ✓ Avaliar a atividade esquizonticida sanguínea de atorvastatina, itraconazol, posaconazol, pterocarpanquinonas e derivados e bioextratos de *A. nitidum*, em camundongos experimentalmente infectados com o *P. berghei*;

- ✓ Avaliar a atividade gametocitocida *in vitro* de híbridos derivadas do artesunato com mefloquina (MEFAS) e com primaquina (PRIMAS), contra gametócitos maduros (estágios IV-V) do *P. falciparum*;

- ✓ Avaliar a capacidade de MEFAS e PRIMAS em interferir na produção de gametas masculinos e femininos, através dos ensaios *in vitro* de exflagelação e ativação do gameta feminino, respectivamente.

4. Materiais e Métodos

Resumimos abaixo alguns métodos utilizados nesse trabalho. Detalhes metodológicos mais específicos estão descritos em cada um dos artigos, em anexo, publicados e/ou submetidos para publicação, como parte do item 5.

4.1. Compostos selecionados para testes de atividade antimalárica

4.1.1. Fármacos selecionados em estudos de modelagem molecular

Os fármacos comercialmente disponíveis, selecionados por estudos computacionais de ancoramento molecular com a enzima LDH, foram adquiridos da seguinte forma: (i) itraconazol (Sporanox®), Janssen-Cilag (produzido pela Brainfarma), comercialmente disponível também como um fármaco genérico; (ii) atorvastatina foi doada por Farmanguinhos como composto purificado, e, (iii) posaconazol, gentilmente cedido pelo Laboratório de Parasitologia Molecular do Centro de Pesquisas René Rachou (CPqRR) e pela Merck *Research Laboratories* (lote #IRQ-PAZ-10-X-103). As estruturas químicas dos fármacos estão representadas na **Figura 5**.

As moléculas selecionadas por estudos de *docking* com a enzima TopoI foram sintetizadas pela Dra. Camila D. Buarque, do Departamento de Química da Pontifícia Universidade Católica do Rio de Janeiro. Foram sintetizados seis isoflavonoides, dos quais quatro pterocarpanquinonas (LQB118, LQB216, LQB221 e LQB222), um N-tosyl-azapterocarpanquinona (LQB192) e um N-tosyl-azapterocarpan (LQB223) (Netto *et al*., 2010; Buarque *et al*., 2011; Costa *et al*., *international patent application* WO2010054452-A1). As estruturas químicas dessas moléculas sintéticas estão representadas na **Figura 6**.

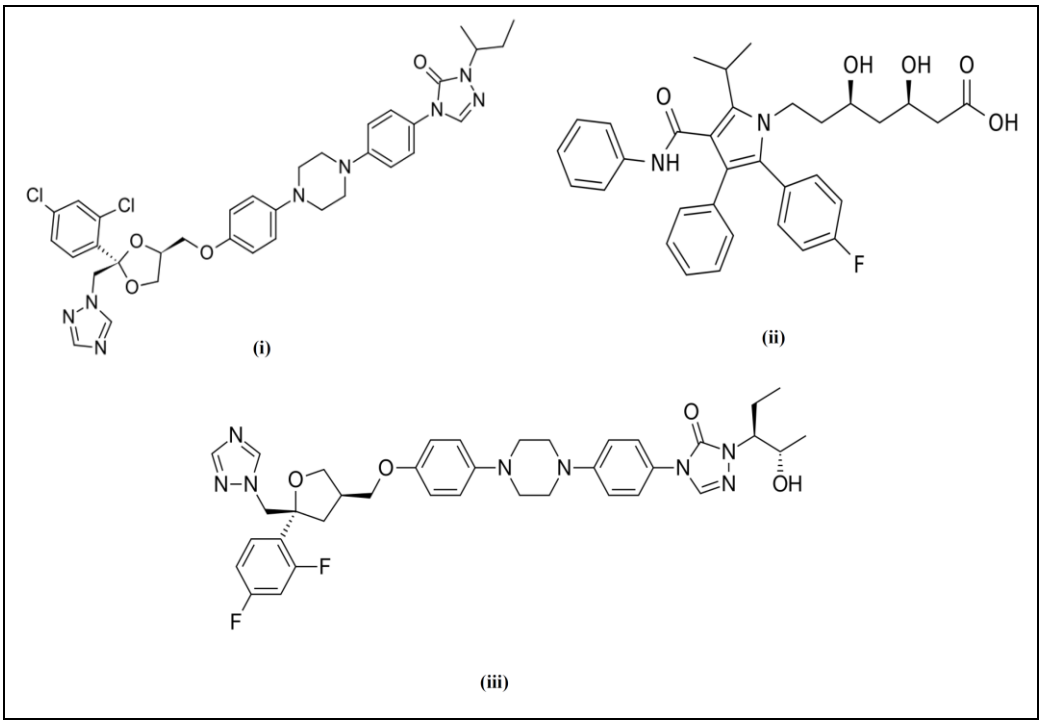


Figura 5- Estruturas químicas dos fármacos: (i) itraconazol, (ii) atorvastatina e (iii) posaconazol.

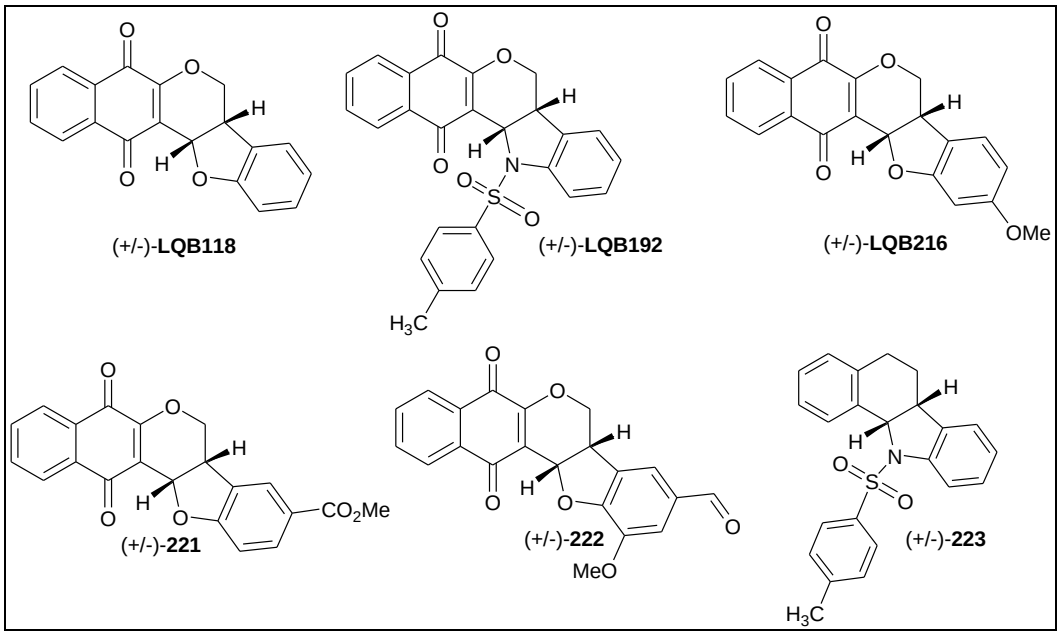


Figura 6- Estruturas químicas das pterocarpanquinonas (LQB118, LQB216, LQB221 e LQB222), N-tosyl-azapterocarpanquinona (LQB192) e N-tosyl-azapterocarpan (LQB223).

4.1.2. Extratos e frações da planta medicinal *A. nitidum*

Extratos e frações de *A. nitidum*, uma planta rica em alcaloides indólicos, foram obtidos a partir de material vegetal coletado na Fazenda da Universidade Federal do Amazonas (UFAM), Manaus, em duas ocasiões diferentes. A exsicata da espécie foi depositada no Herbário do Instituto Nacional de Pesquisas da Amazônia, número de deposito/registro 181832. A coleta e a caracterização química da planta, bem como a obtenção dos extratos e seu fracionamento, foi realizada no laboratório coordenado pela Profa. Dra. Maria Meneses Pereira, na Faculdade de Ciências Farmacêuticas da UFAM.

4.1.3. Moléculas hibridas

As moléculas MEFAS e PRIMAS foram preparadas e enviadas pela Dra. Núbia Boechat, do Instituto de Tecnologia em Fármacos de Farmanguinhos/FIOCRUZ. MEFAS, ou artesunato de mefloquina (**Figura 7**), e PRIMAS, ou artesunato de primaquina (**Figura 8**), são dois sais híbridos sintetizados a partir dos antimaláricos AS e MQ e AS e PQ, respectivamente.

MEFAS, previamente testada como esquizonticida sanguíneo pelo nosso grupo (de Pilla Varotti *et al*., 2008) foi patenteada em Farmanguinhos (N. Boechat *et al*., *international patent application* WO 2005/100370 A1).

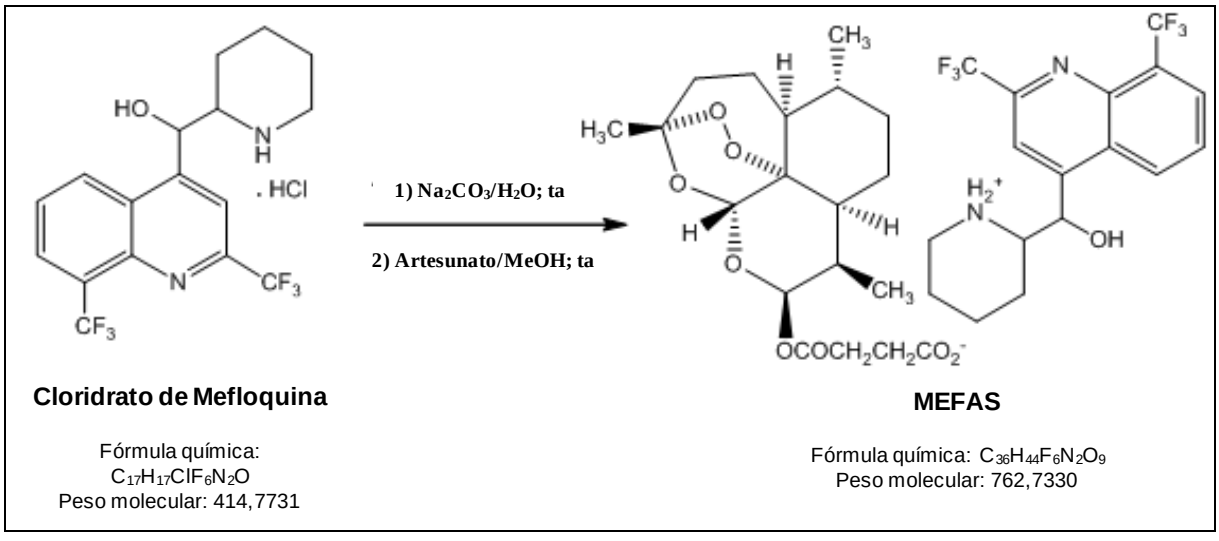
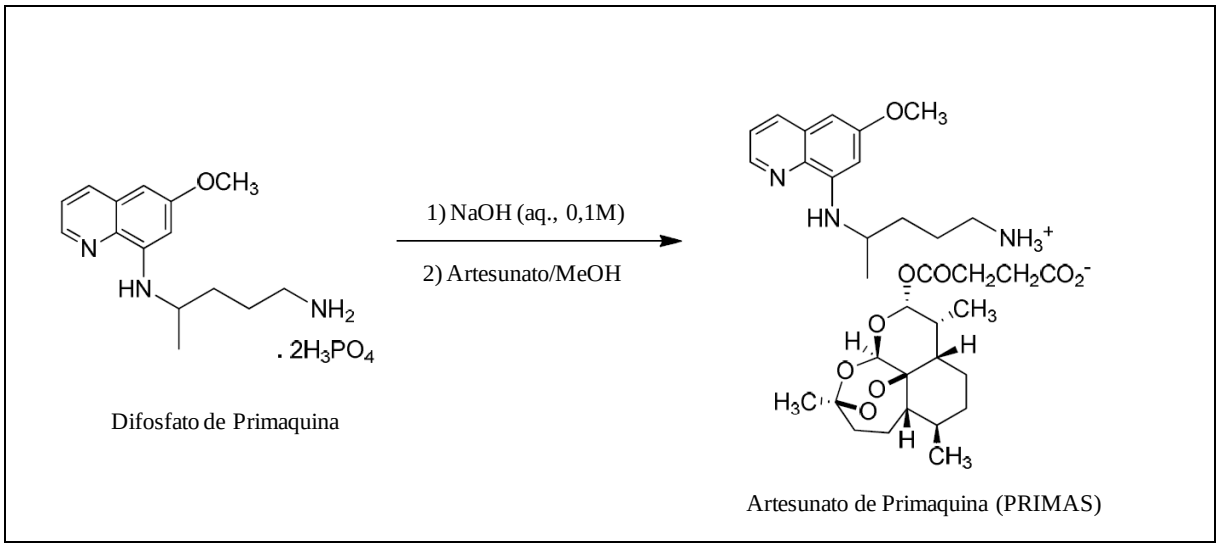


Figura 7- Síntese e estrutura química de MEFAS.



Difosfato de Primaquina

Artesunato de Primaquina (PRIMAS)

Figura 8- Síntese e estrutura química de PRIMAS.

4.2. Solubilização dos compostos

Para a realização dos testes os fármacos, moléculas sintéticas, extratos e frações de *A. nitidum* foram solubilizados de acordo com as especificações e informações cedidas pelos químicos colaboradores, ou pela literatura. Na maior parte dos casos foi utilizado o solvente dimetilsulfóxido (DMSO) (Sigma-Aldrich) na concentração final de até 0,02% para testes *in vitro* e 3% para testes *in vivo*. O antimalárico controle, CQ, bem como os compostos utilizados no teste de citotoxicidade *in vitro*, foi diluído em meio de cultura RPMI (Sigma-Aldrich) incompleto [RPMI 1640 suplementado com 25mM de Hepes (Sigma-Aldrich), 21mM de bicarbonato de sódio (Sigma-Aldrich), 11mM de glicose (Sigma-Aldrich) e 40μg/mL de gentamicina (Schering-Plough, Kenilworth, New Jersey, EUA)]. As soluções-teste foram preparadas no dia da realização dos experimentos.

4.3. Testes *in vitro* com formas sanguíneas do *P. falciparum*

4.3.1. Cultivo contínuo das fases intraeritrocitárias

Os parasitos da cepa 3D7 (sensível à CQ), originalmente recebidos da *New York University Medical School* e do clone W2 (resistente à CQ) (Oduola *et al*., 1988) foram cultivados em hemácias humanas A^+ sob condições estabelecidas por Trager & Jensen, (1976), com pequenas modificações, utilizando um protocolo previamente padronizado no

Laboratório de Malária do CPqRR (de Andrade-Neto *et al*., 2004). Os parasitos foram cultivados em placas de petri (Corning, Santa Clara, CA, EUA), com hematócrito a 5%, em meio de cultura RPMI (item 4.2) suplementado com 10% (v/v) de soro humano A^+ inativado. As placas foram mantidas em dessecadores a 37°C, nos quais a concentração adequada de oxigênio foi obtida pela combustão de uma vela. Diariamente, foram realizadas trocas do meio de cultura e a parasitemia monitorada em esfregaços, fixados com metanol, corados com Giemsa e visualizados em microscópio óptico com objetiva de imersão (100x).

4.3.2. Sincronização dos parasitos de cultivo para os testes *in vitro*

Os cultivos com predomínio de anéis, utilizados nos ensaios de quimioterapia, foram sincronizados conforme descrito por Lambros & Vanderberg, (1979). Resumidamente, o meio de cultura foi retirado da placa de petri e 10mL de uma solução de sorbitol 5% e glicose 0,5% foram adicionados ao sedimento contendo o sangue parasitado. O conteúdo foi transferido para um tubo tipo Falcon de 15mL, incubado a 37°C por 10min e centrifugado por 5min, 70*g* à temperatura ambiente. O sobrenadante foi retirado e o sedimento ressuspendido com meio RPMI completo, e o hematócrito ajustado para 5%. Essa solução foi novamente transferida para uma placa de petri, e deixada em repouso por aproximadamente 10min para que as hemácias sedimentassem. Posteriormente, foi realizado esfregaço sanguíneo para determinação da parasitemia. O hematócrito e a parasitemia, pré-determinados para cada teste, foram ajustados com a adição de hemácias e meio RPMI completo em quantidades adequadas.

4.3.3. Preparo das placas para os ensaios de quimioterapia

Culturas sincronizadas foram distribuídas em microplacas de 96 poços adicionando-se 180µL/poço de meio contendo: (i) 1% de parasitemia e 1% de hematócrito para o teste de incorporação de [^{3}H]-hipoxantina; (ii) 0,05% de parasitemia e 1,5% de hematócrito para o teste ELISA anti-HRPII, e, (iii) 0,5% de parasitemia e 2% de hematócrito para o teste ELISA anti-*Pf*LDH. Os compostos e fármacos a serem testados foram adicionados em diferentes concentrações na placa contendo os parasitos (20µL/poço). Os poços controles continham hemácias normais não infectadas (controle negativo), no caso dos ensaios de [^{3}H]-hipoxantina e ELISA anti-*Pf*LDH, ou hemácias infectadas em meio de cultivo sem adição dos compostos

(controle positivo). Foram ainda utilizados em cada experimento poços contendo o antimalárico padrão, CQ, testada em paralelo. Cada composto ou fármaco foi testado em triplicata.

4.3.4. Teste de incorporação de [^{3}H]-Hipoxantina

Os parasitos foram previamente cultivados em meio isento de hipoxantina por pelo menos 72h e então sincronizados como descrito acima (item 4.3.2). Após o preparo das microplacas com os compostos teste e controles (item 4.3.3), a mistura parasito-compostos teste e controles foi incubada por 24h a 37°C e, após esse período, a cada poço foi adicionado 20μL de solução de [^{3}H]-hipoxantina (5μCi/poço) (PerkinElmer, Waltham, MA, EUA), retornando as placas para mais 18h de incubação a 37°C (Desjardins *et al*., 1979). Após o segundo período de incubação, as microplacas foram acondicionadas a -70°C (por 6 a 10h) para promover a lise das hemácias. As amostras então foram aspiradas pelo coletor de células Harvester 96 Mach III (TomTec Imaging Systems GmbH, Unterschleissheim, Germany), em membranas (PerkinElmer), secas em microondas por 3min em potência média e acondicionadas em embalagem plástica apropriada, na qual foram adicionados 4mL de líquido de cintilação. A concentração de [^{3}H]-hipoxantina incorporada aos parasitos foi avaliada nas membranas através da leitura da radioatividade incorporada no equipamento Microbeta 1450 (PerkinElmer), em contagem por minuto, a qual é proporcional à viabilidade do parasito, obtida como 100% nos controles não tratados.

4.3.5. Teste ELISA anti-*Pf*LDH

Foram preparadas duas placas de 96 poços: a placa-teste, contendo os parasitos e os fármacos testados (item 4.3.3), e outra placa (Maxysorp, Nunc, Denmark), na qual foi realizada a reação de ELISA. Após o preparo, as placas-teste foram incubadas por 48h a 37ºC e congeladas e descongeladas três vezes (-70°C) para promover a lise das hemácias.

Para realização do teste, 100μL/poço do monoclonal primário (monoclonal anti-pLDH *falciparum specific* 17E4 *purified murine* IgG, Vista Diagnostics International LLC®, EUA) (1,0μg/mL) foram adicionados à placa. Após incubação por 14 a 16 horas a 4°C, a placa foi bloqueada com uma solução de PBS-BSA 1% (Sigma-Aldrich), e incubada à temperatura ambiente por 4h. A placa foi lavada quatro vezes com PBS/Tween 20 a 0,05% (PBS-T). As

amostras hemolisadas foram diluídas (1:100) em uma solução de PBS-BSA 1%, adicionadas à placa (100µL/poço) e incubada por 1h a 37ºC, em câmara úmida. Após esse período, a placa foi lavada quatro vezes com PBS-T e 100µL do monoclonal secundário (monoclonal anti-pLDH Pan-malaria 19g7 *biotin purified* IgG, *biotinylated with* sulfo-NHS-LC-Biotin, Vista Diagnostics International LLC®, EUA) diluído 1:5000, foram adicionados aos poços. Após 1h de incubação a 37ºC, em câmara úmida, a placa foi lavada quatro vezes com PBS-T e 100µL/poço da solução de estreptavidina (Sigma-Aldrich), marcada com peroxidase, (1:1000) foram adicionados, e a placa novamente incubada por 30min à temperatura ambiente. A placa foi lavada quatro vezes com PBS-T e 100µL/poço de TMB (KPL, Gaithersburg, MD, EUA) - uma mistura (v/v) de solução de substrato de peroxidase (3,3,5,5- tetramethylbenzidine e 0,02% H_2O_2) - foram adicionados. A reação foi interrompida pela adição de 50µL/poço de ácido sulfúrico 1M e a leitura da absorbância realizada à 450nm em um leitor de ELISA (SpectraMax 340PC, Molecular Devices, Sunnyvale, CA) (Druilhe *et al*., 2001).

4.3.6. Teste ELISA anti-HRPII

Foram preparadas duas placas: a placa-teste, contendo os parasitos e os fármacos testados (item 4.3.3), e outra placa (Maxysorp, Nunc, Denmark), na qual foi realizada a reação de ELISA. Após o preparo, as placas-teste foram incubadas por 72h a 37ºC. Após 24h, o conteúdo de seis poços (controle positivo), foi retirado e congelado a -20°C para ser utilizado posteriormente como *background*. Após 72h de incubação, as placas foram congeladas e descongeladas duas vezes (-70°C) para que houvesse a lise das hemácias.

Para realização do teste de ELISA, 100µL/poço do anticorpo primário (MPFM-55A *monoclonal mouse* IgM anti-*Plasmodium falciparum* HRPII, ICLLAB®, EUA) a 1,0µg/mL, foram adicionados à placa de ensaio. Após incubação por 14 a 16 horas a 4°C, o conteúdo dos poços foi descartado, uma solução de bloqueio (PBS-BSA 2%) adicionada (200µL/poço) e a placa incubada à temperatura ambiente por 2h. Após a incubação, o conteúdo dos poços foi novamente descartado e a placa lavada três vezes com PBS-T. A cada poço da placa de ELISA foram adicionados 100µL das amostras da cultura hemolisada. Em seis poços da placa foram adicionados 100µL dos controles congelados nas primeiras 24h (*background*) e, em seguida, a placa foi incubada por 1h à temperatura ambiente, em câmara úmida. A placa foi lavada três vezes com PBS-T e 100µL/poço do anticorpo secundário (MPFG55P HRP *conjugated monoclonal* IgG (h+1) anti-*Plasmodium falciparum*, ICLLAB®, EUA), diluído a

1:5000, foram adicionados a cada poço. Após incubação da placa à temperatura ambiente por 1h, em câmara úmida, essa foi lavada três vezes com PBS-T e 100µL de TMB foram acrescentados. A placa foi incubada por 5 a 10min à temperatura ambiente, ao abrigo da luz, e a reação interrompida adicionando-se 50µL/poço de uma solução de ácido sulfúrico 1M. A leitura foi realizada à 450nm em um leitor de ELISA (Noedl *et al*., 2002).

4.3.7. Determinação da concentração inibitória de 50% do crescimento do parasito

A inibição do crescimento de 50% dos parasitos (IC_{50}) foi determinada através de curvas dose-resposta, em função da regressão não linear obtida. Para isso foi utilizado o programa *Origin* (versão 8.0) (OriginLab Corporation, Northampton, MA, EUA), onde o valor de IC_{50} foi determinado.

4.4. Testes *in vitro* com gametócitos do *P. falciparum*

Os ensaios realizados com as formas sexuadas (gametócitos) do *P. falciparum* foram conduzidos no Centro de Investigações de Doenças Tropicais, *Diseases of the Developing World* (DDW), GlaxoSmithKline R&D, Madrid, Espanha, como parte do doutorado sanduíche pelo Programa Ciência sem Fronteiras. O trabalho teve a supervisão da Dra. Esperanza Herreros Avilés, diretora da *Malaria Discovery Performance Unit*, e chefe do grupo de pesquisa responsável por estudos relacionados ao bloqueio da transmissão da malária.

4.4.1. Cultivos contínuos de gametócitos do *P. falciparum*

Parasitos sanguíneos do *P. falciparum*, cepas NF54 (MR4, MRA-1000) e 3D7 (MR4, MRA-102), foram cultivados a 37ºC, em baixas concentrações de oxigênio, como anteriormente descrito (Trager & Jensen, 1976). A gametocitogênese foi avaliada nas culturas iniciadas conforme descrito por Ifediba & Vanderberg, (1981), com algumas modificações. Resumidamente, as culturas em estágios de anéis foram sincronizadas (dia zero) através da lise do sedimento adicionando-se 5% de sorbitol durante 10min a 37ºC (Lambros & Vanderberg, 1979). A parasitemia foi então ajustada para 0,5% e o hematócrito para 4% no volume final de 40mL de meio de cultura RPMI 1640 (Sigma-Aldrich) suplementado com

50mg/L de hipoxantina (Sigma-Aldrich), 2g/L de bicarbonato de sódio (Sigma-Aldrich), 5% soro humano A^+ e 2,5mg/mL de AlbuMAX II (Duffy & Avery, 2013). O meio de cultura foi trocado diariamente e a parasitemia monitorada através de esfregaços sanguíneos corados com Giemsa, realizados nos dias sete e 14. Parasitemias de aproximadamente 6% de gametócitos maduros foram observadas no 14º dia de cultivo.

4.4.2. Purificação dos gametócitos

No 14º dia de cultura, quando a maioria dos gametócitos estava madura (estágios IV e V), os parasitos foram concentrados com Nycoprep™ 1.077 (Axis-Shield); o sedimento foi então lavado, ressuspendido em 10mL de meio completo, transferido lentamente para tubo Falcon 15mL contendo 5mL de Nycoprep pré-aquecido e seguido por centrifugação (20min, 800*g*). A banda formada, contendo os gametócitos, foi coletada (cerca de 5mL), lavada, centrifugada, e o sedimento ressuspendido em 5mL de meio de cultura. Os gametócitos e as soluções utilizadas foram mantidos a 37°C durante todo o procedimento, utilizando uma chapa de aquecimento e banho seco QBD2 (Grant). O sangue com os gametócitos foi então transferido para uma Coluna-LS (Miltenyi Biotech) para realizar o segundo processo de purificação, utilizando para isso, um separador magnético (VarioMACS, Miltenyi Biotech). A coluna foi lavada com 5mL de meio de cultura para eluir o restante das hemácias, os gametócitos imediatamente removidos da coluna com auxílio do êmbolo e ressuspendidos em 5mL de meio.

4.4.3. Ensaio de Bioluminescência ATP

Uma vez purificados, os gametócitos foram quantificado em câmara de Neubauer, diluídos a $5x10^4$ gametócitos/poço e transferidos para placas de 96 poços (100µL/poço) ou diluídos a $1,25x10^4$ gametócitos/poço e transferidos (50µL/poço) para placas de 384 poços (Greiner), contendo os compostos teste pré-dispensados na concentração final de 0,5% de DMSO. As placas foram incubadas em câmara úmida por 48h a 37°C. O nível de ATP foi determinado utilizando-se o reagente BacTiter-Glo™ (Promega), de acordo com as recomendações do fabricante. Esse ensaio gera um sinal luminoso produzido pela reação de luciferase, a qual consiste na mono-oxigenação de luciferina, catalisada pela luciferase na presença de Mg^{2+}, ATP e oxigênio molecular.

4.4.4. Ensaio de exflagelação dos gametas

Culturas de gametócitos no 14º dia foram transferidas para meio de cultura RPMI suplementado com 10% de soro humano A^+ no dia do ensaio. A exflagelação da cultura foi avaliada utilizando-se uma amostra de 200μL da cultura. Após centrifugação o sedimento foi ressuspendido em 15μL de meio de oocineto (meio RPMI suplementado com 25mM Hepes, 50mg/L de hipoxantina, 2g/L de bicarbonato de sódio, 100μM de ácido xanturênico e 20% de soro humano A^+) e, em seguida, transferido para uma lâmina descartável *fastread*. Após 14min a exflagelação foi observada em microscópio (objetiva 100x) e a cultura foi considerada adequada para o ensaio se pelo menos 30 centros de exflagelação/campo foram observados.

Para a realização do ensaio, tubos tipo eppendorf de 1,5mL contendo 100μL de meio de cultura e o composto a ser testado, foram pré-aquecidos a 37°C em banho seco. Cem microlitros da suspensão de cultivo de gametócitos foram transferidos para cada tubo de ensaio, que foram incubados a 37°C (3% O_2, 5% CO_2, 92% N_2). Após 48h, a exflagelação foi avaliada, sendo possível identificar os centros de exflagelação como pontos brancos intensos (regiões de movimento) em um fundo preto, que foram quantificados como previamente descrito (Delves *et al*., 2013).

4.4.5. Ensaio de ativação do gameta feminino

Gametócitos maduros (estágio V) foram utilizados no ensaio de ativação do gameta feminino (Delves *et al*., 2012). Os gametócitos foram purificados por sedimentação por gradiente diferencial Nycoprep, como descrito acima. A banda recolhida foi lavada com meio RPMI pré-aquecido e os gametócitos quantificados, sendo a concentração ajustada para $8x10^3$ gametócitos/poço e, então, adicionados (100μL/poço) em placas de 384 poços pré-dispensadas contendo os compostos. As placas foram então incubadas durante 48h a 37°C (3% O_2, 5% CO_2, 92% N_2). A ativação do gameta feminino foi realizada através da redução da temperatura para 26ºC, e da adição de 10μL/poço de meio de oocineto. Foi então adicionado o anticorpo anti-*Pf*s25 (concentração final de 0,5μg/mL) (Barr *et al*., 1991) conjugado com o fluorocromo Cy3 (GE Healthcare), e as placas incubadas a 26°C (protegidas da luz) durante 24h, em uma câmara de gás (3% O_2, 5% CO_2, 92% N_2).

Os gametas femininos, quando ativados, expressam a proteína *Pf*s25 na superfície da membrana. Utilizando um anticorpo monoclonal anti-*Pf*s25, os gametas ativados puderam ser detectados por meio de microscopia de fluorescência, no confocal Opera® (Perkin Elmer, EUA). Tiostrepton e o azul de metileno foram utilizados como controles positivos da inibição.

4.5. Ensaios *in vitro* de citotoxicidade

4.5.1. Cultivo de células

Duas linhagens celulares foram utilizadas: (i) HepG2 A16 (ATCC, Manassas, VA. EUA), derivada de um hepatoblastoma primário humano (Calvo-calle *et al*., 1994), cedida pelo Dr. Virgílio E. do Rosário, do Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, e, (ii) BGM, células de rim de macaco, doada pela Universidade Federal de Minas Gerais. As células foram cultivadas como recomendado (Calvo-Calle *et al*., 1994). As células congeladas (-70°C) foram descongeladas a 37°C e seu conteúdo transferido para um tubo tipo Falcon de 50mL contendo RPMI incompleto e então centrifugadas à temperatura ambiente a 80*g* por 5min. O sobrenadante foi descartado e o sedimento foi ressuspendido em meio RPMI suplementado com 5% de Soro Bovino Fetal (SBF) (Gibco/Invitrogen, Carlsbad, CA, EUA) e 40mg/L de gentamicina (Schering-Plough). As células foram transferidas para garrafas de cultura de 75cm^2 (Corning) e mantidas em estufa com 5% de CO_2, 95% de umidade e 37°C. O meio das garrafas foi substituído a cada dois dias. Após confluência de 80%, a cultura de células foi repicada, ou utilizada na realização de ensaios de citotoxicidade. Quando necessário, o congelamento foi realizado em ampolas de criopreservação com uma solução contendo 95% de SBF e 5% de DMSO.

4.5.2. Ensaio de citotoxicidade

Os ensaios de citotoxicidade foram realizados em triplicatas, conforme descrito por Madureira *et al*., (2002). Anteriormente à tripsinização, a garrafa de cultura de células foi lavada com meio sem SBF, tripsinizadas utilizando-se 1mL de uma solução de tripsina-EDTA a 0,25% (Gibco/Invitrogen) e incubadas a 37°C por 3min para que as células se descolassem da garrafa. Ao conteúdo resultante da tripsinização foram adicionados 10mL de meio completo, que foi centrifugado a 80*g* por 5min. O sobrenadante foi descartado e o sedimento

ressuspendido em meio completo com 5% SBF. Após contagem, as células foram distribuídas em microplacas de 96 poços ($4x10^5$ células/100µL/poço) e incubadas em estufa de CO_2 a 37°C por 24h para que as células se aderissem à placa. Em seguida, 100µL de meio completo contendo diferentes concentrações dos compostos foram adicionados aos poços e as placas incubadas por 24h a 37°C, 5% de CO_2 e 95% de umidade. Ao final deste período, 20µL de uma solução de brometo 3-(4,5-Dimetiltiazol-2-yl)-2,5-difeniltetrazol (MTT) (Sigma-Aldrich), um sal tetrazólico, na concentração de 5mg/mL e ressuspendido em RPMI sem vermelho de fenol, foram adicionados aos poços da placa (Denizot & Lang, 1986). Após 4h de incubação com o MTT, o sobrenadante foi retirado e os cristais de formazan, presentes no fundo da placa, na coloração púrpura, foram diluídos em uma solução de DMSO (100µL/poço). As microplacas foram lidas em um leitor de ELISA, utilizando-se filtro de 570nm e um filtro diferencial de 630nm. A concentração letal para 50% das células (MDL_{50}), na presença dos compostos-testes e do antimalárico controle, foi determinada quando comparada à célula cultivada sem a presença de compostos (considerada 100% de viabilidade).

4.5.3. Índice de seletividade

O índice de seletividade (IS) foi obtido calculando-se a razão entre o valor de MDL_{50} pelo valor de IC_{50} (Bézivin *et al*., 2003), sendo considerado não tóxico valores maiores que 10.

4.6. Testes em camundongos inoculados com formas sanguíneas do *P. berghei*

4.6.1. Comitê de Ética para uso de animais

Os aspectos metodológicos envolvendo o uso de animais de laboratório neste estudo foram aprovados pelo Comitê de Ética para Uso de Animais da Fundação Oswaldo Cruz-FIOCRUZ (CEUA LW-23/13).

4.6.2. Teste de esquizonticidas sanguíneos *in vivo*

Camundongos suíços Webster, fêmeas, pesando 20±2g, provenientes do biotério de produção do CPqRR, foram inoculados com hemácias infectadas com *P. berghei*, cepa NK65, originalmente recebida da Universidade de Nova Iorque (EUA), e mantida em camundongos por passagens sanguíneas semanais. Foi realizado o teste supressivo descrito por Peters *et al*., (1975) e modificado por Carvalho *et al*., (1990). Cada camundongo foi inoculado com $1x10^5$ hemácias parasitadas (0,2mL), por via intraperitoneal (1º dia de experimento). Aproximadamente 24h após a inoculação, anteriormente ao tratamento, os animais foram divididos aleatoriamente em grupos de seis camundongos por gaiola. Em cada experimento, os animais foram distribuídos em dois grupos controles, um não-tratado e um tratado com CQ, e grupos testes, com 3 a 6 animais por grupo. Nos 2º, 3º e 4º dias do experimento, os camundongos foram tratados por via oral com os compostos teste, em diferentes concentrações. A parasitemia foi avaliada nos 5º, 7º e 9º dias de experimento pela contagem dos parasitos em esfregaços sanguíneos em microscópio óptico sob objetiva de imersão (100x).

A redução da parasitemia foi calculada em relação aos grupos controles não-tratados. Redução de 30% do crescimento dos parasitos, quando comparado o grupo controle com o grupo teste, foi considerada como indicador de uma amostra ativa (Andrade-Neto *et al*., 2003).

4.7. Determinação da parasitemia

Esfregaços sanguíneos de cultivos, ou de camundongos, foram secos ao ar, fixados com metanol e corados com solução recém diluída de Giemsa na proporção de duas gotas para cada 1mL de solução salina tamponada pH 6,8. Após 10min, as lâminas foram lavadas em água corrente, secas ao ar e examinadas ao microscópio óptico em objetiva de imersão (100x). A parasitemia foi determinada através da contagem do número de hemácias infectadas, sendo contadas: de 2000 a 3000 hemácias no caso de parasitemias entre 5 a 20%, aproximadamente 1000 hemácias nas parasitemias maiores de 20%, e 6000 hemácias nas menores que 5%. A avaliação foi feita pela estimativa do número total de hemácias/campo microscópico sendo contados os parasitos em 50 a 100 campos. A parasitemia foi expressa em porcentagem de hemácias parasitadas.

4.8. Análise Estatística

O Programa GraphPad Prism 5 foi utilizado para a comparação estatística dos valores de IC_{50} gerados nos ensaios *in vitro* e das porcentagens de redução da parasitemia nos ensaios *in vivo*. Valores de $p<0,05$ foram considerados estatisticamente significativos.

5. Resultados e Discussão

Os resultados obtidos durante o doutorado serão apresentados na forma de artigos publicados ou submetidos à publicação.

5.1. Atividade antimalárica de compostos selecionados por estudos de modelagem molecular (Anexos I e II)

A abordagem utilizada para a obtenção desses compostos foi desenvolvida em colaboração com o grupo do Dr. Tanos França, do Instituto Militar de Engenharia, responsável pelos estudos *in silico* de modelagem molecular realizados tanto com a enzima LDH como com a TopoI.

Embora a *Pf*LDH não seja um alvo direto da CQ, dados experimentais tem mostrado que esse fármaco se liga fracamente, mas de maneira estável, no sítio de ligação do NADH, o cofator da enzima (Read *et al*., 1999; Menting *et al*., 1997). A enzima LDH plasmodial (pLDH) do *P. vivax, P. malariae* e *P. ovale* exibe 90-92% de similaridade com a *Pf*LDH, sendo, portanto, um alvo de estudo promissor em cultivos de formas sanguíneas do *P. falciparum*. Baseado nessas informações, ferramentas computacionais de modelagem molecular com a enzima LDH, essencial à sobrevida e desenvolvimento dos parasitos, foram utilizadas para selecionar compostos com pontuação *MolDock* semelhante à do NADH.

Para a seleção dos melhores candidatos a inibidores da *Pf*LDH, foram estudados 50 fármacos comercialmente disponíveis. Os compostos que apresentaram os valores de energia de ligação mais estáveis, comparados ao NADH, foram o itraconazol, a atorvastatina e o posaconazol. Nos estudos de *software*, esses fármacos interagiram com os resíduos presentes no sítio ativo da enzima *Pf*LDH, sugerindo haver uma inibição competitiva com o NADH, além de também apresentar uma forte estabilidade de ligação no sítio ativo da enzima plasmodial.

A hipótese estabelecida nos estudos computacionais de modelagem molecular foi confirmada *in vitro* contra o *P. falciparum* clone W2 (resistente à CQ), por meio de ensaios imunoenzimáticos ELISA anti-*Pf*LDH e anti-HRPII. Os ensaios ELISA com anticorpos monoclonais dirigidos contra uma enzima e uma proteína específicas para o *P. falciparum* foram utilizados baseados em dados prévios que confirmaram a sensibilidade dos mesmos (Druilhe *et al*., 2001; Noedl *et al*., 2002).

Na ordem de atividade, o posaconazol foi o mais ativo (IC_{50} = 2,6 e 5,3µg/mL), seguido do itraconazol (IC_{50} = 9,3 e 9,2µg/mL) e da atorvastatina (IC_{50} = 13,1 e 13,8µg/mL), nos ensaios anti-*Pf*LDH e anti-HRPII, respectivamente. Os fármacos foram também testados em camundongos infectados com *P. berghei*, administrados por via oral, durante três dias consecutivos. Na dose de 20mg/kg, a atorvastatina e o posaconazol foram ativos, apresentando 41 e 46% de redução da parasitemia, respectivamente, e o itraconazol parcialmente ativo, com 30% de redução.

A contradição observada pelo resultado obtido *in vitro* e *in vivo* com o itraconazol, parcialmente ativo *in vivo*, pode ser atribuída ao fato do medicamento ser comercializado na forma de microesferas, a única disponível. As microesferas são formas farmacêuticas sólidas com tamanho milimétrico, que tem a função de possibilitar a veiculação do princípio ativo, melhorando a absorção de fármacos não absorvidos pelo trato gastrointestinal. A microesfera, inadequada para administração oral aos camundongos, foi triturada e uma suspensão foi preparada. Permanece ainda a ser esclarecido se o resultado revela que a ineficácia do composto se deve a sua inativação por enzimas gástricas, ou se é resultante da sua má absorção, ambas consequentes da forma de administração utilizada.

Seguindo o mesmo racional, estudos teóricos de ancoramento molecular foram realizados com as enzimas topoisomerases de DNA, essenciais durante a replicação, transcrição, recombinação e reparo do DNA, as quais tem atraído considerável interesse no desenvolvimento de novos fármacos contra a malária (Bodley *et al*., 1998; Roy *et al*., 2011).

As pterocarpanquinonas do tipo 1, moléculas previamente estudadas quanto a atividade antineoplásica (Buarque *et al*., 2011; Drwall *et al*., 2011; Bacelar *et al*., 2013) e antiparasitária (Portes *et al*., 2012; Ribeiro *et al*., 2013), possuem mecanismo de ação relacionado com a inibição da topoisomerase em linhagens tumorais. Com base nessa premissa, foram realizados estudos de modelagem e dinâmica molecular de seis novas pterocarpanquinonas (LQB118, LQB192, LQB216, LQB221, LQB222, LQB223) na estrutura 3D de *Pf*Topo e *Hss*TopoI comparando-as com inibidores conhecidos das enzimas, camptotecina e topotecano (Bodley *et al*., 1998; Staker *et al*., 2002; Roy *et al*., 2011).

Os resultados de ancoramento molecular mostraram uma boa energia de ligação para todas as moléculas no sítio ativo das enzimas *Pf*TopoI e *Hss*TopoI, sendo a LQB223 a molécula com valor de energia de ligação mais estável com a enzima plasmodial. Os resultados de atividade biológica, *in vitro* e *in vivo*, corroboraram os dados virtuais; a LQB223, com a maior energia de ligação, apresentou o melhor valor de índice terapêutico

(MDL_{50}/IC_{50}), ~100, e reduziu em até 80% a parasitemia de camundongos infectados com *P. berghei* e tratados com 100mg/kg do composto teste.

Os dados apresentados confirmam que os estudos de dinâmica molecular são uma importante ferramenta utilizada na busca de novos fármacos, pois permitem selecionar candidatos específicos para enzimas e/ou proteínas essenciais à sobrevivência do parasito. Determinar os alvos moleculares específicos anteriormente aos estudos biológicos permite a seleção de compostos potencialmente ativos contra os parasitos, os quais tem o provável mecanismo de ação já sugerido.

5.2. Atividade de *A. nitidum*, planta medicinal utilizada na Amazônia para tratar a malária humana (Anexo III)

O gênero *Aspidosperma* (Apocynaceae) se destaca por apresentar diferentes propriedades medicinais (Bolzani *et al*., 1987; Pereira *et al*., 2007). Cascas das plantas de espécies desse gênero são comumente utilizadas na Região Amazônica na forma de infusões, tônicos, afrodisíacos, antitérmicos e contra a malária humana (Brandão *et al*., 1992). A aparente ausência de contra indicações dessas infusões tem contribuído para a difusão do uso das cascas de *Aspidosperma* (Ferreira *et al*., 2004). Dentre as espécies, *A. olivaceum*, utilizada para tratar febres em algumas regiões do Brasil, demonstrou atividade terapêutica específica contra *P. falciparum*, com altos valores de IS *in vitro*. O alcaloide aspidoscarpina, responsável pela atividade da planta, foi isolado (Chierrito *et al*., 2014).

A espécie *A. nitidum*, conhecida como "carapanaúba", é utilizada para tratar várias outras doenças, acreditando-se, na Amazônia, que o chá da casca desta espécie pode curar a malária. Recentemente, essa informação foi relatada em estudos realizados em comunidades quilombolas do município de Oriximiná, Pará (Oliveira DR 2009). O estudo químico realizado com extratos do cerne de *A. nitidum* permitiu o isolamento de um alcaloide inédito, a braznitidumina (Pereira *et al*., 2007).

No presente trabalho, foi caracterizada a atividade antiplasmodial de extratos e frações de *A. nitidum* avaliando-se *in vitro* sua atividade contra o *P. falciparum* cepa W2, resistente à CQ, nos métodos tradicional, incorporação de [^{3}H]-hipoxantina e ELISA anti-HRPII. Extratos e frações provenientes da casca do caule foram ativos (IC_{50} < 12μg/mL), confirmando o uso de infusões dessa parte da planta para o tratamento da malária humana na Região Amazônica. Mais importante, demonstramos a baixa citotoxicidade dos bioprodutos ativos. Os extratos e

frações dessa planta medicinal também foram ativos em camundongos infectados com *P. berghei* (cepa NK65), sensível e resistente à CQ. A braznitidumina, um novo alcaloide isolado do extrato etanólico bruto, não demonstrou atividade antiplasmodial.

Muitas vezes o extrato ou a fração são ativos, mas não uma ou mais substâncias isoladas. Nesse caso, a atividade sinérgica observada na mistura de compostos presentes no extrato é benéfica e deve ser preservada. No entanto, a possibilidade de testar compostos já purificados, como a braznitidumina, entre outros, pode validar ou não essa hipótese. Estudos prévios demonstraram que a atividade antimalárica do pó das folhas secas da planta inteira de *A. annua* é pelo menos cinco vezes mais eficaz do que uma dose equivalente de ART pura (Elfawal1 *et al*., 2015).

A atividade antimalárica da espécie *A. nitidum*, relacionada à mistura de alcaloides presentes no extrato bruto da casca da planta, não pode ser atribuída a uma substância pura isoladamente (braznitidumina). O uso de infusões da casca da planta para o tratamento da malária, portanto, se justifica. Isso já foi observado, por exemplo, na cinchonina, com efeitos sinérgicos quando combinadas a quinina e a quinidina, ou ambas (Druilhe *et al*., 1988). Portanto, em alguns casos, um único alcaloide pode não possuir atividade tão significativa quando comparado à combinação com outros alcaloides, como com *A. nitidum* aqui demonstrada. Os elevados valores de IS desses compostos validam o uso dessa planta no tratamento da malária humana; a mistura de compostos presentes no extrato bruto reduz a probabilidade de resistência dos parasitos, visto que uma grande variedade de moléculas, que tem como alvo diferentes sítios ativos, dificulta o desenvolvimento de resistência.

5.3. Estudo da atividade bloqueadora da transmissão da malária das moléculas híbridas MEFAS e PRIMAS (Anexo IV)

O AS, um antimalárico com ação rápida e intensa, porém com meia vida curta (escala de horas), e a MQ, um potente antimalárico de meia vida longa (escala de semanas) (WHO 2006), compõem uma associação amplamente adotada na Ásia para o tratamento dos casos do *P. falciparum* multi-resistente (Wiseman *et al*., 2006). Considerando a importância do desenvolvimento de novos fármacos associada ao conceito de moléculas híbridas, a presença de dois ou mais sítios estruturais com diferentes ações biológicas como uma das estratégias atuais utilizadas na formulação de novos antimaláricos (Meunier 2008), testamos a atividade de MEFAS e de PRIMAS, dois sais híbridos de AS com MQ e com PQ, respectivamente, desenvolvidos em Farmanguinhos pelo grupo da Dra. Núbia Boechat.

Considerando ser a atividade gametocitocida secundária à atividade esquizonticida sanguínea no processo de descoberta de novos fármacos (Baird & Surjadjaja, 2011) a proposta foi pesquisar a atividade gametocitocida das moléculas híbridas MEFAS e PRIMAS. Sabidamente ativas contra os estágios sanguíneos assexuados do *P. falciparum* e na malária experimental em camundongos (de Pilla Varotti *et al*., 2008), MEFAS e PRIMAS foram utilizadas como base para a seleção de moléculas com dupla atividade, buscando um novo fármaco que tenha o efeito bloqueador da transmissão adicional à atividade esquizonticida sanguínea, o qual deve ser encaminhado para ensaios clínicos nas áreas endêmicas.

Os gametócitos maduros (estágios V) são o único estágio do parasito capaz de infectar os mosquitos e representam um elo fundamental na transmissão da malária ao vetor; logo, eliminá-los ou comprometer sua viabilidade irá impedir o ciclo do parasito e a infecção de novos indivíduos (Delves *et al*., 2013). A atividade gametocitocida de novos compostos pode ser avaliada em cultivos *in vitro* de gametócitos do *P. falciparum* produzidos em larga escala (Lelièvre *et al*., 2012; D'Alessandro *et al*., 2013), proposta desse trabalho submetido a publicação.

Em ensaios conduzidos durante o doutorado sanduíche na GlaxoSmithKline R&D, com culturas de gametócitos maduros (estágios IV e V) do *P. falciparum*, foi avaliada a atividade gametocitocida das moléculas híbridas MEFAS e PRIMAS. MEFAS, com comprovada atividade esquizonticida sanguínea (de Pilla Varotti *et al*., 2008), possui também atividade gametocitocida, agindo contra gametócitos do *P. falciparum in vitro*. PRIMAS, ao contrário de MEFAS, não melhorou a atividade gametocitocida do AS nos ensaios *in vitro*, dado atribuído à necessidade da PQ (presente no híbrido) ter que ser metabolizada *in vivo* para ser ativa.

Visto que gametócitos masculinos e femininos respondem de forma diferente aos antimaláricos (Delves *et al*., 2013), foi avaliada também a capacidade das moléculas híbridas em inibir a formação de gametas masculinos e femininos. O ensaio de ativação do gameta feminino baseia-se na expressão específica da proteína *Pf*s25 na superfície da membrana do gameta ativado. A expressão da proteína é detectada através da análise de imagem e de um algoritmo que quantifica o número de gametócitos femininos que se transformaram em gametas viáveis, fornecendo o número exato de gametas femininos ativados/poço e permitindo o cálculo da porcentagem de inibição. Tiostrepton e azul de metileno foram utilizados como controles positivos da inibição da ativação dos gametas.

O tiostrepton é um antibiótico inibidor do proteassoma do parasito, dificultando a degradação de proteínas. Apesar da baixa atividade descrita contra as formas assexuadas do

parasito, tiostrepton possui moderada atividade *in vitro* contra gametócitos jovens (Aminake *et al*., 2011). Por outro lado, foi mostrado que esse fármaco inibe a formação de gametócitos masculinos e femininos em concentrações submicromolares (Delves *et al*., 2013).

O azul de metileno, primeiro composto sintético a ser utilizado como fármaco (Buchholz *et al*., 2008), é um análogo estrutural da riboflavina. O seu exato mecanismo de ação ainda não é totalmente conhecido; sabe-se que este fármaco interfere na detoxificação do grupo heme (Delves 2012). Muitos estudos mostraram que o azul de metileno é um potente inibidor de gametócitos jovens *in vitro* (Adjalley *et al*., 2011, D'Alessandro *et al*., 2013, Delves *et al*., 2013), e também previne a formação de oocistos no mosquito vetor (Delves 2012).

MEFAS inibiu a ativação do gameta feminino com um valor de IC_{50} inferior aos fármacos AS e MQ, e apresentou-se cerca de trinta vezes mais ativa do que o azul de metileno, fármaco conhecido pela potente atividade contra gametócitos. Em contraste, PRIMAS não foi mais potente do que o AS em inibir a formação do gameta feminino, sendo o valor de IC_{50} de PRIMAS maior do que o AS.

O ensaio de exflagelação visa avaliar o potencial de compostos em bloquear a transmissão da malária através da inibição da produção dos gametas masculinos (ou microgametas). A ativação dos gametócitos masculinos é avaliada pelo processo de exflagelação, com rápida extrusão de estruturas tipo-flagelos de movimentos rápidos, em movimentos ondulantes saindo dos eritrócitos infectados. Esse processo ocorre naturalmente poucos minutos após a ingestão de sangue infectado durante o repasto sanguíneo da fêmea do mosquito anofelino vetor. Os gametócitos masculinos ativados causam movimentos nos eritrócitos adjacentes, enquanto as células mais distantes permanecem imóveis; esse movimento tem sido utilizado como critério de identificação e quantificação de centros de exflagelação, que pode ser realizada através de vídeos, em intervalos de tempo capazes de detectar o movimento dos eritrócitos, utilizando-se um aumento de 100x, ou por microscopia óptica (40x). Diidroartemisinina (DHA) foi utilizada como controle positivo correspondendo a 100% de inibição da exflagelação.

MEFAS foi doze vezes mais ativa do que o AS e inibiu 99% da exflagelação, como a DHA, em uma dose 20 vezes inferior. PRIMAS inibiu a exflagelação em uma concentração inferior a da PQ, mas não inferior ao AS.

Os dados obtidos nos permitem concluir que MEFAS e PRIMAS podem ser considerados possíveis candidatos no controle da malária humana como antimaláricos de

atividade dupla, esquizonticidas sanguíneos e bloqueadores da transmissão, capazes, portanto, de curar a infecção e reduzir a prevalência da malária.

6. Conclusão

Neste trabalho diferentes modelos experimentais foram utilizados para o estudo da atividade antiplasmodial, visando novos antimaláricos com atividade esquizonticida sanguínea e bloqueadora da transmissão. Ensaios *in vitro* com formas sanguíneas assexuadas e sexuadas de cultivos do *P. falciparum*, bem como testes em camundongos com malária experimental pelo *P. berghei*, nos permitiram encontrar diversos compostos ativos. Nossos resultados permitiram chegar às seguintes conclusões:

✓ Os estudos de dinâmica molecular, *docking* e ensaios biológicos, realizados em paralelo, permitiram a seleção de fármacos já comercializados (atorvastatina, posaconazol e itraconazol), e de novas moléculas ativas (LQBs) contra o *P. falciparum*, atuando nas enzimas *Pf*LDH e *Pf*TopoI, essenciais à sobrevivência do parasito sendo, por isso, potenciais candidatos à um novo antimalárico, com mecanismo de ação já sugerido;

✓ A atividade antimalárica em camundongos e a baixa citotoxicidade dos extratos brutos e frações isoladas de *A. nitidum* evidenciam seu potencial terapêutico, validando seu uso etnofarmacológico. Surge então o grande interesse em torná-la, após testes de segurança clínica, um fitoterápico comercial, confirmando assim seu uso na medicina tradicional popular contra a malária, extensivo e antigo na Amazônia Brasileira;

✓ Dois sais híbridos, MEFAS e PRIMAS, apresentaram potente atividade gametocitocida e inibiram a formação de gametas masculinos e femininos do *P. falciparum*, o que impede a continuidade do ciclo evolutivo do parasito no mosquito vetor. Ambos são candidatos a fármacos antimaláricos de ação dupla, ou seja, capazes de não só curar a infecção, como também de bloquear a transmissão da doença.

7. Referências

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Anexos- Artigos publicados ou submetidos

Anexo A- Artigo publicado PlosOne

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PLoS one

Antimalarial Activity of Potential Inhibitors of *Plasmodium falciparum* Lactate Dehydrogenase Enzyme Selected by Docking Studies

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Abstract

The *Plasmodium falciparum* lactate dehydrogenase enzyme (*Pf*LDH) has been considered as a potential molecular target for antimalarials due to this parasite's dependence on glycolysis for energy production. Because the LDH enzymes found in *P. vivax*, *P. malariae* and *P. ovale* (pLDH) all exhibit ~90% identity to *Pf*LDH, it would be desirable to have new anti-pLDH drugs, particularly ones that are effective against *P. falciparum*, the most virulent species of human malaria. Our present work used docking studies to select potential inhibitors of pLDH, which were then tested for antimalarial activity against *P. falciparum in vitro* and *P. berghei* malaria in mice. A virtual screening in DrugBank for analogs of NADH (an essential cofactor to pLDH) and computational studies were undertaken, and the potential binding of the selected compounds to the *Pf*LDH active site was analyzed using Molegro Virtual Docker software. Fifty compounds were selected based on their similarity to NADH. The compounds with the best binding energies (itraconazole, atorvastatin and posaconazole) were tested against *P. falciparum* chloroquine-resistant blood parasites. All three compounds proved to be active in two immunoenzymatic assays performed in parallel using monoclonals specific to *Pf*LDH or a histidine rich protein (HRP2). The IC_{50} values for each drug in both tests were similar, were lowest for posaconazole (<5 μM) and were 40- and 100-fold less active than chloroquine. The compounds reduced *P. berghei* parasitemia in treated mice, in comparison to untreated controls; itraconazole was the least active compound. The results of these activity trials confirmed that molecular docking studies are an important strategy for discovering new antimalarial drugs. This approach is more practical and less expensive than discovering novel compounds that require studies on human toxicology, since these compounds are already commercially available and thus approved for human use.

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Introduction

Malaria is the most lethal parasitic disease in the world, annually affecting approximately 500 million people and resulting in 800,000 deaths, mostly in African sub-Saharan countries [1]. Brazil registered 306,000 cases of malaria in 2009, most of which were in the Amazonian region, as diagnosed and treated by Ministry of Health officers [2], [3]. Transmission occurs through the bite of *Anopheles* mosquitoes infected with the parasite and five different species may affect humans. *Plasmodium falciparum* is the most pathogenic species and may cause severe malaria and death in untreated nonimmune individuals, especially children under five [4].

The antimalarial treatment recommended for *P. falciparum* consists of drug combinations containing artemisinin derivatives (ACT) with other antimalarials, including quinoline compounds, such as amodiaquine and mefloquine. The quinolines act mainly by inhibiting hematin polymerization, thus intoxicating the parasite with the ferriprotoporphyrinic groups generated by hemoglobin degradation [5]. Other antimalarials used in ACT, for example, pyrimethamine and proguanil, inhibit the tetrahydrofolic acid cycle and thus eliminate an important cofactor for DNA synthesis. Despite the arsenal of drugs available for malaria treatment, the disease remains a worldwide public health problem. *P. falciparum* quickly develops resistance under selective drug pressure [5]. *P. vivax*, the most prevalent human parasite worldwide, has been shown to be resistant to chloroquine, including in Brazil [3]. Continuous efforts on the development of new antimalarials are required, and our primary method has been to use different approaches, such as testing natural products and synthetic molecules, as reviewed [6], [7].

Drug-resistant malaria parasites are believed to emerge through mutations in the active sites of drug targets [5] or from biochemical changes in the drug receptors [8]. The continued search for new molecular targets for drug design should broaden the therapeutic arsenal and strategies to fight drug resistance in

human malaria. The lactate dehydrogenase enzyme from *P. falciparum* (*Pf*LDH) has been considered as a potential molecular drug target. Although the primary mechanism of action of quinoline drugs is by inhibition of heme polymerization [9], other molecular targets have been reported as being important for the improvement of their biological effectiveness against *P. falciparum*. Chloroquine interacts specifically with *Pf*LDH in the NADH binding pocket, occupying a position similar to that of the adenyl ring cofactor and hence acting as a competitive inhibitor for this critical glycolytic enzyme [9], [10], [11], [12].

The LDH enzyme catalyzes the interconversion of pyruvate to lactate in the final step of glycolysis, which is required for energy production in living cells. Ferriprotoporphyrin IX (hematin), one of the products of hemoglobin degradation by malarial parasites, intoxicates the parasite by competing with NADH for the active site of *Pf*LDH; parasite survival depends on polymerization of hematin to hemozoin, which remains active in the food vacuole of the parasite and causes parasite death [9]. The quinoline derivatives are believed to form complexes with the dimeric hematin, preventing the formation of hemozoin [11].

Analogs of NADH have been identified as new potential inhibitors to *Pf*LDH in DrugBank [13]. In previous docking studies, Molegro Virtual Docker software (MVD)® yielded higher docking accuracy than other docking programs; the accuracies were: MVD, 87%; Glide, 82%; Surflex, 75%; and FlexX, 58% [14]. A total of 50 compounds were selected based on their interactions with an active site similar to that of NADH; the three (itraconazole, atorvastatin and posaconazole) compounds that presented the best theoretical results were tested *in vitro* against *P. falciparum* blood parasites and against malaria in mice.

Results

Docking studies

The results of docking studies using the MolDock Scores observed for NADH and the 50 compounds chosen from the DrugBank [13] are summarized in Table 1. The superposition of NADH, as observed in the cavity of the crystallographic structure of *Pf*LDH, and the best conformation obtained theoretically for itraconazole are shown in Figure 1. This result suggests that the software reproduced the appropriate conformation of NADH inside its binding pocket in the *Pf*LDH active site.

The compounds atorvastatin and posaconazole also fitted well in the NADH pocket (data not shown), showing the best docking energy values, that is, closest to NADH (which has a docking energy of -249.6 kcal·mol^{-1}). These three compounds were selected for further *in vitro* tests because they are commercially available for human use as well.

The active site residues that interact with NADH, itraconazole, atorvastatin and posaconazole inside *Pf*LDH are shown in Table 2, and the H-bonds between each compound and the *Pf*LDH active site are shown in Figures 2 and 3. The H-bond energy values were -1.9 kcal·mol^{-1}, -5.0 kcal·mol^{-1} and -6.5 kcal·mol^{-1} for atorvastatin, itraconazole and posaconazole, respectively, and are all higher than that observed for NADH, which is able to make more H-bonds in the binding pocket than the studied compounds.

Activity of itraconazole, atorvastatin and posaconazole against *P. falciparum*

The *in vitro* tests against chloroquine-resistant *P. falciparum* clone W2 showed that itraconazole, atorvastatin and posaconazole were active. In two different immunoenzymatic assays (ELISA) with monoclonals anti-*Pf*LDH or anti-HRP2, the three compounds inhibited parasite growth at low doses. Posaconazole was the most

Table 1. MolDock Scores observed for NADH and the 50 compounds chosen from the DrugBank [13].

Drugs	MolDock Score (kcal.mol^{-1})	Drugs	MolDock Score (kcal.mol^{-1})
NADH	−249.6	Cefotaxime	−143.2
Itraconazole	−218.5	Valaciclovin	−142.4
Atorvastatin	−209.3	AMP	−136.5
Posaconazole	−201.6	Cephapirin	−136.5
Rescinnamine	−201.0	Cefuroxime	−135.2
Cefpiramide	−195.0	Cefalotin	−132.9
Eprosartan	−177.0	Abacavir	−130.4
Cefditoren	−177.2	Entecavir	−130.4
Ergotamine	−175.0	Capecitabine	−129.1
Cefmenoxime	−173.3	Topotecan	−128.6
Dicloxacilin	−165.0	Tenoxican	−123.9
Novobiocin	−162.5	Genifloxacin	−120.6
Ceftriaxone	−157.4	Cefdinir	−117.2
Cefamandole	−154.9	Moxifloxacin	−116.4
Ceftazidine	−156.8	Clorafabine	−114.8
ATP	−155.2	Adenosine	−113.6
Cefamandole	−154.9	Zidovudine	−111.7
Dasatinib	−154.5	Nelarabine	−111.3
Droperidol	−152.3	Cladribine	−111.0
Cefixime	−151.0	Pentostanine	−110.7
S-Adenozylmetionine	−150.7	Vidarabine	−110.6
Paliperidol	−150.4	Trifluridine	−102.2
Rysperidone	−146.9	Zalcitabine	−94.4
Cefmetazole	−146.9	Gemcitabine	−92.1
Bethamidine	−144.3	Floxuridine	−91.4

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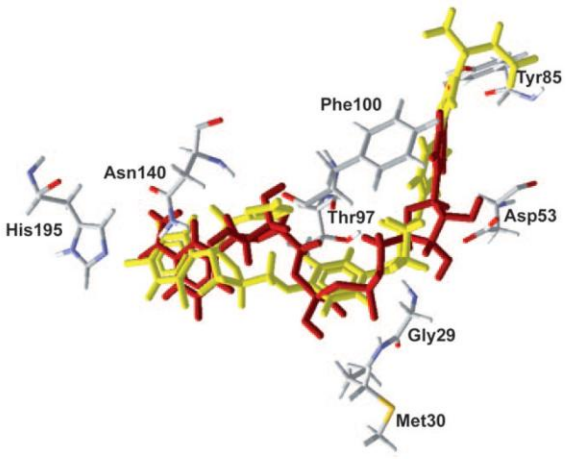

Figure 1. Superposition of the best conformation of itraconazole (in yellow) and NADH (in red) in the active site pocket of *P. falciparum* enzyme lactate dehydrogenase (*Pf*LDH).
doi:10.1371/journal.pone.0021237.g001

Table 2. Docking results for atorvastatin, posaconazole and itraconazole inside *Pf*LDH.

Drugs	MolDock Score (kcal.mol^{-1})	Hbond Score (kcal.mol^{-1})	Number of H-Bonds	Residues of the NADH binding site interacting with the ligands
NADH	−249.6	−29.3	22	Asn140, His195, Val138, Phe100, Gly99, Thr97, Gly32, Ile31, Met30, Gly29, Asp53, Ile54, Tyr85
Atorvastatin	−209.3	−1.9	6	Asp53, Thr97, Gly32, Ile31, Gly99
Itraconazole	−218.5	−5.0	5	Asn140, Gly29, Tyr85
Posaconazole	−201.6	−6.5	8	Gly99, Gly29, Met30, Ile31, Asn140, Ser 245, Glu122

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active compound. Moreover, in one of the four experiments, the IC_{50} was 2.6 µM in the ELISA anti-*Pf*LDH; the average of IC_{50} from the experiments is shown in Table 3, corroborating our computer analysis and docking calculations (Table 1).

Antimalarial tests in mice with malaria due to *P. berghei*

The compounds active *in vitro* were next tested in mice infected with *P. berghei*. Due to limitations in the availability of the purified compounds, they were first tested at doses of 20 mg/kg. All compounds inhibited parasite growth, especially atorvastatin and posaconazole, which reduced parasitemia by 41% and 46%, respectively, compared to untreated group (Table 4). Further tests confirmed the activity of atorvastatin (data not shown) and posaconazole. For the latter, a commercially available oral suspension of posaconazole, formulated for human use (Noxafil®) was acquired at a pharmacy in the USA and tested at 50 and 100 mg/kg. It reduced parasitemia in the treated mice by 45% and 71%, respectively, as compared to untreated controls (Table 4).

However, itraconazole reduced parasitemia by only 30% whereas, as expected, chloroquine cured the animals at 20 mg/kg. By day 30, the last day of the trial, all chloroquine-treated mice were still alive and had negative blood smears for malaria parasites. Thus, this antimalarial is significantly more active than the tested drugs.

Discussion

Although *Pf*LDH is not a direct chloroquine target, experimental data have shown that this enzyme binds to chloroquine [10], [12]. Based on this information, we studied 50 commercially available compounds as candidates to *Pf*LDH inhibitors. The compounds that presented the closest binding energy values to NADH, which we considered to be the best results, were itraconazole, atorvastatin and posaconazole. In our software simulation, these compounds also interacted with the residues present in the *Pf*LDH active site, suggesting a competitive inhibition with NADH. The selected compounds also presented strong stability inside the *Pf*LDH active site; thus, they could also dock in the NADH binding pocket of *Pf*LDH. This theoretical hypothesis proved correct in light of our experimental data from *in vitro* assays performed with *P. falciparum*. Indeed, the selected compounds, itraconazole, atorvastatin and posaconazole, were all active *in vitro*. The results with two different tests were similar: one used monoclonals specific to a *P. falciparum* parasite protein (HRP2) and the other used monoclonals against the *Pf*LHD enzyme.

Posaconazole, an inhibitor of ergosterol biosynthesis [15], was the most active compound against *P. falciparum*; it also was the most effective compound against murine malaria caused by *P. berghei*. Because posaconazole was the most promising compound *in vitro* and *in vivo* in the present antimalarial study, we conducted a second test using higher doses of the compound, this time in the

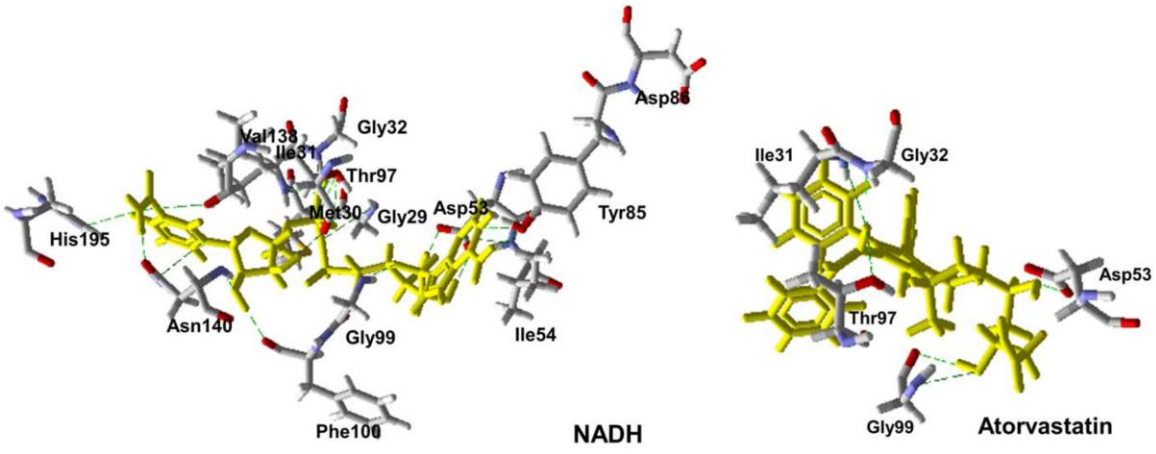

Figure 2. H-Bonds (in green) observed for NADH and atorvastatin (in yellow) with the active site residues of *Pf*LDH.
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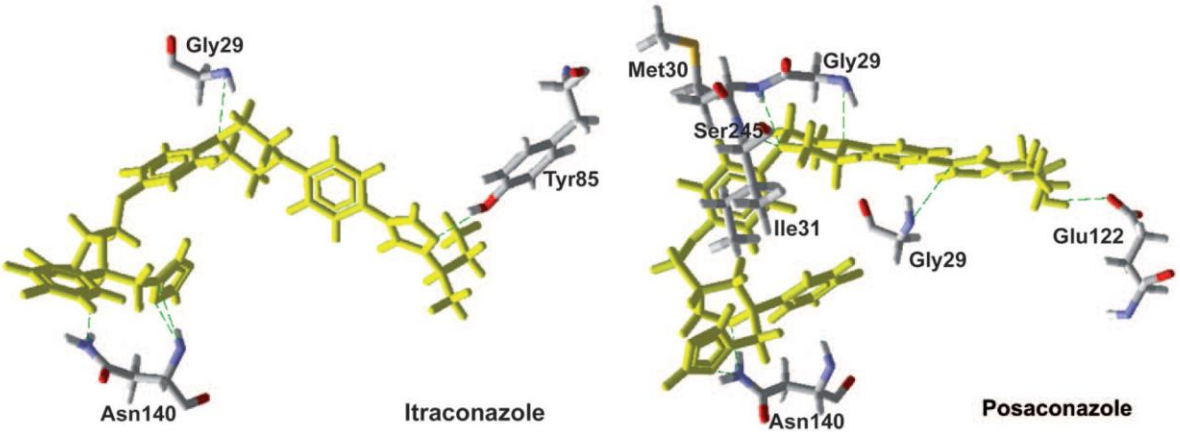

Figure 3. H-bonds (in green) observed for Itraconazole and Posaconazole (in yellow) with the active site residues of *Pf* LDH.
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form of a commercially available (USA) oral suspension for human use and confirmed its activity in mice. We hope to use this drug in subsequent human malaria trials. In other models, the *in vivo* activity of posaconazole depends on the interleukins IFN-γ and IL-12, such as in the case of *Trypanosoma cruzi* infections in mice [16]. Posaconazole has been considered a candidate for clinical trials in human Chagas disease caused by this hemoprotozoan parasite [15].

Itraconazole, acquired in tablet form and purified for the tests described herein, also caused a strong inhibition of *P. falciparum* growth *in vitro*; however, it was only partially active against *P. berghei* malaria in mice. The fact that the animals were not treated with the same pharmaceutical form (pellets) available for human use may explain its failure. Alternatively, it may have not been absorbed, may have been inactivated in the animal digestive tract or used in an insufficient dose. These possibilities should be further explored.

Atorvastatin, despite being over 100-fold less active than chloroquine *in vitro*, appears to be an attractive compound for the development of new antimalarials because its mechanism of action involves *Pf*LDH. In a recent work, atorvastatin activity was tested in combination with quinine and had a synergistic activity, enhancing antimalarial effects [17]. In addition, as recently shown, atorvastatin was able to reverse the binding of *P. falciparum* infected human erythrocytes (cytoadherence) to endothelial cells *in vitro* [18]. This drug is likely to become a good candidate for the treatment of severe malaria, specially if used together with other antimalarials. However, the ideal drug combinations and doses for human use are yet to be defined, either for ACT or with another antimalarial.

Itraconazole, like posaconazole and atorvastatin, does not require further testing for human toxicology and bioavailability because it is already approved and available for human use worldwide. Itraconazole is used against fungal infections and atorvastatin is the main component of the medicine Lipitor®, which is widely used to reduce cholesterol levels. Whether they can be useful for malaria treatment will depend on their possible synergisms with other antimalarials because only drug combination therapy is recommended for the control of malaria to avoid further selection for drug resistance. It would be desirable to perform further tests with the three compounds, applying them in different treatment routes and in ACT.

The *Plasmodium* lactate dehydrogenase (pLDH) enzymes found in all four species of human malaria parasites have been cloned, expressed and analyzed for structural and kinetic properties that may be explored for drug development. The pLDH from the species *P. vivax*, *P. malariae* and *P. ovale* exhibit 90–92% identity to *Pf*LDH. The catalytic residues and the cofactor sites are similar in the pLDH from *P. falciparum* and *P. malariae*, and the pLDH from *P. vivax* and *P. ovale* share one substitution. Homology modeling of the pLDH from *P. vivax*, *P. ovale* and *P. malariae* using the crystal structure of *Pf*LDH as a template yielded similar structures [19]. Thus, it would be desirable to have new anti-pLDH drugs that are effective against major species of human *Plasmodium* because cases of chloroquine-resistant *P. vivax* have already been reported [3], [20]. In addition, except for the catalytic residues (Arg171, Arg109, and the dyad His195/Asp168), *Pf*LDH has different active sites and substrate specificity loop residues than the human LDH isoforms (hLDH), reflecting a relative displacement of the nicotinamide ring and a volume increase of the active site compared with *Pf*LDH; *Pf*LDH displays kinetic differences with hLDH, suggesting that *Pf*LDH is a unique antimalarial target [19].

Table 3. *In vitro* activity of atorvastatin, itraconazole, posaconazole and chloroquine against *P. falciparum* as evaluated through their 50% inhibitory concentration (IC_{50}) in immunoenzymatic assays (ELISA) performed with monoclonal antibodies against a parasite protein (*Pf*HRP2) or the enzyme lactate dehydrogenase (*Pf*LDH).

Drugs	IC_{50} (μM) Mean ± SD in ELISA tests and CQ ratio*			
	Anti-*Pf*LDH	CQ activity ratio**	Anti-HRP2	CQ activity ratio**
Atorvastatin	13.1±4.5	262	13.8±3.2	115
Itraconazole	9.3±0.8	186	9.2±1.6	77
Posaconazole	2.6±0.3	52	5.3±1.8	44
Chloroquine-CQ	0.05±0	-	0.12±0.09	-

*Average of 4 experiments.
**Ratio of chloroquine activity, significantly higher that of test compounds.
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Table 4. Inhibition of *P. berghei* growth in mice infected with blood parasites that were then treated orally with atorvastatin, itraconazole, posaconazole or and chloroquine for three consecutive days in two independent experiments.

Drugs	Dose (mg/kg)	% Parasitemia Reduction*		Activity
		Exp. 1	Exp.2**	
Atorvastatin	20	41	40	Yes
Itraconazole	20	30	ND	Partial
Posaconazole	20	46	ND	Yes
	50	ND	45	Yes
	100	ND	71	Yes
Chloroquine	20	100	100	Yes

ND = not done.
*Reduction of parasitemia at day five of the experiment in relation to untreated controls (n = 4 to 6 mice per group).
**In the second experiment, posaconazole was diluted from a commercial oral suspension (Noxafil®).
Only the chloroquine-treated mice survived to day 30 post-inoculation; all the other mice died, despite the suppression of parasitemia in relation to the control mice.
doi:10.1371/journal.pone.0021237.t004

The study data suggest that the mechanism of parasite growth inhibition by the different compounds results from drug competition with NADH for the *Pf*LDH and that different methods used to measure drug activity against *P. falciparum in vitro* were equally efficient. The activity of itraconazole, atorvastatin and posaconazole, selected through docking studies and confirmed in biological assays, indicates that docking is an appropriate strategy for antimalarial discovery; furthermore, this technique is likely to be less expensive that traditional screening methods, especially because these compounds are commercially available and approved for human use. The best association(s) between these compounds and other antimalarials remains to be determined. Further improvements in the structures of the lead compounds could include additional pharmacophoric groups that can interact with more amino acids of the NADH pocket, leading to new and more effective antimalarials. Dynamic studies of these drugs binding with *Pf*LDH using more accurate methods can be used to evaluate the interactions between these drugs and the enzyme.

Materials and Methods

Molecular docking

The 3D structures of the *Pf*LDH complex with NADH and the substrate oxamate were obtained from the Protein Data Bank (PDB ID: 1LDG) [21]. For the docking studies, 50 compounds from DrugBank [13], all structural analogs of NADH, were selected using the search algorithm of the website and accessing the NADH chart in DrugBank through the option "show similar structures." This option uses a locally developed simplified molecular input line entry specification (SMILES) string comparison method to identify related structures and perform structure similarity searches. All structures are converted into SMILES strings, and a substring-matching program (similar to BLAST) is used to identify similar structures. The scoring scheme is based simply on the number of character matches for the longest matching substring [13]. The selected compounds were submitted to docking studies using MVD® [14]. The candidates with the best conformational and energetic results were selected for further experimental tests. MVD® [14] was used to calculate the interaction energies between ligands and macromolecular systems from the 3D structures of the protein and ligands. The algorithm used was the MolDock Score, an adaptation of the Differential Evolution (DE) algorithm [14]; the MolDock Score energy, E_{score}, is defined by Equation 1, where E_{inter} is the ligand-protein interaction energy and E_{intra} is the internal energy of the ligand. E_{inter} is calculated according to Equation 2.

$$E_{score} = E_{inter} + E_{intra} \tag{1}$$

$$E_{\text{inter}} = \sum_{i=ligant} \sum_{j=protein} [E_{PLP}(r_{ij}) + 332.0 \frac{q_i q_j}{4r_{ij}^2}] \tag{2}$$

The E_{PLP} term is a "piecewise linear potential" [22] that uses two different parameters, one for the approximation of the steric term (van der Waals) between atoms and another for the potential for hydrogen bonds; it describes the electrostatic interactions between charged atoms [14]. E_{intra} is calculated according to Equation 3.

$$E_{\text{intra}} = \sum_{i=ligant} \sum_{j=protein} [E_{PLP}(r_{ij})] + \sum_{flexible\,bonds} A[1-\cos(m\theta-\theta_o)] + E_{clash} \tag{3}$$

The first term in Equation 3 calculates all the energies involving pairs of atoms of the ligand, except those connected by two bonds. The second term represents the torsional energy, where θ is the torsional angle of the bond. The average of the torsional energy bond contributions is used if several torsions can be determined. The last term, E_{clash}, assigns a penalty of 1,000 kcal·mol^{-1} if the distance between two heavy atoms (more than two bonds apart) is smaller than 2.0 Å, ignoring infeasible ligand conformations [14].

Drug samples for pharmacological tests

Itraconazole [(2*R*,4*S*)-*rel*-1-(butan-2-yl)-4-{4-[4-({[(2*R*,4*S*)-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-lmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)piperazin-1-yl]phenyl}-4,5-dihydro-1*H*-1,2,4-triazol-5-one], which is commercially available as a generic >compound (Sporanox®, Janssen-Cilag) and is produced by Brainfarma, was purified into the crystallized form as previously described [23]. Atorvastatin [(3*R*,5*R*)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid] was provided by Farmanguinhos-FIOCRUZ as a purified compound. Posaconazole [4-(4-(4-(4-(((3r,5r)-5-(2,4-difluorophenyl)-5-(1,2,4-triazol-1-ylmethyl)oxolan-3-yl)methoxy)phenyl)piperazin-1-yl)phenyl)-2-((2s,3s)-2-hydroxypentan-3-yl)-1,2,4-triazol-3-one] was a kind gift from the Laboratory of Molecular Parasitology-FIOCRUZ, Belo Horizonte/MG, Brazil, where the compound has been studied against *T. cruzi* [16]. A second sample was later acquired in a drug store in USA to repeat the tests as Posaconazole oral suspension (Noxafil®) 200 mg/5 mL, Lot # 001R5H (Schering-Plough Research Institute, Kenilwort, New Jersey, USA).

All test drugs were assayed against *P. falciparum* diluted in dimethyl sulfoxide (DMSO 0.02% v/v) (Sigma-Aldrich, St. Louis, MO, EUA) from a 10 mg/mL stock solution and further diluted with RPMI 1640 (Sigma-Aldrich) supplemented with Hepes

25 mM (Sigma-Aldrich), sodium bicarbonate 21 mM (Sigma-Aldrich), glucose 11 mM (Sigma-Aldrich), glutamine 2% (Sigma-Aldrich) and gentamicin 40 mg/mL (Schering-Plough, Kenilworth, New Jersey, EUA). For each test well, the controls consisted of the parasite in culture without drug addition or with chloroquine at various concentrations.

Continuous cultures of *P. falciparum*

The chloroquine-resistant and mefloquine-sensitive *P. falciparum* W2 clone [24] was maintained in continuous culture at 37°C in human erythrocytes (A^+) in Petri dishes (Corning, Santa Clara, CA, USA) using the candle jar method [25] and grown in complete medium (RPMI 1640 supplemented with 10% human sera blood group A^+), with daily medium changes. Parasite samples were also stored frozen in liquid nitrogen.

Antimalarial tests *in vitro* against blood stages of *P. falciparum*

The effect of the test drugs against *P. falciparum* was determined from curves of inhibition of parasite growth *in vitro* as described previously [26] with some modifications [27]. Before the tests, the ring-stage parasites were concentrated in sorbitol-synchronized blood [28] and the suspension of infected red blood cells (iRBC) was adjusted for parasitemia and hematocrit following the specifications for each test; parasites were then distributed (180 µL/well) into a 96-well microtiter plate (Corning, Santa Clara, CA, EUA). All compounds were tested in triplicate for each dose in parallel with chloroquine, the standard antimalarial. The antiplasmodial activity was then measured using: (i) an ELISA anti-HRP2 test as previously described [29] and (ii) an ELISA anti-*Pf*LDH (*double-site enzyme-linked lactate dehydrogenase assay*) [30].

The effect of antimalarial drugs is initially characterized by the inhibition of parasite growth in drug-exposed cultures in comparison to a drug-free control culture. When performed using serial drug dilutions, sigmoid dose-response curves are generated and enable the determination of the 50% inhibitory concentration (IC_{50}).

ELISA anti-HRP2

The production of HRP2, a histidine- and alanine-rich protein, by *P. falciparum* parasites was tested *in vitro* as described previously [29]. Briefly, a suspension of iRBC with sorbitol-synchronized parasites, was adjusted to 0.05% parasitemia and 1.5% hematocrit, placed in 96-well plates containing the test and control drugs at various concentrations and incubated for 72 h under the culture conditions described above. After 24 h, the contents of the six control wells (parasites in drug-free medium) were harvested in microtubes and frozen for later use to further exclude the background value (i.e., the production of HRP2 during the first 24 h of incubation) by subtracting the average value obtained from these wells from the wells with the test and control drugs. After 72 h of incubation, the plates were frozen and thawed twice to lyse the erythrocytes.

To perform the test, a clean plate (Maxysorp, Nunc, Denmark) was first coated with 100 µL of the primary antibody anti-HRP2 (MPFM ICLLAB-55A®, USA) at 1.0 µg/mL. Following overnight incubation at 4°C, the monoclonal was discarded and replaced with 200 µL/well of the blocking solution PBS-BSA 2% (Phosphate Buffered Saline and Bovine Serum Albumin) (Sigma-Aldrich). Following a new incubation at room temperature for 2 h, the plate was washed three times with PBS/Tween20 at 0.05% (PBS-T). Then, each pretreated well received 100 µL of *P. falciparum* parasite culture (as described above), which was prehemolyzed by freeze-thawing at −70°C. In each test, two hemolyzed control sets of six wells each were used; one containing the 24 h cultures (background), the other with the 72 h parasite cultures. After incubation for 1 h at room temperature, the plate was again washed three times with PBS-T, incubated with 100 µL/well of the secondary antibody (MPFG55P ICLLAB®, USA), diluted 1:5,000 times, and again incubated for 1 h at room temperature. After three more washes with PBS-T, each well received 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) chromogen (KPL, Gaithersburg, MD, EUA) and was incubated for 10 min at room temperature in the dark; the reaction was stopped with 50 µL/L of 1 M sulfuric acid and the absorbance was immediately read at 450 nm in a spectrophotometer (SpectraMax340PC^{384}, Molecular Devices).

ELISA anti-*Pf*LDH

The anti-*Pf*LDH test was performed as described previously [30]. Briefly, cultures of *P. falciparum* were adjusted to 0.5% parasitemia and 2% hematocrit, placed in 96-well plates with the test drugs or control antimalarial drugs at different concentrations. The plates were then incubated under the same culture conditions as described above for 48 h, frozen and thawed thrice to lyse erythrocytes.

To perform the anti-*Pf*LDH test, a clean plate was first coated with 100 µL/well of the primary antibody anti-*Pf*LDH (17E4 Vista Diagnostics International LLC®, USA) at 1.0 µg/mL. Following overnight incubation at 4°C, the monoclonal was then discarded and replaced with 300 µL/well of the blocking solution (PBS-BSA 1%); the plate was then incubated at room temperature for 4 h and washed four times with PBS-T. The precoated plate received 100 µL of the *P. falciparum* parasite cultures in each well (as above), which was prehemolyzed by freeze-thawing and diluted 1:100 times in PBS/BSA 1%. For each test, two hemolyzed control sets of six wells each were used, one containing the parasite in cultures without drug addition and the other with uninfected red blood cells (RBCs).

The plate containing the parasite lysate and the monoclonal was again incubated for 1 h at room temperature, washed four times with PBS-T and then incubated with 100 µL/well of the secondary antibody (19g7, Vista Diagnostics International LLC®, USA) diluted 1:5,000 times. After 1 h of incubation at room temperature and four washes with PBS-T, each well received 100 µL of streptavidin-HRP conjugate (Sigma-Aldrich) diluted 1:1,000 and incubated for 30 min at room temperature. The plate was washed four times with PBS-T and incubated with 100 µl/well of TMB chromogen followed by 10 min of incubation at room temperature in the dark. The reaction was stopped with 50 µL/L of 1 M sulfuric acid and the absorbance was immediately read at 450 nm in a spectrophotometer (SpectraMax340PC^{384}, Molecular Devices).

Ethical committee approval for animal use

Methodological issues involving the use of laboratory animals in this study were approved by the Ethics Committee for Animal Use, the Oswaldo Cruz Foundation - Fiocruz (CEUA L-0046/08).

Antimalarial tests in mice infected with *P. berghei*

The antimalarial chemotherapy suppressive tests were performed as previously described [31], with modifications [32]. The *P. berghei* NK65 strain, a chloroquine-sensitive parasite, was stored at −70°C and also maintained by weekly blood passages in outbred Swiss mice. For the chemotherapy experiments, adult female mice weighing 20±2 g were inoculated intraperitoneally with 1×10^5 iRBC and kept together in a cage. Twenty-four hours

after parasite inoculation, the mice were randomly distributed, six mice per cage, and then orally treated with the test and control compounds daily for three consecutive days. The drugs were freshly diluted with water, DMSO 3% or RPMI and given orally (200 µL per animal) at doses of 20–100 mg/kg. Posaconazole was used at a dose of 20 mg/kg body weight in the first experiment and at 50 and 100 mg/kg in the second experiment. Chloroquine-treated and untreated control groups were included in each test. Thin blood smears were taken starting at day five after parasite inoculation, Giemsa stained and examined microscopically. Drug activity was determined on the basis of the average parasitemia per group of mice. The percent inhibition of parasite multiplication in the treated groups was compared to the untreated controls and the parasite inhibition growth was calculated based on the percent parasitemia in the groups according to equation 4 [32].

$$Percent\ parasitemia = \frac{PC - PTG}{PC} x100 \qquad (4)$$

Where: PC is the parasitemia in the control group and PTG is the parasitemia in the test group. Drugs that reduced parasitemia by 29–40% were considered partially active; a reduction of >40% was considered active. Overall mortality was monitored daily until day 30 post-infection in all groups.

Supplementary Material. A table with the energy values of the main HBond interactions between residues of NADH binding site and NADH, atorvastatin, itraconazole and posaconazole is available as Table S1.

Supporting Information

Table S1 HBond Energies between residues of NADH binding site and NADH, atorvastatin, itraconazole and posaconazole.
(DOC)

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Author Contributions

Conceived and designed the experiments: JP-C WAC AAO TCCF AUK. Performed the experiments: JP-C WAC. Analyzed the data: JP-C WAC. Contributed reagents/materials/analysis tools: TCCF AUK. Wrote the paper: JP-C WAC TCCF AUK.

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Anexo B- Artigo publicado PlosOne

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Theoretical and Experimental Studies of New Modified Isoflavonoids as Potential Inhibitors of Topoisomerase I from *Plasmodium falciparum*

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Abstract

DNA topoisomerase I from *Plasmodium falciparum* (*Pf*Topo I), a potential selective target for chemotherapy and drug development against malaria, is used here, together with human Topo I (*Hss*Topo I), for docking, molecular dynamics (MD) studies and experimental assays. Six synthetic isoflavonoid derivatives and the known *Pf*Topo I inhibitors camptothecin and topotecan were evaluated in parallel. Theoretical results suggest that these compounds dock in the binding site of camptothecin and topotecan inside both enzymes and that LQB223 binds selectively in *Pf*Topo I. *In vitro* tests against *P. falciparum* blood parasites corroborated the theoretical findings. The selectivity index (SI) of LQB223 ≥98 suggests that this molecule is the most promising in the group of compounds tested. *In vivo* experiments in mice infected with *P. berghei* showed that LQB223 has an antimalarial activity similar to that of chloroquine.

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Introduction

Malaria is the most lethal parasitic disease, causing 219 million cases and 660,000 deaths annually, mainly in African sub-Saharan countries [1]. Brazil registered 306,000 cases of malaria in 2009, most of which were in the Amazon Region and caused by *Plasmodium vivax* followed by *P. falciparum* [2]. This species may cause severe malaria and death in untreated individuals, especially children under five [3], in addition, it quickly develops resistance under selective drug pressure [4]. Presently, *P. falciparum* is resistant to most available drugs; its low susceptibility to artemisinin-combined therapy (ACT) is also registered, especially in Southeast Asia countries [5]. *P. vivax*, highly prevalent worldwide, now shows resistance to chloroquine (CQ) in several countries, including in Brazil [2,6]. There is no effective vaccine available against malaria [7,8], thus the disease control relies on individual protection against the mosquito vector bites, and specific drug chemotherapy. Continuous efforts to develop new antimalarials and other control measures are required. The search for better treatments based on new molecular targets should broaden the therapeutic arsenal and allow strategies to fight drug resistance in human malaria [9–11].

DNA topoisomerases (Topo) are enzymes involved in DNA replication, transcription, recombination and repair. Although the human Topo I (*Hss*Topo I) presents 42% of similarity with *P. falciparum* Topo I (*Pf*Topo I) [11], this enzyme may represent a potential selective target to be explored for drug development against malaria, the aim of our present work. We used docking, molecular dynamics and experimental assays with six synthetic isoflavonoid derivatives, shown in Figure 1 [12–14], in comparison to the known Topo I inhibitors camptothecin and topotecan [15–17]. Four are pterocarpanquinones (LQB118, LQB216, LQB221, and LQB222), one is N-tosyl-azapterocarpanquinone (LQB192), and one is N-tosyl-azapterocarpan (LQB223). The results show that LQB223 was the most promising compound, thus, a potential *Pf*Topo I inhibitor and promising new antimalarial lead compound.

Methodology

Docking and molecular dynamics simulations

The 3D structures of the compounds in Figure 1 were built and optimized using the Gaussian 03W package [18], and the initial geometries optimized by the method DFT/B3LYP/6-31G [18,19]. The 3D structure of *Hss*Topo I in complex with topotecan and a 22 base pair DNA duplex used here is available in the Protein Data Bank (PDB) [20] under the code 1K4T [17]. The 3D structure of *Pf*Topo I (Figure S1) was built in the Swiss Model server [21], according to the procedure reported by Roy *et al* [16], using as templates the PDB structures of *Hss*Topo I 1K4T [17] and 1A36 [22]. The coordinates of crystallographic water molecules,

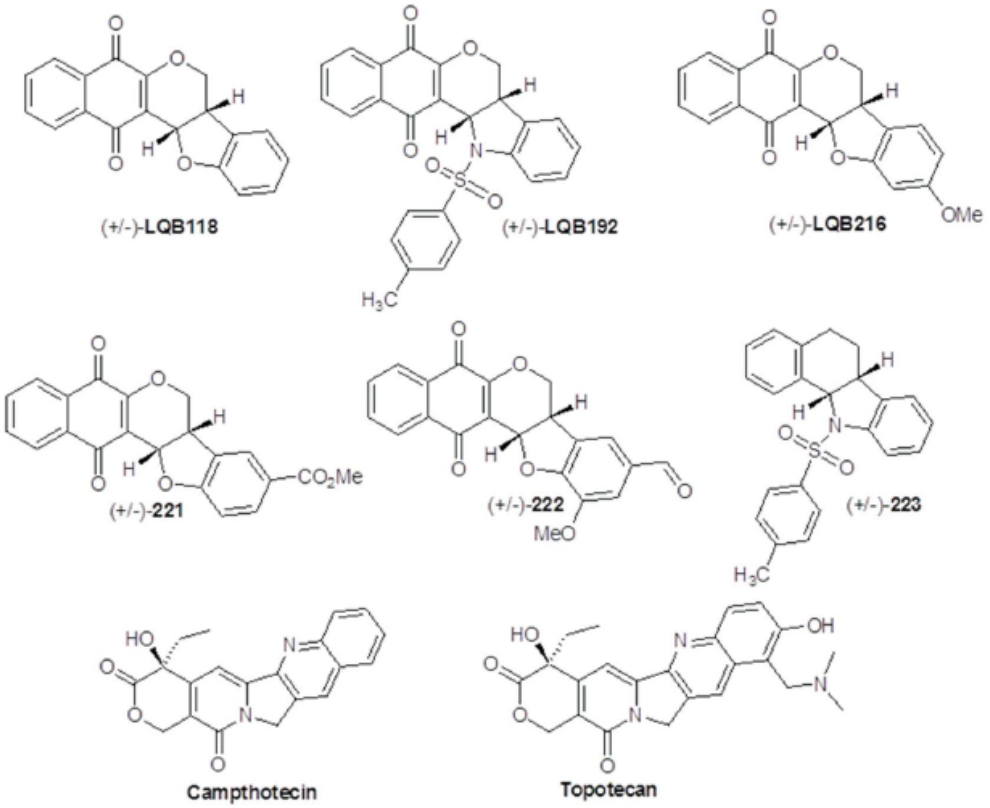

Figure 1. Chemical structures of the new modified isoflavonoids (LQBs), camptothecin and topotecan (Topo I inhibitors).
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topotecan and DNA from 1K4T were copied into the model to obtain the *Pf*TopoI-DNA covalent complex for the docking study.

The docking energies were calculated using the software Molegro Virtual Docker® (MVD) [23]. Our studies were performed considering the covalent complex. Calculations were carried inside a constraint sphere of radius 12 Å with the topotecan as the center, considering the residues within a radius of 15 Å from the center of the sphere as flexible. For the other residues, the rigid docking methodology was performed. Due to the stochastic nature of the docking algorithm, about 20 runs were carried out for each compound, and, 30 poses were found by analyzing the overlap and interactions between ligand and protein. The docking protocol used was previously validated by re-docking topotecan into its own crystallographic structure inside *Hs*TopoI. The best poses of LQB223, based on the lowest values of MolDock Score [23], obtained from the docking studies inside *Hs*TopoI and *Pf*TopoI and complexed with DNA were submitted to additional steps of MD simulations, carried out using the GROMACS 4.5.3 package with the AMBER force field 99SB [24]. The system to be simulated was put into a space-filling box, which was replicated periodically in the x, y and z directions. These systems were studied using the single-point charge (SPC) water model [25]. The minimization algorithms used were performed according to a protocol previously published [26]. The minimized complexes were then submitted to MD simulations for 15 ns using a NpT ensemble, following the same protocol [26]. All Arg and Lys residues were assigned with positive charges and, the residues Glu and Asp were assigned with negative. The short-range Lennard-Jones energies (LJ-SR) and Coulombic potentials within R-coulomb (Coul-SR) were calculated using the g_energy program available in Gromacs, extracting information from .edr files generated during the MD simulations.

Continuous cultures of *P. falciparum* and antiplasmodial tests

A CQ-resistant and mefloquine-sensitive *P. falciparum*, clone W2 [27] and a CQ-sensitive strain 3D7, were cultivated as described [28], in human erythrocytes (A^+), in complete medium (RPMI 1640 supplemented with 10% human sera blood group A^+) and tests followed a protocol [29] slightly modified as follows. Human erythrocytes (A^+) and serum blood group A^+ were kindly donated by Center of Hemotherapy and Hematology of Minas Gerais (HEMOMINAS - http://www.hemominas.mg.gov.br), under the mutual cooperation term number 18/09.

The ring-stage parasites sorbitol-synchronized [30] were adjusted for parasitemia and hematocrit, according to the specifications for the test, then distributed in 96-well microtiter plates (Corning, Santa Clara, CA, EUA), previously prepared and containing the diluted compounds in dimethyl sulfoxide (DMSO) (0.02%) aqueous stock solution, in triplicates for each dose. CQ, the standard antimalarial, was tested in parallel each time. The drug activity was determined in relation to parasite cultures in complete medium and no drugs, as described [31]. Parasite growth was measured through the anti-HRPII test [32] in cultures adjusted to 1.5% hematocrit and 0.05% parasitemia, with monoclonal antibodies (MPFM-55A and MPFG-55P) commercially acquired (ICLLAB®, USA) and the TMB

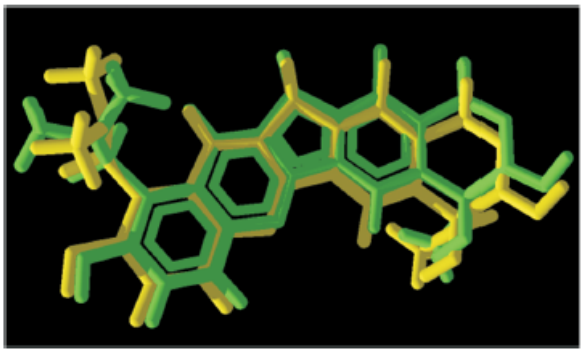

Figure 2. Superposition of topotecan (yellow) to its crystallographic structure (green) after re-docking calculations.
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chromogen (3,3′,5,5′-Tetramethylbenzidine) from KPL (Gaithersburg, MD, EUA). The reaction was stopped with 50 µL/L of 1 M sulfuric acid, absorbance read at 450 nm in a spectrophotometer (SpectraMax340PC384, Molecular Devices). Sigmoid dose-response curves were generated with curve-fitting software (Microcal Origin Software 5.0, Inc.), used to determine the 50% inhibitory concentration of the parasite growth (IC_{50}), and activity calculated by comparing growth in drug-exposed cultures and the drug-free control cultures.

Cytotoxicity tests

The cytotoxicity tests were performed against normal kidney glomerular cells from green monkey (BGM) as follows. The cells were cultured under a 5% CO_2 atmosphere, at 37°C, in 75 cm^2 sterile flasks in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, and 40 mg/L gentamicin. When confluent, the cell monolayer was washed with culture medium, trypsinized, distributed in flat-bottomed 96-well plates (5×10^3 cells/well), and incubated for 18 h at 37°C for cell adherence [33]. The compounds (20 µL) were added to the cell plates, at various concentrations (1000 to1 µg/mL), incubated for 24 h, then received a solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg/mL; 20 µL/well), followed by another 3 h incubation, to evaluate mitochondrial viability. The supernatants were removed, DMSO added to each well (100 µL), carefully mixed to solubilize the formazan crystals. The optical density was determined at 570 and 630 nm (background) (SpectraMax340PC384, Molecular Devices). Cell viability was expressed as the percentage of the control absorbance in the untreated cells after subtracting the appropriate background. The minimum lethal dose for 50% of the cells (MLD_{50}) was determined [34] and the selectivity index (SI) calculated as the ratio between cytotoxicity and activity [35].

Antimalarial tests against *P. berghei* in mice

The use of laboratory animals was approved by the Ethics Committee for Animal Use of the Oswaldo Cruz Foundation - Fiocruz (CEUA LW-23/13), being in accordance with the principles of the Brazilian Society of Laboratory Animal Science (SBCAL - http://www.cobea.org.br/).

The antimalarial suppressive test in mice, as previously described [36], was slightly modified [37]. Briefly, Swiss outbred adult female mice, 20±2 g weight, were inoculated with *P. berghei* CQ-sensitive (NK65) by intraperitoneal route, 1×10^5 freshly infected red blood cells (iRBC) per animal. Two to 24 h later, the mice were randomly distributed to be submitted to drug treatment by oral route, during three consecutive days, once a day; two control groups were used, one non treated and another treated with CQ (either 5 or 15 mg/kg).

The molecule LQB223, freshly diluted with 3% DMSO in water immediately before treatment, was given by gavage, in doses of 100 and 50 mg/kg in 200 µL volume per animal. Blood smears were prepared at days fifth and seventh after parasite inoculation, methanol-fixed, stained with Giemsa, and examined microscopically for parasitemia determination. The inhibition of parasite growth (IPG) in the treated groups was evaluated by comparison with the non-treated mice, through the equation: IPG = 100 minus the mean parasitemia in the treated mice, multiplied by 100, and divided by the mean parasitemia in non-treated controls. Molecules reducing parasitemia by 30% or more were considered active.

Results

Docking

The re-docking calculations showed superposition of topotecan to its crystallographic structure (Figure 2) with a Random Mean

Table 1. *In vitro* activities (IC_{50}) of Topo I inhibitors determined in assays against *P. falciparum* blood parasites, CQ-sensitive (3D7) or CQ-resistant (W2), and cytotoxicity against a monkey kidney cell line (BGM) measured as the minimal lethal dose for 50% of cells (MDL_{50}).

Molecule	MDL_{50} (µg/mL) Mean ± SD	W2		3D7	
		IC_{50} (µg/mL)* Mean ± SD	SI**	IC_{50} (µg/mL)* Mean ± SD	SI**
LQB118	≤3.9	0.17±0.1	23	0.16±0.03	24
LQB192	68.7±8.4	2.9±2.5	24	3.8±1	18
LQB216	24.2±3	0.4±0.2	60	0.21±0.09	115
LQB221	5.1	0.63±0.8	8	0.25±0.1	20
LQB222	≤3.9	1.8±1.6	2	3.5±0.4	1
LQB223	≥1000	10.2±1.2	98	7.8±3	128
Chloroquine	ND	0.138	-	0.03	-

The selectivity index (SI) is a ratio between toxicity and activity.
*IC_{50} ≤10 µg/mL are considered as active; 11–20 µg/mL as partially active (PA) and > 20 µg/mL as inactive.
**SI based in results from three experiments; values bellow 10 are indicative of drug toxicity. ND= not done.
doi:10.1371/journal.pone.0091191.t001

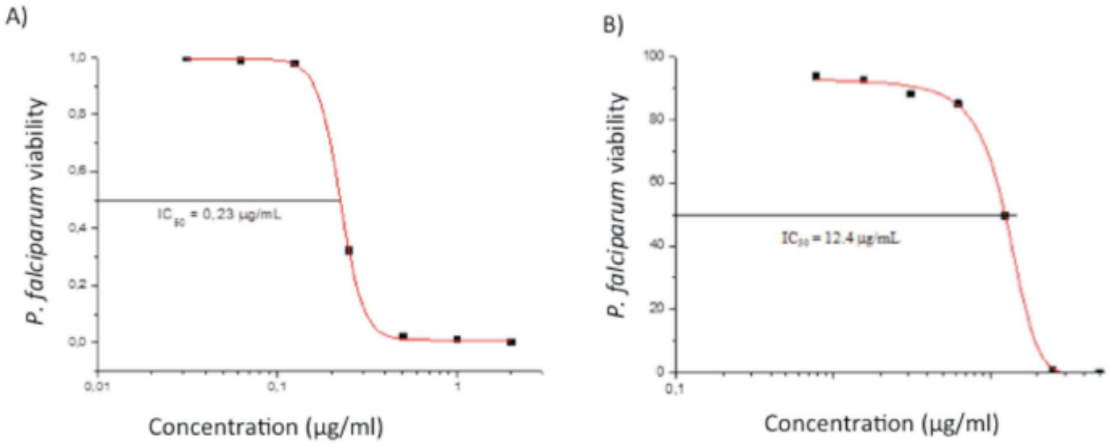

Figure 3. Dose response effect of LQB216 (A) and LQB223 (B), tested in parallel against the CQ-resistant *P. falciparum*.
doi:10.1371/journal.pone.0091191.g003

Square Deviation (RMSD) of 0.54 Å which, according to literature, is acceptable [19,38,39]. LQB223 presented the most comparable docking values to camptothecin and topotecan (Table S1), thus the most promising and selective inhibitor for *Pf*TopoI. The results suggest that LQB223 may establish better interactions with *Pf*TopoI (-187.5 cal mol^{-1}) than with *Hss*TopoI (-171.2 Kcal mol^{-1}). The main residues involved in the interactions with LQB223 (Table S1) were different for *Hss*TopoI (Ans352, Glu356, Arg364, Tyr452 and Lys425) and *Pf*TopoI (Arg312, Lys512, Asp513, Gln698 and Thr701).

In vitro and in vivo studies against malaria parasites

The assays of drug activity were performed in three independent experiments, using *P. falciparum* iRBC with parasites CQ-resistant (W2 clone) and CQ-sensitive (3D7 strain), in the anti-HRPII test. All topoisomerase I inhibitors were active in micromolar range (Table 1). The most active compounds were LQB118 and LQB216 (IC_{50} 0.17 and 0.4 µg/ml for W2, and, 0.16 and 0.21 for 3D7 parasites); nevertheless, LQB223 was less active against both, the 3D7 ($IC_{50} = 7.8 \pm 3$ µg/mL), and W2 parasites ($IC_{50} = 10.2 \pm 1.2$ µg/mL). The IC_{50} values for CQ were 70 ng/ml (W2 clone) and 3.5 ng/ml (3D7 strain) in our previous work [9,10]; these parasite susceptibilities to CQ were herein confirmed in parallel experiments (Table 1). The growth inhibition curves and the IC_{50} values for molecules LQB216 and LQB223, against the CQ-resistant *P. falciparum*, are illustrated in Figure 3.

Compounds LQB118, LQB192, LQB221 and LQB222, although active, were toxic to BGM cells; thus, in spite of their activity *in vitro*, they displayed low selectivity indexes (SI). Compound LQB223, in spite of being less active, showed higher SI (128 and 98), respectively, for the 3D7 and W2 parasites; the second best compound was LQB216, with SI of 115 and 60, respectively, for these parasite lines.

The most promising compound, LQB223, was then tested in mice infected with *P. berghei* malaria induced by blood forms. Given by gavage, in daily doses of 100 or 50 mg/kg, for three days, LQB223 reduced parasitemia, respectively, in 67% and 30% on the fifth day of infection (Table 2).

Molecular dynamics

In order to validate the docking methodology and to understand the drug selectivity observed *in vitro* with LQB223, this promising compound was further studied considering the dynamic behavior inside the topotecan binding sites of *Hss*TopoI and *Pf*TopoI. The temporal RMSD calculations were carried out on all atoms of each complex to give frames every 200 ps during the 15 ns of

Table 2. *P. berghei* (NK65 chloroquine-sensitive strain) parasitemia and reduction at days 5 and 7 of infection, in mice treated with either LQB223 or chloroquine in relation to control non-treated mice.

Treatment with	Dose (mg/kg)	Mean Parasitemia ± SD (% Reduction)	
		5^{yh} Day	7^{th} Day
Exp.1			
Control non-treated	**0**	0.5±0.2	15±4.4
Chloroquine	**5**	0 (100%)	7.3±3 (52%)
LQB223	**100**	0.15±0.2 (67%)	11±3 (28%)
Exp.2			
Control non-treated	**0**	1.7±0.8	21±8
Chloroquine	**15**	0±0.0 (100%)	0±0.0 (100%)
LQB223	**50**	1.2±0.5 (30%)	18.3±1.5 (13%)

doi:10.1371/journal.pone.0091191.t002

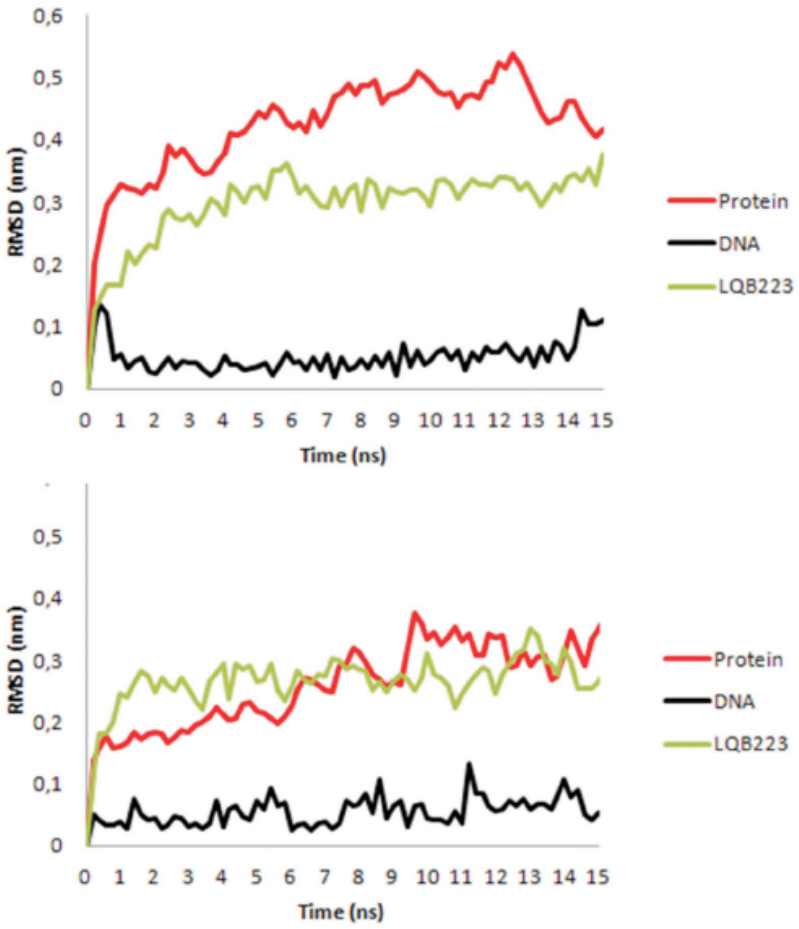

Figure 4. Temporal RMSD values for the LQB223, DNA an *Pf*Topol (upper) an *Hss*Ropol (lower). These values were counted in the time range of 0–15,000 ps, at each 20 ps.
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simulation. Figure 4 shows the equilibration of each system during the first 1 ns.

The MD studies show that LQB223 presents distinct dynamical behavior inside *Pf*TopoI and *Hss*TopoI. Considering Lennard-Jones short-range (LJ-SR) and Coulombic short-range (Coul-SR) potential energy calculations between protein, DNA, and ligands, it was observed that LQB223 interacts differently inside *Pf*TopoI and *Hss*TopoI (Table 3). LQB223 displays relatively lower energy pattern inside *Pf*TopoI compared to *Hss*TopoI. The simulations also show that the interactions energies for the interaction between LQB223 and DNA are lower in the *Hss*TopoI binding site.

Discussion

Previous studies identified some flavonoids as DNA intercalators, *Hss*TopoI and *Pf*TopoI inhibitors, suggesting that topoisomerase I may be a molecular target for these compounds [40,41]. All LQBs previously prepared presented antineoplasic [12,41,42] and antiparasitic [43,44] properties *in vitro* [12,42,43,44] and *in vivo* [44]. Considering that and their similarity with the two known Topo I inhibitors camptothecin and topotecan (Figure 1), they are expected to be potential inhibitors of *Pf*TopoI. The best way to provide this theory would be performing experimental analysis of the LQBs inside the *Pf*TopoI. As our group does not have access to the enzyme, we performed computational analysis to understand the dynamic of these compounds, which give us an idea of inhibition and toxicity. But, additional experimental work should be performed to validate *Pf*TopoI as a molecular target for the LQBs. The docking results suggest that the LQBs fit well into the binding sites of camptothecin and topotecan, although the absolute values were lower than those obtained with the inhibitors. The Topo I-DNA-drugs complex demonstrate that all LQBs intercalate at the site of DNA cleavage and are stabilized by π-stacking interactions (data not shown), suggesting they act as uncompetitive inhibitors, as previously reported for topotecan [17]. However, only LQB223 is likely to be potentially selective, considering the differences observed in the docking energies for this compound in *Pf*TopoI and *Hss*TopoI (Table S1). This molecule also presented the closest docking energies to topotecan and camptothecin. The location of LQB223 inside *Pf*TopoI suggests that the interactions with the residues Arg312 (11.4 Kcal mol^{-1}), Asp513 (−6.0 Kcal mol^{-1}), Gln698 (−11.5 Kcal mol^{-1}), and DNA (−145.6 Kcal mol^{-1}) are relevant for drug selectivity.

Most Topo I inhibitors tested showed a similar activity profile against *P. falciparum* parasites CQ-resistant (W2) or CQ-sensitive (3D7); however, only two compounds were promising as new antimalarial targets (LQB223 and LQB216), active against both

Table 3. Lennard-Jones energies short-range (LJ-SR) and Coulombic potential within R-coulomb Coul-SR) between LQB223, protein, DNA and water during MD simulations.

	*Hss*Topo1 (kJ mol^{-1})				*Pf*Topo1 (kJ mol^{-1})			
	Coul-SR	LJ-SR	Coul-SR + LJ-SR	Estimated Error	Coul-SR	LJ-SR	Coul-SR + LJ-SR	Estimated Error
Protein-LQB223	2.4	−29.8	−27.4	4.1	1	−42.4	−41.4	5.0
DNA-LQB223	−6.6	−144.3	−150.9	7.7	−4.95	−121.9	−126.8	6.5
Water-LQB223	−5.9	−59.61	−65.5	5.4	−5.68	−73.5	−79.1	7.1
Total			−243.8				−247.3	

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strains, and displayed the highest therapeutic index, i.e., SI of 98 and 60 for W2, and 128 and 115 for 3D7. Compound LQB222 was the most toxic, with SI bellow 10 for both strains.

Data *in vitro* for LQB223, the only *N*-tosyl-azapterocarpan with the best SI value, corroborate the docking results, and explain the data *in vivo*, when it also inhibited malaria in mice infected with *P. berghei*.

MD results suggest that this molecule fits well in the binding site during the simulation, showing the system stabilization. The differences observed in the short-range potential energy calculations suggest a reasonable explanation for the selectivity of LQB223 for *Pf*TopoI over *Hss*TopoI observed in the experimental studies. The MD simulations showed that the energies of interaction between LQB223 and the protein are more favorable inside *Pf*TopoI, corroborating with the docking results. However, in contrast with the docking calculations, MD studies indicate that LQB223 interacts better with DNA in *Hss*TopoI instead of *Pf*TopoI. An analysis of the RMSF indicates that the fluctuation of the residues of *Pf*TopoI is higher than in *Hss*TopoI (Figure S2). This result is similar with previous published data showing that the residues in the plasmodial enzyme displays a larger flexibility than the human enzyme when docked with camptothecin [45] and raises the possibility of other molecular targets being responsible for the *in vitro* and *in vivo* activity observed for LQB223.

The search for new inhibitors of specific drug targets of *P. falciparum* has been explored as a promising way to develop new antimalarials [9,46,47]. The differences in the binding sites between *Pf*TopoI and *Hss*TopoI suggest this enzyme as a suitable selective target for the drug design against *P. falciparum*. According to the *in vitro* results, all LQBs are potential candidates to *Pf*TopoI inhibitors, being the LQB223 the most promising compound, considering that this molecule presented the highest SI and was also active *in vivo*. It suggests LQB223 as a promising lead compound to develop new antimalarials. The interactions with DNA and residues Arg312, Asp513 and Gln698 of *Pf*TopoI may be explored in further studies of new potential inhibitors of *Pf*TopoI.

Supporting Information

Figure S1 3D protein model of *Pf*TopoI.
(TIF)

Figure S2 Per-residue Root Mean Square Fluctuations (RMSF) of the *Hss*Topo1 and *Pf*TopoI.
(TIF)

Table S1 Interaction energies between the LQBs, DNA and the residues of *Hss*TopoI and *Pf*TopoI.
(PDF)

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Author Contributions

Conceived and designed the experiments: WAC JP-C ASP CDB PRRC TCCF AUK. Performed the experiments: WAC JP-C ACCA CDB BRMA. Analyzed the data: WAC TCCF ASP AUK. Contributed reagents/materials/analysis tools: TCCF ASP CDB PRRC AUK. Wrote the paper: WAC JP-C ACCA BRMA ASP CDB PRRC TCCF AUK.

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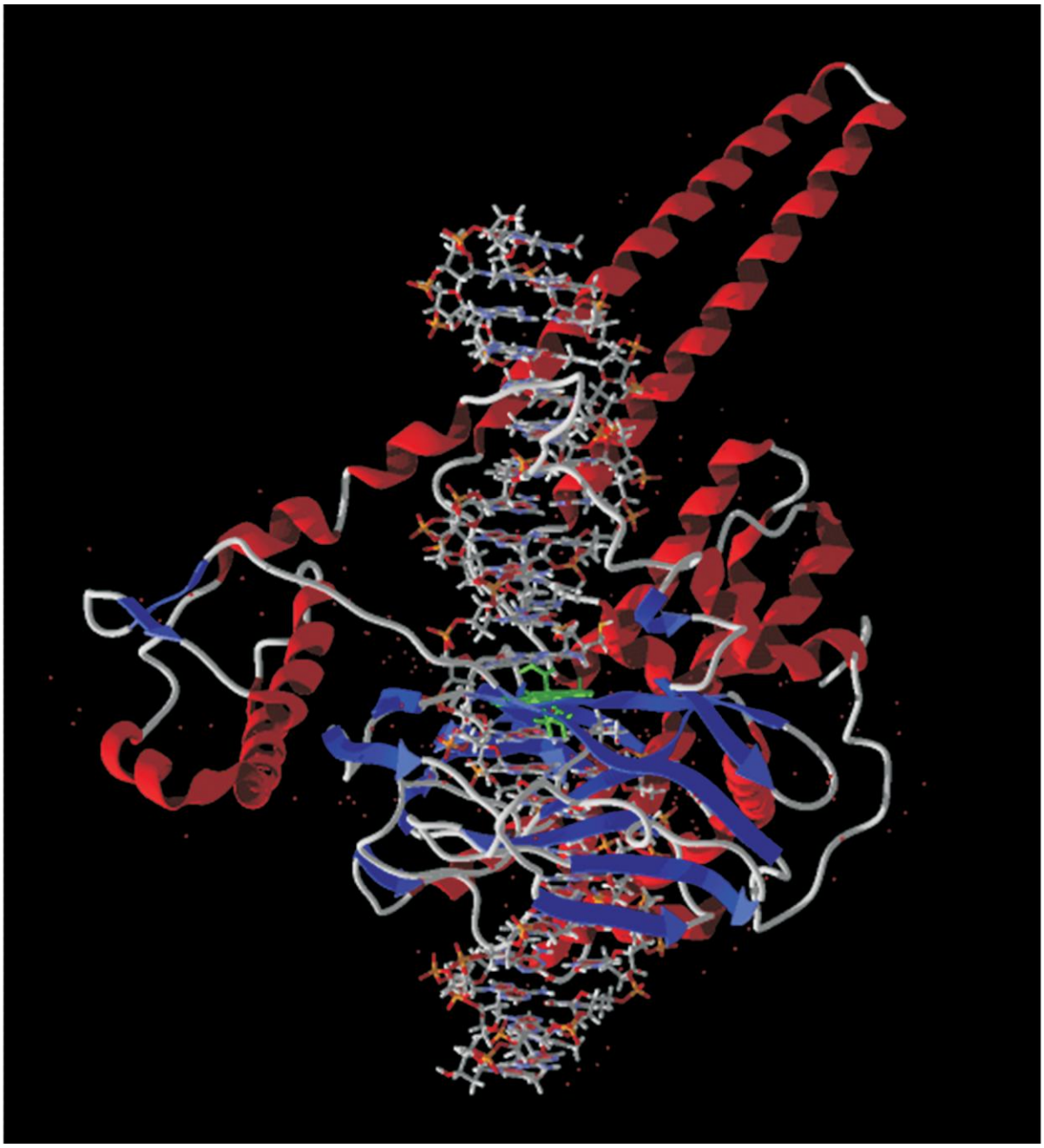

Figure S1. 3D protein model of *Pf*TopoI.

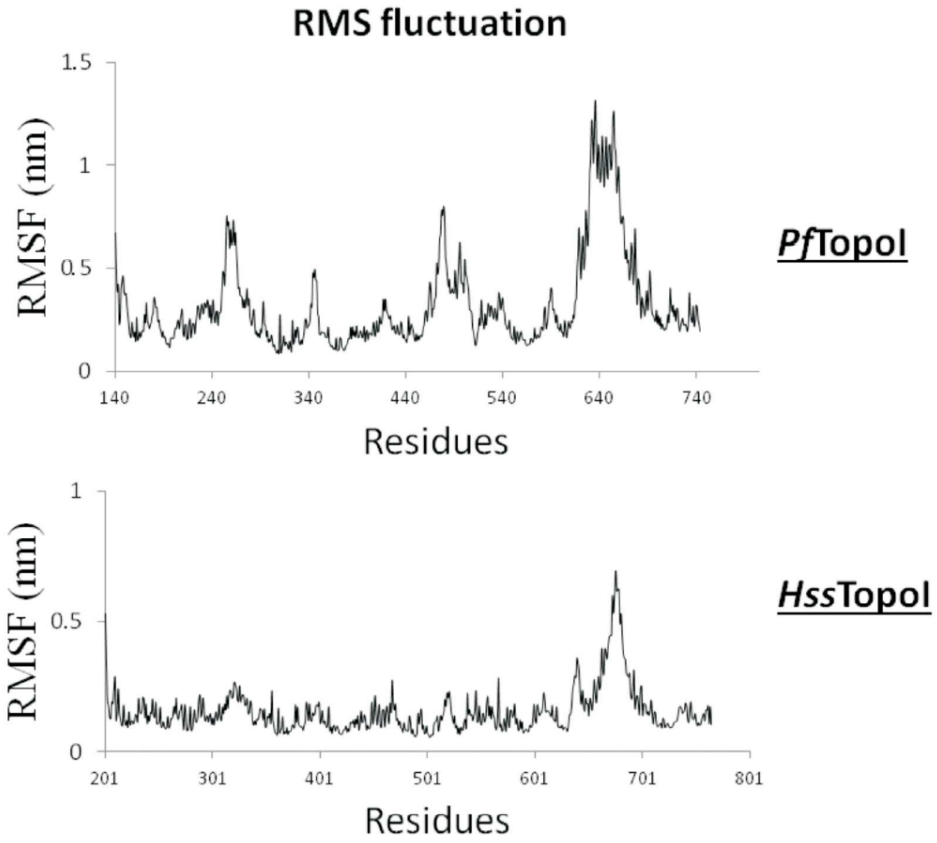

Figure S2. Per-residue Root Mean Square Fluctuations (RMSF) of the *Hss*Topo1 and *Pf*TopoI.

Table S1. Interaction energies between the LQBs, DNA and the residues of *Hss*TopoI and *Pf*TopoI.

Supplementary Material

Table S1. Interaction energies between the LQBs, DNA and the residues of *Hss*TopoI and *Pf*TopoI.

Compound	*Hss*Topo1			*Pf*Topo1		
	Total Energy (kcal mol^{-1})	Residue	Energy (kcal mol^{-1})	Total Energy (kcal mol^{-1})	Residue	Energy (kcal mol^{-1})
Topotecan	-237.5	DNA	-186.4	-238.6	DNA	-187.9
		Asp533	-10.7		Asp513	-10.4
		Arg364	-8.6		Glu304	-8.8
		Glu356	-5.8		Arg312	-8.3
		Thr718	-3.9		Gln698	-7.1
		Asn352	-3.8		Lys512	-3.4
Camptothecin	-211.1	DNA	-175.7	-212.7	DNA	-175.6
		Asp533	-9.6		Asp513	-10.0
		Arg364	-9.3		Arg312	-9.0
		Lys532	-4.9		Gln698	-7.1
		Thr718	-4.5		Lys512	-4.8
		Asn722	-1.4		Glu304	-0.8
LQB192	-95.6	DNA	-92.5	-96.4	DNA	-91.9
		Lys425	-9.9		Arg312	-9.9
		Trp416	-5.7		Asp513	-6.2
		Asn352	-5.0		Gln698	-5.0
		Tyr426	-4.4		Lys512	-2.0
		Arg364	-2.4		Ile515	-0.8
LQB223	-171.2	DNA	-132.5	-187.5	DNA	-165.3
		Asn352	-9.3		Gln698	-11.5
		Arg364	-4.3		Arg312	-11.4
		Glu356	-4.1		Asp513	-6.0
		Tyr452	-3.8		Lys512	-2.5
		Lys425	-3.0		Thr701	-1.8
LQB118	-144.4	DNA	-133.2	-143.4	DNA	-133.6
		Arg364	-10.2		Arg312	-10.1
		Asp533	-6.9		Asp513	-6.8
		Asn722	-2.8		Lys512	-2.5
		Lys532	-2.5		Gln698	-2.4
		Thr718	2.0		Thr702	-0.5
LQB221	-160.0	DNA	-121.6	-156.2	DNA	-122.2
		Lys425	-13.9		Lys376	-12.5
		Tyr426	-5.7		Tyr377	-6.4
		Asn352	-4.9		Tyr367	-6.3
		Glu356	-4.3		Arg312	-4.0
		Trp416	-4.1		Glu304	-3.8
LQB222	-154.4	DNA	136.3	-155.4	DNA	-136.8
		Arg364	-11.9		Arg312	-11.6
		Asp533	-7.0		Asp513	-6.7
		Lys532	-3.4		Glu698	-4.4
		Thr718	-3.3		Lys512	-3.5
		Ile535	-1.4		Ile695	-2.1
LQB216	-146.9	DNA	-134.1	-145.8	DNA	-133.6
		Lys425	-6.3		Lys376	-6.7
		Glu356	-4.1		Glu304	-3.9
		Arg364	-3.9		Arg312	-3.3
		Glu418	-3.0		Asp369	-3.0
		Phe361	-0.5		Asn372	-0.5

Anexo C- Artigo publicado Memórias do Instituto Oswaldo Cruz

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Aspidosperma (Apocynaceae) plant cytotoxicity and activity towards malaria parasites. Part I: *Aspidosperma nitidum* (Benth) used as a remedy to treat fever and malaria in the Amazon

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Infusions of Aspidosperma nitidum *(Apocynaceae) wood bark are used to treat fever and malaria in the Amazon Region. Several species of this family are known to possess indole alkaloids and other classes of secondary metabolites, whereas terpenoids, an inositol and the indole alkaloids harmane-3 acid and braznitidumine have been described in* A. nitidum. *In the present study, extracts from the wood bark, leaves and branches of this species were prepared for assays against malaria parasites and cytotoxicity testing using human hepatoma and normal monkey kidney cells. The wood bark extracts were active against* Plasmodium falciparum *and showed a low cytotoxicity in vitro, whereas the leaf and branch extracts and the pure alkaloid braznitidumine were inactive. A crude methanol extract was subjected to acid-base fractionation aimed at obtaining alkaloid-rich fractions, which were active at low concentrations against* P. falciparum *and in mice infected with and sensitive* Plasmodium berghei *parasites. Our data validate the antimalarial usefulness of* A. nitidum *wood bark, a remedy that can most likely help to control malaria. However, the molecules responsible for this antimalarial activity have not yet been identified. Considering their high selectivity index, the alkaloid-rich fractions from the plant bark might be useful in the development of new antimalarials.*

Key words: *Aspidosperma nitidum* - medicinal plants - human malaria - *Plasmodium falciparum* - selectivity index

Malaria affects millions of individuals in tropical and subtropical regions of the world and infection with *Plasmodium falciparum* continues to cause an economic burden in several areas (Sachs & Malaney 2002) and to cause death among non-treated individuals, particularly children, non-immune travellers and pregnant women (WHO 2011). Approximately 219 million cases of malaria and 660,000 deaths were reported worldwide in 2010 (WHO 2012a).

Medicinal plants offer an alternative in treating malaria, particularly among populations exposed to endemic transmission in sub-Saharan African countries and in certain regions of Latin America (Bourdy et al. 2004, Willcox & Bodeker 2004, Adebayo & Krettli 2011, Adebayo et al. 2012). Although synthetic drug combinations are the basis for malaria treatment in Brazil, plants are still used as remedies in the Amazon and elsewhere and represent a potential source for compounds in the development of new antimalarial drugs (Krettli 2009).

Due to the spread of drug-resistant parasites globally, the treatment of malaria is becoming increasingly difficult (WHO 2010). Thus, the identification of safe and selective antimalarial drugs derived from plants has been stimulated by the World Health Organization and is a research priority of several groups, including ours (Krettli et al. 2001, 2009, Aguiar et al. 2012a). Indeed, medicinal plants afforded the discovery in the XIX century of the first remedies from the bark of *Cinchona* spp, later shown to be rich in alkaloids, particularly quinine, the basis of such aminoquinoline synthetic drugs as chloroquine, amodiaquine and mefloquine. Another class of important antimalarials is based on artemisinin, a compound found in *Artemisia annua*, a medicinal plant native to China (WHO 2006). Unfortunately, clinical failures have also been reported in patients with *P. falciparum* treated with artemisinin derivatives in the recommended artemisinin combined therapy (WHO 2012b).

The testing of products from medicinal plants in animal models and in parasite cultures has resulted in the identification of potent molecules, as recently reviewed (Krettli et al. 2001, Krettli 2009, Aguiar et al. 2012b).

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An example is *Cecropia pachystachya* (Cecropiaceae), which is used against fever and malaria in several parts of Latin America, with tormentic acid and beta-sitosterol shown to be responsible for the antimalarial activity (Uchoa et al. 2010). Other examples include *Aspidosperma nitidum* (Benth) and *Aspidosperma excelsum* (Muell Arg), widely cited as remedies in the treatment of malaria, among other species of the genus *Aspidosperma* (Apocynaceae), which comprises various medicinal species used as bark infusions against malaria and other diseases (Brandão et al. 1992, Weniger et al. 2001, Ferreira et al. 2004, Tanaka et al. 2007, de Oliveira et al. 2011).

Reviews on the chemistry of *Aspidosperma* plants cite indole alkaloids (Pereira et al. 2007, Guimarães et al. 2012) and a new alkaloid, braznitidumine, from *A. nitidum* was described, isolated and characterised by one of the present authors (Meneses-Pereira et al. 2006). In this study, we verify the biological activity of wood bark extracts and fractions in vitro against chloroquine-resistant (CQR) *P. falciparum* parasites and in a *Plasmodium berghei*-infected mouse malaria model.

MATERIALS AND METHODS

Plant materials - Wood bark, branches and leaves were harvested between 2004-2012 from the same adult tree (Fig. 1) growing at the Federal University of Amazonas farm located in Manaus, state of Amazonas, Brazil. A plant voucher coded 181832 has been deposited at the National Institute of Amazon Research herbarium. Using the plant material collected in 2004, a new alkaloid molecule named braznitidumine was previously described by one of the present authors (MMP) (Meneses-Pereira et al. 2006).

Extraction and chromatographic profiles of the active fractions - The materials of *A. nitidum* were dried, powdered and extracted using previously described protocols (Marques et al. 1996). The wood bark powder (2 kg) was divided into two equal parts: one was extracted by static maceration with ethanol (EtOH) for seven days at room temperature (RT) and the other with methanol (MeOH). After solvent removal by rotary evaporation, 23.4 g (2.3%) and 32 g (3.2%) of the EtOH and MeOH-A crude extracts, respectively, were obtained. The EtOH crude extract and the chloroform and ethyl acetate fractions obtained were used for the biological tests described.

A new sample of wood bark (1 kg) was extracted with MeOH by maceration with agitation at 40°C for 48 h and yielded 34.4 g (3.4%) of the crude extract (MeOH-B). Extracts of the wood bark, leaves and branches were also prepared using either the Soxhlet technique (EtOH and acetone) or maceration at RT with agitation (EtOH and water).

The wood bark collected in 2012 (1.5 kg) was macerated at RT with 1.6 L of MeOH for 48 h to yield, after solvent removal, 85.5 g (5.7%) of MeOH-C crude extract. This extract was then subjected to an acid-base fractionation protocol to obtain alkaloid-rich fractions (Fig. 2). The yields of each fraction are also shown in Fig. 2. The shaded box indicates the biological activity and positive reaction with Dragendorff's reagent.

The MeOH-C crude extract and its fractions (FO III, FO IV and precipitate) were subjected to a chromatographic analysis using a high performance liquid chromatography (HPLC) in with a Shimadzu apparatus (Shimadzu Co, Kyoto, Japan) composed by of a pump (model LC-10AD) and diode array detector (model SPDM20A) scanning from 200-500 nm, controlled by a CBM 20A. The sam-

Fig. 1: *Aspidosperma nitidum*, a medicinal plant named *carapanaúba*, from which the wood bark material was collected and used to prepare extracts and fractions that displayed high antimalarial activity and low toxicity, in vitro and in vivo.

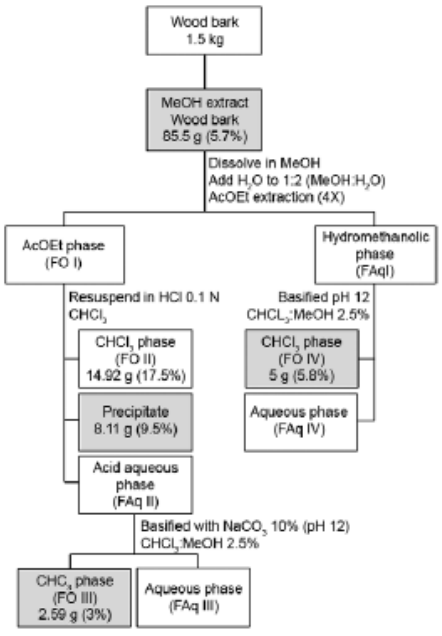

Fig 2: fractionation scheme of *Aspidosperma nitidum* methanolic wood bark extract and its fractions showing activity against malaria parasites highlighted in the boxes. AcOEt: ethyl acetate; $CHCl_3$: chloroform; HCl: hydrochloric acid; MeOH: methanol.

ples were injected into a 20-l loop coupled to a Rheodyne (IDEX Health & Science, Oak Harbor, WA, USA) valve and analysis separated over with an Ascentis RP18 column (Supelco Inc, Bellefonte, PA, USA) with a particle size of 5 micrometers. The mobile phase was a: MeOH-water (both with 0.1% formic acid) gradient from 30-50% in 20 min, reaching 100% MeOH at 25 min. The data were acquired and analysed using the soft-ware LC-Solutions (Shimadzu North America, Columbia, MD, USA).

Antimalarial tests against P. berghei in mice - The protocol for animal use was approved by the Ethical Committee (CEUA LW-23/13) at the Oswaldo Cruz Institute.

A previously described antimalarial suppressive test in mice (Peters 1965) was slightly modified (Carvalho et al. 1991). Briefly, Swiss outbred adult female mice, 20 ± 2 g, were inoculated with *P. berghei* by an intraperitoneal route, 1×10^5 freshly infected red blood cells (iRBC) per animal, using either chloroquine-sensitive (CQS) (NK65) or CQR parasites selected by successive treatment (Krettli et al. 1969). Two-24 h later, the mice were randomly distributed and subjected to drug treatment by an oral route once a day for three consecutive days. Two control groups were used: one not treated and another treated with chloroquine (either 15 mg/kg for animals with CQS parasites or 100 mg/kg for animals with CQR parasites).

The plant extracts and fractions were freshly diluted with 3% dimethyl sulphoxide (DMSO) in water immediately before gavage using different doses in a 200-µL volume per animal. Blood smears were prepared at the fifth and seventh days after parasite inoculation, MeOH-fixed, stained with Giemsa and examined microscopically for parasitaemia determination.

The inhibition of parasite growth (IPG) in the treated groups was evaluated by a comparison with the level of parasitaemia in the non-treated mice using the equation IPG = 100 minus the mean parasitaemia in the treated mice, multiplied by 100 and divided by the mean parasitaemia in the non-treated controls. The compounds reducing parasitaemia by 40% or more were considered active, those reducing 30-40% as partially active and reductions of less than 30% as inactive.

Continuous cultures of P. falciparum and antiplasmodial tests - CQR and mefloquine-sensitive *P. falciparum*, clone W2 (Oduola et al. 1988), was cultivated using the candle jar method (Trager & Jansen 1976), with minor modifications (Andrade-Neto et al. 2004). The continuous culture was maintained in human erythrocytes (A^+) at 37ºC in complete medium (RPMI-1640 supplemented with 10% human sera blood group A^+), which was changed daily. The human erythrocytes (A^+) and serum blood group A^+ were kindly donated by the Centre of Haemotherapy and Haematology of Minas Gerais under a mutual cooperation agreement (protocol 18/09).

Immediately prior to the tests, the blood parasites were concentrated through sorbitol synchronisation of the ring stages (Lambros & Vanderberg 1979). The blood suspension was adjusted for parasitaemia and haematocrit according to the specifications for each test, then distributed (180 µL/well) into 96-well microtitre plates (Corning, Santa Clara, CA, USA) containing the diluted extracts, fractions and braznitidumine (20 µL/well) in triplicate for each dose. Chloroquine, the standard antimalarial, was tested in parallel each time.

The activity of the plant extracts and fractions against *P. falciparum* was determined relative to the control cultures without antimalarial drugs as described (Rieckmann et al. 1978) and measured as follows: (i) the anti-HRPII test previously described (Noedl et al. 2002), (ii) the [^{3}H]-hypoxanthine incorporation assay (Desjardins et al. 1979) with slight modifications (Zalis et al. 1998) or (iii) quantitative tests based on optical microscopy, here referred to as the traditional test (Rieckmann et al. 1978).

For the anti-HRPII test, the *P. falciparum* cultures were adjusted to 1.5% haematocrit and 0.05% parasitaemia; anti-HRPII monoclonal antibodies were commercially acquired from ICLLAB (Stuart, FL, USA) (MPFM-55A and MPFG-55P) and the TMB chromogen (3,3′,5,5′-tetramethylbenzidine) was acquired from KPL (Gaithersburg, MD, USA). After stopping the reaction with 50 µL/L of 1 M sulphuric acid, the absorbance was measured at optical density (OD) 450 nm using a spectrophotometer (SpectraMax^{340}PC^{384}) (Molecular Devices, Sunnyvale, CA, USA).

For the [^{3}H]-hypoxanthine incorporation assay, *P. falciparum* cultures were maintained in a medium without hypoxanthine for at least four days and the haematocrit and parasitaemia were adjusted to 1%. [^{3}H]-hypoxanthine (0.5 µCi) was acquired from PerkinElmer (Waltham, MA, USA). After 42 h of incubation with the drug, the cells were harvested [Tomtec 96-Harvester (Tomtec Inc, Handem, CT, USA)] on glass-fibre filters (Wallac Ou, Turku, Finland), placed in sample bags (Wallac) and immersed in scintillation fluid (Optiphase super mix, Wallac). The radioactive emission was counted using a 1450 Microbeta reader (Wallac).

For the traditional test, a suspension of RBCs with 1% parasitaemia, previously synchronised, was distributed into a 96-well microtitre plate (100 µL/well) and cultured in complete medium (RPMI-1640 containing 10% human serum, 2% glutamine and 7.5% $NaHCO_3$). After 48 h, blood smears were prepared, coded, stained with Giemsa and examined at 1,000X magnification. The parasitaemia of the controls (considered as 100% growth) was compared to that of the test cultures and the percentage IPG was calculated.

For all tests, the extracts and fractions were dissolved in aqueous DMSO (0.02%) stock solution and the antiplasmodial activity was calculated by comparing the IPG in the drug-exposed cultures to that in the drug-free control culture. In tests performed using serial drug dilutions, sigmoid dose-response curves were generated with curve-fitting software (Microcal Origin Software v.5.0), which enabled the determination of the 50% inhibitory concentration (IC_{50}).

Cytotoxicity tests - The human hepatoma cell line (HepG2) was originally received from the New University of Lisbon and the monkey kidney cell line (BGM) was obtained from the Federal University of Minas Gerais. The cells were cultured in 75-cm^2 plates with

RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum and 40 mg/L gentamicin in a 5% CO_2 atmosphere at 37°C. For the in vitro tests, a confluent cell monolayer was trypsinised, washed with culture medium, distributed in a flat-bottomed 96-well plate (5 x 10^3 cells/well) and incubated for 18 h at 37°C to ensure cell adherence.

Cytotoxicity was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Denizot & Lang 1986) assay. Briefly, the HepG2 and BGM cell lines were incubated with 20 µL of the extracts and fractions at different concentrations (1,000-15 µg/mL) for 24 h in a 5% CO_2 and air atmosphere at 37°C. For the MTT assay, which evaluates mitochondrial viability, 20 µL of MTT solution (5 mg/mL) was added and the plates were incubated for another 3 h. After incubation, the supernatant was carefully removed from the wells, followed by the addition of 100 µL DMSO, with thorough mixing. The OD at 570 nm and 630 nm (background) was determined using an ELISA reader (SpectraMax^{340}PC^{384}).

The cell viability was expressed as the percentage of control absorbance obtained from the untreated cells after subtracting the absorbance from the appropriate background. The minimum lethal dose for 50% of the cells (MLD_{50}) was determined as previously described (Madureira et al. 2002). This value was used to calculate the selectivity index (SI) defined by the ratio between the MDL_{50} and IC_{50} values (Bézivin et al. 2003).

Statistical analysis - The data are expressed as the mean ± standard deviation for the in vivo tests. The statistical significance of the survival curves was evaluated by an ANOVA test followed by a Bonferroni post-test and the mean parasitaemia by a Student's *t* test followed by a Mann-Whitney *U* post-test. Both analyses were performed using GraphPad Prism 5.0 software. Only $p < 0.05$ or less was considered statistically significant.

RESULTS

The plant material in the present study was collected between 2004-2012 from the *A. nitidum* specimen shown in Fig. 1. Extracts from the wood bark, leaves and branches were prepared and evaluated in biological assays with *P. falciparum* and mice infected with *P. berghei* in addition to assays to evaluate cytotoxicity. The EtOH crude extract of the wood bark collected in 2004 and its fractions, as obtained through liquid-liquid partitioning, were able to reduce the parasitaemia in mice infected with *P. berghei* by 55% (Table I). These are the same extracts and fractions used in a previous chemical investigation that resulted in the identification of several compounds, including harman-3-carboxylic acid and braznitidumine (Meneses-Pereira et al. 2006) which was inactive against *P. falciparum* in vitro (data not shown).

None of the crude extracts or fractions was cytotoxic to HepG2 or BGM cells; the MDL_{50} values of all the crude bark extracts and fractions were similar or better when tested on normal cells, with the exception of FO IV (Table II).

The crude plant extracts showed significant antimalarial activity. Thus, new plant material from this specimen was collected and extracts were prepared for further fractionation and in vitro tests against *P. falciparum*, as shown in Table III. The data showed that the *A. nitidum* crude extracts and fractions had similar IC_{50} values in the traditional test (data not shown), [^{3}H]-hypoxanthine incorporation and anti-HRPII test with monoclonal antibodies. The most active extracts were those obtained from the wood bark, whereas those from the branches and leaves were inactive or only slightly active (EtOH

TABLE I

Parasitaemia and reduction in mice inoculated with *Plasmodium berghei* infected erythrocytes after oral treatment daily, for three consecutive days, with *Aspidosperma nitidum* ethanolic wood bark extract and its fractions

Plant extract and ethanol fractions used for mice treatment by oral route[a]	Dose (mg/kg)	Mice parasitaemia n (% reduction)[b]		Activity
		Day 5	Day 7	
Crude ethanolic extract	125	11 (48)	25 (49)	Yes
	250	11 (48)	22 (55)	Yes
Chloroform fraction	125	17 (19)	33 (33)	Partial
	250	12 (43)	18 (63)	Yes
Ethyl acetate fraction	125	18 (14)	36 (27)	No
	250	16 (24)	30 (39)	Partial
Chloroquine (control drug)	15	0 (100)	10 (80)	Yes
Non-treated	None	21 (0)	49 (0)	Control

a: crude extract and fractions obtained through liquid-liquid partition (Marques et al. 1996) using the plant wood bark collected in 2004; *b*: reduction of parasitaemia calculated in relation to control infected non-treated mice (n = 3 mice for treated groups and n = 4 mice for non-treated control group).

crude extract of leaves) (Table III). The best activity was observed with the crude extracts from the wood bark, with IC_{50} values between 4.6-12.4 µg/mL. The calculated SI, a ratio between toxicity and activity in vitro, varied from 24-152.

The acid-base liquid-liquid partition of the MeOH-C extract afforded three alkaloid-rich fractions (FO III, FO IV and precipitate) that were active against *P. falciparum*, with IC_{50} values as low as 1.6 µg/mL (for FO III). These fractions showed a positive reaction with Dragendorff's reagent and their analysis by HPLC-DAD confirmed the presence of braznitidumine in each (Fig. 3). Regardless of the in vitro assay used, the IC_{50} values were low, ranging from 1.6-5.5 µg/mL in the [^{3}H]-hypoxanthine assay, 2.3-7.6 µg/mL in the anti-HRPII assay and 2.8-6.8 µg/mL in the traditional test. None of the fractions was toxic to either HepG2 or BGM cells. Among the three fractions tested, the highest SI was found with the precipitate, which showed values of 200 in HepG2 and 364 in BGM cells in the [3H]-hypoxanthine assay and 145 (HepG2) and 263 (BGM) in the anti-HRPII assay (Table III).

TABLE II

Cytotoxicity of *Aspidosperma nitidum* extracts and fractions against a monkey kidney cell line (BGM) and a human hepatoma cell line (HepG2) determined by MTT expressed as the minimal lethal dose for 50% of cells (MDL_{50})

	MDL_{50} (µg/mL) mean ± SD	
Crude extracts and fractions	BGM	HepG2
Bark extracts[a]		
Ethanolic	≥ 2000	654 ± 40
Methanolic-A	964 ± 1.4	255 ± 9
Methanolic-B	≥ 2000	≥ 1000
Methanolic-C	427 ± 243	600 ± 14
Fractions from MeOH-C[b]		
Fraction FO III	301 ± 175	62 ± 18
Fraction FO IV	89 ± 53	169 ± 50
Precipitate	≥ 2000	1103 ± 371
Leaves extracts		
Ethanolic	≥ 2000	≥ 1000
Acetonic	≥ 2000	≥ 1000
Aqueous	≥ 2000	≥ 1000
Branches extracts		
Ethanolic	≥ 2000	280 ± 30
Aqueous	≥ 2000	≥ 1000

a: the different crude extracts were prepared from plant material by static maceration at room temperature [methanol (MeOH)-A and MeOH-C] or by maceration with agitation at 40°C (MeOH-B); *b*: fractions from MeOH-C were obtained through an acid-base liquid-liquid partitioning protocol to produce alkaloid-rich fractions (Fig. 2); MTT: (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide; SD: standard deviation. Three tests were performed with each cell line.

The alkaloid-rich fractions were tested in mice infected with *P. berghei* and were active in two independent experiments (Table IV). The MeOH-C crude extract reduced *P. berghei* parasitaemia by up to 67% with the dose of 100 mg/kg; the FO III, FO IV and precipitate fractions, at 50 mg/kg, also reduced parasitaemia by 66%, 65% and 57%, respectively. Although a dose of 100 mg/kg decreased parasitaemia to a slightly reduced extent for unclear reasons, survival was significantly increased in the mice treated with the MeOH-C crude extract and precipitate and in the mice receiving 50 mg/kg of the FO III and FO IV fractions (Table IV).

In another independent experiment using mice infected with *P. berghei* CQR, the EtOH crude extract was protective at a dose of 125 mg/kg, with the best results on the fifth day after inoculation, with 54% reduction in parasitaemia (Table V). Although chloroquine significantly reduced parasitaemia and increased survival, it was not curative in the mice treated with a high dose (100 mg/kg) compared with the untreated control mice (Fig. 4).

The potential of the active MeOH and EtOH crude wood bark extracts to induce mutagenic and genotoxic effects was evaluated with Ames tests (Ames et al. 1975). The tests were performed at the Genotox-Royal Institute, state of Rio Grande do Sul, Brazil (contract GT00752 and GT00719) using five strains of *Salmonella typhimurium* with different mutations at concentrations of up to 3,000 µg/plate. The plant extracts were tested in the absence and presence of the metabolising rat liver fraction and none exhibited a potential for mutagenicity or genotoxicity.

DISCUSSION

Plants have been used throughout history as remedies in most cultures and are the basis for many pharmaceuticals currently in use. It is estimated that 80% of the tropical and subtropical populations of the world depend on herbal remedies to treat diseases and sources of new, safer and effective compounds with medicinal properties are investigated (Willcox et al. 2011). Several antimalarial formulated phytomedicines have been proposed as possible alternatives to costly and, therefore, unaffordable therapies (Willcox & Bodeker 2004); however, this practice occurs to a lesser extent in Brazil, as the Ministry of Health provides specific diagnoses and synthetic antimalarials (portal.saude.gov.br/portal/arquivos/pdf/guia_pratico_tratamento_malaria_brasil_2602.pdf]. Nonetheless, several reference laboratories are located in and outside the malaria-endemic areas that are targeted for control (portal.saude.gov.br/portal/arquivos/pdf/centros_de_referencia.pdf).

A recent broad compilation of plant species reported in the worldwide literature cites plants as treatments for fever and/or malaria and the names of 1,277 species in 160 families are listed; among these, 956 species belonging to 140 plant families are found in the Amazon alone (Willcox & Bodeker 2004). In Brazil, a country with great biodiversity and rich ethnic and cultural diversity, the traditional knowledge of medicinal plants has been reported and medicinal plants have been investigated (Brandão et al. 1992, Andrade-Neto et al. 2007, Oliveira et al. 2009,

Mota et al. 2012), as previously reviewed (Krettli et al. 2001, Krettli 2009, Aguiar et al. 2012a), constituting important sources that should be better explored.

Different native populations exposed to malaria transmission in the Amazon Region (including Brazil, Venezuela, Colombia and Guiana) and other Latin American countries use plant remedies as antimalarials, e.g., in Colombia (Garavito et al. 2006, López et al. 2009) and Bolivia (Muñoz et al. 2000) and among traditional healers in Brazil (Carvalho et al. 1991).

Among *Aspidosperma* species, *A. excelsum* and *A. nitidum* are widely cited as curative relative to fever and malaria (Brandão et al. 1992, de Oliveira et al. 2011) and communities living in areas of the state of Pará cite *A. nitidum* and *A. rigidum* (Rusby) and "Indian beer" or *Ampelozyziphus amazonicus* (Rhamnaceae) as antimalarials. Although "Indian beer" has been proven to act as a prophylactic in rodent malaria, in mice with sporozoite-induced infection and in vitro with sporozoite cultures (Andrade-Neto et al. 2008), its various crude extracts were clearly proven to be inactive against blood parasites in vitro and in vivo (Krettli et al. 2001). Crude extracts and fractions from other *Aspidosperma* species native to regions outside the malaria transmission area (in the state of Minas Gerais) have been proven to be active against malaria blood parasites (Oliveira et al. 2009, Dolabela et al. 2012). Similarly, extracts from *Aspidosperma vargasii* and *Aspidosperma desmanthum* were also shown to be active and are believed to be a source of economically affordable drugs against malaria.

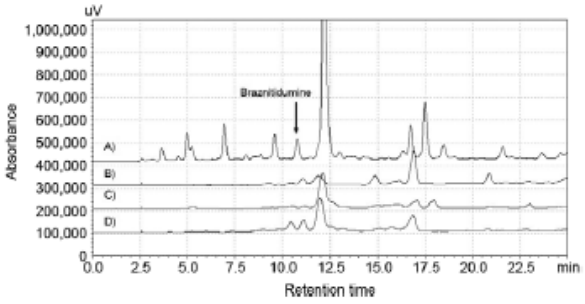

Fig. 3: extracted chromatograms comparison at 254 nm of MeOH crude extract (A) and fractions, precipitate (B), FO III (C) and FO IV (D).

The present work confirms that crude extracts from the wood bark of *A. nitidum* show intense activity against malaria parasites in mice. Indeed, wood bark is the part of the plant used to prepare infusions to treat malaria in the Amazon Region. Based on our present results that the pure

TABLE III

In vitro activity of *Aspidosperma nitidum* extracts and fractions against *Plasmodium falciparum* blood forms measured as the dose inhibiting 50% inhibitory concentration [(IC_{50}) in µg/mL] of parasite growth and selectivity index (SI), a ratio between cytotoxicity and activity [lethal dose for 50% of cells (MDL_{50})/IC_{50}]

Crude extracts and fractions	[^{3}H]-hypoxanthine			Anti-HRPII		
	IC_{50} mean ± SD[b]	SI[a]		IC_{50} mean ± SD[b]	SI[a]	
		BGM	HepG2		BGM	HepG2
Bark extracts						
Ethanolic	7 ± 3	286	83	4.6 ± 4.4	435	126
Methanolic-A	8.3 ± 1.6	116	31	10.7 ± 1.3	90	24
Methanolic-B	10 ± 3.6	200	100	6.6 ± 2.2	303	152
Methanolic-C	12.4 ± 1.9	34	48	10.8 ± 0.4	40	56
Fractions from MeOH-C[c]						
Fraction FO III	1.6 ± 0.06	188	39	2.3 ± 1.5	70	30
Fraction FO IV	3.3 ± 0.4	30	51	4.8 ± 2.1	19	35
Precipitate	5.5 ± 0.2	364	201	7.6 ± 4.4	263	145
Leaves extracts						
Ethanolic	22 ± 0.7	91	29	25.3 ± 6.0	79	25
Acetonic	≥ 50	Inactive		≥ 50	Inactive	
Aqueous	≥ 50	Inactive		≥ 50	Inactive	
Branches extracts						
Ethanolic	≥ 50	Inactive		≥ 50	Inactive	
Aqueous	≥ 50	Inactive		≥ 50	Inactive	

a: SI based on the results of cytotoxicity shown in Table II (SI ≤ 10 is indicative of toxicity); *b*: the samples considered active had IC < 12 µg/mL (partially active had 12-20 µg/mL and > 20 µg/mL were considered inactive); *c*: fractions from methanol (MeOH-C) were obtained through an acid-base liquid-liquid partitioning protocol to produce alkaloid-rich fractions (Fig. 2); BGM: monkey kidney cell line; HepG2: hepatoma cell line. Three tests were performed with each methodology.

TABLE IV

Antimalarial activity of *Aspidosperma nitidum* extracts and fractions from wood barks against *Plasmodium berghei* in mice in one experiment evaluated seven days after intraperitoneal infection induced by blood parasites

Mice group treated with[a]	Dose (mg/kg)	Parasitaemia mean ± SD (% reduction)[b]	Mice survival in days (mean ± SD)
Non-treated	None	2.2 ± 0.4	22.8 ± 2.7
Treated with			
MeOH-C[c]	100	0.7 ± 0.2 (67)	24.2 ± 3.9[d]
Fraction FO III	50	0.7 ± 0.3 (66)	21.4 ± 2.3[d]
	100	1.1 ± 0.6 (48)	22.8 ± 5.0
Fraction FO IV	50	0.8 ± 0.2 (65)	21.4 ± 2.3[d]
	100	1.0 ± 0.9 (54)	23.0 ± 4.5
Precipitate	50	0.9 ± 0.5 (57)	23.8 ± 4.3
	100	1.0 ± 0.3 (53)	24.2 ± 2.0[d]

a: treatment with extracts and fractions from the plant barks collected in 2012; *b*: reduction of parasitaemia in relation to control untreated mice (n = 5); *c*: fractions from methanol (MeOH)-C were obtained through an acid-base liquid-liquid partitioning protocol to produce alkaloid-rich fractions (Fig. 2); *d*: $p < 0.05$ in relation to the non-treated control group; SD: standard deviation.

TABLE V

Activity of ethanolic crude extract from wood bark of *Aspidosperma nitidum* against *Plasmodium berghei* chloroquine-resistant in mice after treatment during three consecutive days in parallel with the antimalarial chloroquine

Treatment with	Dose (mg/kg)	Parasitaemia mean ± SD (% reduction)[a] Day 5	Day 7
Control non-treated	0	2.6 ± 0.7 (0)	5.2 ± 2.3
Chloroquine	100	0.9 ± 0.3 (65)	3.7 ± 0.6 (29)
EtOH crude extract	62.5	1.8 ± 0.5 (31)	3.9 ± 0.2 (25)
	125	1.2 ± 0.5 (54)	3.3 ± 0.4 (37)
	250	1.4 ± 1 (46)	4.8 ± 1 (8)

a: reduction of parasitaemia calculated in relation to control infected non-treated mice (n = 4); SD: standard deviation.

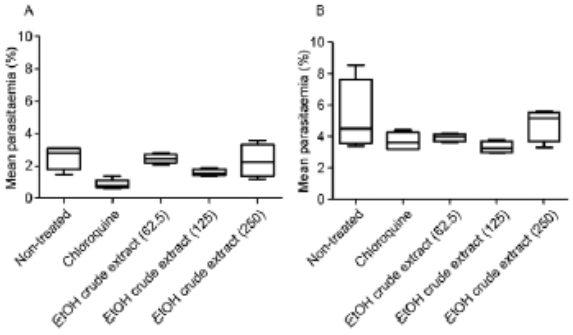

Fig. 4: mean parasitaemia by *Plasmodium berghei* in mice infected with chloroquine-resistant parasites following treatment for three consecutive days with the test compounds, evaluated on the fifth (A) and seventh (B) days after inoculation. EtOH: ethanol.

alkaloid braznitidumine described in the plant (Meneses-Pereira et al. 2006) was inactive in vitro, it is probable that a mixture of alkaloids detected in the active fractions of the crude extract of this plant are responsible for the antimalarial activity. New tests with braznitidumine need to be performed in mice, but this trial will require a large amount of this alkaloid, which is difficult to obtain.

In conclusion, the active *A. nitidum* crude bark extract showed the best activity against malaria parasites, the lowest cytotoxicity in vitro and no potential for mutagenicity or genotoxicity. Other species with proven antimalarial activity, such as *Aspidosperma ramiflorum*, *Aspidosperma pyrifolium*, *Aspidosperma olivaceum*, *Aspidosperma macrocarpon* and *Aspidosperma tomentosum*, have a lower therapeutic index, but have resulted in the chemical characterisation of new compounds systematically tested in vitro for anti-*P. falciparum* activity, with a low cytotoxicity and a less impressive therapeutic index (our unpublished observations). The alkaloids from several of these species have been characterised and shown to be active against *Leishmania* sp. parasites (Cunha et al. 2012). *A. nitidum* was by far the most active species, a finding that makes this traditional remedy useful for the control of malaria. However, further studies are required for drug development, as the present data indicate that the activity of the fractions and extracts may be due to a mixture of alkaloids rather than a single substance (braznitidumine). Nevertheless, drug mixtures are known to be important for delaying the appearance of drug-resistant malaria parasites.

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Anexo D- Artigo submetido Antimicrobial Agents and Chemotherapy

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In vitro transmission blocking potential of hybrid compounds derived from Artesunate with Mefloquine (MEFAS) or Primaquine (PRIMAS) and drug synthesis

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Running Head: *In vitro* blocking potential of MEFAS and PRIMAS

ABSTRACT

One of the goals of malaria control and eradication is to block parasite transmission to vector mosquitoes. Both male and female gametocytes of *Plasmodium*, responsible for parasite transmission from the mammalian host to the mosquitoes, represent specific targets for transmission-blocking interventions, and show different susceptibility to the antimalarials. Most drugs target the asexual stages of the parasite, without reducing gametocytemia, thus, developing drugs to target both stages is essential. An intense blood schizonticidal activity of MEFAS, a synthetic hybrid salt derived from mefloquine (MQ) and artesunate (AS), has been previously reported. Its activity and that of PRIMAS, synthesized from primaquine (PQ) and AS, are now evaluated against mature gametocytes of *P. falciparum*, assessed by the ATP bioluminescence assay; the functional capability of male mature gametocytes to exflagellate, and that of female gametocytes to activate and form gametes expressing *Pf*s25, are also evaluated. After drug exposure, stage IV-V gametocytes were more sensitive to MEFAS than to AS and MQ, tested in parallel, whereas PRIMAS was as active as AS against the gametocytes, displaying similar or higher IC_{50} values than AS in all assays. MEFAS exhibited a specific activity up to fifteen times higher against female and male gametocytes than AS, and also than the control drugs, methylene blue, thiostrepton and dihydroartemisinin. In conclusion, PRIMAS did not improve the activity of AS against mature gametocytes *in vitro* and the effect of MEFAS on asexual parasites was complemented by a pronounced reduction in gametocyte carriage, supporting MEFAS as a dual-acting antimalarial.

KEYWORDS: human malaria control, transmission blocking, hybrid compounds, dual-acting antimalarial, *Plasmodium falciparum*.

Introduction

Malaria is the deadliest and most prevalent parasitic disease, especially infections by *Plasmodium falciparum*; whereas *P. vivax* is responsible for high morbidity (1, 2). Despite the technological, scientific development and international efforts to control malaria, it remains a major public health problem. In 2013, the disease caused 198 million cases and 584,000 deaths, mostly in regions of Africa and children under five (3). Attempts of eradication should include transmission-blocking antimalarials capable to eliminate *Plasmodium* gametocytes, the sexual forms of the parasite responsible for the transmission from the mammalian host to the vector (4).

The mature *Plasmodium* gametocytes (stage V) are responsible for parasite transmission to the *Anopheles* mosquito host (5) and the asexual blood stages of the parasites are responsible for the clinical symptoms of the disease (6). An ideal antimalarial should cure the symptomatic infection and also block the disease transmission, preventing the selection of resistant parasites and avoiding their spread (7). Drugs that block transmission are particularly important in the case of *P. falciparum* malaria, whose gametocytes survive longer than the asexual stages (8). These parasite stages develop over a period of 12 days. The initial stages (I to III) are sensitive to schizonticidal antimalarials, but become insensitive to most drugs at the end of their maturation (stages IV and V), except to primaquine (PQ) (9, 10, 11).

PQ is used as a transmission-blocking drug in areas of transmission of *P. falciparum* (12). But because of its toxicity, causing gastrointestinal problems and severe hemolytic anemia in patients deficient in the glucose-6-phosphate dehydrogenase (G6PD) enzyme, PQ use requires medical supervision (13, 14). New drugs that blocks malaria transmission have been developed, like bulaquine, considered less toxic than PQ in G6PD deficient individuals (15). Bulaquine exerts its gametocytocidal activity faster than PQ (16).

The development of antimalarial hybrids based on covalent linking drugs into a single molecule with different biological functions, and distinct pharmacophores, may reduce the toxicity and improve activity, as compared to the isolate compounds (17, 18). Thus, the synthetic antimalarial hybrid MEFAS, a salt derived from mefloquine (MQ) and artesunate (AS), proven to be less toxic than the combination of AS and MQ, was also more effective against *P. falciparum* (chloroquine-resistant, clone W2, the chloroquine-sensitive 3D7 strain), and in mice with *P. berghei* malaria, leading to cure (19). Two other AS hybrid derivatives covalently bound with the PQ were more effective as blood schizonticide than the isolated molecules; one of them exhibited about 99% inhibition of hepatic forms development in C57BL/6 mice infected with *P. berghei* sporozoites (20).

In this work, PRIMAS, a hybrid salt composed by the ionic bonding between PQ and AS, was synthesized in an attempt to reduce the toxicity of PQ and to improve its potential of blocking parasite transmission, and evaluated in parallel with MEFAS, against *P. falciparum* mature gametocytes in order to identify their transmission-blocking potential.

Methods

Synthesis

The PRIMAS has been produced in laboratory scale: the PQ diphosphate salt (2 g) was hydrolyzed by the reaction with an aqueous sodium hydroxide (40 mL; 0.1 M); after extraction with dichloromethane (CH_2Cl_2, 3x 20 mL); a brown oil was obtained (1.02 g; 90% yield). Then, to a solution of AS (1 g; 2.6 mmol) in methanol (10 mL) the PQ base was added (0.69 g; 2.7 mmol) in methanol (6 mL). The mixture was stirred at room temperature for 4 h and the reaction was followed by TLC (thin layer chromatography) using as eluent a mixture of hexane/ethyl acetate (7:3). The PRIMAS salt was dried under vacuum and a brown solid was obtained (1.5 g, 88% yield). PRIMAS was characterized, using 1H NMR (400 MHz; DMSO), 13C NMR (100 MHz; $CDCl_3$) spectra and mass spectra. Melting point was determined in a Büchi model B-545 apparatus. The FT-IR absorption spectra were recorded in a Shimadzu mode IR Prestige-21 spectrophotometer through KBr reflectance. Mass spectra were recorded using a Waters micromass ZQ. NMR spectra were recorded on a Bruker HC400 instrument: internal standards used were tetramethylsilane for 1H and 13C. Melting point (°C): 84-86. data FTIR (KBr, νmax/cm-1): 3384 (NH_3^+), 2925, 2871, 1747 , 1615 and 1575; 1H - NMR (400 MHz, DMSO-d6) δ: 8.53 (dd, J = 4.2 and 1.6 Hz, 1H); 8.07 (dd, J = 8.2 and 1.5 Hz, 1H); 7.43 (dd, J = 8.2 and 4.2 Hz, 1H); 6.48 (d, J = 2.4 Hz, 1H); 6.28 (d, J = 2.4 Hz, 1H); 6.14 (d, J = 8.2, 1H); 5.64 (d, J = 9.7 Hz, 1H); 5.50 (s, 1H); 3.82 (s, 3H); 3.63 (m, 1H); 2.71 (m, 2H); 2.51 (m, 4H); 2.28 (m, 1H); 2.18 (m, 1H); 1.98 (m, 1H); 1.75 (m, 7H); 1.54 (m, 2H); 1.32 (m, 9H); 1.16 (m, 2H) and 0.90 (m, 8H); 13C - NMR (100 MHz; DMSO-d6) δ: 11.72 (CH_3); 19.94 (CH_3); 20.06 (CH_3); 20.91 (CH_2); 24.07 (CH_2); 25.03 (CH_2); 25.41 (CH_3); 30.07 (CH_2); 30.86 (CH_2); 31.55 (CH_2); 32.94 (CH_2); 33.57 (CH) ; 33.74 (CH); 35.80 (CH); 35.85 (CH_2); 44.47 (CH); 46.83 (CH_2); 51.00 (CH); 54.88 (OCH_3); 80.06 (COO); 90.45 (O-CH-O); 91.29 (O-CH-O); 91.49 (CH-Ar); 91.57 (CH-Ar); 103.44 (O-C-O-O); 122.01 (CH-Ar); 129.47 (C-Ar); 134.46 (C-Ar); 134.71 (CH-Ar); 144.14 (CH-Ar); 144.52 (C-Ar); 158.90 (O-C-Ar); 171.02 (C=O); 174.25 (COO-). EM [M^+]: 644.

Parasites in cultures

Two *P. falciparum* strains, 3D7 (MR4, MRA-102) and NF54 (MR4, MRA-1000), were cultured using a method modified from (21) in a 3% O_2, 5% CO_2, 92% N_2 atmosphere at 37°C (22). The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents.

Gametocytogenesis

Gametocyte cultures of each strain were initiated as described (23), with minor variations: on day 0, cultures were synchronized at the ring stage by lysis of the erythrocytes pellet with 5 volumes of a 5% sorbitol solution for 10 min at 37°C. Asexual blood cultures were initiated at 0.2% parasitemia and 12% hematocrit in a 10 mL culture medium, consisting of RPMI 1640 (Sigma-Aldrich) supplemented with hypoxanthine 50 mg/L (Sigma-Aldrich), sodium bicarbonate 2 g/L (Sigma-Aldrich), 5% pooled human A^+ serum and 2.5 mg/ml AlbuMAX II solution (Sigma-Aldrich). The medium was changed daily for 15 days without fresh erythrocytes addition. From day 6 on, the volume was doubled (hematocrit diminishes in half); from day 12 to 15, cultures were treated with 50 mM N-acetyl-D-glucosamine (GLcNA, Sigma-Aldrich) and 50 ng/mL of bistratene A (provided by JF Biard, University of Nantes, France) to remove asexual forms. To assess gametocyte production and maturation, the percentage of asexual forms and gametocytes were counted on Giemsa-stained smears.

Purification

Cultures showing mainly stage IV-V gametocytes (from day 14 on) were purified using a Nyco-Prep^{TM} 1.077 cushions (Axis-Shield, Norway). The culture pellet was washed, resuspended in 10 mL of medium and placed slowly onto 5 mL of Nycoprep cushion in a 15 mL Falcon tube. The tube was centrifuged at 800 g for 20 minutes. The interface was collected, washed, spun down, and the pellet was resuspended in 5 mL of medium. During the entire procedure, gametocytes were maintained at 37°C using a QBD2heater block (Grant). A Giemsa-stained smear was prepared and the suspension containing the gametocytes was loaded onto a LS-Column (Miltenyi Biotech, UK) for the second purification step using a VarioMACS magnetic separator (Miltenyi Biotech, UK). The column was washed with 5 mL of medium to elute the remaining red blood cells (RBCs); the gametocytes were immediately flushed out by removing the column from the holder, adding 5 mL of medium, and firmly applying the plunger supplied with the column.

ATP bioluminescence assay

This assay was performed as described (24). Once purified, mature gametocytes were counted using a Neubauer chamber and diluted (5 x 10^4 gametocytes per well), then transferred to a 96-well plate in a final volume of 100 μL or diluted (1.25 x 10^4 gametocytes per well), then transferred to a 384-well pre-dispensed plate containing the testing drugs (50 μL/well). The dilutions of each drug were added and the plates were incubated (at 37°C in a 3% O_2, 5% CO_2, 92% N_2 atmosphere) for 48 h. The ATP level in each well was determined using the BacTiter-GloTM reagent (Promega) according to manufacturer's recommendations. The BacTiter-GloTM assay generates a ''glow-type'' luminescent signal produced by the luciferase reaction, which consists of mono-oxygenation of luciferin catalyzed by luciferase in the presence of Mg^{2+}, ATP, and molecular oxygen.

Female gametocyte activation assay

Stage V gametocyte cultures were used to perform the Female gametocyte activation assay (25). Pre-dispensed plates containing the testing drugs were incubated with gametocytes for 48 h at 37ºC (3% O_2, 5% CO_2, 92% N_2). Activation was performed by a drop in temperature to 26ºC and the addition of ookinete medium (RPMI medium with 25 mM HEPES, 50 mg/L hypoxanthine, 2 g/L sodium bicarbonate, 100 μM xanthurenic acid, 20% human serum). At this point, the addition of antibody anti-*Pf*s25 (26) (0.5 μg/mL, final concentration) conjugated with Cy3 fluorochrome (GE Healthcare) enables female gamete detection by fluorescence microscopy. Activated cultures were then incubated (protected from light) at 26°C for 24 h, inside a gassed chamber (3% O_2, 5% CO_2, 92% N_2).

Female *P. falciparum* gametocytes round up when activated and the *Pf*s25 protein distributes widely in the membrane of the gamete. Using a monoclonal antibody against this protein, activated female gametocytes were specifically detected by fluorescence microscopy under 10X magnification and using a Cy3 filter.

Thiostrepton and methylene blue (MB) were used as positive control compounds. Despite the low activity described against asexual forms of the parasite (27), it has been shown that thiostrepton inhibits the formation of male and female gametes at submicromolar concentrations (25, 28). MB is a potent inhibitor of young and mature gametocytes *in vitro* (11, 25, 28, 29), and also prevents the formation of oocysts in the mosquitoes (30).

Exflagellation assay

On day 14, the gametocyte cultures grown in medium supplemented with 5% AlbuMAX II and 5% human serum, were transferred to medium supplemented with 10% human serum the day of the assay. The exflagellation was tested by withdrawing a 200 μL sample of gametocyte culture and briefly centrifuging the cells. The cell pellet was resuspended in 15 μL of ookinete medium, then introduced into a chamber of a FastRead disposable hemocytometer slide (Immune Systems). After 14 min, exflagellation was observed at a magnification of 100X. The culture was deemed suitable for the assay if at least 30 exflagellation centers per field were counted.

To set up the assay, sterile 1.5 mL tubes containing 100 μL culture medium and compound to be tested, were prewarmed to 37°C in a heater block. Once the cells had settled on the bottom of the flask, half the gametocyte culture medium was then removed to concentrate them, and then the cells were resuspended in the remaining medium. A hundred microliters of the cell suspension was quickly dispensed into each assay tube. Tubes were incubated at 37°C in a humidified incubator (3% O_2, 5% CO_2, 92% N_2). After 48 h, the exflagellation was assessed. By recording a 4-s, 20-frame time-lapse image, subtracting each frame from the previous, and then combining the subtracted images, it is possible to identify the exflagellation centers as intense white dots (regions of movement) on a black background (regions that are still). Applying standard image processing the exflagellation centers were identified and counted as described (28). The assay was performed with small batches, as exflagellation is a time-dependent event.

Determination of half-minimum inhibitory concentration (IC_{50})

The 50% inhibition of *P. falciparum* sexual stage development was calculated from dose-response curves using GraphPadPrism 5 software. The compounds used for all the assays included dihydroartemisinin (Sigma-Aldrich), artesunate (Sigma-Aldrich), epoxomicin (Sigma-Aldrich), thiostrepton (Sigma-Aldrich), methylene blue (Sigma-Aldrich), mefloquine (Sigma-Aldrich), primaquine (Farmanguinhos), PRIMAS (Farmanguinhos) and MEFAS (Farmanguinhos). The compounds were dissolved in DMSO (final concentration 0.5%).

Statistical analysis

Differences between IC_{50}s were evaluated using GraphPadPrism 5, nonparametric T-test. A p value of < 0.05 was considered to be statistically significant.

Results

The new hybrid salt PRIMAS herein synthesized, similarly to MEFAS, was obtained from the basic PQ and AS (**Fig. 1**). The melting point obtained for the PRIMAS was 84-86°C, which is significantly different from those of PQ base an oil, PQ diphosphate (195-205°C) and AS (139-140°C). The mass spectrum of PRIMAS exhibits EM [M^+] = 645. This salt was characterized by ^{1}H-NMR spectrum of the new salt which not showed the signal at 12.26 ppm attributed to OH observed in the original spectrum of AS. Furthermore, the FTIR spectrum of PRIMAS does not showed the absorption at 3276 cm^{-1} corresponding to O-H observed in the spectrum of AS, 3379 (NH_3^+), 2926, 2873, 1745 (stretch C=O ester and carboxylate together in the same band), 1612 and 1573 (related to angular deformation of ammonium).

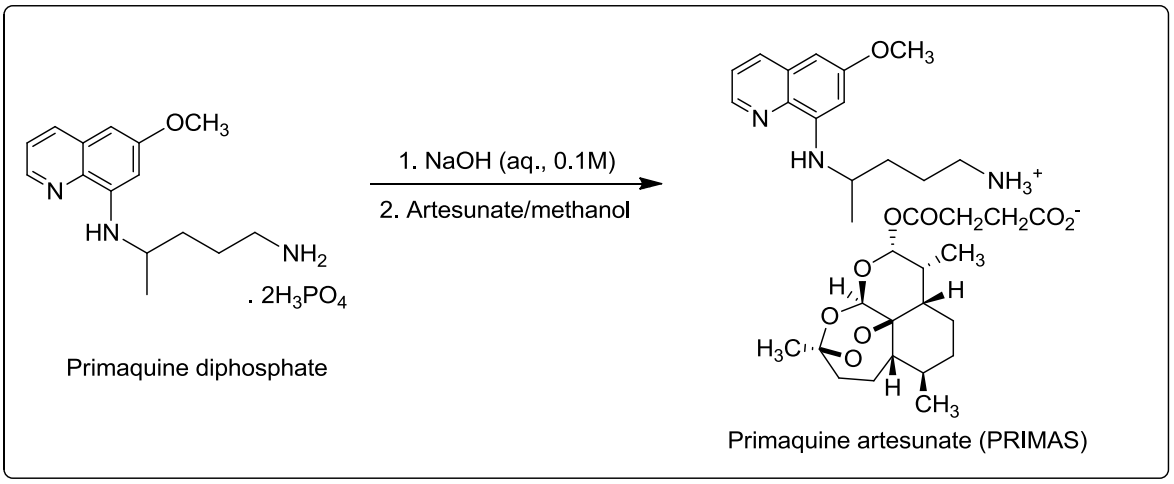

FIG 1 Synthesis and structure of PRIMAS.

The procedure of the gametocyte test starts with the production and purification of mature gametocytes (IV-V) and their exposure to compounds, during 48 h, after which their viability is measured using a sensitive ATP bioluminescence-based assay. This determines the level of intracellular ATP content and allows to calculate the IC_{50} from dose-response curves.

PRIMAS and MEFAS were tested in parallel with the standard antimalarials AS, MQ, PQ, and with epoxomicin, a proteasome inhibitor, which has been recently described as a good gametocytocidal agent, killing stage V gametocytes in the nanomolar range (31). MEFAS and PRIMAS were active against gametocytes at low concentrations (IC_{50} 4.6 ± 1.8 and 4.3 ± 0.4 μM, respectively), and more active than the original drugs AS (IC_{50} = 12.7 ±

10.7 μM), MQ (IC_{50} = 16.5 ± 2.8 μM) and PQ (IC_{50} = 42 ± 10.2 μM). PQ was considered inactive.

The high standard deviation value found for IC_{50} values of AS can be explained by the instability of the compound. No statistical difference was observed for the IC_{50} values between PRIMAS and AS (**Table 1**).

TABLE 1 *In vitro* activity of standard antimalarials (artesunate, mefloquine and primaquine), epoxomicin, and the hybrid molecules MEFAS and PRIMAS, against *P. falciparum* mature gametocytes, evaluated in the ATP bioluminescence-based assay.

Compounds	***Pf*3D7 Gametocytes IC_{50} (μM)**			
	Exp1	**Exp2**	**Exp3**	**Average ± SD**
Epoxomicin	0.0016	0.0006	0.0006	0.0009 ± 0.0006
Artesunate	25	7.1	6.0	12.7 ± 10.7[c]
Mefloquine	13.4	18.8	17.2	16.5 ± 2.8[c]
Primaquine	>50	45.4	30.5	42 ± 10.2
MEFAS[a]	2.5	5.8	5.4	4.6 ± 1.8[c]
PRIMAS[b]	4.02	4.1	4.7	4.3 ± 0.4

[a]A hybrid of artesunate and mefloquine, we previously described in de Pilla Varotti *et al*., 2008.

[b]A hybrid of artesunate and primaquine.

[c]($p < 0.05$)

The female activation assay is based on the specific expression of the *Pf*s25 protein at the surface of the female activated gametocyte, which is detected using an image analyzer. A further algorithm quantifies the number of female gametocytes that have developed successfully into gametes.

MEFAS inhibited the female gametocyte activation with an IC_{50} lower than AS (IC_{50} = 0.02 ± 0.01 μM and 0.3 ± 0.2 μM, respectively) and MQ (IC_{50} = 5.6 ± 2.1 μM). MEFAS was around thirty times more active than MB (0.71 ± 0.08 μM), drug known for the potent activity against gametocytes. Furthermore, MEFAS showed specific activity against female

gamete formation with IC_{50} value over a hundred times lower than that in the gametocytocidal assay. The difference between the IC_{50} values of MEFAS and AS was not statistically significant. In contrast, PRIMAS was not more potent than AS in the ability to inhibit female gamete formation. The IC_{50} value of PRIMAS was higher than the AS (0.7 ± 0.3 and 0.3 ± 0.2 μM, respectively). PQ was inactive in this assay (IC_{50} ≅ 30 μM). PRIMAS was more active in this assay with IC_{50} value six times lower than that in the ATP assay. The results are summarized on **Table 2**.

Exflagellation of the male gametocyte can be detected and quantified in an assay of computer-aided, through identification of erythrocytes movement surrounding an exflagellating male gametocyte called "exflagellation center", as described in methods. MEFAS and AS were tested in 10-fold dilutions, from 500 nM and 2 μM as initial concentration, respectively, since they showed IC_{50} value less than 0.125 μM in a preliminary test. MQ, PQ and PRIMAS were tested from 2 μM to 0.125 μM in 2-fold dilutions. Dihydroartemisinin (DHA) was tested at 10 μM to ensure 100% inhibition of exflagellation.

The assay was performed using 14-days old cultures; control using DMSO 0.5% exhibited 87.2 ± 0.98 exflagellation centers. The results showed that all samples were active. MEFAS (IC_{50} = 11 ± 1.7 nM) was twelve times more active than AS (IC_{50} = 0.13 ± 0.003 μM) and inhibited 99% of exflagellation, as DHA, in a twenty-fold lower dose; DHA showed a 99% inhibition at 10 μM, as a positive control for the assay. MQ and PQ were also active, with IC_{50} of 0.57 ± 0.2 and 1.25 ± 0.12 μM, respectively. PRIMAS (IC_{50} = 0.17 ± 0.001 μM) inhibited the exflagellation in a concentration lower than PQ, but not than AS.

This data was confirmed in another experiment, in which MEFAS (IC_{50} = 22.6 ± 4.7 nM) was five times more active than AS (IC_{50} = 0.11 ± 0.01 μM). Control using DMSO 0.5% exhibited 59.8 ± 16 exflagellation centers. The results are also summarized on **Table 2**.

TABLE 2 Inhibition of female and male gametes by methylene blue, artesunate, mefloquine, primaquine, and the hybrid molecules MEFAS and PRIMAS, as compared to untreated controls.

Compounds	IC_{50} (μM) Average ± SD	
	Female Gamete	**Male Gamete**
Methylene Blue	0.71 ± 0.08	NT
Artesunate	0.3 ± 0.2	0.12 ± 0.01
Mefloquine	5.6 ± 2.1	0.57 ± 0.2
Primaquine	29.2 ± 4.6	1.25 ± 0.12
MEFAS	0.02 ± 0.01	0.017 ± 0.008
PRIMAS	0.7 ± 0.3	0.17 ± 0.01

NT = not tested.

Discussion

The transmission of *Plasmodium* to *Anopheles* mosquitoes depends on the ability of the parasites to differentiate into mature viable gametocytes, which are also the responsible for spreading the resistant parasites (7). To block the disease transmission, drugs should target gametocytes, either by killing them or by functionally preventing their progressive maturation (25, 28).

Despite the significant advances achieved in the prevention and treatment of malaria in recent years, the success remains below the goals of the World Health Organization for control and elimination. For elimination strategies to be effective, it is essential to limit disease transmission, achieved through reduction of infective parasites in the host and/or decrease of gametocyte transmission to the mosquitoes (7).

With the recent reports of delayed rates of parasite clearance after administration of artemisinin derivatives (32, 33), the development of novel antimalarials that target multiple stages of the parasite life cycle (34), similarly to artemisinin in blocking both infection and transmission (35), will help to overcome drug resistance and achieve malaria eradication. Indeed, AS combined with MQ reduced gametocyte carriage among recrudescent *P. falciparum* infections by 18-fold compared to MQ (36).

In an attempt to optimize dual-active antimalarials, we studied two rather potent schizonticidal hybrid molecules, PRIMAS and MEFAS, and report herein their ability to interfere with the sexual stages of *P. falciparum in vitro*. Since male and female gametocytes respond differently to a range of antimalarials, and the current gametocyte screening assays do not give readout on the sex specificity of compound action (28), we also evaluated the specific activity of MEFAS and PRIMAS against male and female gamete formation.

The combination of AS and MQ is currently used in areas with drug-resistant *P. falciparum* parasites (37). Although AS clears parasitemia in shorter time periods than any other antimalarial drug (38), it does not cure the infected patients (37). In addition, MQ causes undesirable side effects (39) and is rather expensive (40). Both are important problems considering that malaria affects mostly populations in poor endemic countries. MEFAS, besides being more active than the isolated antimalarials MQ and AS (19) displayed greater activity against mature *P. falciparum* gametocytes. MEFAS was more active against male and female gametocytes, inhibiting exflagellation and female activation, respectively, *in vitro* at nanomolar concentrations. These data suggest that MEFAS has a profound effect on the final and critical steps of gametocyte maturation and gamete formation.

MEFAS has also been shown to increase the levels of cytoplasmatic Ca^{2+} (19). Since Ca^{2+} has been shown to be involved in several biological processes of *Plasmodium* such as regulation of sexual parasite differentiation in the mosquito vector (41), it is likely to be a mechanism of drug activity. Importantly, a rise in Ca^{2+} is responsible for (i) the response of *P. berghei* gametocytes to stimulation by xanthurenic acid (41); (ii) its entry in the cell cycle and release of the flagellated microgametes (42); (iii) and the emergence of male and female gametocytes from their host (41). Later stages differentiation is dependent on phosphorylation of *Pf*Map2, an atypical mitogen activated protein kinase, by *Pf*CDPK4 (*P. falciparum* Calcium-Dependent Protein Kinase), just before male gametes become motile and are released (43). This may explain the profound effect of MEFAS on the final and critical steps of gametocytes maturation and exflagellation.

Considering the possibility of a dual mode of action typical of the hybrid drugs, MEFAS probably acts interfering with calcium homeostasis within the parasite by a direct interaction with *Pf*ATP6, as suggested for endoperoxides (44). MEFAS is also able to act on the digestive vacuole, disrupting the pH gradient, as a result of its endoperoxide bridge and aminoquinoline entities (19).

PRIMAS, the synthetic hybrid drug described herein, was active against *P. falciparum* gametocytes but not more than AS. In [^{3}H]-hypoxanthine incorporation assay performed

against *P. falciparum* 3D7 asexual stages, PRIMAS (IC_{50} = 0.007 ± 0.001 μM) was more active than the standard antimalarials PQ (IC_{50} = 11.6 ± 1.3 μM) and AS (IC_{50} = 0.01 ± 0.001 μM) (data not shown). However, this improvement in activity was not observed against gametocytes in which PRIMAS was active against *P. falciparum* gametocytes but not more than AS.

The inhibitory activity of PQ against exflagellation and its lack of activity against *P. falciparum* mature gametocytes may be attributed to the fact that asexual-stage parasites rely on glycolysis to generate ATP, whereas sexual ones rely on electron transport chain (ECT) (45) that takes place in the mitochondria. This organelle has already been pointed as the target of PQ (46, 47), which is likely to bind to Q_0 or Q_1 site of the cytochrome bc1 complex (47) and therefore disrupt ECT, whose major role is to provide dihydroorate dehydrogenase. This enzyme is involved in the *de novo* pyrimidine synthesis (48) and abortion of this essential biological process leads to parasite's death. Furthermore, exflagellation involves several steps that require energy, such as DNA *de novo* synthesis (49) followed by three rounds of mitosis (50), a cell-division process highly dependent on ATP (51). The participation of kinases is also essential for exflagellation, as demonstrated for *Pf*CDPK4 in *P. falciparum* parasites (52, 53). Female gametogenesis is less dramatic and does not require DNA synthesis (28). Furthermore, *P. falciparum* displays a sex ratio of approximately one male gametocyte to every three to five females (54, 55).

PRIMAS did not improve the activity of AS against mature gametocytes *in vitro*, but it may need to be metabolized to be active, like PQ does to be active *in vivo*, a hypothesis yet to be assessed. Having its activity confirmed, PRIMAS can be used to prevent gametocyte maturation, overcome the problem of chloroquine resistance and therefore help controlling *vivax* malaria.

MEFAS, in addition to its intense activity against the asexual stages of the parasites, has an outstanding anti-*P. falciparum* gametocytocidal activity *in vitro*, likely to occur also *in vivo*. The potential of MEFAS as a dual-acting antimalarial should also occur against *P. vivax*, and if so, it would be useful to overcome the burden of this human malaria parasite. Therefore, MEFAS should be used in clinical studies due to its dual antimalarial activity.

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Anexo E- Artigo publicado em colaboração

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In Vivo Antimalarial Activity and Mechanisms of Action of 4-Nerolidylcatechol Derivatives

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4-Nerolidylcatechol (1) is an abundant antiplasmodial metabolite that is isolated from *Piper peltatum* roots. *O*-Acylation or *O*-alkylation of compound 1 provides derivatives exhibiting improved stability and significant *in vitro* antiplasmodial activity. The aim of this work was to study the *in vitro* inhibition of hemozoin formation, inhibition of isoprenoid biosynthesis in *Plasmodium falciparum* cultures, and *in vivo* antimalarial activity of several 4-nerolidylcatechol derivatives. 1,2-*O*,*O*-Diacetyl-4-nerolidylcatechol (2) inhibited *in vitro* hemozoin formation by up to 50%. In metabolic labeling studies using [1-(*n*)-^{3}H]geranylgeranyl pyrophosphate, diester 2 significantly inhibited the biosynthesis of isoprenoid metabolites ubiquinone 8, menaquinone 4, and dolichol 12 in cultures of *P. falciparum* 3D7. Similarly, 2-*O*-benzyl-4-nerolidylcatechol (3) significantly inhibited the biosynthesis of dolichol 12. *P. falciparum in vitro* protein synthesis was not affected by compounds 2 or 3. At oral doses of 50 mg per kg of body weight per day, compound 2 suppressed *Plasmodium berghei* NK65 in infected BALB/c mice by 44%. This *in vivo* result for derivative 2 represents marked improvement over that obtained previously for natural product 1. Compound 2 was not detected in mouse blood 1 h after oral ingestion or in mixtures with mouse blood/blood plasma *in vitro*. However, it was detected after *in vitro* contact with human blood or blood plasma. Derivatives of 4-nerolidylcatechol exhibit parasite-specific modes of action, such as inhibition of isoprenoid biosynthesis and inhibition of hemozoin formation, and they therefore merit further investigation for their antimalarial potential.

Despite large investments of resources and scientific advances in molecular, cellular, and clinical research on malaria, clinically effective vaccines are still far from being available as tools for the control and eradication of malaria. Early diagnosis, case management, and especially drug-based therapy are important tools for the control of this disease (1). In 2013, an estimated 198 million cases of malaria were reported, and 584,000 deaths were attributed to this disease worldwide. Today, morbidity and mortality due to malaria remain at unacceptable levels, and great challenges must be surmounted in order to attain global targets set for malaria control (2).

The control of malaria has become gradually more complex due to the spread of *Plasmodium* spp. that are resistant to the antimalarials presently used in therapy (3). In recent years, *Plasmodium falciparum* resistance to artemisinin and its derivatives has been detected in four southeast Asian countries (2). *Plasmodium vivax* is the most important malaria parasite outside the African continent, and *in vivo* resistance and *in vitro* resistance of this parasite to chloroquine have been the subject of a growing number of recent reports (4, 5). Thus, new chemical entities that may overcome the mechanisms of resistance and offer significant advances over existing drug regimens are in urgent demand.

The sequencing of the *P. falciparum* and *P. vivax* genomes has led to the identification of a growing list of potential drug targets (6). A number of molecular targets are associated with the distinct functions of different organelles present in the asexual blood phases of *Plasmodium* spp. The parasite digestive vacuole and the apicoplast are among the important organelles, due to the intensities and specificities of their metabolic activities, which are absent in human beings, thus making the metabolic processes in these organelles interesting targets for antimalarial drugs (3).

The digestive vacuole is the organelle wherein the intense degradation of erythrocyte hemoglobin occurs, thus providing *Plasmodium* spp. with amino acids for protein synthesis. A by-product of hemoglobin digestion is heme, which is toxic to the parasite. Heme polymerizes rapidly forming hemozoin (malaria pigment) in the interior of the digestive vacuole (7). The inhibition of hemozoin formation is considered to be one of the main mechanisms of action of several antimalarial drugs in clinical use, such as chloroquine and artemisinin (8, 9).

The apicoplast is an organelle similar to the plastid and is pres-

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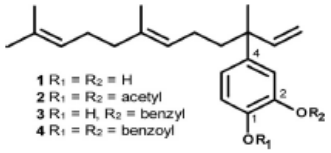

FIG 1 Structures of 4-nerolidylcatechol (**1**) and semisynthetic derivatives **2** to **4**.

ent in species of the phylum *Apicomplexa*, such as *Plasmodium* spp. In the apicoplast, unique metabolic pathways are present (biosynthesis of fatty acids, isoprenoids, iron-sulfur clusters, and heme) (10). One of the main metabolic activities of the apicoplast is the synthesis of isoprenoid compounds. In mammals, C_5 isoprene (hemi-terpene) precursors to isoprenoids are synthesized by the mevalonate pathway (MVA). *Plasmodium* spp. use the non-MVA (NMVA), or methylerythritol phosphate (MEP), pathway to synthesize these five-carbon isoprenoid precursors (11). Numerous intermediates and enzymes of this pathway have been characterized, and light has been shed on the physiological importance of the isoprenoid pathway and role of biosynthesized isoprenoid metabolites in *Plasmodium* survival (12–15). The NMVA (MEP) pathway is absent in humans and essential to *Plasmodium* survival. It is thus a potential source of targets for the development of novel antimalarials (16).

The apicoplast and digestive vacuole are distinct cellular compartments (organelles). Their physiological processes are targeted by different drugs. However, fosmidomycin exhibits dual mechanistic actions against *P. falciparum*. This compound inhibits the biosynthesis of isoprenoids in *Plasmodium* spp. and interferes in the prenylation of proteins of the digestive vacuole, thus affecting the overall process of hemozoin formation in the parasite (17).

Plant terpenes exhibiting *in vitro* antiplasmodial activity have been evaluated in *P. falciparum* to establish whether they may exert effects on the isoprenoid biosynthetic pathway. Thus, nerolidol causes total inhibition of *P. falciparum* trophozoite development at the schizont stage, and *in vitro* 50% inhibitory concentrations (IC_{50}s) in the range of 760 ± 23 nM (mean ± standard deviation) have been reported for nerolidol against *P. falciparum*. Terpenoid compounds like nerolidol, farnesol, and linalool have been shown to strongly inhibit the biosynthesis of both dolichol and the isoprene side chain of ubiquinones. Also, terpenes can inhibit the isoprenylation of proteins in the intraerythrocytic stages of *P. falciparum* in a specific manner that does not affect overall protein biosynthesis (18).

Traditionally used plants are sources of important antimalarial compounds, such as quinine and artemisinin and other secondary metabolites exhibiting potential as antimalarials (19, 20). The Amazon region, where malaria is endemic, is a rich source of antimalarial substances obtained from traditionally used antimalarial plants (21). 4-Nerolidylcatechol (**1**) is a major secondary metabolite of mixed terpene-phenylpropanoid biosynthetic origins found in the roots of *Piper peltatum*, a traditionally used antimalarial shrub from the Amazon region (Fig. 1). *P. peltatum* has been domesticated, and its roots (containing ca. 5% [wt/wt] compound **1**) can yield an estimated 27 kg of compound **1** per hectare. Thus, this compound is potentially available for large-scale applications (22, 23). It exhibits *in vitro* activity against the chloroquine-pyrimethamine-cycloguanil-resistant K1 strain of *P. falciparum* (IC_{50} = 0.67 μM) (24) and low oral and subcutaneous activity against *Plasmodium berghei* in mice (maximal effect was 63% inhibition at oral doses of 600 mg per kg of body weight per day) (25). Also, deactivated plasma taken from healthy mice that had orally ingested this compound exhibited high *in vitro* inhibition of *P. falciparum*, thus providing evidence for the presence of antiplasmodial metabolites in mouse blood as a result of oral ingestion of compound **1**.

Natural product **1** is unstable under ambient conditions or in a freezer. Mono- and di-*O*-alkyl and di-*O*-acyl derivatives of this compound have been introduced and exhibit improved chemical stability. These derivatives exhibit a range of *in vitro* inhibitory activities against the K1 strain of *Plasmodium falciparum* (IC_{50} = 0.67 to 23 μM) (26, 27), antioxidant activity comparable to food preservatives BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene), and low toxicity to normal cells (28).

Derivatives of compound **1** exhibit catechol structures that can adopt planar conformations that *a priori* could make these derivatives excellent binders of heme (inhibitors of hemozoin formation). Also, like nerolidol, farnesol, and linalool and other terpenes that inhibit terpenoid synthesis in *P. falciparum*, derivatives of compound **1** have a linear terpenyl (nerolidyl) side chain that *a priori* could make these compounds promising inhibitors of isoprenoid synthesis in *Plasmodium* spp.

The aim of this work was to investigate the potential *in vivo* antimalarial activity of 4-nerolidylcatechol (**1**) derivatives and explore two independent mechanisms of possible antiplasmodial action of these compounds. Herein, derivatives **2** to **4** (Fig. 1) were assayed for (i) inhibition of *in vitro* hemozoin formation, (ii) *in vitro* inhibition of the biosynthesis of isoprenoid metabolites in *P. falciparum* cultures, and (iii) *in vivo* antimalarial activity in *P. berghei*-infected mice. The acute oral toxicity and presence of compound **2** in mouse blood after oral ingestion were investigated along with the *in vitro* recovery of this compound from human and mouse blood and plasma.

MATERIALS AND METHODS

Chemical substances. Natural product **1** makes up 5% or more of the dry weight of the mature roots of *P. peltatum* and was obtained by extraction of the dry, ground roots with chloroform and ethanol (1:1), evaporation of the solvents, and column chromatography on the resulting extract as previously described (25). The three semisynthetic derivatives **2** to **4** studied herein were synthesized from freshly purified compound **1** in straightforward synthetic procedures that have been published previously (26, 29). Briefly, 1,2-*O*,*O*-diacetyl-4-nerolidylcatechol (**2**) was prepared by diacetylation of compound **1** in acetic anhydride/pyridine. 2-*O*-Benzyl-4-nerolidylcatechol (**3**) was obtained by benzylation with benzyl bromide, and 1,2-*O*,*O*-dibenzoyl-4-nerolidylcatechol (**4**) was obtained by reaction with benzoyl chloride. After each of these reactions, column chromatography and preparative thin-layer chromatography were used for isolation and purification of the products whose structures were determined based on nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) techniques. The purities of the products were evaluated by thin-layer chromatography and liquid chromatography coupled to mass spectrometry, and they were considered to be >95% (26). The commercial drug standards chloroquine diphosphate (Sigma-Aldrich, Steinheim, Westphalia, Germany) and quinine sulfate (Sigma-Aldrich, Steinheim, Westphalia, Germany) were used as controls in the biological tests.

***In vitro* culture of *Plasmodium falciparum*.** The *in vitro* culture experiments were performed using the 3D7 clone of the NF54 strain (chloroquine sensitive) and the K1 *P. falciparum* strain. Parasites were cultured using the Trager and Jensen method (30) with modifications (24). The parasites were cultured at 37°C under a low-oxygen atmosphere (5% ox-

ygen, 5% carbon dioxide, and the remainder nitrogen) in A+-type erythrocytes at a hematocrit of 3 to 5%. Roswell Park Memorial Institute (RPMI) 1640 culture medium (Sigma-Aldrich) supplemented with 0.5% Albumax I (Gibco) was used. Synchronized ring-phase cultures were obtained by two consecutive treatments at intervals of 48 h with a 5% (wt/vol) solution of D-sorbitol (Sigma-Aldrich) as described by Lambros and Vanderberg (31). The development and growth of parasites were analyzed on smears of cultures stained with Panótico (Laborclin, Pinhais, Paraná, Brazil).

***In vitro* inhibition test.** A microtest was performed using the method introduced by Rieckmann et al. (32) with modifications (24). Briefly, 20 mM stock solutions of compounds **1** to **4** were initially prepared in dimethyl sulfoxide (DMSO) and then serially diluted (1:3) in culture medium (RPMI 1640) to obtain 7 final concentrations (100 to 0.14 μM). Chloroquine and quinine drug standards (7 final concentrations, 2.0 to 3.4×10^{-3} μM) were used as controls. Each sample of diluted compound was tested in triplicate in a 96-well plate containing a suspension of ring-stage (synchronized) parasitized red blood cells (pRBCs) with a hematocrit of 2% and 1% initial parasitemia. The final volume in each well was 200 μl. Wells containing pRBCs in culture medium and 2% DMSO were used as controls of parasite growth. The plates were incubated for 48 h at 37°C under the culture conditions described above. After incubation, smears of the contents of each well were prepared on microscope plates and colored with Panótico and examined using an optical microscope. The number of pRBCs present in a total of 2,000 red blood cells was counted. The parasitemia was expressed as a percentage. The half-maximal concentration (IC_{50}) responses were calculated using Origin 8.1 software (Origin Lab). All tests were performed in triplicate in a total of three independent experiments. One-way statistical analysis of variance (ANOVA) was performed, followed by Dunnett's *post hoc* test (Prism; Graph Pad, CA). A P value of <0.05 was considered statistically significant.

Animals and ethical approval. Adult female BALB/c mice (22 ± 3 g of body weight) were used for the acute toxicity assay and antimalarial *in vivo* tests and received water and food *ad libitum*. *In vivo* tests were performed using Guidelines for Ethical Conduct in The Care and Use of Animals of the National Institute for Amazon Research (INPA). This work was authorized by INPA's Commission of Ethics for the Use of Animals (CEUA 062/2012).

***In vivo* suppressive test with *Plasmodium berghei*.** Evaluation of the *in vivo* antimalarial activity was performed based on the Peters 4-day suppressive test (33) with modifications (25). Briefly, 0.2 ml of infected blood suspension containing 1×10^5 *P. berghei* NK65 pRBCs was inoculated intraperitoneally in mice. The animals were randomly divided into groups of 5 individuals. Animals in test groups were treated orally or subcutaneously with compound **2** or **4** at doses of 600 to 10 mg kg^{-1} day^{-1}. Positive-control group animals were treated orally or subcutaneously with 10 mg kg^{-1} day^{-1} of chloroquine, and negative-control group animals received 0.2 ml of vehicle (2% DMSO in water). Animals were treated for 4 days starting 24 h after inoculation with *P. berghei*. Parasitemia levels were assessed by examining Giemsa-stained thin blood smears from each animal via an optical microscope on days 5 and 7. Overall mortality was monitored daily in all groups during a period of 40 days following inoculation. The difference between the average parasitemia of negative-control groups (100%) and test groups was calculated as the percentage of parasite growth suppression (PGS) according to the following equation: $PGS = 100 \times [(A - B)/A]$, where A is the average parasitemia of the negative-control group and B corresponds to the parasitemia of the test group. Each sample was tested in 3 independent experiments. For comparisons of average parasitemia at different time points, analysis of variance was performed with a *post hoc* Mann-Whitney test for comparison of the means with Microcal Origin 8.1 software (Origin Lab, Northampton, MA).

***In vivo* acute toxicity assay.** Acute toxicity of 1,2-*O*,*O*-diacetyl-4-nerolidylcatechol (**2**) was determined in healthy mice based on the Organization for Economic Cooperation and Development (OECD) *Guidelines for the Testing of Chemicals for Acute Oral Toxicity* (34). Briefly, this involved gavage administration of compound **2** at doses of up to 2,000 mg kg^{-1} in groups of three mice. Compound **2** was diluted in 2% DMSO–distilled water solution and administered in a single 200-μl dose. The negative-control group received 200 μl of 2% DMSO solution. Animals were observed individually during the first 30 min and periodically over 24 h. Special attention was paid over the first 4 h and daily thereafter for 14 days.

Inhibition of hemozoin formation assay. The hemozoin formation inhibition assay was performed as described by Ncokazi and Egan (35) with modifications (36). Briefly, solutions with different concentrations (20 to 0.156 mg ml^{-1}) of compounds **2** and **4** were transferred (20 μl) in triplicate to a 96-well oval-bottomed plate. Next, bovine hematin (Sigma-Aldrich, Germany) solution (101 μl; 1.68 mM in 0.1 M sodium hydroxide) was added to each well followed by addition of pH 5 sodium acetate buffer (12 M; 58 μl) with constant stirring at 60°C. After incubation at 60°C for 60 min, the plate was centrifuged at 500 × *g* for 8 min. The supernatant was discarded and the crystals of hemozoin were redissolved in 200 μl of 0.1 M sodium hydroxide and transferred to a 96 flat-bottomed well plate. The reaction was monitored on a spectrophotometer (Spectra Max 340 PC384; Molecular Devices) at 405 nm, and the results were expressed as the percent inhibition of hemozoin formation. The optical density of the untreated controls corresponded to 100% hemozoin formation. Chloroquine was used as a positive control. Each substance was tested in three independent experiments.

Treatment of *P. falciparum* with compounds 2 and 3 and metabolic labeling. To evaluate the effects of compounds 2 and 3 on the biosynthesis of isoprenoids in *P. falciparum*, a protocol described by Rodrigues Goulart et al. (18) was used. Synchronized cultures of young trophozoites (ring form) of *P. falciparum* 3D7 exhibiting parasitemia of ca. 10% were treated or not treated for 48 h with compound **2** or **3** at a final concentration of 4 μM and metabolically labeled with 3.1 μCi ml^{-1} of [1-(*n*)-^{3}H]geranylgeranyl pyrophosphate triammonium salt {[1-(*n*)-^{3}H]GGPP; 16.5 μCi $mmol^{-1}$; Amersham} during the last 18 h. The *P. falciparum* schizonts obtained after the incubation period were purified using a discontinuous Percoll gradient (37). The schizonts (80 μl) were freeze-dried, and then lipid extraction was performed with hexane (three times, 0.5 ml). The extracts were combined and dried under a stream of nitrogen and resuspended in 500 μl of hexane. Each extract was divided into 2 aliquots for high-performance liquid chromatography (HPLC) analyses of dolichol 12 or isoprene chains linked to coenzyme Q as described below. In all experiments, the same quantities of treated and untreated parasites were analyzed.

Reverse-phase HPLC (RP-HPLC). Aliquots of the hexane extracts of treated and untreated schizonts were monitored for radioactivity. Samples of hexane extracts were suspended in 250 μl of methanol and analyzed by HPLC using a Phenomenex Luna C_{18} column (250 by 4.6 mm), Gilson HPLC 322 pump, and Gilson 152 variable UV-visible detector. Purified fractions were obtained on a Gilson fraction collector FC203B, and UNIPOINT System software was used to analyze chromatograms.

For analysis of dolichol 12, the following gradient elution system was used at a flow rate of 1.5 ml min^{-1}: 9:1 methanol/water (solvent A) and 1:1:2 hexane/isopropanol/methanol (solvent B) were mixed in a linear gradient from 5 to 100% solvent B over 25 min. The column was further eluted for 5 min with 100% solvent B. The eluent was monitored at 210 nm. Fractions were each collected for 0.5 min (0.75 ml). The mobile phase was evaporated, scintillation liquid was added, and radioactivity was evaluated on a Beckman 5000β-radiation scintillation apparatus. Polyprenols were coinjected as standards in the same HPLC elution and fraction-collecting procedure (18).

For analysis of ubiquinones, the hexane extracts of treated and untreated schizonts were dried and resuspended in methanol and coinjected with Q_8 (ubiquinone with eight isoprene units) and menaquinone standards. An isocratic methanol/ethanol (1:1) elution system was used at a

flow rate of 1.0 ml min^{-1} with fractions being collected at intervals of 0.5 min. The detector was operated at 275 nm. After evaporation of the mobile phase, scintillation liquid was added to each purified chromatographic fraction, and the fractions were analyzed for radioactivity by using a Beckman 5000β-radiation scintillation apparatus (15).

Data analysis. Comparative statistical analysis of peak areas from HPLC chromatograms of samples treated with compounds **2** and **3** versus untreated samples were performed for both dolichol 12 and ubiquinones. After evaluating the normality of the population, Student's *t* test was applied to the data, taking as the null hypothesis (H_0) the equality of the means between control and treated populations (with determination of 95% confidence limits). The average inhibition was then estimated with a significance of 95%.

Protein inhibition assay. For the protein synthesis inhibition assay, asynchronous cultures of *Plasmodium falciparum* were treated (4 μM) or not treated for 48 h with **2** and **3** and marked with [1-^{14}C]sodium acetate (3.1 μCi ml^{-1}; 56 mCi $mmol^{-1}$; Amersham) during the last 18 h. Then, the different intraerythrocytic stages of *P. falciparum* were purified as mentioned above. After purification, each stage (ring, trophozoite, and schizont) was lysed with twice its volume of an ice-cold solution made up of 10 mM Tris-HCl (pH 7.2), 150 mM sodium chloride, 2% (vol/vol) Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, 1 mM N-(*p*-tosyllysine) chloromethyl ketone, and leupeptin (1 μg ml^{-1}). After incubation for 15 min at 4°C, the lysates were centrifuged at 10,000 × *g* for 30 min and the supernatants were stored in liquid nitrogen for further analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Gel electrophoresis. SDS-PAGE was performed in 12.5% gels as described elsewhere (38). The same number of substance-treated or untreated parasites as mentioned above were dissolved in sodium dodecyl sulfate sample buffer and applied to each well for analysis. All gels were treated with Amplify (Amersham), dried, and exposed to Kodak X-Omat film with intensifying screen sets at −70°C for 60 days.

UFLC conditions for pharmacokinetic evaluation. A Shimadzu ultrafast liquid chromatography (UFLC) Prominence system (Kyoto, Japan) consisting of a binary LC-20AT gradient pump, SPDM-20A diode-array detector (DAD), and SIL-20A automatic injector system was used to analyze blood, blood plasma, compound **2**, and mixtures of these prepared as described below. A Shim-Pack XR-ODS column (50 by 2.0 mm [inner diameter], 2 μm; Shimadzu) was used at room temperature. The mobile phase consisted of 0.1% aqueous (Milli-Q) mass spectral-grade formic acid (Fluka Analytical) (A) and 0.1% formic acid in acetonitrile (LiChrosolv, Merck, Darmstadt, Germany) (B) starting at 70% B for 2 min, then a linear increase to 100% B over 8 min, 100% B for 2 min, and a linear decrease to 70% B over 4 min (column reequilibration). Total run time was 16 min. The flow rate was 0.4 ml min^{-1} with a 15% split for mass spectral analysis (see below). The injector volume was 5 μl. Mass spectral-grade isopropanol (Chromasolve; Fluka Analytical) and acetonitrile (1:1) were used in the injector (C).

Mass spectrometer conditions for pharmacokinetic evaluation. A Bruker Daltonics MicroTOF-QII mass spectrometer (Bremen, Germany) with quadrupole-time of flight (TOF) analyzer exhibiting 17,500 full width at half maximum (FWHM) resolution and multichannel detector plate was used as the detector for the UFLC analysis described above to analyze blood, blood plasma, compound **2**, and mixtures of these prepared (below). An electrospray ionization (ESI) source in positive mode was operated using the following parameters: capillary voltage, 4,500 V; end plate offset, −500 V; nebulizer pressure, 2.0 × 10^5 Pa; dry heater temperature, 180°C; dry gas flow, 6.0 liters min^{-1}. The mass range analyzed was *m/z* 100 to 1,000. UFLC-MS analyses were controlled and processed, respectively, by using a Bruker Daltronics Hystar 3.2 system and Data Analysis 4.1 software.

Blood and blood plasma interactions with compound 2. The aim of this procedure was to evaluate potential metabolic reactions between diacetyl derivative **2** and human and mouse blood and blood plasma. It was performed by adapting recovery and analytical procedures from previous pharmacokinetic studies on compound **1** and other compounds (39–41). Mouse or human blood plasma was the supernatant liquid (500 μl) obtained after centrifuging noncoagulated, sodium citrate-treated blood at 1,750 × *g* for 10 min. A stock solution (1.0 mg ml^{-1}) of compound **2** in isopropanol was prepared. This solution (10 μl) was diluted 10-fold by addition to mouse or human blood plasma (90 μl). Next, isopropanol (1,000 μl) was added, and the resulting solution was vortexed (15 min) and then centrifuged (10 min, 1,750 × *g*). The supernatant liquid was transferred to a clean microcentrifuge tube and then was completely dried under a stream of nitrogen. The residue was dissolved in isopropanol (500 μl), filtered (0.22-μm porosity), and analyzed by UFLC-MS. This procedure was performed with human type A+ blood plasma and blood plasma from a healthy mouse. As a control for each experiment (blank), a sample of plasma was treated with vehicle (without compound **2**) and was otherwise processed as above.

The procedure described above was also performed by adding compound **2** to whole (complete) mouse or human blood. A solution of compound **2** in isopropanol (40 μl, 1.0 mg ml^{-1}) was mixed with whole blood (360 μl), and after gentle manual homogenizing in a clean microcentrifuge tube (10 min), the mixture was centrifuged (10 min, 1,750 × *g*). The supernatant liquid (100 μl) was transferred to a new clean microcentrifuge tube. Isopropanol (1,000 μl) was added, and the resulting solution was vortexed (10 min) and then centrifuged (10 min, 1,750 × *g*). The supernatant liquid was transferred to a new clean microcentrifuge tube and completely dried under a stream of nitrogen. The residue was dissolved in isopropanol (500 μl), filtered (0.22-μm porosity), and analyzed by UFLC-MS (39). As a control (blank) for each experiment, a sample of complete blood was treated with vehicle (without compound **2**) and was otherwise processed as above. Three independent experiments were performed.

UFLC-HRMS analysis of mouse blood after oral administration of compound 2. To study alterations in the composition of mouse blood after oral ingestion of compound **2**, 50 and 600 mg kg^{-1} of compound **2** diluted in 2% DMSO–distilled water solution were administered to healthy mice (200 μl) by gavage. One hour after administration, mice were anesthetized with 200 μl of xylazine-ketamine (1 ml kg^{-1}), and blood (500 μl) was removed from mice by cardiac puncture and stabilized with sodium citrate (anticoagulant). The citrated blood was centrifuged (10 min, 1,750 × *g*). The plasma (100 μl) was transferred to a clean microcentrifuge tube and isopropanol (1.0 ml) was added. The mixture was vortexed (10 min) and then centrifuged (10 min, 1,750 × *g*). The supernatant liquid was transferred to another microcentrifuge tube and was completely dried under a stream of nitrogen. The residue was dissolved in isopropanol (500 μl), filtered (0.22-μm porosity), and analyzed by UFLC-HRMS. Two independent experiments were performed.

RESULTS

***In vitro* antiplasmodial activity.** IC_{50}s and standard deviations for 4-nerolidylcatechol (**1**) and derivatives **2** to **4** against *P. falciparum* K1 and 3D7 strains are summarized in Table 1. This is the first report on the *in vitro* antiplasmodial activity (IC_{50} = 4.8 and 5.5 μM against the K1 and 3D7 strains, respectively) of compound **2**. Compounds **1**, **3**, and **4** were evaluated against the 3D7 strain here for the first time. In general, derivatives **2** to **4** were less active *in vitro* than natural compound **1**. The cytotoxicity/antiplasmodial selectivity indices of compounds **1**, **3**, and **4** were calculated based on previously published cytotoxicity data against normal cells (28). The concentration (4.0 μM) of compounds **2** and **3** used to evaluate the effects on the *in vitro* incorporation of isoprene precursors into dolichols, ubiquinones, or proteins was established based on these *in vitro* inhibition results.

***In vivo* antimalarial activities.** Compounds **2** and **4** were evaluated for *in vivo* antimalarial activity using the Peter's suppression

TABLE 1 *In vitro* IC_{50} values of 4-nerolidylcatechol (**1**) and semisynthetic derivatives **2** to **4** against *Plasmodium falciparum* K1 and 3D7 strains and selectivity indices

Compound	IC_{50}[a] (μM)			
	P. falciparum[a]		Mouse embryonic fibroblasts[b]	SI[c]
	K1	3D7		
1	0.67 ± 0.1	0.59 ± 0.2	31.5	47/53
2	4.85 ± 1.2	5.57 ± 1.0	<15.7	<3.2/2.8
3	7.05 ± 2.0	5.94 ± 1.8	>124	>17/>21
4	28.73 ± 10.3	38.07 ± 15.6	95.8	3.3/2.5
Chloroquine diphosphate	0.13 ± 0.1	0.05 ± 0.02	ND[d]	ND
Quinine sulfate	0.16 ± 0.1	0.11 ± 0.1	ND	ND

[a] IC_{50} values were calculated by probit analysis as described in Materials and Methods. Student's *t* test was applied to results. Values are expressed as means ± standard deviations ($n = 3$ experiments).
[b] Mouse embryonic fibroblasts were of the 3T3L1 line. Data in this column for compounds **1**, **3**, and **4** were previously published (28).
[c] SI, selectivity index, calculated with the formula IC_{50} (fibroblasts)/IC_{50} (*P. falciparum*) and is reported for the K1 and 3D7 strains.
[d] ND, not determined.

test. The results are presented in Table 2. At 50 mg kg^{-1} day^{-1}, a dose often used to identify potential new drugs in murine malaria (42, 43), compound **2** was considered partially active (44, 45), as it suppressed *P. berghei* growth by >30% on days 5 and 7 for both routes of administration. At the highest dose (600 mg kg^{-1} day^{-1}), subcutaneous and oral administration of compound **2** led to 72 and 64% suppression of parasitemia, respectively, on the fifth day after treatment began. Compound **2** was inactive at doses of 10 mg kg^{-1} day^{-1}. In general, compound **4** exhibited lower suppression of parasitemia than compound **2**. Thus, maximal inhibition by compound **4** was 48% on the fifth day when administered subcutaneously. A decrease in activity on the seventh day was in general observed compared to the corresponding activity on the fifth day at all doses tested and for both routes of administration. Survival times of infected animals were not significantly increased compared to untreated controls in any of the groups that were treated with compound **2** or **4**, although animals treated orally with 600 mg kg^{-1} day^{-1} of compound **2** exhibited an average survival time of 22 ± 2 days. Control groups treated with 10 mg kg^{-1} day^{-1} of chloroquine diphosphate exhibited 99 to 100% suppression of parasitemia on both days of observation and survival rates of >40 days. The median lethal dose (LD_{50}) of compound **2** was >2 g kg^{-1}, since no animal death or sign of intoxication was observed at any of the assayed doses.

Compound **2** was not detected by LC-MS in the plasma of healthy mice 1 h after administration of 50 or 600 mg kg^{-1} by gavage. This compound was also not detected by UFLC-HRMS after *in vitro* dilution (0.1 mg ml^{-1}; 0.25 mM) in whole blood or blood plasma of mice. Also, no metabolites of compound **2** were discernible by UFLC-HRMS in human or mouse blood plasma, human or mouse complete blood, or in the blood of mice 1 h after oral ingestion of compound **2**. Compound **2** was detected by UFLC-HRMS after *in vitro* dilution in human blood and human blood plasma as a chromatographic peak having a retention time of 6.3 min and characteristic $[M + H - C_2H_2O]^+$, $[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$, and $[M + K]^+$ adduct/fragment ions (data not presented).

Inhibition of hemozoin formation. Figure 2 presents inhibition data for compounds **2** and **3** and chloroquine. Compound **2** inhibited hemozoin formation by 50% at a concentration of 20 mg ml^{-1} (50 mM), and inhibitory activity decreased as a function of concentration. Compound **3** exhibited low inhibition of hemozoin formation (maximum of 20% inhibition at the highest concentration tested). At concentrations of 40 mg ml^{-1} (ca. 100 mM), these compounds were insoluble and could not be tested.

TABLE 2 *In vivo* suppression of *Plasmodium berghei* parasitemia in mice after oral and subcutaneous treatment with compound **2** or **4**[a]

Compound and dose (mg/kg/day)	% parasitemia ± SEM (% inhibition of parasite growth)[b]				Avg survival time ± SD (days)	
	Oral		Subcutaneous			
	Day 5	Day 7	Day 5	Day 7	Oral	Subcutaneous
Compound **2**						
600	0.9 ± 0.3 (64)	1.2 ± 0.73 (56)	0.7 ± 0.4 (72)	0.9 ± 0.3 (70)	22 ± 2	22 ± 3
200	1.0 ± 0.5 (62)	1.43 ± 0.7 (48)	1.0 ± 0.3 (60)	1.0 ± 0.2 (66)	21 ± 3	19 ± 3
50	1.5 ± 0.6 (44)	1.9 ± 0.4 (32)	1.8 ± 0.8 (33)	1.9 ± 0.7 (37)	18 ± 4	19 ± 4
10	2.1 ± 0.6 (23)	2.4 ± 0.7 (12)	2.7 ± 0.4 (0)	3.8 ± 0.7 (0)	20 ± 3	17 ± 2
Compound **4**						
200	1.5 ± 0.6 (46)	2.4 ± 0.6 (28)	1.4 ± 0.7 (48)	2.7 ± 0.8 (17)	21 ± 3	20 ± 2
50	2.0 ± 0.7 (27)	2.5 ± 1.0 (23)	1.9 ± 0.9 (32)	3.4 ± 0.9 (0)	20 ± 2	19 ± 4
10	3.0 ± 0.8 (0)	3.4 ± 0.9 (0)	2.8 ± 0.6 (0)	3.6 ± 1.2 (0)	19 ± 1	17 ± 4
Chloroquine diphosphate						
10	0.00 (100)	0.02 (99)	0.03 (99)	0.03 (99)	>40	>40
Control	2.7 ± 0.9	3.3 ± 0.7	2.6 ± 0.6	3.0 ± 0.9	19 ± 4	21 ± 3

[a] The experiment was performed following the Peters protocol (33).
[b] The percentage (mean ± standard error of the mean) of parasitized red blood cells of a total of 5 mice in three independent experiments. The percent inhibition of parasite growth (parasitemia reduction) was compared to results for untreated control mice. The Mann-Whitney test was used to evaluate statistical differences between groups. A *P* value of <0.05 was considered statistically significant.

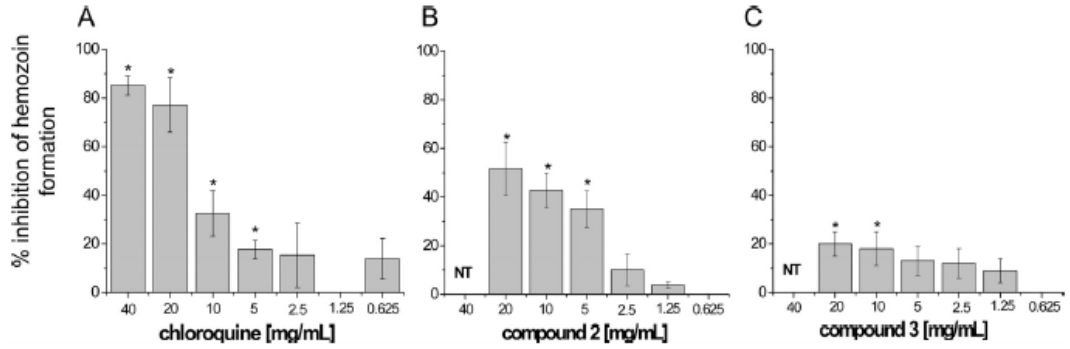

FIG 2 Inhibition of hemozoin formation by chloroquine (A), 1,2-*O*,*O*-diacetyl 4-nerolidylcatechol (compound **2**) (B), and 2-*O*-benzyl 4-nerolidylcatechol (compound **3**) (C). Significant differences compared to drug-free controls are indicated in each graph by an asterisk ($P \leq 0.05$). NT, not tested (insoluble).

Effects on the biosynthesis of isoprenoids and proteins in *P. falciparum*. The effects of compounds **2** and **3** on the biosynthesis of isoprenoids in *P. falciparum* were determined by metabolic labeling with the precursor [1-(*n*)-^{3}H]GGPP in treated parasites (with 4 μM compound **2** or **3**) or untreated parasites for 48 h. The schizont extracts were analyzed by RP-HPLC for the presence of dolichol containing 12 isoprene units and ubiquinones. Figure 3 presents the radioactivities of the fractions corresponding to the retention times of dolichol 12, ubiquinone 8, and menaquinone 4 of the hexane extract obtained from the cultures in the schizont stage. The radioactivities of fractions from the tests were compared with those of the corresponding fractions from untreated controls. The latter fractions were considered to have 100% (maximal) incorporation of the radiolabeled precursor [1-(*n*)-^{3}H]GGPP. The statistical analysis applied is described in Material and Methods.

Biosynthesis of dolichol 12 was inhibited by 41 and 45% by compounds **2** and **3**, respectively, in the schizont stage. Also, compound **2** significantly inhibited the biosynthesis of ubiquinone and menaquinone by 36 and 41%, respectively. When the cultures were treated with compound **3**, no significant difference in the biosynthesis of prenylated quinones compared to untreated controls was observed. SDS-PAGE analysis of proteins from ring stages, trophozoites, and schizonts from cultures labeled with [1-^{14}C]sodium acetate and treated or not treated with compound **2** or **3** revealed that there was no inhibition of protein synthesis by these compounds (see Fig. S1 in the supplemental material).

DISCUSSION

In previous work, natural compound **1** administered subcutaneously in mice at high doses (200 to 600 mg kg^{-1} day^{-1}) suppressed *P. berghei* by 0 and 41 to 61%, respectively, on days 5 and 7 after inoculation. Also, at oral doses of 200 to 600 mg kg^{-1} day^{-1} compound **1** suppressed *P. berghei* on the fifth and seventh days by 15 to 63 and 49 to 60%, respectively (25). The subcutaneous and oral activities observed for derivative **2** (Table 2) represent improved *in vivo* antimalarial activities compared to those reported previously for compound **1**. The suppression of parasitemia observed for compound **2** (≥30%) is indicative of partial antimalarial activity (42, 43). The development of derivatives of **1** exhibiting greater *in vivo* antimalarial activity would therefore be desirable. In this vein, it is important to keep in mind the possible limitations

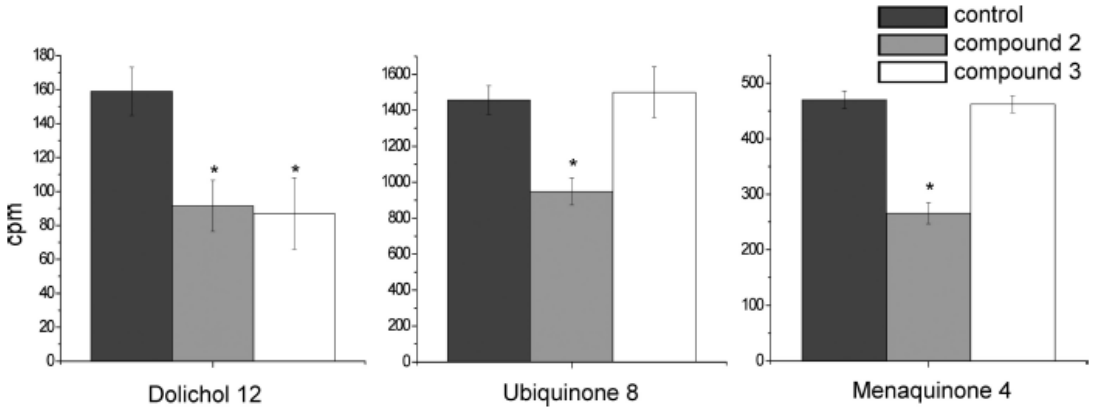

FIG 3 The radioactive peaks corresponding to the retention times of dolichol 12 (18), ubiquinone 8, and menaquinone 4 (15) from the hexane extract of the schizont stage, untreated or treated for 48 h with 4.0 μM 1,2-*O*,*O*-diacetyl 4-nerolidylcatechol (compound **2**) or 2-*O*-benzyl-4-nerolidylcatechol (compound **3**) after purification by RP-HPLC as described in Materials and Methods. Metabolic labeling was performed with [1-(*n*)-^{3}H]geranylgeranyl pyrophosphate. A one-way ANOVA was applied, and *P* values of <0.05 were considered statistically significant. An asterisk indicates a significant difference compared to untreated controls.

of optimizing activity based on rodent malaria as a model for human malaria infections. For example, clinically viable drugs, such as quinine (against *P. berghei* ANKA, 50% effective dose [ED_{50}] = 34 mg kg^{-1} day^{-1} [46] and slow clearance [47]) and artemisinin derivatives, exemplified by artesunate (recrudescence below 80 mg kg^{-1} day^{-1} via oral against *P. vinckei*) (48) are not optimal for treating rodent malaria parasites but are of unquestionable therapeutic value in humans.

The pharmacokinetic profile of natural compound **1** has been determined in Sprague-Dawley rats (49), and bioavailability was found to be 2.7% based on blood analyses 10 to 180 min after oral administration of 10 mg kg^{-1}. Plasma concentrations of compound **1** after intravenous injection (100 mg kg^{-1}) exhibited a large distribution and rapid elimination rate, as expected for highly lipophilic drugs such as compound **1**. Here, the pharmacokinetics of compound **2** could not be determined, presumably due to metabolism and or strong interactions of compound **2** with mouse blood and mouse blood plasma components. Interestingly, the *in vitro* tests demonstrated that compound **2** could be recovered from human blood and human blood plasma.

Several classes of antimalarial drugs are known to cause an increase in the concentration of free heme (toxic to *Plasmodium* spp.) through the inhibition of hemozoin formation. Chloroquine is the classic drug known to inhibit hemozoin formation (9, 50). The inhibition of hemozoin formation observed for derivative **2** may be related to structural features of this compound and direct interactions with heme. Compound **2** may adopt highly planar conformations and undergo noncovalent van der Waals and or π-stacking interactions with heme (50). Furthermore, the catechol oxygen atoms in derivatives of compound **1** may interact strongly with iron in heme (as is believed to occur with the endoperoxide oxygen atoms of artemisinin [8]) and undergo complexation and redox reactions (28). The plausibility of these interactions needs corroboration through docking studies and spectroscopic analysis of heme/hemozoin-drug intermediates.

In *Plasmodium* spp., isoprenoids are biosynthesized via the MEP pathway in the interior of the apicoplast. *P. falciparum* biosynthesizes essential intermediates and final products important to its survival via the isoprenoid pathway, such as carotenoids, vitamin E, ubiquinones, menaquinones, and dolichols, as well as geranylated and farnesylated proteins (13–17). Through the incorporation of the radioactive metabolic precursor [1-(*n*)-^{3}H]GGPP in cultures that were treated or untreated with compounds **2** or **3**, it was possible to determine the inhibitory effects of these substances on the biosynthesis of dolichol, menaquinone, and ubiquinone metabolites in schizonts of the 3D7 strain of *P. falciparum*. The inhibition of isoprenoid biosynthesis in *P. falciparum* by compounds **2** and **3** was considered good because it occurred at concentrations (4 μM) lower than the IC_{50}s, while overall protein biosynthesis was unaffected. No significant inhibition of isoprenoid biosynthesis by compounds **2** or **3** was observed in ring and trophozoite stages of *P. falciparum* (data not presented). This result is related to the relatively low metabolic production of isoprenoids at these early stages in parasite development (12, 18).

The strong inhibition of dolichol 12 biosynthesis in schizonts (Fig. 3) can be explained by the interference of compounds **2** and **3** in the isoprene chain elongation mechanism in dolichol biosynthesis, as has been suggested for the isoprene chains attached to the benzoquinone ring (51). The reduction in the availability of dolichol 12 produced by these derivatives can presumably interfere directly in posttranslational protein modifications (12, 16) and other metabolic processes that require the presence of dolichols.

Compound **2** strongly inhibited the biosynthesis of the prenylated metabolites ubiquinone and menaquinone in *P. falciparum* (Fig. 3). This is significant, as ubiquinones and menaquinones are linked to the survival of *Plasmodium* spp. in the host. Ubiquinone, also known as coenzyme Q, is an important electron carrier which is actively synthesized in the mitochondria of *Plasmodium* parasites and exerts an important protective antioxidant effect in the parasite (52, 53). An octaprenyl pyrophosphate synthase has been found in the intraerythrocytic stages of *P. falciparum* that is responsible for the biosynthesis of the isoprene side chains attached to the benzoquinone ring of ubiquinones. The recombinant version of this synthase exhibited marked similarity to that of the native, partially purified octaprenyl pyrophosphate synthase from schizont-stage parasites and was inhibited by nerolidol with a K_i of 10 nM (51). Vitamin K_2, also known as menaquinone (MQ), is in a class of fat-soluble vitamins that regulates metabolic pathways. In radiolabeling experiments of the direct precursor, Tonhosolo et al. (15) demonstrated that vitamin K_2 is synthesized by *P. falciparum* and acts as an important electron receiver of the respiratory chain. Also, inhibition of menaquinone production by Ro 48-8071, a known 1,4-dihydroxy-2-naphthoate prenyltransferase inhibitor, resulted in decreased parasite growth. Given the importance of menaquinone and ubiquinone to parasite survival, future work should focus on the specific molecular targets upon which derivative **2** directly acts during the biosynthesis of these compounds.

The inhibition of hemozoin formation and the inhibition of isoprenoid biosynthesis in *Plasmodium* generally involve independent molecular targets (6, 7, 16). However, work by Howe et al. (17) and others has demonstrated that the prenylation of the small ATPase Rab5 is associated with small hemoglobin-containing vacuoles that collectively represent >50% of hemoglobin uptake in trophozoites and schizonts (54). Disruption of posttranslational prenylation of Rab5 by fosmidomycin in turn disrupts the localization of Rab5 in vacuolar membranes and is associated with abnormal food vacuole morphologies. *P. falciparum* is believed to perform *de novo* synthesis of the terpenoid fragments it requires for ubiquinone and prenylated protein synthesis (17). It is conceivable that besides interfering in the biosynthesis of terpenoids in the apicoplast, 4-nerolidylcatechol derivatives **2** and **3** may interrupt the posttranslational prenylation of Rab5 and other proteins associated with normal vacuolar membrane formation in *P. falciparum*. However, the action of **2** and **3** on prenyltransferases or digestive vacuole formation mechanisms in *P. falciparum* was not demonstrated herein. 4-Nerolidylcatechol derivatives biochemically inhibited the synthesis of isoprenoids by inhibiting the incorporation of precursors in the metabolic pathway in the live parasite. Also, these derivatives chemically inhibited hemozoin formation *in vitro*, especially compound **2**, which exhibits inhibition similar to chloroquine, thus providing indirect evidence for this mechanism of action. Derivatives of 4-nerolidylcatechol potentially could act on different targets, in the apicoplast and in the food vacuole, in distinct mechanistic processes. Future work should investigate the possibility that derivatives of **1** are inhibitors of prenyl transferases that are important to digestive vacuole physiology and function.

4-Nerolidylcatechol (**1**) is a major component of infusions and

other extracts prepared from the roots and leaves (22-25, 55) of the traditionally used caapeba plant (*Piper peltatum* and *P. umbellata*) (56). Caapeba extracts are active *in vitro* against *P. falciparum* and inactive or variably active against *P. berghei* in mice (57–62). Interestingly, the deactivated blood plasma taken from healthy rodents after oral ingestion of caapeba extracts inhibits *P. falciparum in vitro* (59). We isolated compound **1** from *P. peltatum* extracts and demonstrated that it exhibits good *in vitro* inhibition against *P. falciparum* (24, 25, 29) and, after oral ingestion, activates murine blood plasma against *P. falciparum* (25). However, as discussed above, *P. berghei* exhibits low sensitivity to caapeba extracts and 4-nerolidylcatechol and, as seen here, to derivatives of **1**. In similar circumstances, diamidines were tested in the *P. vinckei* model because *P. berghei* is almost insensitive to these drugs. Further studies on 4-nerolidylcatechol derivatives should explore *P. vinckei*-infected mouse or *P. falciparum*-infected humanized mouse (HuMouse) models (63).

Natural compound **1** exhibits *in vitro* antiplasmodial activity that is desirable as a starting point for a chemistry program for the development of new antimalarials; however, its *in vivo* antimalarial activity and stability are not satisfactory. In general, derivatives of compound **1** exhibit decreased *in vitro* antiplasmodial activity and improved stability and *in vivo* antimalarial activity compared to **1**. Nonetheless, the levels of *in vivo* activity observed for compound **2** are not satisfactory and may be negatively influenced by poor pharmacokinetics.

Derivatives of 4-nerolidylcatechol inhibit *in vitro* biosynthesis of vital isoprenoid metabolites in *P. falciparum*. New derivatives with improved activity, solubility, and pharmacokinetics are needed. Poor pharmacokinetics associated with low water solubility (and fast clearance) were also problems identified for the low-polarity natural antimalarial artemisinin, and these properties were markedly improved by the development of the water-soluble derivative sodium artesunate (64). Also, formulations of derivatives of compound **1** may help to improve bioavailability. In this vein, an inclusion complex of compound **1** and derivatized cyclodextrin has been prepared that exhibits improved water solubility (65). The development of more soluble, structurally diverse 4-nerolidylcatechol derivatives and formulations could be important strategies for increasing bioavailability and the *in vivo* antimalarial activities of these compounds.

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Supplemental Material

For the protein synthesis inhibition test, asynchronous cultures of *Plasmodium falciparum* were treated or not treated for 48 h with compounds 2 or 3 and marked with [1-^{14}C]-sodium acetate during the last 18 h. Then, the different intraerythrocytic stages of *P. falciparum* were purified as mentioned in the text, for analyses using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After purification, each stage (ring, trophozoite and schizont) was lysed with twice its volume of an ice cold solution made up of 10 mM Tris-HCl (pH 7.2), 150 mM NaCl, 2% (v/v) Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, 1 mM N-(*p*-tosyllysine) chloromethyl ketone, and leupeptin (1 µg/mL). After incubation for 15 min at 4°C the lysates were centrifuged at 10,000 g for 30 min and the supernatants were stored in liquid nitrogen for further analysis by SDS-PAGE.

Gel electrophoresis: SDS-PAGE was performed in 12.5% gels as described previously (1). The same number of substance-treated or untreated parasites as mentioned above were dissolved in sodium dodecyl sulfate sample buffer and applied to each well for analysis. All gels were treated with Amplify (Amersham), dried, and exposed to Kodak X-Omat film with intensifying screen sets at -70°C for 60 days.

The SDS-PAGE analysis of proteins from rings, trophozoites and schizonts from cultures labeled with [1-^{14}C]-sodium acetate and treated and not-treated with **2** and **3** are presented in Fig. S1. The intensity of the bands demonstrates that there was no inhibition of protein synthesis by the compounds tested.

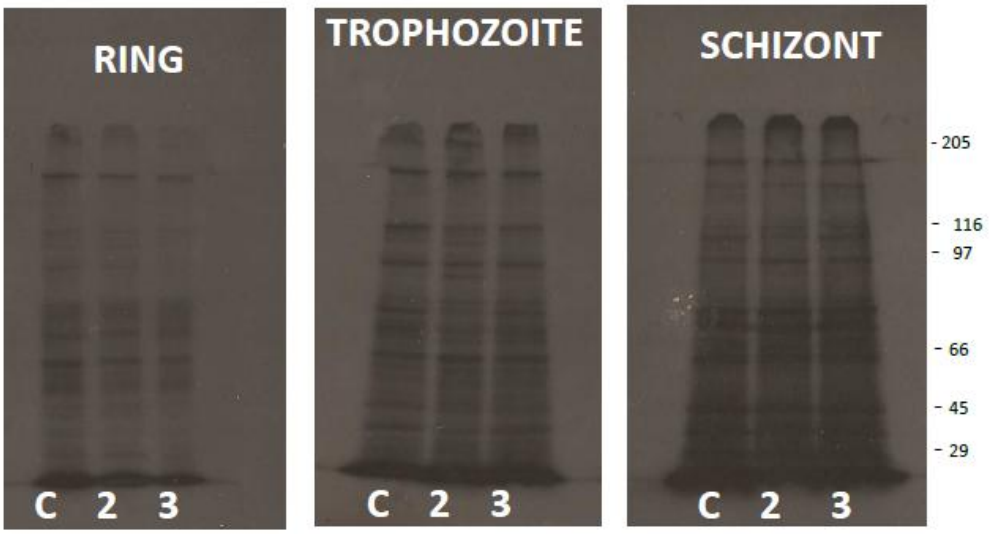

FIG S1 SDS-PAGE of proteins obtained from the cultures of *P. falciparum* in the treated and untreated ring, trophozoite and schizont stages after 36 h with 4.0 μM of 4-nerolidilcatecol derivatives 2 and 3 and then metabolically labeled for the last 12 h with [1-^{14}C]-sodium acetate. C – untreated control; **2** - treatment with 1,2-*O*,*O*-diacetyl 4-nerolidylcatechol; **3** – treatment with 2-*O*-benzyl 4-nerolidylcatechol. Molecular mass standards are indicated on the right. This is a representative experiment of two independent assays.

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