Universidade Federal de Minas Gerais
Faculdade de Medicina
Programa de pós-graduação em Medicina Molecular

Monitoramento da resistência do *Plasmodium falciparum* e *P. vivax* aos antimaláricos em Rondônia e busca de novos esquizonticidas sanguíneos e teciduais para controle e tratamento da doença.

Anna Caroline Campos Aguiar

Belo Horizonte
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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 à obtenção do título de doutorado em Medicina Molecular.

Orientadora: Professora Dra. Antoniana Ursine Krettli

Belo Horizonte

Outubro/2014

UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA MOLECULAR

ATA DA DEFESA DE TESE DA ALUNA
ANNA CAROLINE CAMPOS AGUIAR - 2011743383

Realizou-se, no dia 03 de outubro de 2014, às 14:00 horas, Centro de Pesquisas *René Rachou*, da Universidade Federal de Minas Gerais, a defesa de tese, intitulada *Monitoramento da resistência do Plasmodium falciparum e P. vivax aos antimaláricos em Rondônia e busca de novos esquizonticidas sanguíneos e teciduais para controle e tratamento da doença.*, apresentada por ANNA CAROLINE CAMPOS AGUIAR, número de registro 2011743383, graduada no curso de BIOMEDICINA, como requisito parcial para a obtenção do grau de Doutora em MEDICINA MOLECULAR, à seguinte Comissão Examinadora:Prof(a). Antoniana Ursine Krettli - Orientadora (UFMG), Prof. Alejandro Miguel Katzin (USP), Prof(a) Taís Nóbrega de Sousa (FIOCRUZ/RENE RACHOU), Prof(a). Luzia Helena Carvalho (FIOCRUZ/RENE RACHOU), Prof(a). Eliana Maria Mauricio da Rocha (UFSJ).

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, 03 de outubro de 2014.

Prof.ª Antoniana Ursine Krettli (Doutora)

Prof. Alejandro Miguel Katzin (Doutor)

Prof.ª Taís Nóbrega de Sousa (Doutora)

Prof.ª Luzia Helena Carvalho (Doutora)

Prof.ª Eliana Maria Mauricio da Rocha (Doutora)

UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA MOLECULAR

FOLHA DE APROVAÇÃO

Monitoramento da resistência do Plasmodium falciparum e P. vivax aos antimaláricos em Rondônia e busca de novos esquizonticidas sanguíneos e teciduais para controle e tratamento da doença.

ANNA CAROLINE CAMPOS AGUIAR

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

Aprovada em 03 de outubro de 2014, pela banca constituída pelos membros:

Prof(a). Antoniana Ursine Krettli - Orientador
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Belo Horizonte, 3 de outubro de 2014.

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SUMÁRIO

RESUMO

A malária humana é a doença parasitária mais importante e um dos maiores problemas de saúde pública do mundo. Seu controle é dificultado pela falta de uma vacina eficaz, problemas no combate ao inseto, mas, sobretudo, pelo surgimento e disseminação de parasitos resistentes aos antimaláricos disponíveis. O tratamento espécie-específico permanece como principal estratégia visando a redução da morbidade e da mortalidade ocasionados pela doença. A busca de novos antimaláricos contra diferentes estágios do parasito torna-se essencial, tema abordado neste trabalho de tese. Na pesquisa de novos esquizonticidas sanguíneos, dois análogos da cloroquina (BAQ e MAQ) foram testados quanto a sua capacidade de inibir *ex vivo* o desenvolvimento de isolados de *P. vivax* e *P. falciparum*, coletados de pacientes diagnosticados em Porto Velho, Rondônia. Ambas as espécies foram sensíveis a MAQ e BAQ, com IC_{50} na ordem de nanomolar. Essa suscetibilidade *ex vivo* dos parasitos foi testada em paralelo contra os antimaláricos cloroquina (CQ), mefloquina (MQ) e artesunato (AS). A maioria dos isolados de *P. vivax* foi sensível à CQ (IC_{50} médio de 32nM), mas os isolados de *P. falciparum* apresentaram baixa sensibilidade *ex vivo* à CQ (IC_{50} médio de 70nM); todos os isolados foram sensíveis ao AS e à MQ. Polimorfismo de nucleotídeo único foi avaliado nos genes *mdr*1 e *crt*, ambos relacionados com a resistência dos parasitos aos antimaláricos. Mutações de troca foram encontradas no gene *pf*mdr1 nos códons 184 (100%), 1042 (100%) 1246 (100%) e 1034 (84%). Mutações no gene *pf*crt, códons 72 e 76, foram encontradas em 100% dos isolados. No entando, os isolados de *vivax* não apresentaram mutações. Na busca de novos esquizonticidas teciduais, 15 tiazolidinonas derivadas da primaquina (PQTZs) foram avaliadas contra formas exoeritrocíticas (FEE) de *P. berghei*-ANKA *in vitro* inibindo o desenvolvimento dos esporozoítos nos hepatócitos, com redução significantemente na porcentagem de células infectadas e tamanho dos esquizontes. No entanto, somente o composto 4m foi ativo *in vivo*, reduzindo 95% o desenvolvimento hepático das FEE dos animais tratados e aumentando o período pré-patente da malária em 3 dias. Os PQTZs foram avaliados quanto à capacidade de bloquear a transmissão, nos modelos da malária aviária (*P. gallinaceum* e *Aedes fluviatilis*) e de roedores (*Anopheles stephensi* e *P. berghei*), após tratamento dos hospedeiros vertebrados com os compostos. Os compostos 4b, 4c, 4g, 4m, 4o, assim como a primaquina, inibiram 100% a infecção dos mosquitos anofelinos. O PQTZ 4m apresentou a melhor atividade contra FFE *in vitro* e *in vivo* e bloqueou 100% a transmissão, portanto, este composto deverá ser avaliado em modelos com

malária pelo *P. cynomolgi* e/ou *P. vivax*, visando o desenvolvimento de um novo medicamento para o tratamento das recaídas da malária humana.

ABSTRACT

Human malaria is the most important parasitic disease and a major public health problem. Its control remains difficult due to the lack of an effective vaccine, difficulties in the vector control and, especially, the emergency and spread of parasites resistant to most available antimalarial drugs. The specific drug treatment is a major strategy to reduce morbidity and mortality owed to malaria. The research for new antimalarials against different parasitic stages is essential and was studied in the present work. Two chloroquine analogs (BAQ and MAQ) were evaluated *ex vivo* against *P. vivax* e *P. falciparum* isolates from patients diagnosed in Porto Velho, Rondônia. The isolates were sensitive to MAQ and BAQ, presenting IC_{50} at nanomolar dose. The *ex vivo* sensitivity of those isolates was tested, in parallel, against the antimalarials chloroquine (CQ), mefloquine (MQ) and artesunate (AS). Most of *P. vivax* isolates were sensitive to CQ (IC_{50} of 32nM) whereas the *P. falciparum* isolates exhibited low sensitivity to CQ (IC_{50} of 70nM); all isolates were sensitive to AS and MQ. Single Nucleotide Polymorphisms were evaluated at *mdr*1 and *crt* genes, both related with antimalarial parasite resistance. Mutations were found in *pf*mdr1 gene at codons 184 (100%), 1042 (100%) and 1246 (100%), 1034 (84%). For the *pf*crt gene, mutations were observed at codons 72 and 76 in all *P. falciparum* isolates. The *P. vivax* isolates did not present mutations. As tissue schizonticidals, fifteen primaquine derivatives (PQTZs) were evaluated against the exoritrocitic forms (EEF) of *P. berghei*-ANKA *in vitro*. The compounds inhibited the development of sporozoites into the hepatocytes, and reduced the number and the size of EEF *in vitro*. Only 4m was active *in vivo* reducing 95% the development of EEF and increasing the malaria pre-patent period in 3 days. The PQTZs were evaluated for their ability to block the malaria transmission in avian malaria (*P. gallinaceum* in chickens and *Aedes fluviatilis*) and rodent malaria (*P. berghei* in mice and *Anopheles stephensi*) using the vertebrate host treated with the compounds. The compounds 4b, 4c, 4g, 4m and 4o inhibited 100% infection of *Anopheles* mosquitoes by *P. berghei*, like primaquine, tested in parallel. The PQTZs 4m showed the best activity against EEF *in vitro* and *in vivo* and exhibited the best blocking transmission activity. This compound has yet to be evaluated against late EEF in models using *P. cynomolgi* malaria and/or *P. vivax*, aiming to develop a new drug to treat human malaria relapses.

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LISTA DE ABREVIATURAS

ACT	*Artemisinin Combined Therapy*
AS	Artesunato
BAQ	Biquinolínicos
BGM	Linhagem celular renal de macaco
CEM	*Campanha de Erradicação da Malária*
CEPEM	Centro de Pesquisas em Medicina Tropical
CPqRR	Centro de Pesquisas René Rachou
CQ	Cloroquina
crt	*Chloroquine resistance transporter*
CSP	Circumesporozoíta
DDT	Dicloro-difenil-tricloroetano
DMEM	Dulbecco Modificado por Eagle
DMSO	Dimetilsulfóxido
EEF	Formas Exoeritrocíticas
FEE	Formas exoeritrocíticas
FIOCRUZ	Fundação Oswaldo Cruz
G6PD	Glicose-6-fosfato desidrogenase
GE	Gota Espessa
GSH	Glutationa na forma reduzida
HepG2 A16	Linhagem celular hepática
HRPII	*Histidine rich protein II*
IC_{50}	Concentração inibitória de 50% do crescimento do parasito
MAQ	Monoquinolícos
MDL_{50}	Dose letal mínima de 50% das células
*mdr*1	*Multi-drug resistance* 1
MQ	Mefloquina
MTT	Sal tetrazólico 3-(4,5-dimetiltiazol-2-yl)-2,5 difenitetrazol)
MS	Ministério da Saúde
OMS	Organização Mundial da Saúde
PBS	*Phosphate buffered saline*
PBS-T	PBS/Tween 0,05%

PCR	Reação da Cadeia em Polimerase
PPP	Período pré-patente
PQ	Primaquina
PQTZs	Primaquina-Tiazolidinonas
rpm	Rotação por minuto
RPMI	Meio de cultura (*Roswell Park Memorial Institute*)
SBF	Soro Bovino Fetal
SNPs	Polimorfismo de nucleotídeo único
SVS	Secretaria de Vigilância Sanitária
TMB	3,3′,5,5′-Tetramethylbenzidine
UFAL	Universidade Federal do Alagoas
UFPel	Universidade Federal de Pelotas
VD	Vacúolo digestivo
WHO	*World Health Organization*

1. INTRODUÇÃO

1.1. A malária e os fatores epidemiológicos

A malária, uma doença causada por protozoários do gênero *Plasmodium*, continua sendo um dos principais problemas de saúde do mundo, sendo causa de intensa morbidade e óbitos. A doença ocorre em 109 países, sendo endêmica nas regiões tropicais e subtropicais da África, sudeste da Ásia e América Latina (WHO, 2010). Metade da população mundial (3,3 bilhões de pessoas) está exposta à transmissão da malária em áreas de risco (Hay *et al*., 2009), no ano de 2012 foram registrados 207 milhões de casos e 627 mil mortes, a maioria deles na África (WHO, 2013). A doença resulta ainda em pobreza nas populações de países com altas taxas de prevalência, segundo Sachs e Malaney (2002).

São cinco as espécies de plasmódios causadores da malária humana, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* e *P. knowlesi*. O *P. falciparum* causa a febre denominada terçã maligna, e o *P. vivax* a terçã benigna, no entanto, há vários casos de malária grave por *P. vivax* descritos (Genton *et al*., 2008; Poespoprodjo *et al*., 2009; Alexandre *et al*., 2010; Andrade *et al*., 2010; Lanca *et al*., 2012) aparentemente relacionados à multi-resistência medicamentosa (Tjitra *et al*., 2008; Wélé *et al*., 2011). No Brasil, há três espécies causadoras da malária humana: *P. falciparum*, *P. vivax* e *P. malariae* (Secretaria de Vigilância Sanitária - SVS, 2009); 99% dos casos ocorrem na Amazônia Legal (Acre, Amapá, Amazonas, Maranhão, Mato Grosso, Pará, Rondônia, Roraima e Tocantins), onde as condições ambientais favorecem a proliferação dos mosquitos anofelinos vetores da doença. Em 2013, foram registrados aproximadamente 178 mil casos de malária, aproximadamente 85% causados por *P. vivax* (Ministério da Saúde - MS, 2014).

1.2. Ciclo biológico dos parasitos da malária

O ciclo de vida dos plasmódios de mamíferos compreende dois hospedeiros, o mosquito *Anopheles* spp, onde ocorre a reprodução sexuada do parasito, e o vertebrado, no qual ocorre a reprodução assexuada do parasito, inicialmente no fígado e posteriormente nos eritrócitos. A transmissão se inicia quando o mosquito inocula o parasito, na forma de esporozoíto, durante o repasto sanguíneo. Em camundongos infectados com *P. berghei*, cerca de 10% dos esporozoítos permanecem e evoluem na pele, no sítio de inoculação onde

originam merozoítos infectantes (Gueirard *et al*., 2010). Mas, mesmo no *P. berghei*, a maioria dos esporozoítos evolui no fígado, podendo invadir e migrar através dos hepatócitos antes de se desenvolver em um vacúolo parasitóforo (Mota *et al*., 2001). Ocorre a divisão esquizogônica tecidual assexuada nos hepatócitos, de onde os esquizontes maduros liberam merozoítos para a corrente sanguínea, através de um processo de brotamento de vesículas denominadas merosomos (Sturm *et al*., 2006). Esta etapa do ciclo é denominada fase exoeritrocítica. Nas infecções por *P. vivax* e *P. ovale*, formas latentes, os hipnozoítos, permanecem nos hepatócitos por tempo variável causando recaídas da doença (Krotoski, 1982).

Os merozoítos hepáticos infectam os eritrócitos multiplicando também por reprodução assexuada, liberando merozoítos a cada 48h (ou após 72h no caso do *P. malariae*). Há desenvolvimento sincrônico no ciclo eritrocítico com rompimento das hemácias infectadas, causando as febres cíclicas, típicas da malária. Alguns merozoítos se diferenciam em gametócitos masculino e feminino, os quais só evoluem nos mosquitos suscetíveis.

Minutos após o repasto sanguíneo pelo inseto vetor ocorre a exflagelação do gametócito masculino (ou microgametócito) com liberação dos microgametas no lúmen do intestino médio dos mosquitos, evento regulado por fatores como diminuição da temperatura do sangue, aumento do pH e exposição dos parasitos ao ácido xanturênico, um subproduto do metabolismo do triptofano (Billker *et al*., 1998). Ocorre a fusão dos gametas masculino e feminino resultando no zigoto, uma estrutura móvel (oocineto), que atravessa a membrana peritrófica e se localiza entre epitélio do intestino médio e a lâmina basal, onde é denominado oocisto; milhares de esporozoítos são formados no seu interior, por reprodução esporogônica e escapam 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 proteína circumesporozoíta (CSP) (Touray *et al*., 1992) e a proteína adesiva relacionada à trombospondina (TRAP) (Vlachou *et al*., 2006). Os esporozoítos são inoculados pelo mosquito vetor no vertebrado ao exercer o hematofagismo; originam as formas exoeritrocíticas e reiniciam o ciclo do parasito no hospedeiro suscetível.

Recentes avanços em técnicas moleculares tem permitido a observação direta de esporozoítos e sua evolução no hepatócito, a partir da utilização de parasitos fluorescente e bioluminescente (Menard *et al*., 2013).

1.3. Controle da malária

Várias estratégias foram utilizadas no controle da malária, durante a Campanha de Erradicação da Malária (CEM) iniciada em 1965, visando a eliminação do vetor e erradicação da malária com: aplicação intradomiciliar do inseticida dicloro-difenil-tricloroetano (DDT), controle químico dos focos de criação dos mosquitos, amplo uso de cloroquina (CQ) no tratamento curativo e profilático e do sal cloroquinado. A primaquina (PQ) foi utilizada para controle das recaídas tardias por *P. vivax*.

A CEM foi coordenada pela Organização Mundial de Saúde (OMS) e resultou na eliminação da malária em extensas áreas do mundo, incluindo do território brasileiro (nas regiões Nordeste, Sudeste, Centro-oeste e Sul) onde pelo menos 40 milhões de pessoas viviam sob o risco de transmissão da doença. Mas esse sucesso não ocorreu na Amazônia Legal, onde as características ambientais na região favorecem a intensa proliferação do vetor e a extensa floresta tropical úmida no desenvolvimento do parasito no mosquito, com alta transmissão extradomiciliar. O surgimento do *P. falciparum* resistente à CQ e os problemas de logística levaram à interrupção da CEM, em 1970 (Loiola *et al*., 2002).

Houve depois outras tentativas no controle da malária, sendo criada em 1970 a Superintendência de Campanhas de Saúde Pública (SUCAM), que não se dedicava exclusivamente ao controle da malária. Foram criados planos mais específicos como o Projeto de Controle da Malária na Bacia Amazônica, em 1989, com investimentos do Banco Mundial, com estruturação dos serviços locais de saúde, melhora da capacitação nos serviços de diagnóstico e tratamento da malária. O Plano de Intensificação das Ações de Controle da Malária foi criado em 1999, e subsequentemente, o Programa Nacional de Controle da Malária, com objetivos de fornecer diretrizes para controlar a doença, reduzindo a sua incidência e a gravidade dos casos, com eliminação da transmissão em áreas urbanas e diminuição da letalidade (MS, 2003).

Atualmente a malária continua causando empobrecimento na África, Ásia e em algumas regiões da Amazônia e dezenas de óbitos, ocorrendo, sobretudo fora da região Amazônica, onde há dificuldade no diagnóstico por falta de suspeita clínica dos profissionais da saúde o que retarda o tratamento da doença (Krettli *et al*., 2013; de Pina-Costa *et al*., 2014; Junior *et al*., 2014).

A principal estratégia no controle da malária é a realização do diagnóstico precoce e tratamento espécie-específico imediato dos casos com fármacos adequados. São realizadas constantes ações de medidas de controle do vetor, sobretudo em áreas de transmissão urbanas

e periurbanas, tais como: (i) obras de saneamento; (ii) borrifação intradomiciliar com inseticidas; (iii) uso de mosquiteiros impregnados com inseticidas e (iv) tratamento químico e biológico de criadouros e de espaços abertos (MS, 2012).

O principal método de diagnóstico laboratorial é o exame de microscopia da gota espessa (GE), confeccionado com sangue da polpa digital do paciente e corado com Giemsa, o mais utilizado pelo MS atualmente. Existem testes rápidos para diagnóstico do *P. falciparum*, baseados na pesquisa indireta de proteínas específicas do parasito, com a utilização de anticorpos monoclonais, por exemplo, contra uma proteína rica em histidina (*Histidine Rich Protein II* - HRPII) ou contra a enzima lactato desidrogenase (LDH) (WHO, 1996). A identificação do DNA do parasito, a partir da Reação da cadeia em polimerase (PCR), possibilita o diagnóstico nos pacientes com baixas parasitemias ou assintomáticos (Erdman e Kain, 2008). No entanto, o padrão ouro de diagnóstico permanece sendo a GE, técnica simples e barata que permite a visualização do parasito, a identificação da espécie, do estágio de desenvolvimento, quantificação do parasito, facilitando o controle de cura do paciente (WHO, 2012).

Na busca de alternativas para o controle da malária algumas vacinas promissoras em desenvolvimento estão na fase de testes clínicos nas áreas endêmicas, porém nenhuma está comercialmente disponível para uso humano (Schwartz *et al*., 2012; Campo *et al*., 2014). Outra abordagem para o controle da doença seria desenvolver mosquitos transgênicos e/ou infectados com bactérias capazes de inibir a transmissão (Hughes *et al*., 2014).

1.4. Tratamento da malária humana e principais desafios

Apesar dos vários antimaláricos disponíveis para tratamento da doença (**Tabela 1**), a seleção e disseminação de parasitos resistentes continuam sendo o principal fator que dificulta o controle da malária. Não existe ainda tratamento ideal com um fármaco ativo contra os vários estágios de desenvolvimento dos parasitos (Aguiar *et al*., 2012A).

A CQ, uma 4-aminoquinolina com intensa atividade esquizonticida sanguínea, de baixa toxicidade, baixo custo, excepcionalmente eficaz por via oral contra todas as espécies de *Plasmodium*, teve seu uso interrompido devido ao surgimento e disseminação da resistência pelo *P. falciparum* nas áreas endêmicas. Mas a CQ continua em uso no tratamento de malária não *falciparum* (WHO, 2013), sendo capaz de atravessar a membrana do eritrócito na sua forma não protonada, se acumulando no interior do vacúolo digestivo (VD) do parasito (pH ~ 5,0) na sua forma protonada, onde atua inibindo a formação da hemozoína (Kouznetsov

et al., 2009). Após ligação covalente com o heme, a CQ inibe a geração dos cristais de hemozoína e gera uma alta produção de espécies reativas de oxigênio (Sullivan 2002; Fitch, 2004), causando danos oxidativos às biomoléculas como as proteínas, o DNA e os lipídeos, gerando modificações estruturais irreversíveis, além da perda de função biológica (Radfar *et al*., 2008). Outros efeitos independentes do acumulo da CQ no VD como sua interação com a molécula de DNA, levam a alterações na estrutura e bloqueio da síntese do DNA (Stephen *et al*., 2000). Se descontinuada a pressão da CQ, a sensibilidade dos parasitos ao fármaco é aparentemente revertida (Pereira *et al*., 2011).

Novos derivados da CQ tem sido propostos como candidatos importantes no desenvolvimento de fármacos, como a ferroquina, cuja atividade é comparável a da CQ (Kreidenweiss *et al*., 2006). A ferroquina não apresenta resistência cruzada com a CQ em cepas de *P. falciparum* isoladas de pacientes do Camboja, resistentes à CQ e em *P. yoeli* após a indução dessa resistência em camundongos, por pressão de fármacos (Biot *et al*., 2005; Dubar *et al*., 2008; Biot *et al*., 2009).

Outros derivados do anel quinolínico sintetizados foram muito utilizados no tratamento da malária, como a amodiaquina e a mefloquina (MQ), com ação esquizonticida sanguínea semelhante a da CQ (WHO, 2012). No entanto, por ser rapidamente metabolizada, a amodiaquina apresenta menor atividade antimalárica que a CQ (Churchill, 1985). A MQ, um 4-quinolinometanol derivado da CQ, é mais ativa que essa e foi muito utilizada contra cepas de *P. falciparum* resistentes às 4-aminoquinolinas. Mas, por possuir meia-vida longa, rapidamente selecionou parasitos resistentes. Além disso, a MQ é tóxica e causa efeitos colaterais e sintomas neurológicos importantes durante sua administração (Patchen *et al*., 1989).

Atualmente, a OMS recomenda o uso de combinação de fármacos esquizonticidas sanguíneos no tratamento da malária, como a terapia de combinação com derivados da artemisinina (ACT), utilizada na malária não complicada por *P. falciparum* e em áreas onde os parasitos apresentam resistência à CQ (WHO, 2013). Os derivados semi-sintéticos da artemisinina (artemether, arteether e artesunato) são administrados com outro antimalárico (lumefantrina, amodiaquina, MQ, sulfadoxina-pirimetamina) ou com antibióticos.

Derivados da artemisinina, obtidos por modificações químicas e sintetizados para melhorar suas características farmacocinéticas (Li e Wu, 2003), são de ação rápida, meia vida curta e agem contra gametócitos (Okell *et al*., 2008; Stepniewska e White, 2008). Mas o surgimento de resistência aos mesmos foi observado desde 2004, com os primeiros casos de recrudescência descritos no Camboja (Dondorp *et al*., 2009; Lin *et al*., 2010). Essa resistência

do *P. falciparum* está disseminada no sudeste e sudoeste Asiático (Miotto *et al*., 2013); a menor suscetibilidade do parasito a ACT foi detectada no oeste da África (Ashley *et al*., 2014). Mutações pontuais na proteína "kelch" (kelch13) foram recentemente associadas à resistência dos parasitos, o que poderá facilitar a identificação de parasitos resistentes (Ariey *et al*., 2014).

No Brasil, a primeira opção de tratamento da malária *falciparum* preconizada pelo MS é a combinação de artemether (480mg) com lumefantrina (2880mg), COARTEM®, em quatro dias, associada à PQ, na dose 45mg, para evitar a transmissão. Essas doses são recomendadas para adultos com 50Kg ou mais. No caso da malária não complicada pelo *P. vivax*, a CQ é o medicamento de escolha (1500mg de CQ, divididos em 3 dias), associada com a PQ (210mg, divididos 7 ou 14 dias), para evitar as recaídas tardias da doença (MS, 2010). Em um estudo realizado entre os anos de 1946 e 2004 foi demonstrada a eficiência de 75% da PQ no tratamento supervisionado das recaídas tardias, em pacientes infectados no Brasil. A eficácia da PQ variou de acordo com a região da transmissão do parasito, sendo 100% em isolados da Nicarágua e de 96% em isolados da América Central (Baird e Hoffman, 2004).

Apesar da utilização da CQ no tratamento da malária *vivax*, a resistência desses parasitos ao antimalárico está descrita em áreas da Indonésia e Papua Nova Guiné (Ratcliff *et al*., 2007; Karunajeewa *et al*., 2008; Sutanto *et al*., 2009). Baixos níveis de resistência do *P. vivax* à CQ foram descritos em Myanmar, Corea do Sul, Vietnan, Índia, Turquia, Etiópia e regiões do Sudoeste da África e na América do Sul (Suwanarusk *et al*., 2007; Price *et al*., 2009).

Os esquizonticidas teciduais são utilizados para eliminação dos hipnozoítos, formas hepáticas latentes de esporozoítos, responsáveis pelas recaídas tardias em infecções causadas pelo *P. vivax* (Markus *et al*., 1978). A PQ, uma 8-aminoquinolina, é o fármaco disponível para tratar as recaídas (Mueller *et al*., 2009; Wells *et al*., 2010); outros medicamentos também pertencentes ao grupo das 8-aminoquinolinicos, a tafenoquina e a ebulaquina, estão em testes clínicos visando substituir a PQ no tratamento dessas recaídas (Walsh *et al*., 1999; Walsh *et al*., 2004). A PQ apresenta meia vida curta e requer o uso prolongado para a cura radical (14mg/dia/7 ou 14 dias consecutivos) (Baird e Rieckman, 2003; Baird e Hoffman, 2004; WHO, 2009). Nessas doses a PQ causa efeitos colaterais como desconforto gastro-intestinal, além de anemia hemolítica em indíviduos deficientes da enzima 6-fosfato-desidrogenase (G6PD) (Hill *et al*., 2006; Carmona-Fonseca *et al*., 2009; Baird e Surjadjaja, 2011).

Como *P. vivax* é a espécie mais prevalente no mundo, a descoberta de substitutos menos tóxicos que a PQ é importante (Wells *et al*., 2010); aproximadamente 2,85 bilhões de indivíduos no Sudoeste Asiático, nas Américas Central e do Sul, estão sob o risco de contrair malária causada por essa espécie de plasmódio (Guerra *et al*., 2009). Devido a atividade gametocitocida da PQ, seu uso em dose única é também recomendado para bloquear a transmissão da malária em áreas endêmicas pelo *P. falciparum* (WHO, 2009).

Tabela 1. Antimaláricos de referência e sua atividade contra diferentes estágios do parasito e resistência do *P. falciparum* a esses medicamentos.

Antimalárico	**Estágios do parasito**				**Resistência reportada em *P. falciparum***
	FEE[a]	**HP[b]**	**iRBC[c]**	**GM[d]**	
Amodiaquina			*		Sim
Artemisinina			*	*	Sim
Atovaquona	*		*	*	Sim
Cloroquina			*		Sim
Clindamicina	*		*		Não
Doxycycline	*		*		Não
Lumefantrina			*		Não
Mefloquina			*	*	Sim
Primaquina	*	*	*	*	Sim
Proguanil	*		*	*	Sim
Pirimethamina	*		*	*	Sim
Pironaridina			*	*	Sim
Quinina			*		Não
Sulfadoxina			*	*	Sim
Tetraciclina	*		*		Não

1.5. Marcadores de resistência aos antimaláricos

O gene *mdr*1 (*multi-drug resistance* 1), localizado no cromossomo 5, codifica uma proteína de 162kDa, expressa na membrana do VD do parasito ao longo de todo o seu desenvolvimento intraeritrocítico (Cowman *et al*., 1991). O gene *crt* (*chloroquine resistance transporter*), localizado no cromossomo 7, codifica uma proteína de transporte, também expressa na membrana do VD e que está relacionada com o acúmulo de CQ e a conversão da hematina solúvel em hemozoína (Bray *et al*., 1998).

Os polimorfismos de nucleotídeo único (SNPs) nos genes *pf*crt (C72S, K76T) e no gene *pf*mdr1 (N86Y; Y184F; S1034C; N1042D; D1246Y), descritos no *P. falciparum* estão relacionados a resistência à CQ (Wellems e Plowe., 2001; Sidhu *et al*., 2002; Wongsrichanalai *et al*., 2002). O gene *pf*crt é considerado um bom marcador da resistência; essa mutação está relacionada com mudanças no fluxo intracelular de CQ, com a redução da susceptibilidade dos isolados ao fármaco (Fidock *et al*., 2000). Não foi ainda descrita mutação no gene ortólogo em (*pv*crt). No entanto um aumento de 2 vezes na expressão desse gene está associado a diminuição na suscetibilidade do parasito à CQ (Russell *et al*., 2003).

No gene *mdr*1 de *P.vivax* (Y976F) foi descrita mutação associada com a resistência a CQ, em isolados de pacientes na Papua, Indonésia (Suwanarusk *et al*., 2007). Nesta região há vários relatos de falha terapêutica após o tratamento da malária *vivax* com CQ, 96% dos parasitos analisados apresentaram essa mutação de troca associada com um alto valor de IC_{50} para a CQ 283nM [95% CI: 211-379] nos ensaios *ex vivo* (Suwanarusk *et al*., 2007). Esses dados foram comparados com os de outra região (Thai) onde os parasitos são sensíveis a 44,5nM [95%CI: 31,3–63,4] e o tratamento ainda é eficaz; somente 26% dos isolados apresentaram a mutação (Suwanarusk *et al*., 2007).

Outra mutação no códon 382 (S→C) do gene *pv*dhps, recentemente descrita em isolados de *P. vivax* da região Amazônica Brasileira, está relacionada à suscetibilidade a CQ (Chehuan *et al*., 2013).

Devido a movimentos de migração populacional, o possível surgimento de parasitos resistentes em área endêmicas precisa ser monitorado, sendo relevante o estudo de marcadores da resistência.

1.6. Mecanismos de toxicidade da Primaquina e a busca por substitutos

Devido as altas doses necessárias de PQ para eliminar os hipnozoítos e a flutuação dos níveis plasmáticos dos seus metabólitos após administração oral do fármaco (Bolchoz *et al*., 2001; Bolchoz *et al*., 2002; Baird e Hoffman., 2004), esse medicamento possui efeitos colaterais graves, principalmente em pacientes deficientes da enzima G6PD. Esta enzima desempenha um papel crucial no sistema de defesa antioxidante dos eritrócitos, sendo necessária para a redução do NADP a NADPH, por sua vez necessária para a manutenção da glutationa na forma reduzida (GSH). A GSH neutraliza o estresse oxidativo nos eritrócitos que leva a danos celulares; enquanto sua ausência aumenta a sensibilidade do eritrócito aos agentes oxidantes (Shiraki *et al*., 2011). Em estudos realizados na Tanzânia, a anemia hemolítica relatada pelo uso de PQ foi assinalada mesmo em indivíduos que não apresentam a deficiência da enzima G6PD (Shekalaghe *et al*., 2010).

Dois novos medicamentos do grupo das 8-aminoquinolinicos estão em testes clínicos: a tafenoquina e ebulaquina. A tafenoquina (GlaxoSmithKline®), utilizada em dose única, se encontra em testes clínicos de fase III, sendo utilizada na prevenção das recaídas tardias causadas pelo *P. vivax*. Apesar de apresentar meia-vida longa e bom perfil farmacocinético, a tafenoquina desencadeia hemólise em pacientes deficientes na enzima G6PD (Walsh *et al*., 1999). A ebulaquina, desenvolvida pelo Instituto Central de Pesquisas de Fármacos da Índia, é considerada uma molécula promissora como subtituto da PQ (Mazier *et al*., 2009), sendo aproximadamente quatro vezes menos tóxica do que PQ em cães (Puri e Dutta, 2005). Os níveis de metahemoglobina em voluntários humanos tratados com sete doses de 25mg/Kg do composto não foram alterados. Pacientes que receberam 15mg/Kg de PQ desenvolveram metemoglobinemia. Apesar da ebulaquina ser metabolizada à PQ após administração oral, ela parece ser melhor tolerada por indivíduos deficientes da enzima G6PD (Puri e Dutta, 2005). O desenvolvimento de fármacos com base na modificação da estrutura química da PQ e pró-drogas baseadas na estrutura da PQ, tem sido estudados visando fármacos ativos e com menos efeitos tóxicos (Davanço *et al*., 2012; Mata *et al*., 2012).

2. JUSTIFICATIVA

Embora o desenvolvimento econômico e a implementação de medidas de controle ao longo do século XX tenham eliminado a malária em algumas partes do mundo, esta doença parasitária endêmica nos trópicos, especialmente nas regiões mais pobres do globo, continua responsável pelo maior número de óbitos (WHO, 2013). Além disso, a malária resulta em perdas econômicas e sociais, comprometendo o desenvolvimento de países com alta transmissão (Gallup e Sachs, 2001).

A falta de uma vacina eficaz, a dificuldade de controle do vetor e o surgimento e disseminação de parasitos resistentes à maior parte dos fármacos disponíveis (WHO, 2010; 2013), incluindo os derivados de artemisinina (Miotto *et al*., 2013; Ashley *et al*., 2014), são problemas que dificultam o controle da malária. O diagnóstico precoce e o tratamento imediato e especifico dos indivíduos infectados são as principais estratégias de controle (WHO, 2012). Mas, devido a movimentos de migração populacional e ao possível surgimento de parasitos resistentes aos antimaláricos, essa resistência precisa ser monitorada, utilizando marcadores da resistência (Pratt-Riccio *et al*., 2013). Além disso, a elevada toxicidade da PQ, único fármaco disponível para tratar as recaídas tardias da malária pelo *P. vivax*, a espécie do parasito mais prevalente no mundo, é outro fator complicador do controle da doença, tornando urgente a descoberta de novos antimaláricos (WHO, 2013).

Essa busca tem sido conduzida utilizando diversos modelos experimentais *in vitro* e *in vivo*. Desenvolver novos fármacos ativos contra as diversas formas do parasito e aumentar as opções de antimaláricos disponíveis deve ajudar a solucionar os problemas advindos do desenvolvimento de resistência e da toxicidade de fármacos (Burrows *et al*., 2012).

Neste contexto, análogos de CQ e tiazolidinonas derivadas de PQ (PQTZs), foram avaliadas como potenciais esquizonticidas sanguíneos e teciduais, respectivamente, sendo ainda o perfil de resistência dos pacientes de Porto Velho-RO infectados com *P. vivax* ou *P. falciparum* caracterizado utilizando marcadores de resistência.

3. OBJETIVOS

3.1. Objetivo geral

Buscar novos antimaláricos esquizonticidas sanguíneos, teciduais e bloqueadores da transmissão, utilizando análogos da CQ (BAQ e MAQ) ou derivados da PQ (PQTZs), visando melhorar o controle da malária.

3.2. Objetivos específicos

a. Avaliar *in vitro* a atividade esquizonticida dos análogos da CQ (MAQ e BAQ) e de derivados de PQ (PQTZs), contra *P. falciparum*;

b. Avaliar a atividade citotóxica desses compostos nas linhagens celulares BGM e HepG2;

c. Avaliar a atividade esquizonticida sanguínea de MAQ e BAQ contra o *P. berghei* em camundongos;

d. Testar MAQ e BAQ na inibição da formação de hemozoína *in vitro* e *in silico* no modelo de modelagem molecular, comparando com a CQ;

e. Caracterizar isolados de *P. vivax* e *P. falciparum* obtidos de pacientes ambulatoriais da população de Porto Velho, Rondônia, quanto à sua resistência aos antimaláricos AS, MQ e CQ, e à BAQ e MAQ, a partir de testes *ex vivo*;

f. Avaliar a presença de mutações nos genes *crt* e *mdr*1 dos isolados de *P. vivax* e *P. falciparum* relacionados à resistência a CQ;

g. Avaliar a atividade hemolítica das PQTZs para hemácias humanas normais e deficientes da enzima G6PD, em paralelo com a PQ;

h. Avaliar a atividade dos derivados de PQ contra o ciclo hepático primário do *P. berghe*i;

i. Estudar a atividade das PQTZs no bloqueio da transmissão da malária pelo *P. berghei* comparando os dados da transmissão do *P. gallinaceum*;

4. MATERIAIS E MÉTODOS

4.1. Comitê de ética

O estudo do material biológico de pacientes foi aprovado pelo Comitê de Ética de pesquisas do Centro de Pesquisas René Rachou (CPqRR), Fundação Oswaldo Cruz (FIOCRUZ) (CAAE - 03209212.7.0000.5091). O protocolo para uso de animais de laboratório foi aprovado pelo Comitê de Ética para Uso de Animais (CEUA LW-23/13), pela FIOCRUZ. O uso de eritrócitos humanos foi aprovado pelo Comitê de Ética do Centro de Pesquisas em Medicina Tropical (CEPEM) (CAAE - 007 59912.0.0000.0011).

4.2. Compostos testes

Foram estudados dois análogos da CQ: MAQ, com um anel quinolínico, e BAQ com dois anéis quinolínicos. BAQ foi previamente descrito na literatura (Zhang *et al*., 2008) e teve a sua atividade comparada com MAQ neste estudo. Os análogos foram sintetizados pelo colaborador químico Dr. Mário Meneghetti, na Universidade Federal do Alagoas (UFAL) (**Figura 1**).

Na busca de substitutos da PQ, quinze compostos heterociclos derivados de PQTZs (4b-4p), foram sintetizados na Universidade Federal de Pelotas (UFPel), a partir de reações químicas entre PQ e arenaldeídos. As estratégias de síntese e o rendimento das reações foram publicados pelo grupo (Neuenfeldt *et al*., 2011) e as estruturas ilustradas na **Figura 2**.

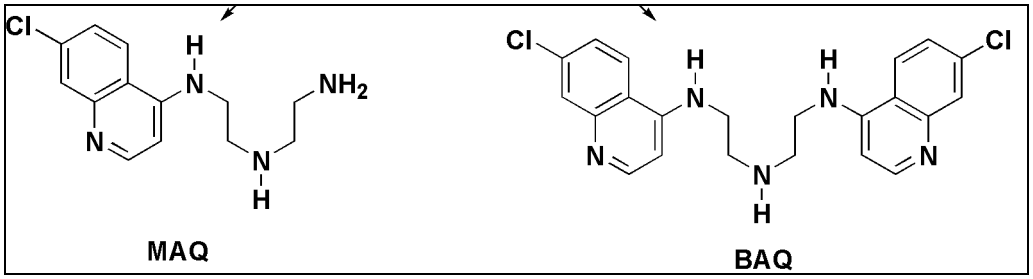
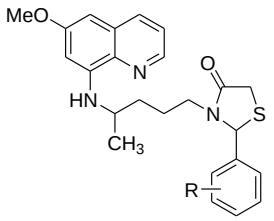


Figura 1. Estrutura química de MAQ e BAQ.



Composto	Radical	**Composto**	Radical	**Composto**	Radical
4b	2-F	**4g**	4-Cl	**4l** - B42A	3-OCH_3
4c	3-F	**4h**	2-NO_2	**4m** - N17A	4-OCH_3
4d	4-F	**4i**	3-NO_2	**4n** - N78B	3-CN
4e	2-Cl	**4j**	4-NO_2	**4o** - P91A	4-CN
4f	3-Cl	**4k**	2-OCH_3	**p** - P92A	4-CH_3

Figura 2. Estrutura química das PQTZs com atividade antiplasmodial.

4.3. Cultivo contínuo da fase eritrocítica do parasito e testes *in vitro*

Nos ensaios de atividade antimalárica foram utilizadas formas sanguíneas de um clone de *P. falciparum* CQ-resistente (W2), cultivadas em hemácias humanas sob condições estabelecidas por Trager e Jensen (1976). Resumidamente, os parasitos foram cultivados em placas de Petri (Corning, Santa Clara, CA, EUA) com hematócrito a 2%, diluídos em meio de cultura RPMI 1640 (Sigma-Aldrich) suplementado com 25mM de Hepes (Sigma-Aldrich), 21mM de bicarbonato de sódio (Sigma-Aldrich), 11mM de glicose (Sigma-Aldrich), 40μg/mL de gentamicina (Schering-Plough, Kenilworth, New Jersey, EUA) e 10% (v/v) de plasma humano A^+ inativado (meio completo). As placas foram mantidas em dessecadores a 37°C ou em mistura gasogênica (5% de O_2, 5% de CO_2 e 90% de N_2), com trocas diárias do meio de cultura e monitoramento da parasitemia em esfregaços sanguíneos fixados com

metanol, corados com Giemsa e visualizados em microscópio óptico com objetiva de imersão (1.000x).

Para realização dos testes, os cultivos com predomínio de anéis foram obtidos através de sincronização dos parasitos como descrito por Lambros e Vanderberg (1979). A solução de sorbitol 5% e glicose 0,5% foi adicionada ao sedimento contendo o sangue parasitado e incubado a 37°C por 10min, seguida de centrifugação (5min, 70g) à temperatura ambiente. O sobrenadante foi retirado e o sedimento ressuspendido em meio completo ajustando-se o hematócrito para 5%. Culturas de parasitos sincronizadas foram distribuídas em microplacas de 96 poços (Corning, Santa Clara, CA, USA) adicionando-se 180µL/poço de meio de cultura RPMI contendo: (i) 1% de parasitemia e 1% de hematócrito para o teste de incorporação de hipoxantina tritiada, ou (ii) 0,05% de parasitemia e 1,5% de hematócrito para o teste de ELISA anti-HRPII. Em placas contendo 20µL dos compostos a serem testados em triplicata e em diferentes concentrações seriadas (400-0.625ng/mL) foi adicionada a suspensão dos parasitos, sendo seis controles com hemácias não infectadas (controle negativo) e seis com hemácias infectadas em poços sem os compostos-testes (controle positivo). O antimalárico padrão CQ foi testado em paralelo em todos os experimentos realizados, em diluições seriadas de 500 a 7,8ng/mL.

4.4. Testes de incorporação de hipoxantina tritiada e anti-HRPII

Para o teste de incorporação de hipoxantina, os parasitos foram cultivados em meio sem hipoxantina por pelo menos 72h. A mistura parasito-compostos teste e controles foi incubada por 24h a 37°C. Após esse período, foram adicionados 20µL de solução de [^{3}H]-hipoxantina à 5µCi (PerkinElmer, Waltham, MA, EUA), as placas incubadas (18h à 37°C) (Desjardins *et al*., 1979) e em seguida mantidas à -20°C (por 6 a 10h) sendo descongeladas para promover a lise das hemácias. As amostras foram aspiradas pelo coletor de células "Harvester 96 Mach III" (Tomtec Imaging Systems Gmbh, Unterschleissheim, Germany) em papéis de filtro (Perkin Elmer), secas em microondas (3min em potência média) e acondicionadas em embalagem plástica, na qual foram adicionados 4mL de líquido de cintilação. A concentração de hipoxantina radioativa incorporada aos parasitos foi avaliada em contagem por minuto (cpm) no equipamento Microbeta 1450 (Perkin Elmer), sendo a incorporação de [^{3}H]-hipoxantina proporcional à viabilidade do parasito.

Para o ensaio imunoenzimático anti-HRPII (Noedl *et al*., 2002) duas placas de 96 poços foram preparadas: uma placa-teste, contendo os parasitos e os compostos a serem

testados; outra pré-sensibilizada com o anticorpo monoclonal anti-HRPII. Após incubação (24h à 37ºC) o conteúdo de seis poços da placa teste (controle positivo) foi retirado e congelado à -20°C, posteriormente utilizado como *background*. A placa foi novamente incubada (48h) depois congelada e descongelada duas vezes à -70°C para a lise das hemácias. A sensibilização das placas (Maxysorp, Nunc, Denmark) foi feita com 100µL do anticorpo primário à 1,0µg/mL (MPFM-55A ICLLAB®, EUA), *overnigth* a 4°C. O conteúdo dos poços foi descartado, foram adicionados 200µL/poço da solução de bloqueio (PBS-BSA 2%), feita incubação à temperatura ambiente (2h) e lavagem da placa por três vezes (PBS-Tween 20 a 0,05%, PBS-T). Em seguida 100µL das amostras da cultura de *P. falciparum* hemolisadas da placa teste foram adicionados na placa sensibilizada, incubada em câmara úmida (1h à temperatura ambiente), lavada três vezes com PBS-T, adicionando-se 100µL do anticorpo secundário (1:5.000 por 1h) (MPFG55P ICLLAB®, EUA). Após lavagem, três vezes com PBS-T, 100µL/poço de uma solução de 3,3′,5,5′-Tetramethylbenzidine (TMB) foi adicionada e a placa incubada por 5 a 10min à temperatura ambiente. A reação foi interrompida adicionando-se 50µL/poço de ácido sulfúrico 1M e a leitura das absorbâncias feita à 450nm em um espectrofotômetro de microplacas (leitor de ELISA) (Spectra Max 340PC384, Molecular Devices).

4.5. Cultivo de linhagens celulares e ensaios de citotoxicidade (MTT e Vermelho Neutro)

As linhagens celulares (Hepa1-6, HepG2 e BGM), adquiridas da ATCC (CRL-1830), foram cultivadas em garrafa de 75cm^2 (Corning, Santa Clara, CA, EUA) em meio Dulbecco Eagle Modificado (DMEM, Gibco/Invitrogen, Carlsbad, CA, EUA), suplementado com 10% de soro bovino fetal (SBF, HyClone Laboratories) e 40mg/L de gentamicina (Schering-Plough) e mantidas em estufa (5% de CO_2, 95% de umidade e 37°C). O meio de cultura foi substituído a cada dois dias (Calvo-Calle *et al*., 1994) até confluência de 80%, quando as células foram repicadas, utilizadas nos ensaios, ou congeladas em ampolas de criopreservação com 95% de SBF e 5% de dimetilsulfóxido DMSO (Sigma-Aldrich, St. Louis, MO, EUA). Antes dos testes as células foram lavadas com meio, tratadas com 1mL de tripsina-EDTA a 0,25% (Gibco/Invitrogen) (37°C por 3min), para seu descolamento. Após adição de 9mL de meio completo e centrifugação (80g por 5min à temperatura ambiente), o sobrenadante foi descartado, o sedimento ressuspendido em meio completo (5% SBF); a suspensão de células ajustada para 5x10^3/mL, sendo 180µL acrescentados a cada poço da microplaca. Após incubação (12 a 16h em estufa de CO_2 a 37°C) para adesão das células, 20µL de meio

completo, contendo diferentes concentrações dos compostos (1000-1µg/mL), foram adicionados em triplicata aos poços da microplaca, sendo a placa incubada 24h, 37°C, e, estufa (5% de CO_2 e 95% de umidade) (Madureira *et al*., 2002).

Para o teste de MTT, 20µL de uma solução de Brometo 3-(4,5-dimetiltiazol-2-yl)-2,5-difeniltetrazol (MTT) (Sigma-Aldrich), na concentração de 5mg/mL foram adicionados aos poços da placa (Denizot e Lang, 1986). Após 3h de incubação, o sobrenadante foi retirado e o corante presente nos fundos dos poços da placa diluído em DMSO (100µL/poço). As microplacas foram então lidas em espectrofotômetro (Spectra Max340PC384, Molecular Devices), utilizando-se filtro de 570nm.

No ensaio de vermelho neutro (Borenfreunda *et al*., 1987) 200µL de solução de vermelho neutro (40µg/mL) foram adicionados a cada poço e incubados (3h, em estufa de CO_2 a 37°C). O sobrenadante foi retirado e 200µL de solução de formaldeído (0,5% v/v) em $CaCl_2$ (1%) foram adicionados (5min à temperatura ambiente), seguido de 100µL de solução de álcool ácido (50% v/v de etanol em 1% v/v de ácido acético). As absorbâncias das microplacas foram lidas em espectrofotômetro (Spectra Max340PC384, Molecular Devices), utilizando-se filtro de 540nm.

4.6. Índice de Seletividade

O índice de seletividade (IS) das amostras foi obtido pela a razão entre o valor de MDL_{50} e o valor de IC_{50}. Valores maiores que 10 foram considerados indicativos de ausência de toxicidade (Bézivin *et al*., 2003).

4.7. Teste de inibição da formação de hemozoína

O ensaio de hemozoína foi realizado em microplacas de 96 poços fundo em "U", como descrito por Kanyile e Egan (2004), testando os compostos (10,1µL) em diferentes concentrações (40-0,62mg/mL), em triplicata, em 101,2µL da solução estoque de hematina a 1,680mM em NaOH a 0,1M. Após homogeneização em 58,7µL de uma solução de acetato de sódio (12,9M, pH 5,0) a microplaca foi incubada (60ºC por 60min), foram adicionados 80µL de solução de piridina (30% v/v em 20mM Hepes, pH 7,5) e a placa incubada à temperatura ambiente. Os sólidos resultantes foram homogeneizados e deixados em repouso (15min) para sedimentação. Posteriormente, 38µL do sobrenadante foram transferidos para outra

microplaca, diluídos em 250µL de solução de piridina (30% v/v em 20mM Hepes, pH 7,5), seguido de leitura a 405nm (SpectraMax340PC384, Molecular Devices). Os resultados foram calculados a partir da comparação da absorbância da curva padrão de solução de hemina e expressos em concentração de heme livre.

4.8. Testes da atividade esquizonticida sanguíneo da malária com o *P. berghei*

Camundongos suíços Webster fêmeas, provenientes do biotério de produção do CPqRR, , com 20±2g de peso, foram inoculados com hemácias infectadas com *P. berghei* (cepa NK65), originalmente recebida da Universidade de Nova Iorque (EUA), mantida em camundongos por passagens sanguíneas semanais. Para os testes de quimioterapia, os camundongos foram inoculados com 10^5 hemácias parasitadas (0,2mL) por via intraperitoneal; aproximadamente 24h depois os animais foram divididos aleatoriamente em grupos de seis camundongos. Em cada experimento foram utilizados dois grupos controles: não-tratado e tratado com CQ. Os camundongos foram tratados diariamente, por via oral, com os compostos testes e com o controle (CQ), em diferentes concentrações, por três dias consecutivos. A parasitemia foi avaliada em microscópio óptico (objetiva de imersão a 1.000x) pela contagem dos parasitos em esfregaços sanguíneos coletados em diferentes dias após a infecção. A atividade antimalárica foi determinada pela percentagem de redução da parasitemia dos animais tratados em relação aos controles, inibição de 30% do crescimento dos parasitos, foi considerada indicadora de uma amostra ativa (Andrade-Neto *et al*. 2003).

4.9. Obtenção dos isolados de *Plasmodium* de pacientes com malária

Os pacientes com malária foram examinados e tratados pelo médico Dr. Dhélio B. Pereira no CEPEM na cidade de Porto Velho, RO. Pacientes acima de 18 anos, dos sexos feminino e masculino, com condições físicas adequadas e enquadrados nos critérios de inclusão, foram convidados a participar do estudo. Os mesmos não apresentavam sintomas de malária grave e/ou outra doença associada, não faziam uso de antimaláricos ou de outros medicamentos no período de 30 dias precedentes a coleta de sangue. Os pacientes que concordaram em participar assinaram o termo de consentimento livre esclarecido (**Anexo IV**) sendo então submetidos a punção venosa, para coleta de 5mL de sangue.

Após coleta do sangue, os glóbulos brancos foram removidos através da passagem por uma coluna de celulose CF11 (Sriprawat *et al*., 2009). Essas células interferem no crescimento do parasito e na caracterização subsequente do DNA purificado. Dois mL do sangue purificado foram congelados em solução de glicerolite para uso posterior; 300µL foram utilizados para extração do DNA e 800µL foram utilizados no ensaio de maturação de esquizontes no cultivo *ex vivo*; o restante do sangue foi armazenado em solução de RNA *later* (Life Technologies, USA) para posterior utilização.

4.10. Preparo das placas de compostos teste

Para a realização dos testes *ex vivo* os análogos de CQ (MAQ e BAQ) e os antimaláricos MQ e AS, foram solubilizados em DMSO (Sigma-Aldrich) para o preparo da solução estoque (10mg/mL); a CQ foi solubilizada em água. As amostras foram preparadas em concentração 10x maior que a inicial utilizada na placa de drogas, sendo 20µL transferidos para a placa teste, submetida à centrifugação (30min a 37ºC) em aparelho de *SpeedVac* (Thermo Scientific, USA) para evaporação do solvente e mantidas à -20ºC, podendo ser utilizada em um período máximo de 6 meses.

4.11. Manutenção dos isolados de *P. vivax* e *P. falciparum* e testes *ex vivo*

Os isolados de sangue humano com *P. vivax* foram mantidos em meio de cultura McCoy´s (Sigma-Aldrich), suplementado com 25mM HEPES, 2mM L-glutamina, 40mg/mL de gentamicina e 20% de plasma humano AB^+. Os isolados de *P. falciparum* foram mantidos em meio de cultivo RPMI (Sigma-Aldrich) suplementado com 25mM HEPES, 2mM L-glutamina, 40mg/mL de gentamicina e 10% plasma AB^+. A susceptibilidade dos parasitos aos antimaláricos (AS, MQ e CQ) e à MAQ e BAQ foi avaliada no *P. falciparum* e no *P. vivax* como descrito por Rieckmann e Antuñano (1971) e Renapurkar e colaboradores (1989), respectivamente, com pequenas modificações (Russell *et al*., 2003). Resumidamente, para cada placa de 96 poços foram preparados 18mL de meio de cultura específico para cada espécie (item 4.6), sendo adicionados 180µL da suspensão por poço seguido da incubação em dessecador a 37ºC (Trager e Jensen, 1976). Após 24, 32, 37 e 42 horas de incubação foram preparadas gotas espessas (GE) dos poços controles para verificação da maturação de pelos menos 40% dos esquizontes. Isolados sem esse mínimo de maturação foram excluídos do

estudo. Após coletadas de todos os poços da placa, as GE foram coradas com Giemsa (5% v/v) por 30min e contadas ao microscópio ótico (100x). A percentagem dos parasitos viáveis nos poços testes, em relação aos controles sem antimaláricos, foi calculada com base no número de esquizontes (mais de 4 núcleos) x 100, no controle sem droga (100% de crescimento).

4.12. Extração de DNA, amplificação e sequencimaneto de genes de resistência por PCR

O DNA genômico do parasito foi extraído utilizando o kit QIAmp (Quiagen, USA), no laboratório de Genética Molecular do Prof. Dr. Luiz Armando Cunha de Marco, Faculdade de Medicina. A dosagem do DNA foi feita em espectrofotômetro a 260/280nm. Foram utilizados 3µL de DNA genômico (30ng/µl), 0,5µl de cada iniciador na concentração de 10pmol/µl, 6,5µl de AmpliTaq Gold® PCR Master Mix (Applied Biosystems, Warrington, UK), em um volume final de 15µl. As condições para amplificação realizadas em um termociclador automático Eppendorf Mastercycler® (Hamburg, Germany) foram padronizadas de acordo com cada iniciador. Foram utilizados os seguintes iniciadores para amplificação dos genes: *pf*crt (gene com mutação associada com resistência à CQ), *pf*mrd1 e *pv*mrd1 (genes de *P. falciparum* e *P. vivax* respectivamente associados com a resistência às múltiplas drogas) (**Tabela 2**).

O produto da PCR, purificado utilizando o Kit Wizard® Genomic DNA, foi sequenciado utilizando o Kit DYEnamic™ ET (Amersham Biosciences, Little Chalfont, UK) em um sequenciador de DNA MegaBace (Amersham). As sequências foram analisadas com o software Sequencher 4.9 (Gene Codes Co, Ann Arbor, MI, USA) (VanBuskirk, *et al*., 2004). Para identificar os possíveis SNPs, as sequências foram comparadas com aquelas disponíveis no banco de dados Plasmo DB.

Tabela 2. Sequência de *primers* utilizados para a caracterização dos polimorfismos gênicos de *P. falciparum* e *P. vivax*.

Gene	**Codóns**	*Primer*	**T(°C)**	**Produto de PCR (pb)**
pfmdr1	86, 184	5'-GAGTTGAACAAAAAGAGTACCGCTGA-3´ 5'-TTTTTCCGTTAATTTATGTTTGTGGTGTCA-3´	55	512
	1043, 1042	5´-TGTCAAGCGGAGTTTTTGCATTTAGT-3´ 5´-TGGTAGTTATGCTGGAAAATTAATGTCCT-3´		299
	1246	5´-GGAGAAACAGGTAGTGGAAAATCAACTT-3' 5´-TTTGGAAGAGAAGATGCAACATTGGAA-3		302
pvmdr1	976	5´-ACTCACTTTATAGTGCTCTTCCTTGTG-3´ 5´- GGACATCAACTTCCCGGCGT- 3´	55	476
pfcrt	72, 76	5´- acagATGGCTCACGTTTAGG -3´ 5'- TTTTGTAACATCCGAAACTCACA -3'	55	162

4.13. Hemólise de eritrócitos humanos normais e deficientes da enzima glicose 6 fosfato (G6PD)

Para os ensaios de hemólise a PQ, diluída em PBS, e os compostos teste diluídos em RPMI com 0,2% (v/v) de DMSO (Sigma-Aldrich) foram utilizados em concentrações variando de 15-1000µg/mL, em placas de 96 poços (Corning). Após incubação (37°C por 2-24h) com 180µL de uma suspensão de eritrócitos (hematócrito 2%) e centrifugação (1000g

por 10min) a absorbância foi medida a 540nm no espectrofotômetro (SpectraMax340PC384, Molecular Devices). Saponina (Sigma-Aldrich) diluída a 0,05% em PBS foi utilizada como controle positivo de 100% de hemólise (Wang *et al*., 2010).

4.14. Isolamento de esporozoítos de *P. berghei*

Mosquitos *A. stephensi* foram obtidos da colônia do insetário da Escola de Saúde Pública da Universidade Jonhs Hopkins (Baltimore, MD). O repasto sanguíneo infectante foi feito em camundongos previamente infectados com *P. berghei* (cepa ANKA) e a produção de oocistos foi avaliada, 12 dias depois, no intestino médio dos mosquitos dissecados em microscópio estereoscópico (100x). As glândulas salivares foram dissecadas 18 a 21 dias pós-alimentação, em meio DMEM, suplementado com fungizona (5mg/mL) e penicilina/estreptomicina 2% (Gibco/Invitrogen, Carlsbad, CA, EUA), sendo rompidas utilizando triturador siliconizado, e os esporozoítos contados em hemocitômetro.

4.15. Atividade de derivados de PQ contra formas exo-eritrocíticas (FEE) do *P. berghei*

Para avaliar *in vitro* a atividade anti-esporozoítas dos PQTZs, 400µL de células da linhagem Hepa 1-6 diluídas em meio de cultura DMEM suplementado com 10% de SBF (Gibco/Invitrogen) na concentração de $4x10^5$/mL foram distribuídas em placas de cultura (6 poços) pré sensibilizadas com solução de colágeno tipo I diluída em água destilada (10µg/cm^2, BD Biosciences). Após incubação a 37ºC *overnight*, para adesão celular e formação de uma monocamada, as células foram incubadas com esporozoítos de *P. berghei*-ANKA ($2x10^4$/poço), como descrito por Sinnis e colaboradores (2013). Os compostos (PQTZs), testados em duplicatas e diferentes concentrações (50-10µg/mL), foram adicionados às células hepáticas 3h após. A placa foi mantida em cultura por 48h (37°C e 5% CO_2) com troca de meio e adição dos fármacos testes e controle (PQ) a cada 12 horas.

Após 48h, as células foram fixadas com formaldeído a 4% *overnight*, permeabilizadas com MeOH (4°C) durante 3h a -20^0C, bloqueadas com 1% de albumina de soro bovino (BSA, Sigma-Aldrich) em PBS 1x durante 1h, seguido pela adição do anticorpo monoclonal (mAb 3D11) contra a proteína CSP do *P. berghei* (http://www.mr4.org/; catalog number MRA-100). Após este tempo foram realizadas três lavagens com PBS-T. As células foram incubadas com o anticorpo secundário (anti-IgG de camundongo) acoplado a Alexa 594 ou 495 durante 1h e

lavadas três vezes com PBS-T. O corante de núcleo DAPI-Prolong (Molecular Probes®) foi adicionado e as células incubadas a 4°C *overnight*. As imagens foram capturadas em microscópio de fluorescência (Nikon 90i) utilizando o software Imagem Pro-Plus (Cybernetics Media), sendo então medido o tamanho das FEEs como descrito (Andrade-Neto *et al*., 2008). O número de células infectadas e o tamanho dos parasitos intracelulares foram comparados com os valores encontrados nos poços do controle não tratado.

4.16. Avaliação do crescimento das FEE no fígado de camundongos tratados

Os PQTZs foram administrados via oral (50mg/kg) em camundongos C57Black6 nos dias 1, 2 e 3 anteriores a infecção com esporozoítos de *P. berghei*-ANKA, via intravenosa. O tratamento com os compostos prosseguiu por dois dias pós-infecção, sendo utilizados seis camundongos por grupo. O fígado de cada camundongo, sacrificado por deslocamento cervical, foi retirado 42h após a infecção, macerado em solução de desnaturação, para posterior extração de RNA com o reagente Trizol. A transcrição reversa a partir de 4µg de RNA foi realizada para se obter cDNA, seguida da PCR para amplificação do gene 18S rRNA, específico de *P. berghei*. Essa amplificação foi visualizada pelo método de coloração com SYBR Green (Bruna-Romero *et al*., 2001) e a inibição do crescimento do parasito calculada a partir da razão entre o número de cópias obtidas no PCR em tempo real no grupo tratado e no grupo controle não tratado. O mesmo esquema de tratamento e infecção com esporozoítos foi realizado em outro grupo de animais, nos quais se avaliou o período pré-patente (PPP) da malária, através do exame de esfregaços de sangue coletados entre os dias 3 e 15 pós-inoculação. A completa proteção foi definida como a ausência de parasitos. Foram utilizados dois grupos controles, um não tratado e um tratado com PQ (10mg/Kg) na dose inibitória de 100% do desenvolvimento de FEE do *P. berghei* em camundongos infectados (Andrade-Neto *et al*., 2008; Singh *et al*., 2010).

5. RESULTADOS

Os resultados dos trabalhos estão apresentados na forma de artigos publicados (**anexos I e II**), submetidos à publicação (**anexo III**), ou como forma de dados ainda não publicados (item 5.4), resumidos abaixo.

5.1. Resumo dos trabalhos Publicados

Anexo I

A atividade antimalárica e mecanismo de ação de novos análogos derivados de CQ contra parasitos resistentes à CQ foram avaliadas. O análogo da CQ monoquinolíco (MAQ), foi sintetizado e comparado com o análogo biquinolínico (BAQ), previamente descrito por Zangh e colaboradores (2008). Os dois compostos foram estudados quanto à sua: (i) atividade *in vitro* contra *P. falciparum*; (ii) toxicidade em duas linhagens celulares, hepatoma humano (HepG2) e célula renal de macaco (BGM); (iii) atividade *in vivo* contra *P. berghei*; (iv) interação com a hematina dimérica (hemozoína sintética) *in vitro*, e (v) interação com a hematina dimérica e com a enzima lactato desidrogenase de *P. falciparum* (*Pf*LDH) no modelo de ancoragem molecular. Para os ensaios de atividade anti-*P. falciparum in vitro* foram utilizados os testes de incorporação de hipoxantina tritiada e com anticorpo monoclonal anti-HRPII, uma proteína rica em histidina e alanina presente no parasito. Os compostos testes e a CQ foram ativos com valores de IC_{50} na ordem de nanomolar (IC_{50} < 500nM). Esses valores foram concordantes nas técnicas de hipoxantina e anti-HRPII, sendo menores para a cepa 3D7-CQ sensível (IC_{50} entre 6,9 e 35nM) que para a cepa W2-CQ resistente (IC_{50} entre 120 e 550nM). MAQ foi mais ativo que BAQ e menos tóxico para as células HepG2 e BGM, resultando em elevados valores de IS (384 a 1038) em relação a BAQ (90). Testados por via oral em camundongos infectados nas doses de 50 e 25mg/kg, MAQ foi mais ativo que BAQ reduzindo entre 70 e 95% a parasitemia até o dia 10 pós-infecção. A CQ, utilizada como controle na dose 20mg/kg, reduziu 100% a parasitemia até o 30º dia de infecção. Na tentativa de elucidar mecanismos de atividade os análogos de CQ e a CQ foram testados *in vitro* para sua capacidade de inibir a formação de hemozoína. MAQ e BAQ inibiram significativamente a formação de hemozoína *in vitro*, apresentando perfil de dose resposta. MAQ foi mais ativo que a CQ e do que BAQ, inibindo a formação de hemozoína a partir da dose de 0,62mg/mL, enquanto CQ e BAQ causaram esta inibição a partir da dose de 2,5mg/mL. Este achado foi

confirmado por estudos de modelagem molecular, realizados no Instituto Militar de Engenharia (IME) em colaboração com o grupo do Dr. Tanos França. Os análogos de CQ e a CQ apresentaram alta pontuação de docagem, indicando uma forte energia de ligação com a hematina dimérica, corroborando com os dados de inibição da formação de hemozoína *in vitro*. A energia de interação também foi avaliada com a enzima *Pf*LDH, na qual a CQ se liga fracamente, mas de maneira estável, no sitio de ligação do NADH, o cofator da enzima. Os compostos exibiram valores de docagem estáveis, assim como a CQ, para o sítio de ligação do NADH, sendo este mais um alvo de ação proposto para MAQ e BAQ. Em conclusão, MAQ e BAQ são moléculas promissoras como antimaláricos e atuam em alvos cruciais para a sobrevivência do parasito, através da inibição da formação de hemozoína e da enzima *Pf*LDH.

Anexo II

A suscetibilidade *ex vivo* aos antimaláricos e a caracterização gênica de isolados de *P. vivax* e *P. falciparum*, obtidos de pacientes em Porto Velho, RO, foi investigada devido à alta morbidade do *P. vivax* (WHO, 2013). A diminuição da suscetibilidade *ex vivo* deste parasito a CQ em várias partes do mundo, incluindo o Brasil (Suwanarusk *et al*., 2007; Price *et al*., 2009; Sutanto *et al*., 2009); a resistência disseminada do *P. falciparum* aos antimaláricos e a suspensão do uso da CQ para o tratamento desta espécie desde a década de 70 (WHO, 2013). Esse estudo teve como objetivo determinar: (i) o perfil de resistência *ex vivo* de isolados de *P. vivax* e *P. falciparum* na área endêmica de Porto Velho, RO aos antimaláricos CQ, MQ e AS; (ii) a presença DE SNPs em diferentes códons dos genes *crt* e *mdr*1, relacionados com a resistência aos antimaláricos. Foram coletados 56 isolados de pacientes, em colaboração com o Dr. Dhelio Batista, no período de agosto de 2012 a março de 2013 no CEPEM. A monoinfecção foi confirmada pela técnica de PCR em tempo real, sendo excluído do estudo um paciente infectado com malária mista (*P. vivax* e *P. falciparum*). Foram analisados isolados de 47 pacientes infectados com *P. vivax* e de oito com *P. falciparum*, um paciente proveniente da África (Angola) e diagnosticado com *P. falciparum* em Belo Horizonte. Nos ensaios *ex vivo* foi possível observar a maturação de 100% (9/9) dos isolados de *P. falciparum* e de 68% (32/47) dos isolados de em cultivos *P. vivax* com duração de até 56 horas. A maioria dos isolados brasileiros de *P. falciparum* foi resistente a CQ (IC_{50} médio de 70nM, variado de 0,19 à 223nM); dois isolados foram mais suscetíveis com IC_{50} de 0,19 e 37nM; dois isolados foram resistentes a MQ (IC_{50} de 50 e 63nM) e todos os isolados foram suscetíveis ao AS (IC_{50} ~ 5,8). Os isolados de *P. vivax* apresentaram valores de IC_{50} médio de

32nM para a CQ, sugerindo a suscetibilidade dos parasitos a este fármaco; somente quatro isolados apresentaram valores de IC_{50} maiores do que 51nM para a CQ. Todos foram sensíveis a MQ e ao AS. O isolado de *P. falciparum* proveniente da África foi suscetível a todos os antimaláricos testados.

O códon 976 do gene *pv*mdr1 foi sequenciado nas 47 amostras para a pesquisa de SNPs. As sequências obtidas foram comparadas com a cepa de referência Salvador I, depositada no banco de dados do Plasmo DB. Os códons 86, 184, 1034, 1042 e 1246 do gene *pf*mdr1 e os códons 72 e 76 do gene *pf*crt foram sequenciados e comparados com a cepa 3D7. Não foram encontradas mutações nos isolados de *P. vivax*. Nas posições 184, 1042 e 1246 do gene *pf*mdr1, 100% dos isolados apresentaram mutação de troca do tipo não sinônimas, na posição 1034 essa frequência foi de 84%. Não foram encontradas mutações na posição 86. Todos os isolados brasileiros apresentaram mutações não sinônimas nos códons 72 e 76 do gene *pf*crt. Nenhuma mutação foi encontrada no isolado africano nos genes *pf*mdr1 e *pf*crt. A alta suscetibilidade dos isolados brasileiros de *P. vivax* aos antimaláricos CQ, MQ e AS e a baixa suscetibilidade dos isolados de *P. falciparum* a CQ corroboraram com as mutações encontradas e ajudam na caracterização do perfil dos isolados circulantes. Este monitoramento é importante, uma vez que movimentos de migração populacional favorecem o surgimento de parasitos resistentes.

5.2. Resumo dos trabalhos não publicados

Anexo III

O *P. vivax* é a espécie mais prevalente no mundo, responsável por elevada morbidade e pelas recaídas tardias ocasionadas pelas formas de esporozoítos hepáticos latentes denominados hipnozoítos (WHO, 2013). A PQ é o único fármaco disponível para o tratamento das recaídas pelo *P. vivax*, utilizada em dose única para o bloqueio da transmissão de *P. falciparum* (Baird *et al*., 2004; WHO, 2013). Este medicamento é pouco tolerado causando efeitos colaterais e hemólise em pacientes deficientes da enzima G6PD (Brewer *et al*., 1962; Carmona-Fonseca *et al*., 2009). Com o objetivo de selecionar derivados de PQ, capazes de bloquear a transmissão da malária, 15 PQTZs (4b-4p) foram avaliados quanto à: (i) hemólise causada em hemácias humanas normais e deficientes da enzima G6PD; (ii) citotoxicidade nas linhagens de células hepáticas humana e murina (HepG2 e Hepa1-6), e de célula renal de macaco (BGM); (iii) toxicidade aguda em camundongos; (iv) bloqueio da

transmissão da malária nos modelos aviário (*P. gallinaceum* e *A. fluviatilis*) e de roedores (*A. stephensi* e *P. berghei*), avaliado pelo número de mosquitos infectados e redução do número de oocistos no intestino médio dos mosquitos infectados cujo o repasto sanguíneo foi feito antes e após tratamento dos hospedeiros vertebrados com os compostos e (v) inibição de formas teciduais exoeritrocíticas, *in vitro* e *in vivo*, após a inoculação, por via intravenosa, de esporozoítos de *P. berghei*. A PQ foi utilizada como controle de todos os ensaios. Alguns PQTZs (4b, 4j, 4k, 4l, 4m, 4o e 4p) foram 20 a 34 vezes menos tóxicos que a PQ; 4f e 4i foram tóxicos, como a PQ, nas linhagens celulares avaliadas. Testados em hemácias humanas normais e deficientes da enzima G6PD os PQTZs não causaram hemólise *in vitro*, ao contrário da PQ, tóxica mesmo para hemácias normais (1000μg/mL), e em doses mais baixas nas hemácias deficientes em G6PD (250μg/mL). O soro de camundongos tratado com PQ causou hemólise nas hemácias deficientes em G6PD, mas os soros de animais tratados com os PQTZs não foram hemolíticos. Os compostos 4b, 4c, 4g, 4h, 4m, 4o e 4p foram administrados diariamente até atingir a dose cumulativa de 1000mg/Kg para avaliação da toxidez aguda; os animais sobreviveram até o dia 30 após a sua administração, na dose de 200mg/kg, enquanto os animais tratados com PQ morreram 20h após sua a administração em dose única (200mg/kg). A atividade bloqueadora da transmissão da malária foi avaliada para os PQTZs, em paralelo com a PQ, fármaco utilizado por causar 100% desse bloqueio. Os compostos 4c, 4g, 4m e 4o reduziram o número de mosquitos infectados e o número de oocistos nos mosquitos. Este dado foi confirmado no modelo murino, no qual estes compostos foram mais ativos, bloqueando 100% a infecção. Possíveis diferenças no metabolismo dos fármacos nas aves ou nos mamíferos podem explicar estas diferenças. Os outros compostos foram inativos nos dois modelos estudados, validando assim o uso da malária de aves e do *A. fluviatilis* na busca de substitutos da PQ. O composto 4m, além de apresentar atividade como esquizonticida sanguíneo, inibiu 100% as FEE *in vitro* e 95% *in vivo* na dose de 50mg/Kg. Neste caso reduziu em três dias o PPP da malária. O composto 4m foi testado em paralelo com a PQ a qual inibe a infecção na dose de 15mg/kg e reduz 100% as FEE *in vitro*. Em conclusão, os PQTZs foram menos tóxicos que a PQ e mais ativos como bloqueadores da transmissão da malária murina (*P. berghei*) quando comparados dados de inibição na malária aviária (*P. gallinaceum*). Os compostos 4b, 4g, 4m e 4o se mostraram potentes bloqueadores da transmissão. Apenas o composto 4m reduziu as FEE *in vitro*, *in vivo* e reduziu o PPP da doença, sendo considerado por isso o composto mais promissor e um antimalárico útil no controle da malária.

5.3. Resumo de dados não submetidos

Atividade *ex vivo* **de análogos de CQ (MAQ e BAQ) contra isolados de pacientes infectados com** *P. vivax* **e** *P. falciparum***.**

A atividade *ex vivo* de MAQ e BAQ foi avaliada contra 47 isolados de *P. vivax* e em nove de *P. falciparum* com mono-infecção confirmada pela reação de PCR em tempo real. Apenas um isolado foi excluído do estudo, por apresentar infecção mista por *P. vivax* e *P. falciparum*; a infecção por *P. malariae* não foi observada. A técnica de PCR em tempo real foi 99% concordante com a microscopia. A maturação nos cultivos *ex vivo* dos parasitos ocorreu em 100% dos isolados de *P. falciparum* e (9/9) e em 68% de *P. vivax* (32/47). Os dados individuais de IC_{50} nos isolados de *P. falciparum* estão na **Tabela 3**. Em um isolado de *P. falciparum*, o perfil dose resposta foi inadequado, impossibilitando definir o valor de IC_{50}. MAQ e BAQ apresentaram valores mais baixos de IC_{50} nos isolados de *P. falciparum* (IC_{50} de 116 e 87nM, respectivamente) que nos isolados de *P. vivax* (IC_{50} de 166 e 169nM, respectivamente). Além disso, a atividade de MAQ foi maior contra os isolados de *P. falciparum* em relação ao clone W2-CQ resistente (IC_{50} de 250nM) (**Tabela 3**). A análise estatística de resistência cruzada entre a CQ e os novos derivados quinolínicos (MAQ e BAQ) foi avaliada pela correlação *Spearman* (GraphPad Prism program). Houve correlação positiva dos IC_{50} nos isolados resistentes de *P. falciparum* para BAQ (r=0,890, p=0,012). Mas não para MAQ (r=0,500, p=0,890) (**Figura 3**). O dado mostra atividade significativa de MAQ em relação à CQ. Cepas de parasitos resistentes a MAQ e BAQ devem ser selecionadas *in vitro* a fim de avaliar se os análogos de CQ atuam por diferentes mecanismos de resistência. Além disso, é necessário testar a atividade de MAQ e BAQ em número maior de isolados, sobretudo no caso do *P. falciparum*.

Tabela 2. Valores de IC_{50} individuais nos isolados de *P. falciparum* de MAQ e BAQ e dos antimaláricos padrão (CQ, MQ e AS).

Amostras	*P. falciparum* IC_{50} (nM)									Média
	W2	P001	P008	P016	P018	P035	P043	P046	P048	
MAQ	250	24,0	12,0	7,5	2,6	3,0	188	223	468,0	116,0
BAQ	60	85,0	75,0	13,0	23,0	185,0	60,0	117,0	138,0	87
Cloroquina	178	42,0	81,0	16,0	0,19	106,0	19,0	Ind*	223,0	70,0
Artesunato	18	5,8	13,0	2,8	0,26	1,82	1,56	1,81	1,4	1,3
Mefloquina	19	2,56	4,00	1,19	23,0	63,0	50,0	5,2	18,0	21,0

*Não foi possível obter perfil dose resposta para determinar o valor de IC_{50}.

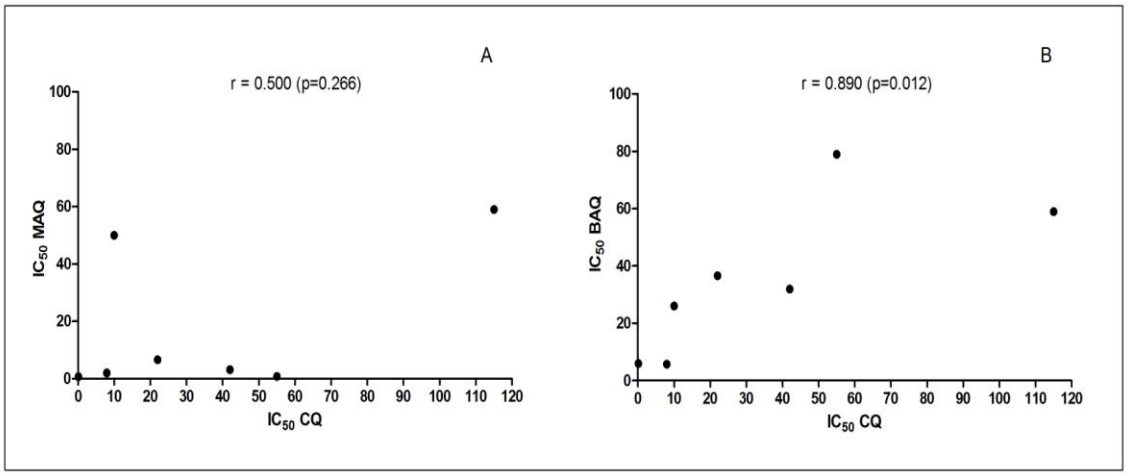


Figura 3. Correlação entre resistência cruzada de isolados humanos de *P. falciparum* aos derivados quinolínicos (MAQ, BAQ) com a CQ.

6. CONCLUSÃO

Neste estudo foi possível caracterizar alta suscetibilidade dos isolados brasileiros de *P. vivax* aos antimaláricos testados (CQ, MQ e AS) e baixa suscetibilidade dos isolados de *P. falciparum* à CQ. Dois novos potentes esquizonticidas sanguíneos análogos de cloroquina, MAQ e BAQ com um e dois anéis quinolínicos, respectivamente, foram também ativos contra esses isolados *ex vivo*. MAQ não apresentou resistência cruzada com a CQ. No entanto, o número de isolados testados foi pequeno, devido à baixa prevalência de *P. falciparum* no Brasil. Entre os possíveis substitutos da PQ, canditados a esquizonticidas teciduais, o composto 4m foi capaz de reduzir parcialmente o número de FEE de P. berghei *in vitro* e *in vivo*; não bloqueou a infecção sanguínea. No entanto 4m atrasou o período pré-patente da malária, diminuiu a parasitemia e a mortalidade dos animais tratados. Além disso, a PQ foi mais ativa que os PQTZs; novos compostos devem ser sintetizados a fim de melhorar sua atividade. Os PQTZs precisam também ser avaliados contra as formas hipnozoítos, exigindo para isso esporozoítos de *P. cynomolgi* e/ou de *P. vivax* para os testes *in vitro*. Sendo ativos, os novos compostos podem ser utilizados em ensaios clínicos no tratamento das recaídas tardias da malária humana *P. vivax*, frequentes nas áreas endêmicas tornando difícil seu controle atual.

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ANEXO I

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Antimalarial Activity and Mechanisms of Action of Two Novel 4-Aminoquinolines against Chloroquine-Resistant Parasites

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Abstract

Chloroquine (CQ) is a cost effective antimalarial drug with a relatively good safety profile (or therapeutic index). However, CQ is no longer used alone to treat patients with *Plasmodium falciparum* due to the emergence and spread of CQ-resistant strains, also reported for *P. vivax*. Despite CQ resistance, novel drug candidates based on the structure of CQ continue to be considered, as in the present work. One CQ analog was synthesized as monoquinoline (MAQ) and compared with a previously synthesized bisquinoline (BAQ), both tested against *P. falciparum in vitro* and against *P. berghei* in mice, then evaluated *in vitro* for their cytotoxicity and ability to inhibit hemozoin formation. Their interactions with residues present in the NADH binding site of *P falciparum* lactate dehydrogenase were evaluated using docking analysis software. Both compounds were active in the nanomolar range evaluated through the HRPII and hypoxanthine tests. MAQ and BAQ derivatives were not toxic, and both compounds significantly inhibited hemozoin formation, in a dose-dependent manner. MAQ had a higher selectivity index than BAQ and both compounds were weak *Pf*LDH inhibitors, a result previously reported also for CQ. Taken together, the two CQ analogues represent promising molecules which seem to act in a crucial point for the parasite, inhibiting hemozoin formation.

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Introduction

Malaria is one of the most prevalent parasitic diseases in the world. It affects approximately 500 million individuals throughout the tropical and subtropical areas of developing countries and causes considerable morbidity and mortality with about 800,000 deaths worldwide each year [1]. *P. falciparum* infection in non-immune adults and children is often associated with severe cerebral malaria. The global importance of this disease, current limitations of vector control and the absence of an effective vaccine, makes the development of therapeutic antimalarial drugs the main strategy of malaria control [2].

CQ is a cost effective antimalarial drug with a relatively good safety profile [3]. However, CQ is no longer used alone due to the emergence and spread of *P. falciparum* CQ-resistant strains and, more recently, of *P. vivax* [4–6]. Artemisinin-based combination therapy (ACT) is the first line treatment in *P. falciparum* malaria (WHO, 2009). However, the limited availability of ACT and the decreased susceptibility of *P. falciparum* to artemisinin derivatives [7–8] have required the development of novel antimalarial drugs [9–11]. Previous studies have described the discovery of novel antimalarial drugs through analysis of medicinal plants [12] and through novel drug synthesis protocols [13–14], however, no new active compound has been shown to be as effective as CQ.

Despite the resistance of *P. falciparum* to CQ, novel drug candidates based on the structure of CQ continue to be considered [15–18]. In the present work, CQ analogs were synthesized as mono- and bisquinoline-based derivatives, referred as MAQ and BAQ, respectively. The main structural aspects considered included the maintenance of the 4-aminoquinoline pharmacophore group and the presence of proton-accepting sites to increase drug bioavailability in the digestive vacuole of the parasite. The compounds were tested: (i) as blood schizonticides against *P. falciparum in vitro* and against *P. berghei* malaria in mice; (ii) for their *in vitro* cytotoxicity; (iii) for their ability to inhibit hemozoin formation; and (iv) for their binding mode to lactate dehydrogenase and dimeric hematin *in silico*.

Results

Synthesis and characterization of CQ analogs

The structure of the two aminoquinoline derivatives, MAQ and BAQ, and their synthesis strategy are depicted in Scheme S1. Both CQ analogs possess the conserved 4-aminoquinoline moiety (a crucial pharmacophore group), and have a chlorine substituent at the 7 position on the quinoline ring, and, both have amino groups on the alkyl chain [19]. MAQ, a new monoquinoline molecule, and BAQ, a previously synthesized bisquinoline already tested against *P. falciparum* and proven to be active [20], were prepared from 4,7-dichloroquinoline and diethylenetriamine. These reactions occurred via a S_NAr synthesis step, which eliminated the use of solvents [21]. MAQ and BAQ were obtained by controlling the stoichiometric relationship between these reagents. The synthesis protocol for the bisquinoline compound BAQ was described previously [20].

BAQ and MAQ were isolated as white solids, which underwent a satisfactory elemental analysis and were fully characterized by NMR and IR spectroscopy. On the ^{1}H NMR spectrum, MAQ showed five of the expected signals of the aromatic region (between 8.32 and 6.50 ppm) and four signals related to the methylenic groups (between 3.47 and 2.78 ppm). The hydrogen signals of the amino groups were either not present or had an integration level lower than the predicted value due to the fast H/D exchange with the deuterated solvent. In the ^{13}C NMR spectrum, it was possible to identify nine signals related to the aromatic carbons (between 152 and 97 ppm) and the four signals associated with the methylenic carbons (50 to 40 ppm). In the infrared spectrum of MAQ, the typical absorption bands for this kind of chemical structure were observed and the ^{1}H NMR spectrum was consistent with the five typical aromatic signals (between 8.25 and 6.46 ppm). However, only two signals related to the methylenic groups were observed due to the symmetry of the molecule (3.45 and 2.95 ppm). In the ^{13}C NMR spectrum of BAQ, the expected nine signals related to the aromatic carbons (between 152 and 97 ppm) and the two possible signals of the methylenic carbons (46.8 and 41.8 ppm) were observed. The infrared spectrum of BAQ exhibited the absorption bands predicted for this structure.

BAQ and MAQ activities against *P. falciparum in vitro*

The 4-aminoquinoline derivatives MAQ and BAQ were compared with CQ for their antiplasmodial activities against the W2 clone of *P. falciparum* (CQ-resistant and mefloquine-sensitive) and against a CQ-sensitive strain 3D7. All compounds showed activity in the nanomolar range in the HRPII and hypoxanthine tests (Table 1). The IC_{50} values were similar in both assays, although somewhat lower for BAQ in the hypoxanthine test with W2 parasites. As expected, the IC_{50} values of BAQ and MAQ were lower with the CQ- sensitive 3D7 strain than with W2 CQ-resistant in most tests.

Neither MAQ or BAQ were toxic against hepatoma (HepG2) or kidney (BGM) cell lines, and important, the new CQ analog MAQ was less toxic than BAQ, based on MTT assay (Table 2) or neutral red method (data not shown). Additionally, the CQ analogs were not lytic to human erythrocytes. Even at doses 100 times higher than the IC_{50} values no hemolysis was detected.

The selectivity index- IS (the ratio between MDL_{50} and IC_{50}) was determined and both compounds had an IS greater than 90. The IS was particularly high for MAQ, 384 and 1038, against BGM and HepG2 cells respectively (Table 2). Nevertheless, neither compound was better then CQ (IS>3083)

BAQ and MAQ were also active *in vivo* against rodent malaria

The antimalarial activity of the new CQ analogs was evaluated in mice with *P. berghei* and is summarized in Table 3. MAQ and BAQ were active *in vivo* at a dose of 50 mg/kg on days 5, 8 and 10 days post-infection, at 25 mg/kg only MAQ was active. MAQ caused a 95% reduction of parasitemia on day 5. However, only CQ caused 100% suppression of parasitemia and increased the animal survival, but the survival differences were not significant from the other groups.

The antimalarial activity of BAQ and MAQ involves inhibition of hemozoin formation

Whether anti *P. falciparum* activity of BAQ and MAQ involves inhibition of hemozoin formation was evaluated using an indirect *in vitro* test. Both compounds significantly inhibited hemozoin formation *in vitro* (Figure 1) in a dose-dependent reaction. The inhibition was more evident with MAQ than with BAQ or CQ (Figure 1A).

Similarly, docking studies with MAQ and BAQ showed that the MolDock Score energies between the antimalarials and the dimeric hematin (Table 4) were approximately –100.000 kcal mol^{-1}. Both aminoquinoline derivatives, similar to CQ, were able to interact with dimeric hematin to form a complex. The best conformations (Figure 2) obtained for the complexes showed that the aromatic rings of both compounds were parallel to the hematin ferriprotoporphyrin group (Figure 3). Weak hydrogen bonds (approximately −5.0 kcal mol^{-1}) corroborate previous studies demonstrating that the hydrophobic interactions were the main contributors for the binding between CQ and the dimer [22–23].

Table 1. The anti-*P. falciparum* activities of BAQ and MAQ determined in parallel with chloroquine, by the ELISA anti-HRPII assay or by ^{3}H hypoxanthine incorporation.

Compounds	W2 (CQ resistant)		3D7 (CQ sensitive)	
IC_{50} (nM)	HRPII	Hypoxanthine	HRPII	Hypoxanthine
BAQ	550±0.0	60±20	35±8	105±7
MAQ	260±20	320±70	26±2	66±5
CQ	120±0.0	180±0.0	6.9±0.9	7.4±1.2

All compounds were active at nanomolar doses in both tests.
IC_{50} = dose that inhibits 50% of blood parasites growth, evaluated in three or four different experiments for each test.
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Table 2. Cytotoxicity of BAQ and MAQ against a human hepatoma cell line (HEPG2) and a monkey kidney cell line (BGM) determined by the MTT assay.

Compounds	MDL_{50} (μM)		Selectivity index*	
	BGM	HepG2	BGM	HepG2
BAQ	50±30	50±0.0	90	90
MAQ	100±0.0	270±10	384	1038
CQ	370±0.0	490±0.0	3083	4083

Data expressed as the minimal lethal dose for 50% of cells (MDL50), used to calculate the selectivity index against either cell.
*Selectivity index = MDL_{50}/IC_{50}.
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Table 3. Antimalarial activity of BAQ and MAQ in mice infected with *P. berghei* after treatment with daily doses of the compounds during three consecutive days.

Compounds	Dose (mg/kg)	Percent reduction of parasitemia*			Time of survival (in days)
		5°	8°	10°	
BAQ	25	64	26	37	25±2
	50	81	75	43	24±1
MAQ	25	91	61	70	24±1
	50	95	71	76	25±1
CQ	20	100	100	100	>30
Control	0	0	0	0	19±7

*Reductions ≤30% were considered as inactive, 30–50% as partially active and ≥50% as active drugs.

The antimalarial action of BAQ and MAQ involves binding to lactate dehydrogenase

Table 5 presents the H-bond energy and MolDock Scores of the residues involved in H-bonds with NADH as well as the protonated forms of chloroquine, BAQ and MAQ docked to PfLDH. Figure 4 shows that these compounds presented the most stable energy conformations in the binding site of NADH. This suggests that they are NADH competitors. The H-bond energy between the protonated chloroquine and the enzyme was -3.8 kcal mol^{-1}, a high value compared with that of NADH H-bond energy (-35.3 kcal mol^{-1}). The energy of interaction between the enzyme and the protonated forms of MAQ were approximately -250.00 kcal mol^{-1}. Furthermore, MAQ like the protonated chloroquine, bound to the active site of PfLDH; BAQ-1 and BAQ-2 presented the lowest energies, which were extremely large when compared to NADH. These findings suggest that all these compounds are weak inhibitors of PfLDH, as previously reported for chloroquine [24].

Discussion

A large series of bisquinoline derivatives containing proton-accepting amino groups on the aliphatic chain moiety has already been described [19] and considered a promising class of compounds for treating drug-resistant malaria parasites. Two CQ analogs MAQ and BAQ were obtained as low-cost antimalarial prototypes, which is important considering that disease transmission is concentrated in developing countries and is most frequent among the impoverished classes of several endemic countries [9]. Comparisons between their antimalarial activities aimed to evaluate the molecular structural-activity relationship between mono- and bisquinoline compounds.

We showed that MAQ and BAQ are active *in vitro* against CQ-resistant and CQ-sensitive *P. falciparum* parasites at nanomolar doses in the hypoxanthine incorporation test and in an enzyme-linked immunosorbent assay (ELISA), using antibodies against the histidine-rich protein 2 (HRP-II), a parasite protein.Our results show that MAQ, a new monoquinoline, has a higher IS, in consonance with the tendency of a decreased toxicity when comparing mono- to bisquinoline analogs. Such *in vitro* activity was confirmed *in vivo* in mice infected with *P. berghei*; MAQ was more effective as proven by parasitemia reduction in response to drug treatment.

Further *in vitro* analysis showed that the compounds interacted with dimeric hematin and inhibited heme polymerization. Although the mechanism of action of CQ is not well understood, several experimental studies describe the interaction between CQ and the dimeric form of heme [25,26,27,28]. This interaction seems responsible for the inhibition of hemozoin formation, a hypothesis further supported by docking studies, which also suggest that all protonated forms of MAQ and BAQ interact with the rings parallel to the heme group, as described previously for CQ [26,27,28,29].

The presence of two quinoline moieties in BAQ increases the probability of hydrophobic interactions when compared with MAQ and also results in lower docking energies for all protonation states of the amino groups. The results of this study suggest that

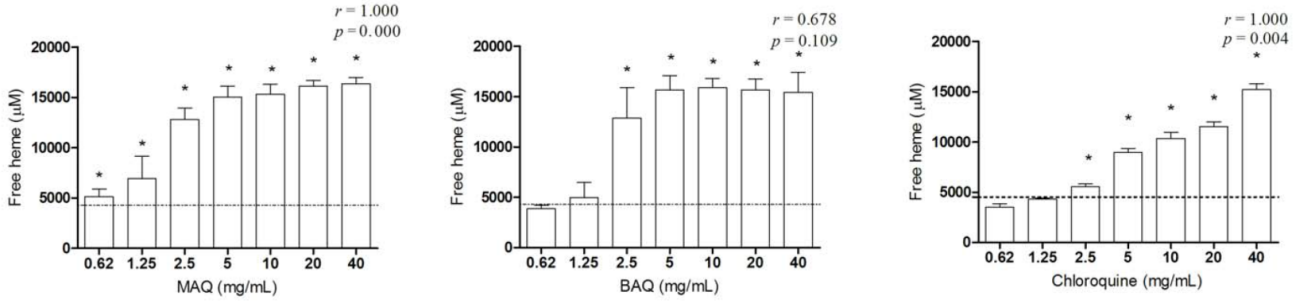

Figure 1. Inhibition of hemozoin formation by the CQ analogs BAQ and MAQ (mean ± SD from triplicates), in two different experiments. Statistical differences as compared to drug-free controls are indicated in each graph by an asterisk ($p<0.05$). The p and r values represent the statistical correlation analyses.
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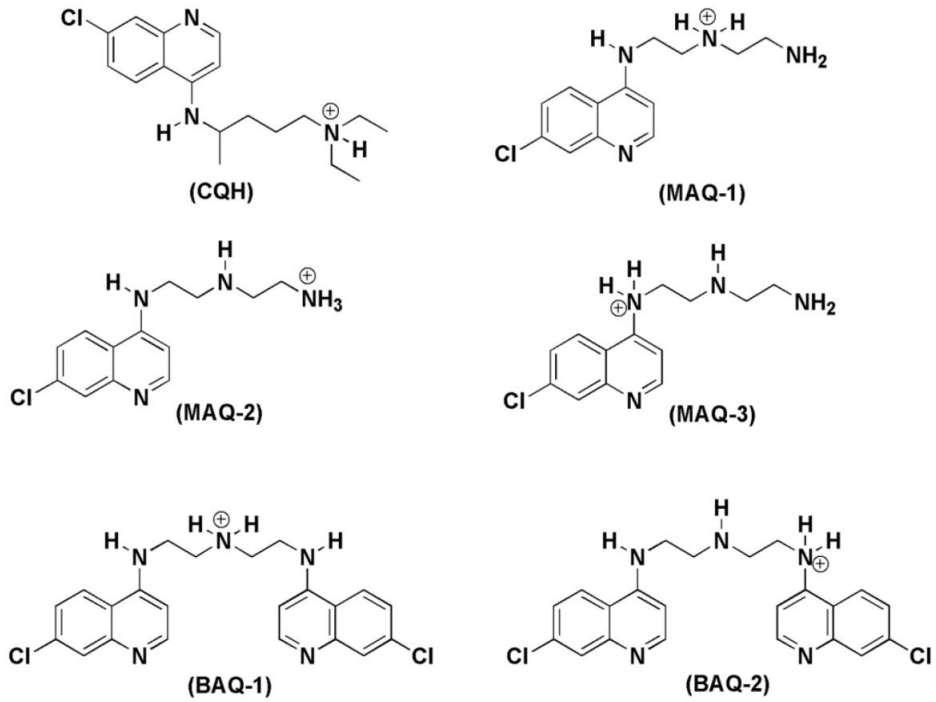

Figure 2. Chemical structures of the protonated forms of the CQ analogs BAQ and MAQ.
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the protonated BAQ at the central nitrogen (BAQ-1) should be more effective at inhibiting heme polymerization.

Experimental and modeling studies have shown that chloroquine binds to PfLDH, suggesting that CQ possesses another mechanism of drug action or a mechanism complementary to the one already proposed [24,29]. Although PfLDH is not a direct chloroquine target, experimental data have shown its weak binding to chloroquine [30]. The existence of this shared target-binding site suggests that PfLDH is related to drug effectiveness. The docking data (Figure 4) suggest that the protonated forms of BAQ and MAQ present the most stable energetic conformations at the binding site of NADH inside PfLDH. The H-bond energy and MolDock Scores, the residues involved in the H-bonds with NADH and the protonated forms of chloroquine as well as BAQ and MAQ binding to PfLDH (Table 5) suggest that these compounds are NADH competitors. The H-bond energy between protonated chloroquine and the enzyme is -3.8 kcal mol^{-1}, a high value compared with the H-bond energy of NADH (-35.3 kcal mol^{-1}). The energies of interaction between the enzyme and the protonated forms of MAQ were approximately -250.0 kcal mol^{-1}. Furthermore, MAQ compounds showed a similar behavior at the active site of PfLDH compared with protonated chloroquine. BAQ-1 and BAQ-2 possessed the lowest energies, which were extremely large compared with NADH. Overall, these findings suggest that all of these compounds are weak inhibitors of *Pf*LDH, as previously reported for chloroquine [22–23].

One of the recent strategies to develop effective antimalarial agents is based on the development of metal-drugs [31,32,33]. Thus, MAQ and BAQ were associated with metals readily available to be tested for their antimalarial activity, in promising ongoing tests (data not shown). Metallocomplexed drugs, considered first-line anticancer drugs [34,35], are increasingly used to develop anti-parasitic agents [36,37,38].

Materials and Methods

Reagents and drug synthesis

All reactions for drug synthesis were performed under a 100% argon atmosphere using a dual vacuum/argon line and standard Schlenk techniques. Reagents and solvents were purchased from Sigma Aldrich and used without further purification. The IR spectra were recorded on a Varian 640-IR with an ATR device and the elementary analyses were conducted at the analytical center, University of São Paulo. The ^{1}H NMR spectra were recorded at 400.130 MHz and the ^{13}C NMR spectra at 100.613 MHz on a Bruker instrument (Bruker Avance 400) and were externally referenced to the tetramethylsilane (TMS). Chemical shifts (δ) and coupling constants (J) were expressed in ppm and Hz, respectively. The melting or decomposition points of the structures were determined on a MQAPF 302 apparatus.

Synthesis of the 4-aminoquinolines, MAQ and BAQ

MAQ, *N^1-(2-aminoethyl)-N^2-(7-chloroquinol-4-yl)ethane-1,2-diamine*, was prepared according to the synthesis procedures described by Musonda and collaborators [39]. Briefly, a mixture of 4,7-dichloroquinoline (0.98 g, 4.9 mmol) and diethylenetriamine (2.7 mL, 25 mmol) was heated at 80°C for 1 h without stirring, following by heating at 135°C for 3 h with stirring. The mixture was cooled to room temperature, alkalized with an aqueous solution of 10% NaOH (30 mL), extracted with hot EtOAc (3×50 mL) and dried over Na_2SO_4. The solvent was removed under vacuum and the residue was crystallized with CH_2Cl_2/hexane, thereby leading to a white solid. Chemical and spectral analyses revealed the following: Yield: 0.52 g, 40%. M.F: $C_{13}H_{17}ClN_4$; M.M.: 264.75 g/mol. Elem. Anal., theoretical: $C_{13}H_{17}ClN_4$, C, 58.98; H, 6.47; N, 21.16, experimental: $C_{13}H_{17}ClN_4.H_2O$, C, 55.23; H, 6.54; N, 19.22. M.P.: 99–100°C; I.R. ($\nu_{máx}$ cm^{-1}): 3256 (Ar- NH); 3065 (CH quinoline

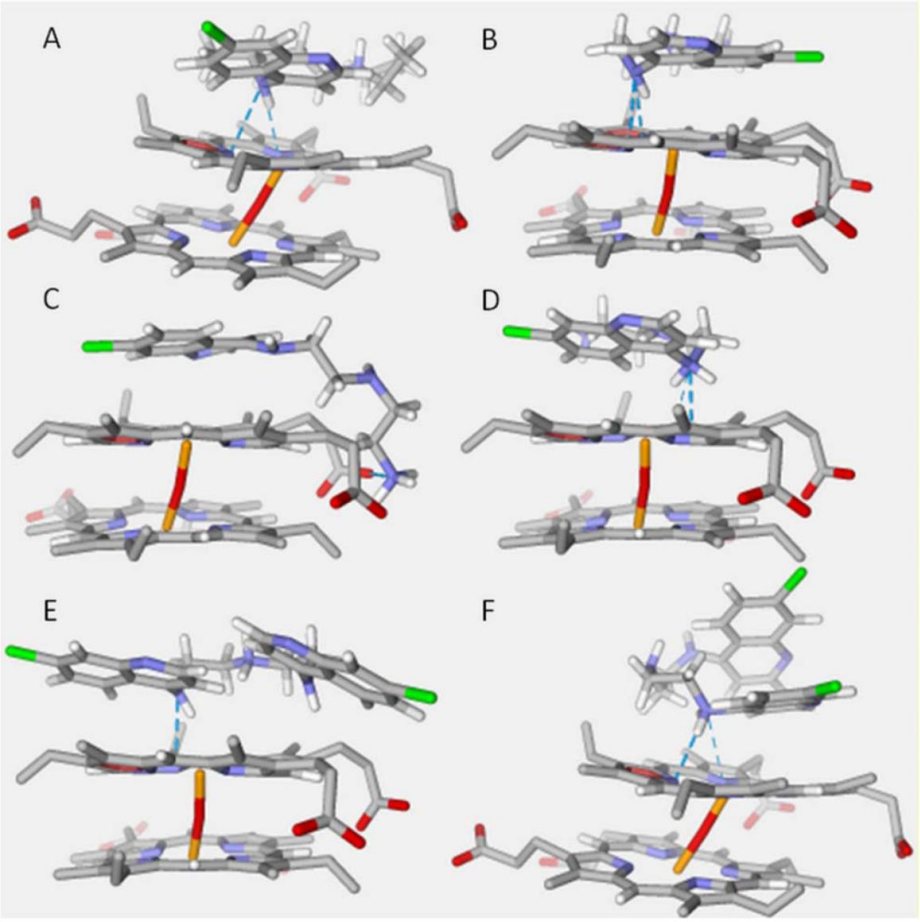

Figure 3. Compounds docked in dimeric hematin. (A) Protonated CQ, (B) MAQ-1, (C) MAQ-2, (D) MAQ-3, (E) BAQ-1, (F) BAQ-2.
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ring); 2929 and 2839 ($CH_{as/s}$ and CH_2); 1607, 1577 and 1528 (C = C quinoline ring); 1449 (CH_2); 1280 (Ar-C-N); 1133 (C-N); 874, 795 and 761 (δ = C-H out-of-plane aromatic ring). RMN ^{1}H (400 MHz, CD_3OD): δ 1.93 (s, NH amine), 2.78 (t, $\mathcal{J}$ = 5.76 Hz, 2H, CH_2-16), 2.85 (t, $\mathcal{J}$ = 5.76 Hz, 2H, CH_2-15), 2.95 (t, $\mathcal{J}$ = 6.38 Hz, 2H, CH_2-13), 3.47 (t, $\mathcal{J}$ = 6.38 Hz, 2H, CH_2-12), 3.70 (ls, NH aniline), 6.50 (d, $\mathcal{J}$ = 5.71 Hz, 1H, Ar-*H* C-3), 7.35 (dd, $\mathcal{J}$ = 9.01 Hz, $\mathcal{J}$ = 2.14 Hz, 1H, Ar-*H* C-6), 7.73 (d, $\mathcal{J}$ = 2.14 Hz, 1H, Ar-*H* C-8), 8.07 (d, $\mathcal{J}$ = 9.01 Hz, 1H, Ar-*H* C-5), 8.32 (d, $\mathcal{J}$ = 5.62 Hz, 1H, Ar-*H* C-2). RMN ^{13}C (100 MHz, CD_3OD): δ 151.1 (C-2), 98.28 (C-3), 151.4 (C-4), 124.6 (C-5), 123.08 (C-6), 134.9 (C-7), 126.2 (C-8), 148.2 (C-9), 117.32 (C-10), 50.03 (C-12), 42.08 (C-13), 39.9 (C-15), 38.46 (C-16).

Table 4. Docking energies of the protonated forms of the CQ analogs BAQ and MAQ.

Structure	H Bond Energy (kcal mol^{-1})	MolDock Score (kcal mol^{-1})
Protonated CQ	−3.43	−105.97
MAQ-1	−1.95	−100.57
MAQ-2	−5.00	−92.16
MAQ-3	−3.50	−76.50
BAQ-1	−2.50	−119.69
BAQ-2	−2.50	−116.89

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BAQ, *N^1-(7-chloroquinol-4-yl)-N^2-{2-[(7-chloroquinol-4-il)amino]ethyl}ethane-1,2-diamine*, was synthesized according to the synthesis procedures described by Zhang and collaborators [40]. Briefly, a mixture of 4,7-dichloroquinoline (1.20 g, 6.00 mmol) and diethylenetriamine (0.22 mL, 2.00 mmol) was heated at 80°C for 6 h without stirring and then at 130°C for 3 h with stirring. The reaction mixture was cooled to room temperature and the resulting solid that formed was washed with an aqueous solution of 10% NaOH (30 mL) and recrystallized with MeOH/water. The solid was recuperated by filtration and dried under vacuum, which lead to a white solid. Chemical and spectral analyses revealed the following: Yield: 0.43 g, 50%. M.F: $C_{22}H_{21}Cl_2N_5$; M.M.: 426.34 g/mol. Elem. Anal., theoretical: $C_{22}H_{21}Cl_2N_5$, C, 61.98; H, 4.96; N, 16.43; experimental: $C_{22}H_{21}Cl_2N_5.4H_2O$, C, 53.03; H, 4.91; N, 14.07. M.P.: 212–215°C; IR. (ν_{max} in cm^{-1}): 3272 (Ar- NH); 3071 (CH quinoline ring); 2901 and 2843 ($CH_{2\ as/s}$); 1608, 1576 and 1531 (C = C quinoline ring); 1441 (CH_2); 1328 (Ar-CN); 1136 (C-N); 865 and 798 (δ out-of-plane aromatic ring). ^{1}H RMN (400 MHz, CD_3OD): δ 2.97 (t, J = 6.22 Hz, 2H, CH_2-13), 3.45 (t, $\mathcal{J}$ = 6.24 Hz, 2H, CH_2-12), 6.45 (d, $\mathcal{J}$ = 5.69 Hz, 1H, Ar-*H* C-3), 7.20 (dd, $\mathcal{J}$ = 9.01 Hz, $\mathcal{J}$ = 2.14 Hz, 1H, Ar-*H* C-6),

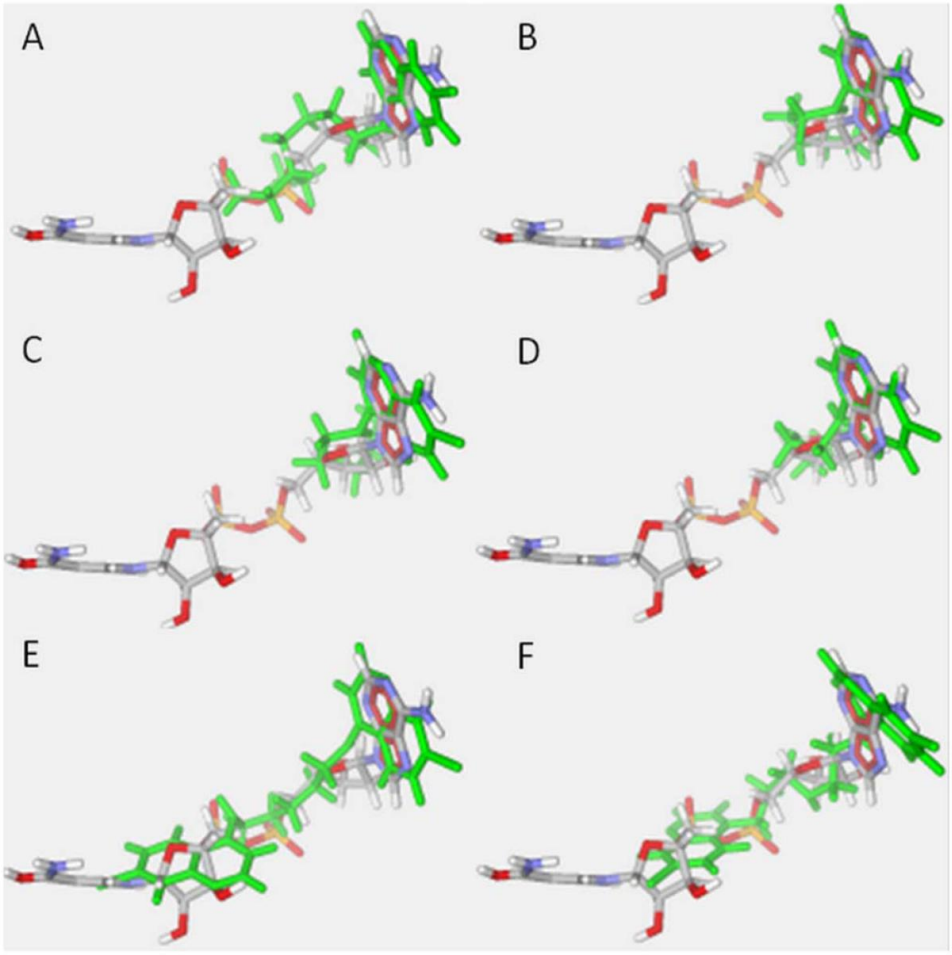

Figure 4. Best conformations of the protonated forms of chloroquine, MAQ and BAQ (in green) in the binding pocket of NADH (in CPK) as generated by the MVD® software. (A) Protonated chloroquine, (B) MAQ-1, (C) MAQ-2, (D) MAQ-3, (E) BAQ-1, (F) BAQ-2.
doi:10.1371/journal.pone.0037259.g004

7.67 (d, J = 2.12 Hz, 1H, Ar-*H* C-8), 7.87 (d, J = 8.97 Hz, Ar-*H* C-5), 8.24 (d, J = 5.61 Hz, 1H, Ar-*H* C-2). RMN ^{13}C (100 MHz, MeOD): δ 150.9 (C-2), 98.3 (C-3), 151.2 (C-4), 124.6 (C-5), 122.6 (C-6), 134.9 (C-7), 126.2 (C-8), 148.14 (C-9), 117.3 (C-10), 46.8 (C-12), 41.8 (C-13).

Continuous cultures of *P. falciparum*

P. falciparum blood parasites (W2 clone, CQ-resistant and mefloquine-sensitive) were maintained in continuous culture as previously described [41] with slight modifications [42]. The 3D7 strain, CQ- sensitive, originally received from the New York University Medical School, was also used. Briefly, the parasites

Table 5. Docking results of NADH and the protonated forms of chloroquine, BAQ and MAQ in the active site of *Pf*LDH.

Ligands	H Bond Energy (Kcal mol^{-1})	MolDock Score (Kcal mol^{-1})	Residues (H Bond interactions)
NADH	−35.3	−432.3	Asp53, Thr97, Gly29, Tyr85, Leu25, Met30, His126, Val55, Glu122, Phe100, His195
Protonated Chloroquine	−3.8	−252.4	Gly99
MAQ-1	−8	−247.5	Asp53, Gly99
MAQ-2	−2.9	−266.2	Asp53, Gly99
MAQ-3	−0.5	−249	Asp53
BAQ-1	−17.4	−337.2	Gly99, Thr97, Asp53, Asn140
BAQ-2	0	−297.3	-

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were kept at 37°C in human erythrocytes (A^+) with complete medium (RPMI 1640 supplemented with 10% human serum blood group A^+, 2% glutamine and 7.5% $NaHCO_3$) in Petri dishes in a candle jar or in a 25-cm culture flask in a defined atmosphere (with 3% O_2, 5% CO_2 and 91% N_2). Human erythrocytes (A^+) and serum blood group A+ were kindly donated by Center of Hemotherapy and Hematology of Minas Gerais (HEMOMINAS). Immediately before the tests, ring stage-parasites were synchronized in sorbitol [43]. Synchronized parasites were stained with orange green in pre-dried coverslips [44] then immediately examined under a fluorescent microscope. The suspension was adjusted for parasitemia and hematocrit as described below for each test. The infected red blood cells were distributed in a 96-well microtiter plate (Corning, Santa Clara, CA, EUA) at 180 µL/well, in which 20 µL of the test drugs and controls were previously diluted at different concentrations (400-0.625 ng/mL) in 0.005% DMSO (v/v) (Sigma, USA).

In vitro assays with *P. falciparum* infected erythrocytes

The effects of BAQ and MAQ on the W2 and 3D7 *P. falciparum* blood cultures were assayed through the incorporation of ^3H hypoxanthine (PerkinElmer, Waltham, MA, EUA) [45] and with monoclonal antibodies to the parasite histidine and alanine-rich protein (HRPII) (MPFM ICLLAB-55A®, MPFG55P ICLLAB®, USA) (Noedl 2002). The [^3H]-hypoxanthine assay was performed at 1% parasitemia and 1% hematocrit and the level of isotope incorporation was read in a beta-counter (PerkinElmer, Waltham, MA, EUA). The anti-HRPII test was performed at 0.05% parasitemia and 1.5% hematocrit and the HRPII quantification was read at 450 nm in a spectrophotometer (SpectraMax340PC^{384}, Molecular Devices). The results of three of these drug activity assays were expressed as the mean of the half-maximal inhibitory dose (IC_{50}) and compared with the drug-free controls. Curve-fitting was performed using Origin 8.0 software (OriginLab Corporation, Northampton, MA, USA) [46].

Inhibition of hemozoin assay

The hemozoin inhibition assay was performed as described by Ncokazi et al., (2002) [47] with some modifications. Briefly, 10.1 µL of the aminoquinoline derivatives at different concentrations (40-0.62 mg/mL) and 101.2 µL of the hematin stock solution (1.68 mM in 0.1 M NaOH) were combined in triplicate in a flat-bottomed 96-well plate. The solutions were further mixed and 58.7 µL of acetate solution (12.9 M, pH 5.0) then added to each well. After incubation at 60°C, 80 µL of 30% (v/v) pyridine solution in 20 mM HEPES, pH 7.5, were added at room temperature. The solids formed were resuspended and allowed to settle for 15 min at room temperature. Then, 38 µL of the supernatant were transferred to a new plate and diluted to 250 µL with a 30% (v/v) pyridine solution (20 mM HEPES, pH 7.5). The color reaction was read in a spectophometer at 405 nm and the results were expressed as the concentration of free heme in each well, which was calculated from the standard absorbance curve of a known hemin solution. CQ was used as a positive control and the *cut off* was determined by the amount of free heme in the drug-free control. Each compound was evaluated in two different experiments.

Antimalarial tests against *P. berghei* in mice

A suppressive parasite growth test was used with *P. berghei*, NK65 strain (originally received from the New York University Medical School) infected mice as described previously [48] with some modifications [49]. Briefly, adult Swiss outbred mice (20±2 g weigh) were inoculated with 1×10^5 red blood cells infected with *P. berghei*, by intraperitoneal route. The infected mice were maintained together for at least 2 h and then divided randomly into groups of 3–5 animals per cage, which were subsequently treated with 25 and 50 mg/kg of each compound diluted in DMSO 3% (v/v) given daily by oral gavage for three consecutive days. Two control groups were used in parallel, one treated with CQ (20 mg/kg) and one with the vehicle. Blood smears from mouse tails were prepared on days 5, 8 and 10 post-infection and then methanol-fixed, stained with Giemsa and examined microscopically. The parasitemia was evaluated and the percent inhibition of parasite growth calculated in relation to the untreated control group (considered 100% growth) using the following equation: [(C - T)/C] ×100; where C is the parasitemia in the control group and T is the parasitemia in the treated group.

The use of laboratory animals was approved by the Ethics Committee for Animal Use of the Oswaldo Cruz Foundation - Fiocruz (CEUA L-0046/08).

Cell cultures and cytotoxicity tests

The human hepatoma cell line (HepG2) was originally received from the New University of Lisbon, and the monkey kidney cell line (BGM) from the Federal University of Minas Gerais. These cells were cultured in 75 cm^2 plates with RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 40 mg/L gentamicine in a 5% CO_2 atmosphere at 37°C. For the *in vitro* tests, a confluent cell monolayer was trypsinized, washed with culture medium, distributed in a flat-bottomed 96-well plate (5×10^3 cells/well) and incubated for 18 h at 37°C to ensure cell adherence.

The cytotoxicity was evaluated with the MTT assay, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [50]. Briefly, the HepG2 and BGM cell lines were incubated with 20 µL of the compounds at different concentrations (1000-1 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) were added and the plates were incubated for a further 3 h. After incubation, the supernatant was carefully removed from the wells, followed by the addition of 100 µL DMSO with thorough mixing. The optical density at 570 and 630 nm (background) was determined on an ELISA reader (SpectraMax340PC^{384}, Molecular Devices).

The neutral red assay was used to evaluate cell viability by the accumulation of dye in the viable cell lysosome [51]. Briefly, the cells were incubated for 2 h with 100 µL of a neutral red solution (100 µg/mL). Then, the supernatants were discard and 200 µL of a formaldehyde (0.5% v/v) and $CaCl_2$ (1%) solution were added, which was followed by another 5 min incubation. Finally, the supernatant was removed, then 100 µL of an alcohol-acid solution added to the sediment. The absorbance was read at 540 nm on an ELISA reader (SpectraMax340PC^{384}, Molecular Devices). Cell viability was expressed as the percentage of control absorbance obtained in untreated cells after subtracting the absorbance of the appropriate background. Lastly, the minimum lethal dose for 50% of the cells (MLD_{50}) was determined as previously described [52].

Hemolysis assay

The test and control compounds were diluted in a 0.2% (v/v) DMSO solution and incubated (0–50 µg/mL) with a human erythrocytes suspension (1% hematocrit) at 37°C for 30 min in a shaking water bath. The mixtures were centrifuged at 1000 g for 10 min and the absorbance of the supernatants was measured at 540 nm in an ELISA reader (SpectraMax340PC^{384}, Molecular Devices). The hemolytic rate was calculated in relation to the

hemolysis of erythrocytes in 0.05% saponin, which was considered to be 100% [53].

Docking energy calculations

The 3D optimized structure of dimeric hematin was taken from our previous studies [29]. The structures of CQ and its analogs were built using the Gaussian View software and optimized using Gaussian 03 [54]. The structural analysis of CQ analogs (BAQ and MAQ) was performed and all possible protonation forms in the biological medium were considered (Figure 2). The geometry optimizations and partial atomic charge distributions were performed using the B3LYP/3-21G level of theory. The calculations for the docking energies were performed with Molegro Virtual Docker (MVD) software [55] as described previously [25]. The algorithm used in the docking studies was the MolDock Score, which is an adaptation of the Differential Evolution (DE) algorithm [53]. The MolDock Score was used because it yields a higher docking accuracy than other state-of-the-art docking algorithms (MVD: 87%, Glide: 82%, Surflex: 75%, FlexX: 58%) [46].

The 3D structure of PfLDH complexed with NADH and the substrate oxamate used in the present work was obtained from the Protein Data Bank (PDB) file 1LDG [56,57]. We performed flexible docking studies (considering the presence of water molecules in the crystal) with the protonated forms of BAQ and MAQ to compare these interactions with protonated chloroquine at the active site of PfLDH.

Supporting Information

Scheme S1 Synthesis of MAQ and BAQ.
(TIF)

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Author Contributions

Conceived and designed the experiments: MRM TCCF AUK. Performed the experiments: ACCA FJBF WAC RMS ASP. Analyzed the data: ACCA FJBF WAC RMS ASP MRM TCCF AUK. Contributed reagents/materials/analysis tools: MRM TCCF AUK. Wrote the paper: ACCA WAC ASP MRM TCCF AUK.

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ANEXO II

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Plasmodium vivax and *Plasmodium falciparum ex vivo* susceptibility to anti-malarials and gene characterization in Rondônia, West Amazon, Brazil

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Abstract

Background: Chloroquine (CQ), a cost effective antimalarial drug with a relatively good safety profile and therapeutic index, is no longer used by itself to treat patients with *Plasmodium falciparum* due to CQ-resistant strains. *P. vivax*, representing over 90% of malaria cases in Brazil, despite reported resistance, is treated with CQ as well as with primaquine to block malaria transmission and avoid late *P. vivax* malaria relapses. Resistance to CQ and other antimalarial drugs influences malaria control, thus monitoring resistance phenotype by parasite genotyping is helpful in endemic areas.

Methods: A total of 47 *P. vivax* and nine *P. falciparum* fresh isolates were genetically characterized and tested for CQ, mefloquine (MQ) and artesunate (ART) susceptibility *in vitro*. The genes *mdr1* and *pfcrt*, likely related to CQ resistance, were analyzed in all isolates. Drug susceptibility was determined using short-term parasite cultures of ring stages for 48 to 72 hour and thick blood smears counts. Each parasite isolate was tested with the antimalarials to measure the geometric mean of 50% inhibitory concentration.

Results: The low numbers of *P. falciparum* isolates reflect the species prevalence in Brazil; most displayed low sensitivity to CQ (IC50 70 nM). However, CQ resistance was rare among *P. vivax* isolates (IC_{50} of 32 nM). The majority of *P. vivax* and *P. falciparum* isolates were sensitive to ART and MQ. One hundred percent of *P. falciparum* isolates carried non-synonymous mutations in the pfmdr1 gene in codons 184, 1042 and 1246, 84% in codons 1034 and none in codon 86, a well-known resistance mutation. For the *pfcrt* gene, mutations were observed in codons 72 and 76 in all *P. falciparum* isolates. One *P. falciparum* isolate from Angola, Africa, showing sensitivity to the antimalarials, presented no mutations. In *P. vivax*, mutations of *pvmdr1* and the multidrug resistance gene 1 marker at codon F976 were absent.

Conclusion: All *P. falciparum* Brazilian isolates showed CQ resistance and presented non-synonymous mutations in *pfmdr1* and *pfcrt*. CQ resistant genotypes were not present among *P. vivax* isolates and the IC_{50} values were low in all samples of the Brazilian West Amazon.

Keywords: Anti-malarials, Resistance, *Plasmodium vivax*, *Plasmodium falciparum*, Chloroquine resistance

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Background

Malaria, one of the most prevalent parasitic diseases in the world, still causes high morbidity and death, mainly in *Plasmodium falciparum*-infected, non-treated patients [1]. *Plasmodium vivax* causes intense morbidity and contributes to significant political, social and economic instability in developing countries of Latin America and Asia [2,3]. CQ is the drug of choice to treat *vivax* malaria in endemic areas of Brazil and primaquine (PQ) is used to avoid late malaria relapses [3]. The recommended dose for adults is 1500 mg of CQ (daily for three days) and 210 mg of PQ (daily for seven days) [4]. *Plasmodium vivax* resistance is now widespread and has rendered CQ ineffective in parts of Indonesia and Papua New Guinea [5-7]. Low levels of resistance have also been reported in Myanmar, South Korea, Vietnam, India, Turkey, Ethiopia, and in regions of Southern Africa and South America [3,8,9]. The occurrence of severe *vivax* malaria and patient's deaths has been reported in Brazil [10-12] raising the possibility of an association between malaria severity and drug resistance [13]. In areas of CQ resistance, treatment of uncomplicated *P. falciparum* malaria is carried out with artemisinin-based combination therapy (ACT) [3]. Drugs that complement ACT include lumefantrine, amodiaquine, AQ, MQ, sulphadoxine-pyrimethamine and antibiotics. In Brazil, the first option for *falciparum* malaria treatment is the combination of artemether (480 mg daily for four days) and lumefantrine (2880 mg daily for four days). PQ (45 mg) is administrated on day one to avoid malaria transmission. These doses are recommended for adults with 50 Kg weight or more [4]. A reduced susceptibility to artemisinin derivatives has been described in *P. falciparum*-treated patients [14,15].

Increasing evidence of a lower *P. vivax* susceptibility to CQ in malaria-endemic areas [16] includes the state of Amazonas [17] and is believed to be associated with malaria's clinical severity [18].

Molecular markers associated with CQ resistance are non-synonymous mutations in the drug/metabolite transporter gene *pfcrt* (C72S, K76T) and in the multidrug resistance protein 1 gene *pfmdr1* (N86Y; Y184F; S1034C; N1042D; D1246Y), described in *P. falciparum* [19-21]. One mutation of the *multidrug resistance gene 1* (Y976F) of *P. vivax* is also associated with parasite susceptibility to CQ [8]. A non-synonymous mutation of the *pvdhps* gene at codon 382 (S382C) was recently associated with *in vitro* susceptibility to CQ [18]. The present study aimed to examine the phenotypic and genotypic chemoresistance profile of *P. falciparum* and *P. vivax* to commonly used anti-malarial drugs in a Brazilian malaria-endemic area in the Amazon Region.

Methods

Subjects

All isolates were collected between August 2012 and March 2013 from patients recruited at the Centre of Malaria Control (CEPEM) in the city of Porto Velho, state of Rondônia, in the Brazilian Western Amazon, where *P. vivax* is highly prevalent. Only patients mono-infected with either *P. falciparum* or *P. vivax* and with high parasitaemia (between 2,000 and 80,000 parasites/μl) were recruited. Patients who used any anti-malarial in the previous month and/or presented severe symptoms of malaria were excluded from this work. The study cohort encompassed 56 patients living in this highly endemic area which is close to Bolivia (Figure 1). Forty seven patients were diagnosed with *P. vivax* and eight with *P. falciparum*. In addition, one isolate of an individual infected with *P. falciparum* with imported malaria (from Africa) was also studied. One patient had mixed malaria (*P. vivax* and *P. falciparum*) and was not included. From each volunteer, a peripheral venous blood sample (5 ml) was collected by venipuncture in heparin-containing tubes and immediately used for the *ex vivo* drug susceptibility assay using pre-prepared plates with the diluted anti-malarials, as described below. DNA was also extracted from peripheral venous blood in EDTA containing tubes for parasite genomic analysis.

Ethical approval

This study was approved by the Ethics Committee, Centro de Pesquisas René Rachou-FIOCRUZ (CAAE –03209212.7.0000.5091). All participants signed a written informed consent before blood collection.

Pre-dosed plates with test and control drugs

CQ, MQ and ART were prepared as 10 μg/mL stock solution in dimethyl sulphoxide (DMSO) in 96-well plates (20 μL per well), then diluted two-fold in RPMI, with variable maximum drug concentration according to each previously determined activity (shown in parentheses), i.e. CQ (854 nM), MQ (724 nM), ART (738 nM), lyophilized and stored at 4°C until further use. Each lot of plates was assayed for quality control just before use, based on the profile response to CQ to the *P. falciparum* laboratory strains W2 and 3D7, for CQ-resistance and -sensitivity, respectively. All plates were prepared in our laboratory, in Belo-Horizonte, transported to the field in dry ice and kept at minus 70°C until use.

Ex vivo drug susceptibility assay

The drug susceptibility of the malaria parasites, from each patient, was measured *in vitro*. For *P. falciparum* the method used was described by Rieckmann and Antuñano [22] and for *P. vivax* by Renapurkar *et al.* [23] with modifications [24]. White blood cells were removed by filtration in a CF 11 cellulose column as described [25]. Immediately before the *ex vivo* drug susceptibility assays, packed red blood cells with the parasites (iRBC) were diluted for a 2% hematocrit, using either complete medium

Figure 1 Rondônia state, West Amazon.

RPMI 1640 medium plus 10% AB human serum, with *P. falciparum* cultures; or the McCoy's 5A medium plus 20% AB human serum, with *P. vivax* samples. The iRBC (200 μl per well) were distributed in the pre-dosed drug plate. For the maturation of parasites, rings to schizonts, the plates were maintained in candle jars at 37°C as described [26], at different incubation times (48–72 hours). The control wells were iRBCs and cultured with drug free complete medium. The incubation parasite-drug was stopped when 40% of the ring stages reached the schizont stage (at least four distinct nuclei per parasite) in the drug-free control wells (n = 6 per plate). Thick blood films were then made from each well, dried, stained with 5% Giemsa solution for 30 min, and examined microscopically. The number of schizonts per 100 asexual stage parasites was determined for each drug concentration and then normalized by comparing with the schizont number in the drug-free control wells (considered as 100%). The half-maximal drug inhibitory response (IC_{50}) was estimated by curve fitting using software (OriginLab Corporation, Northampton, MA, USA) and comparing with parasite growth in the drug-free controls.

Table 1 Set of primer sequences used to characterize gene polymorphisms by *Plasmodium falciparum* and *P. vivax*

Gene	Codons	Primer	AT* (°C)	PCR products (bp)
pfmdr1	86, 184	5′-GAGTTGAACAAAAAGAGTACCGCTGA-3′	55	512
		5′-TTTTTCCGTTAATTTATGTTTGTGGTGTCA-3′		
	1043, 1042	5′-TGTCAAGCGGAGTTTTTGCATTTAGT-3′		299
		5′-TGGTAGTTATGCTGGAAAATTAATGTCCT-3′		
	1246	5′-GGAGAAACAGGTAGTGGAAAATCAACTT-3′		302
		5′-TTTGGAAGAGAAGATGCAACATTGGAA-3		
pvmdr1	976	5′-ACTCACTTTATAGTGCTCTTCCTTGTG-3′	55	476
		5′- GGACATCAACTTCCCGGCGT- 3′		
pfcrt	72, 76	5′- acagATGGCTCACGTTTAGG –3′	55	162
		5′- TTTTGTAACATCCGAAACTCACA -3′		

*Annealing temperature.

Table 2 Characteristics of the study population with malaria in the Amazon-Brazil

	P. falciparum	*P. vivax*
Number of patients	**9**	**47**
Male (age)	N = 6 (41 ± 4)	N = 37 (37 ± 10)
Female (age)	N = 3 (22 ± 3)	N = 10 (47 ± 13)
Fever at the time of blood collection	9	42

Real time PCR for *Plasmodium* detection

Genomic DNA of the parasites was extracted using QIAamp DNA kit (QIAGEN, Chatsworth, CA, USA) and then subjected to real time PCR (Applied Biosystems®). The 18S rRNA gene was chosen as target gene since it contains both highly conserved and variable regions (at least five copies of the gene are dispersed on separate chromosomes of the *Plasmodium* genome). Each 20 μl reaction mix contained 2 μl of sample DNA, 10 μl FastSTART DNA SYBR Green reagent (Roche), 6.5 mM MgCl2 (final concentration), and 0.5 mM concentrations of each primer (5′-TAACGAACGAGATCTTAA-3′and 5′-GTTCCTCTAAGAAGCTTT-3′). The PCR conditions consisted of an initial denaturation at 95°C for 10 min, followed by amplification for 40 cycles of 10 sec at 95°C, 5 sec at 50°C, and 20 sec at 72°C, with fluorescence acquisition at the end of each extension step. Amplification was immediately followed by a melt programme, consisting of 2 min at 95°C, 2 min at 68°C, and a stepwise temperature increase of 0.2°C/sec until 90°C, with fluorescence acquisition at each temperature transition. Fluorescence was analysed using F1/F2 settings, which improved the detection of *P. falciparum* (a cutoff of 35 cycles was used to define *Plasmodium*-positive samples). A melting curve analysis was used to determine the species-specific mean melting temperature (Tm) based on values determined from the respective positive controls [27].

Analysis of the *crt* and *mdr1* genes

The *pfcrt* and *mdr1* genes were amplified by PCR with specific primers for each region (Table 1).The PCR reactions were performed with 3 μl of DNA at 30 ng/μl, mixed with 6.5 μl of AmpliTaq Gold® PCR Master Mix (Applied Biosystems, Warrington, UK), 0.5 μl of each primer at 10 pmol/μl, on a final volume of 15 μl. The samples were placed in an Eppendorf Mastercycler® (Hamburg, Germany) at 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec and a final extension time at 72°C for 5 min. PCR products were purified using Wizard PCR DNA and Gel Band Purification Kit (Promega, Madison, USA) following manufacturer's protocol and visualized on a silver-stained 6.5% polyacrylamide gel. Purified DNA fragments were then sequenced using the dideoxy method. Sequence data were analysed using Sequencher 4.9 software (Gene Codes Co, Ann Arbor, MI, USA). Sequences were then compared with those on Plasmo DB gene bank.

Results

Anti-malarial susceptibility

Ex-vivo drug susceptibility was assessed in field isolates from all 56 patients with a single species infection, either *P. vivax* (n = 47) or *P. falciparum* (n = 9); eight of the latter were Brazilian isolates freshly collected in the state of Rondônia, in the Brazilian Western Amazon (Figure 1) and one was from an individual returning from Angola, Africa, diagnosed and studied in Belo Horizonte, Brazil, outside the endemic malaria region.

Adequate parasite growth was achieved in 100% (9/9) of the *P. falciparum* and in 68% (32/47) of *P. vivax* isolates. The characteristics of these isolates are summarized in Table 2. The majority of *P. falciparum* Brazilian isolates studied were considered resistant to CQ (IC_{50} 70 nM; range, 0.19 to 223 nM); two susceptible showed IC_{50} values of 0.19 and 37 nM; two isolates were considered resistant to MQ (IC_{50} of 50 and 63 nM); but, all isolates were susceptible to ART, since the highest IC_{50} was 5.8 nM.

Among the *P. vivax* isolates the results of drug susceptibility to CQ suggests no resistance; the median IC_{50} was 32 nM; ranging between 3 to 69 nM (Table 3); four isolates (P010, P024, P026, P042) less susceptible to CQ showed IC_{50} values higher than 51 nM. In further analysis 32 were examined in the thick smears for the stages of the parasite blood forms; three patients had approximately 30% of parasites in trophozoite stage, which is considered a high rate. And, they present high IC_{50} values to ART

Table 3 Overall *ex-vivo* sensitivity of Brazilian isolates for each drug, according to the species tested, and for laboratory *P. falciparum* (W2 clone) line, chloroquine resistant

Drug	*P. falciparum*					*P. vivax* Clinical field isolates	
	W2	Fc27*	*African*	Clinical field isolates			
		IC_{50} (nM)		n	Median IC_{50} (nM)**	n	Median IC_{50} (nM)
Chloroquine	178	39	19	9	70 (0.19-223; 80)		32 (3–69; 64)
Artesunate	18	16	1.5	9	1.3 (0.26-5.8; 2.82)	32	21 (0.08-137; 17.4)
Mefloquine	19	11	46	9	21 (1.19-63; 80)		57 (5.0-113; 82)

*Date from Marfurt et al. [28,29]. **The numbers inside of the parenthesis means: lowest IC_{50}, higher IC_{50} and interquartile range.

Table 4 Anti-malarial activity *in vitro* in 15 different human *P. vivax* isolates for chloroquine (CQ), artesunate (ART) and mefloquine MQ)

P. vivax isolates	% of trophozoites	Drugs/IC_{50} Nm		
		CQ	ART	MQ
P006	22	29	15	11
P010	20	61**	11	45
P015	9	13.5	11	6
P022	15	8	11	60
P024	32*	69**	34	113
P025	93*	3.0	28	11
P026	29	51**	137	42
P028	11	6.6	2.8	11
P029	16	34	2.5	5.5
P031	10	15	8.0	8.2
P033	8	23	23	24
P034	17	29	9.6	47
P038	42*	8.5	1.3	2
P037	32*	6.5	9.4	39
P039	88*	6.8	5.2	26
P042	80*	62**	23	15
Mean (±SD)	-	23 + 9	32 + 37	32 + 28

*Isolates that presented more than 30% trophozoites. **Isolates that were considered less sensitive to CQ.

and MQ (Table 4). However this number is not enough to show a strong association between stage-specificity and drug activities and future studies are needed. The only African isolate studied was susceptible to the anti-malarials tested (Table 3).

Characterization of the *CRT* and *MDR1* resistance

The parasite profiles of molecular resistance for *P. vivax* isolates, using the frequencies of SNPs (Single Nucleotide Polymorphism) in *pvmdr1* codon 976, were examined in 47 samples (Table 1). The product was compared with the genomic sequence of Salvador I as the reference wild type found in the Plasmo DB gene bank, and no SNPs were found. For *P. falciparum* isolates, frequencies of SNPs in *pfmdr1* codons 86, 184, 1034, 1042 and 1246 are shown in Table 5. In addition, codons 72 and 76 of *pfcrt* gene were also evaluated (Table 5). Mutant alleles at positions 184, 1042 and 1246 on pfmdr1 gene were present in 100% of the samples. In codon 1034, the frequency of mutation was 84% and no mutations were found in codon 86. The *pfcrt* gene carried SNPs in codons 72 and 76 in all Brazilian isolates. All isolates presented non-synonymous mutations (Table 5). The *P. falciparum* isolate from outside Brazil (Angola, Africa) had no SNPs on the *pfcrt* or *pfmdr1* genes.

Table 5 Prevalence of molecular markers associated with *P. falciparum* resistance to chloroquine

Gene – codon	% of mutations	Mutations	Protein change
PfMDR1			
86	0%	-	-
184	100%	TAT > TTT	Tyrosine > Phenylalanine
1034	84%	AGT > TGT	Serine > cysteine
1042	100%	AAT > GAT	Asparagine > Aspartic acid
1246	100%	GAT > TAT	Aspartic acid > Tyrosine
PfCRT			
72	100%	AAA > ACA	Lysine > Treonine
76	100%	TGT > TCT	Cysteine > Serine

Discussion

Due to the strong impact of chemo-resistance among the malaria parasites to most drugs used in the control of the disease, monitoring the development of resistant phenotypes and genotyping are priorities wherever endemic malaria is present. The *in vitro* methods used to examine anti-malarial drug sensitivity provide a profile of *Plasmodium* sensitivity to a variety of drugs, simultaneously assayed. However, a lower *in vitro* sensitivity of a parasite isolate does not imply drug-resistance *in vivo*, as other factors can interfere, which are not determined by *in vitro* tests. The *ex vivo* tests provide an outline of resistant-circulating phenotypes for each tested drug, provided that an adequate number of patients are examined in a given area. This was not the case for *P. falciparum* in our study: only nine patients were evaluated, reflecting the reduced transmission of this parasite species in Brazil [30]. Determining parasite genotype, performed in parallel, provides further information since mutations in the *pfcrt* gene alter the CQ flux and/or reduce drug efficacy [31]; and provides data for policy makers to decide the best drug to be prescribed in that area. Ideally, monitoring anti-malarial chemo-resistance must be continuous since development and spreading of resistance are dynamic events, changing with time and according to human interventions and other factors such as population migration [32].

The antiplasmodial activity and the molecular profile of resistance by anti-malarial standards like CQ, ART and MQ, confirms *P. falciparum* resistance to CQ only, as demonstrated in *ex-vivo* tests against *P. vivax* and *P. falciparum* isolates from patients with naturally acquired malaria in the state of Rondônia in the Brazilian Western Amazon, which is substantiated by sequencing of genes related to resistance to anti-malarial drugs.

There is evidence of *P. vivax* resistance to CQ in the state of Amazonas, specifically in the city of Manaus where an increase in the proportion of *P. vivax* malaria parallels an increase of unusual clinical complications

related to this species [33]. These authors used an *in vivo* test to assess the efficacy of standard supervised CQ therapy. Among 109 volunteers with *P. vivax* who completed the *in vivo* test, 19 had positive blood smears within the 28-days follow-up (one on day 14, three on day 21, and 15 on day 28). All were then required to undergo an alternative therapy with MQ. In another study performed by the same group, a lower *P. vivax* susceptibility to CQ through the attainment of IC_{50} by ELISA assay or using traditional methods [18,34] was examined; they demonstrated that drug-resistance was related to the presence of non-synonymous mutation at *pvdhps*, *pvcrt* and *pvmdr1*.

All *P. vivax* isolates presently studied in the Brazilian Western Amazon were sensitive to CQ in *ex vivo* assays. Although the threshold of IC_{50} to define a sample as resistant to CQ is not well established for *P. vivax*, it has been proposed that the same threshold used for *P. falciparum* should be used, with 100 nM threshold of CQ [35].

The IC_{50} for ART and MQ in *P. vivax* were also examined and they were higher when compared with the values found in *P. falciparum*. It can be due to differences in the stage-specific activities of CQ, MQ and AQ in *P. vivax*, demonstrated here and by Marfut *et al.* [28]. It may be interesting to compare the susceptibility of *P. vivax* in strains from other regions of the world. This issue remains to be further studied using strains from different places.

Only one point mutation for CQ was studied for *P. vivax* in the *pvmdr1* gene codon 976, but other genes were associated with CQ resistance in the Brazilian Amazon, e.g., in the pvdhps gene in codon 382 (S → C) [18].

Considering that the severity of *P. vivax* malaria in the state of Amazonas has been attributed to CQ resistance and to the increased levels of *pvmdr1* and *pvcrt-o* compared to the levels expressed by parasites from patients with mild symptoms [36], these genes copy number could also be evaluated. In addition, the *mdr1* copy number is strongly associated with recrudescence after artesunate-mefloquine administration, and could be used as a surveillance tool for artesunate-mefloquine resistance, as reported in patients in Cambodia [37,38].

In conclusion, in West Amazon, most *P. falciparum* isolates were CQ resistance, a data confirmed by parasite genotyping. No mutations were found for *P. vivax* in the region supporting the lower prevalence of this strain in Brazil.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ACCA and NSA carried out the molecular studies. DBP was the MD who interviewed and treated the patients in the endemic area and ACCA performed the ex vivo and diagnostic exams tests. AUK and LDM conceived the studies, participated in the experimental design and were responsible for the biological tests. AUK was the project leader. All authors read and approved the final manuscript.

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ANEXO III

PRIMAQUINE-THIAZOLIDINONES ARE LESS TOXIC THAN PRIMAQUINE AND BLOCK MALARIA TRANSMISSION: A TOOL TO CONTROL THE DISEASE.

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Abstract

Primaquine (PQ) is an antimalarial used to cure *P. vivax* relapses and, also to block malaria transmission in some endemic countries. However, PQ metabolites cause hemolysis in patients deficient in the enzyme glucose-6-phosphate dehydrogenase (G6PD) and it is not used in areas where this hemoglobinopathy exists. Several PQ-thiazolidinone derivatives (PQ-TZs), synthesized through one-pot reactions from primaquine (PQ), arenealdehydes and mercaptoacetic acid, were evaluated, in parallel with PQ, for hemolysis caused to human erythrocytes, deficient or not in G6PD; for toxicity against cell lines; and for their ability to block malaria transmission. This was evaluated in *Aedes* mosquitoes blood fed in chickens infected with *P. gallinaceum,* and, in *Anopheles* mosquitoes blood fed in mice with *P. berghei,* before and after drug treatment. Selected PQ-TZs were evaluated against the pre-erythrocytic stages of *P. berghei in vitro*, as well as *in vivo*. All PQ-TZs exhibited lower cell toxicity then PQ; none of them caused hemolysis to normal or to G6PD deficient human erythrocytes; neither did sera from mice pretreated with the compounds, as to exclude possible toxic drug metabolites. The compounds **4c, 4g, 4m** and **4o,** significantly inhibited *P. gallinaceum* sporogony although less than PQ; and, they totally inhibited *P. berghei* sporogony in *Anopheles* mosquitoes. The PQ-TZ **4m** had inhibitory activity on the development of early hepatic forms of *P. berghei in vivo* and *in vitro*; mice pre-treated with **4m** and inoculated with sporozoites had a 3 day delay in the pre-patent period. Overall, these data suggest that PQ-TZs are promising molecules to replace primaquine as a blocker of malaria transmission, and, that **4m** might be useful for inhibiting the development of the early EEF in the vertebrate host.

KEYWORDS: primaquine-thiazolidinones; cytotoxicity; G6PD deficient erythrocytes; *P. berghei; P. gallinaceum*; blocking malaria transmission.

Introduction

Plasmodium vivax is the most widespread parasite in the tropical and subtropical areas in the endemic countries of Asia and Americas. Almost three billion people are estimated to be at risk of *P. vivax* malaria (1). Its radical cure requires primaquine (PQ) treatment, together with a blood schizonticide, usually chloroquine (CQ) (2). PQ, an 8-aminoquinoline, is used to eradicate *P. vivax* relapses (3,4). It is also used as a single dose to block malaria transmission in some endemic areas (1). However, PQ is poorly tolerated, and its metabolites cause hemolysis in patients deficient in the enzyme glucose-6-phosphate dehydrogenase G6PD (5,6).

The finding that PQ-imidazolidin-4-one derivatives (**Fig. 1a**) exhibit transmission-blocking antimalarial activity (7) has led to the exploration of the aliphatic terminal amino group of PQ, as a precursor for the synthesis of thiazolidinones (TZ) (**Fig. 1b**). The TZ heterocyclic class exhibits wide applications in medicinal chemistry (8); and some thiazolidinone and thiazinanone-CQ analogs display higher activity to *P. falciparum* than CQ (9).

PQ inhibits sporogony in *P. gallinaceum*, blocking oocyst formation in *Aedes* mosquitoes (10, 11). This avian model is especially important where neither the rodent or simian malaria vectors are available for such studies (12). In the present work 15 PQ tiazolidinone derivatives (PQ-TZs), synthesized through modification of the amino-terminal group (13), were evaluated for: (i) their blocking effect on the sporogonic cycle of avian and rodent malaria; (ii) their ability to interfere with the development of the liver stages of *P. berghei* sporozoites, a rodent malaria model; (iii) toxicity *in vitro*, and *in vivo* in mice, and; (iv) blood schizonticide activity against *P. falciparum*. In all assays PQ was used as the reference antimalarial drug.

Material and Methods

Chemistry

The target compounds were prepared as outlined in Scheme 1 and as previously described (13). The 2-aryl-3-(4-(6-methoxyquinolin-8-ylamino)pentyl)-1,3-thiazolidin-4-compounds, 4b-p were synthesized using a multicomponent reaction of racemic primaquine diphosphate 1, *N*,*N*-diisopropylethylamine (DIPEA), arenealdehydes 2a-o, and an excess of mercaptoacetic acid 3 in refluxing toluene for 2-4 h. The desired heterocycles were obtained

with moderate to good yields (37-89%) after purification of the crude product by column chromatography, on a silica gel column using a mixture of hexane:ethyl acetate (7:3).

Biological assays

The human erythrocytes deficient in the enzyme G6PD were collected according to the Ethics Committee of CEPEM of Rondônia (CAAE- 007 59912.0.0000.0011; October 9, 2012). The laboratory animal were used as approved by the Ethics Committee of Instituto Osvaldo Cruz (FIOCRUZ, CEUA LW-23/13).

***In vitro* cytotoxic effect of PQ-TZ**

The cytotoxicity of PQ and PQ-TZ compounds was assessed, in parallel, against a human hepatoma cell line (HepG2) and a monkey kidney cell (BGM) cultured in 75-cm^2 sterile flasks in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and 40 mg/L gentamicin, under 5% CO_2 at 37 °C. When confluent, the cell monolayer was washed with RPMI culture medium, then trypsinized, distributed in a flat-bottomed 96-well plate (5×10^3 cells/well), and incubated for 18 h at 37 °C for cell adherence, as described (14). The compounds (20 µL) were added to each well at various concentrations (1-1000 µg/mL) and incubated for 24 h under 5% CO_2 at 37 °C. An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (5 mg/mL; 20 µL/well) was added to evaluate mitochondrial viability, and the plates were incubated again for 3 h, in the same culture conditions. The supernatant was carefully removed, and 100 µL of DMSO were added to solubilize the formazan crystals formed. The optical density was determined at 570 and 630 nm (background) (SpectraMax340PC^{384}, 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 as previously described (15); a ratio between the MDL_{50} of PQ-TZ and PQ was calculated (PQ-TZ /PQ).

***In vivo* toxicity of PQ-TZ in mice**

Compounds 4b, 4c, 4g, 4h, 4m, 4o and 4p were administered daily (250 mg/kg) by oral route to mice, in cumulative doses, until reaching the dose of 1000 mg/kg. Survival was

observed daily, until 30 days after treatment (16). PQ was used as a control starting from 50 mg/kg to 200mg/kg, and also administered to the mice in cumulative subsequent doses, by gavage, until the last mice survive or get the maximum dose (1000 mg/kg).

Inhibition of sporogony of *P. gallinaceum* and *P. berghei* in mosquito hosts

The ability of PQ-TZ to inhibit the sporogonic development of *P. gallinaceum* was evaluated, as described (11), in *A. fluviatilis* blood engorged with infected-treated chickens. Before use, the adult mosquitoes were kept 24 h without the sugar diet, to improve their ability to take the blood meal from chickens, since they are essentially anthropophilic mosquitoes. In brief, one-week-old chickens (*Gallus gallus domesticus*) were inoculated with 10^6 *P. gallinaceum* (8A strain) infected red blood cells, parasitemia was monitored by Giemsa-stained blood smear and upon reaching 2% or higher parasitemia, the chickens were used to feed groups of 30-40 mosquitoes, at time 0 h (the control group), and 6h after treatment with each compound by gavage (test group). The PQ-TZ derivatives were tested at 100 mg/kg or at lower doses; PQ, the reference drug, was used in 15 mg/kg, because of its toxicity. In each experiment, an internal control- a non-treated *P. gallinaceum*-infected chick- was also used twice to feed mosquitoes (0 h and 6 h later).

A similar protocol was performed for the studies of sporogony in *A. stephensi* mosquitoes exposed to *P. berghei* infected mice. In this model, the mosquito blood meal was performed either before (0 h) as the control group, or 2h after administration of either the PQ-TZ derivatives (50 mg/kg) or PQ (15 mg/kg), the reference drug, knowing that PQ inhibits totally sporogony.

The test and control mosquito groups were dissected seven days after the blood meal for *P. gallinaceum* and 12 days for *P. berghei*; their midintestine were removed under a stereomicroscope (100x), stained with 0.5% mercurochrome, examined under a light microscope (40x) and the oocysts counted. The inhibition of sporogony was calculated based in the oocysts numbers in the control mosquitoes blood-fed before treatment (0 h), considered as 100% infection, and those found in the mosquitoes blood-fed after drug-treatment. Two criteria were used to evaluate drug activity: the total number of infected mosquitoes, and, the mean number of oocysts per group, based on the exam of 20 mosquitoes in each group. A compound was considered partial active when the number of oocysts and mosquitoes infected was reduced at least 50% in comparison to non-treated control, and active if 100% reduction occurred.

Inhibition growth assay of *P. berghei* liver-stage parasites

This assay was performed as outlined in Sinnis et al (17). Briefly, sporozoite were isolated from infected *A. sthepensi* mosquitoes through dissection of their salivary gland homogenized to release sporozoites. Mosquito debris was removed by centrifuging (100 × g for 4 min at 4°C) in culture medium. The number of sporozoites was counted in a hemocytometer and diluted accordingly, to infect the mouse hepatocyte cell line or to inoculate mice by intravenous route.

Hepatocyte infections were initiated by adding 10,000 *P. berghei* sporozoites (ANKA strain) per well in 6-well culture plates containing mouse Hepa 1-6 cells (seeded a day prior to the experiment) in 0.4 ml complete Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 40 mg/L of penicillin/streptomycin and fungizone (Sigma). Drug solutions at 10, 1 and 0.1 µM were prepared in 0.05% DMSO medium and added 3 hours after the sporozoite infection. The cultures were allowed to grow at 37°C in a 5% CO2 atmosphere for 48 h. Culture medium was changed every 12 h, and fresh compounds were added at the same concentration, to maintain inhibitor pressure throughout the growth period. After 48 hrs the cells were fixed with 4% formaldehyde, permeabilized with cold ethanol overnight, blocked with 1% bovine serum albumin (BSA) in PBS for 2 h, then incubated for 1 h with primary monoclonal antibody 3D11, against the parasite circumsporozoite protein. After the primary antibody incubation, three washes were performed with PBS with 0.05% Tween 20 (PBST) in order to diminish nonspecific binding. The cells were further incubated with a secondary antibody (rabbit anti-mouse IgG) coupled to Alexa 594 (Life Technologies) for 1 h, and washed three times with PBST. A final wash was given with PBS, and coverslips were mounted using DAPI prolong reagent (Life technologies) and sealed with polish nail. Images of liver-stage parasites were captured by fluorescence microscope (Nikon 90i). The Image Pro-Plus program (Media Cybernetics) was used to measure the size of the liver-stage parasites.

***In vivo* assay for growth inhibition of the liver-stage parasites (EEF)**

Mice were drug-treated by oral route, on days minus 3, 2, 1 and zero, where day 0 corresponds to the day of the sporozoite infection by intravenous route. The EEF load was evaluated 42 hours after the sporozoite infections, when the mice were euthanized, and, the

liver total RNA was extracted using Trizol reagent. Reverse transcription from 4 μg RNA was performed to obtain cDNA. In a real-time PCR mix of 50 μl, a cDNA equivalent of 0.5 μg RNA was used. The real-time PCR mix also contained *P. berghei* 18S rRNA specific primers and SYBR green dye. Real-time PCR was performed in a Bio-Rad I cycler, and the copy numbers were calculated, using known amount of plasmid standard having the amplification target sequence (18). Parasite growth inhibition was calculated by dividing the 18S rRNA copy number of the experimental group by that of the untreated control group. The ability of sporozoites to initiate a blood-stage infection was evaluated in another group of mice from which blood smears were prepared on day 3 post-infection until day 15. Parasitemia was determined by Giemsa-stain. Complete protection is defined as the absence of parasites in blood smears up to 2 weeks post challenge with sporozoites.

Results

Tested for toxicity against human hepatoma (HepG2) and kidney green monkey (BGM) cell lines, PQ was more toxic than most PQ-TZs (**Table 1**); the ratio between PQ-TZs and PQ cytotoxicity *in vitro* shows that PQ-TZs 4d, 4j, 4k, 4l, 4m, 4o and 4p were 20 to 34 times less toxic than PQ; only 4f and 4i were as toxic as PQ. In addition, none of the PQ-TZs was toxic to the rodent cell line Hepa1-6 *in vitro*.

The PQTZs were not hemolytic to normal human erythrocytes or to G6PD deficient erythrocytes. However, PQ was hemolytic, even at the lowest dose, on both types of erythrocytes (Fig S1). Sera collected from mice previously treated with PQ (15mg/kg) or with the PQTZ (100mg/kg) were tested in parallel for hemolysis, at several times after drug treatment (15min up to 2h). Only sera from the PQ treated mice, collected 30 minutes after treatment caused some hemolysis, the sera from mice treated with PQTZ did not.

The *in vivo* toxicity of some PQ-TZs compounds was tested in mice receiving either one dose or several consecutive drug doses by gavage. PQ killed all animals 20 hours after administration in a single dose of 200 mg/Kg; the dose of 150 mg/Kg killed the mice at the 2^{nd} day and 100 mg/Kg on day 8; PQ at 50 mg/Kg was the less toxic and 100% of the mice survive until the cumulative dose of 500 mg/Kg, on day 10. (**Fig. S2**). Mice treated with a single dose of 250 and 500 mg/kg of 4b, 4m and 4p, survived for up to 30 days, the last day of observation. Compounds 4c, 4g, 4m, 4o and PQ given in cumulative doses 250 mg/Kg, daily to reach a 1000 mg/Kg dose), caused no death of the mice during the 30 days of observation.

Knowing that PQ completely inhibits *P. gallinaceum* sporogony (10, 11), all the 15 PQ-TZs were tested for their ability to inhibit this process in *Aedes* mosquitoes fed in drug-treated chickens. The midintestines of 20 mosquitoes per group were examined at each time point, before and after chicken treatment, and two parameters were evaluated: (a) the presence of oocysts or/and their numbers in each group of mosquitoes; and, (b) the average number of mosquitoes infected in each group subjected to the test compounds and PQ.

The percent of sporogony reduction was calculated in relation to the group blood-fed in the same infected chicken immediately before drug administration (time zero), considered as 100% sporogony. Most drugs were tested at 100 mg/kg, and among the 15 PQ-TZs compounds four were active (4c, 4g, 4m and 4o) significantly inhibiting malaria sporogony (**Table 2**); the other PQTZs were inactive (**Table S1**). The drugs were considered as partially active when 11% to 50% of the mosquitoes had oocysts, as compared to 90-100% in control mosquitoes blood fed before chicken treatment, as well as a significantly lower number of oocysts (81 to 96% lower) as compared to the control groups. The compounds were considered active when they totally inhibited sporogony, the case of PQ in the dose of 15 mg/kg (**Table 2**) Compound 4m was tested at doses of 50 and 25 mg/kg, and both significantly ($p < 0.001$) inhibited the sporogonic cycle (**Fig. 2**).

The large variations in the oocyst numbers in the mosquitoes did not hamper the selection between partially active (4c, 4g, 4m and 4o) from the inactive (4b, 4d, 4e, 4f, 4h, 4i, 4j, 4n and 4p) compounds, in the *P. gallinaceum* model, in which, only PQ totally inhibited sporogony. But PQ was more cytotoxic and more hemolytic than most PQ-TZs compounds tested.

Using basically the same protocol for *P. gallinaceum*, compounds 4b, 4c, 4g, 4h, 4m, 4n, 4o and 4p were also tested in the mammalian malaria model, i.e., *P. berghei* in *Anopheles* mosquitoes. Compounds 4b, 4g, 4m and 4o completely inhibited oocyst formation and no infected mosquitoes were observed (**Table 2**), whereas 4c, 4h, 4m and 4p were inactive, reducing less than 30% the infections and the oocysts numbers (**Table S2**).

Some PQ-TZ compounds were also tested for the ability in inhibit the *P. berghei* exoerythrocytic forms (EEF) development *in vivo*, 42 hours after mice intravenous injection with sporozoites. Only compound 4m, at 50 mg/kg, inhibited the EEF, reducing the liver parasite burden by 95%, in comparison with non treated control. PQ was more active against the EEF, since lower concentrations tested (3.2, 7.5 and 15 mg/kg), inhibited the liver parasite burden by 75, 93 and 99%, respectively (**Fig. 3**).

In parallel experiments, the mice inoculated with the sporozoites then treated with these compounds, were followed for the evolution of the blood stage infection resulting from the liver stage parasites seeding the blood. The pre patent period of the malaria infection was delayed by 3 days in the group of mice treated with 4m; the treated animal also had an increased survived, in comparison with non-treated controls (**Fig. 4a and 4b**). These results were expected, based on the fact that compound 4m significantly inhibited the *in vitro* sporozoite development in hepatoma cultures *in vitro* (**Fig. 5**).

The toxicity of 4m and 4o, the two best drugs (classified according to both parameters, the average oocyst numbers and proportion of infected mosquitoes), was about 30x lower than that of PQ (**Table 1**). These molecules were synthesized in large scale to be used in the Ames test (19) against five strains of *Salmonella typhimurium* with different mutations. They were evaluated for mutagenic potential at concentrations up to 3,000 µg/plate, in the absence and presence of the metabolizing rat liver fraction. Both compounds were negative for mutagenicity and they did not induce reverse mutations in the strains tested. The tests were performed at the Genotox-Royal Institute (www.institutoroyal.com.br), São Paulo, Brazil, contract number GT00721.

PQ-TZs were also tested as blood schizonticides *in vitro* against cultures of *P. falciparum* chloroquine-resistant parasites, and, in mice infected with blood stages of *P.berghei*, using strandard protocols (12). The results are shown in the supplementary data (**Table S3 and S4**).

Discussion

PQ is used to prevent *P. vivax* relapses and to block malaria transmission. However, the toxicity of PQ metabolites to the human host, especially its hemolytic activity in G6PD deficient erythrocytes (6), limits it use.

Only 11% of the 435 published antimalarial drug trials have been performed against *P. vivax*, aiming the hypnozoites treatment (20); there are two compounds in clinical trials to replace PQ, i.e., tafenoquine, active in a single dose but also hemolytic for G6PD erythrocytes in deficient patients (21); and, bulaquine, a pro-drug in phase II clinical trials, less hemolytic than PQ in individuals G6PD deficient, and also more potent than PQ to treat *P. vivax* late relapses (10). The role of G6PD in red cells is to provide reductive potential in the form of NADPH (22). In G6PD-deficient red cells the NADPH supply is adequate in the steady state, but falls short upon exposure to PQ. It is possible that the PQ metabolite causes an oxidative

challenge that the G6PD normal red cells would tolerate, but not the G6PD-deficient red cells. However, the stepwise sequence of the events from oxidative attack to hemolysis have not been fully elucidated (23).

The situation of *P. vivax* malaria and drug resistance has changed dramatically in the last decade; almost 90% of all cases are due to *P. vivax.* In malaria patients, PQ is used during 14 days to prevent late relapses (24). In addition, aiming to decrease malaria transmission in Brazil , all patients with malaria, regardless of the parasite species diagnosed, receive 3 days of PQ orally, in order to prevent malaria transmission to mosquitoes feeding on them (25). This measure is believed to have been essential to lower *P. falciparum* transmission, where the incidence of G6PD deficiency is lower than in other malaria endemic countries in Asia and elsewhere (26).

The primary motivation for our studies of PQ-TZ derivatives was to determine their toxicities and activities as blockers of malaria transmission. The modification on terminal amino group of PQ represents a great improvement in terms of drug bioavailability of PQTZs, by blocking the metabolism of the primary amino group. Pharmacokinetic studies showed that in the human body the carboxy-PQ is the main PQ metabolite, a compound less active than PQ (25). Pro-drugs of PQ (27, 28), double-drugs (29, 30) and bis-PQ (31), have been studied to prevent the metabolism, as well as to reduce its toxicity. Some new PQ analogues were synthesized as PQ-trioxaquines (32). The chemical and enzymatic stable PQ-imidazolidinones (**Fig. 1b**) turns then a novel type of 8-amino-quinoline antimalarial (34, 34).

Diverse biological activities have been described for thiazolidinones, including antimicrobial (35, 36) anti-inflammatory (37), anti-tumor (38), anti-HIV (39), Alzheimer's disease as muscarinic receptor 1 agonist (40), and, most important, as anti-malarial (10). Similar to the imidazolidinone-PQ derivatives, the PQ-TZs are unlike to act as pro-drugs since the thiazolidin-4-one ring is stable at low pH. In consequence by blocking the amino free group, PQ-TZs compounds may prevent the formation of the cyclic pyrrolidine reported by Pybus et al (41). Thus, several PQ-TZs were tested, directly *in vitro*, on G6PD deficient human erythrocytes, and on normal erythrocytes and in neither cell did they caused hemolysis, unlike PQ (**Fig. S1**).

Several papers describe the protection of the quinoline ring, at the 5 position, to avoid the formation of toxic 5-hydroxy-PQ metabolite (42-44). Although the structure of PQ-TZ does not influence directly the 5-position and the 5-hydroxy-PQ might be formed, others report that the modification of primary amino group decreases drug toxicity, through unknown mechanisms (45). The fact that most of the 15 PQ-TZ compounds were significantly

less toxic than PQ against human hepatoma, mice hepatocytes and monkey kidney cell lines, corroborates this information.

There was no significant influence of the nature of the aryl substituent on the ability of PQ-TZs to inhibit sporogony in the mosquito host. However, the 4-position seems to be important for drug activity on malaria sporogony. Accordingly, this position was substituted in three of the four most active compounds (4g, 4m and 4o) (**Table 2**). Compound 4m, which contains an electron-releasing methoxy group, inhibited malaria sporogony at a lower dose of 25 mg/kg, but it was not as active as PQ. However, the other compounds were not tested at lower doses, and, compound **4g** was the most toxic compared to 4m and 4o.

In spite of the irregular production of oocysts observed in this and in other malaria models (46), there were statistically significant differences among the groups of mosquitoes blood fed in animals pre-treated with the compounds considered active. However, in all experiments, only PQ completely inhibited oocyst formation in the *P. gallinaceum*. Considering *P. berghei* model, compounds PQ, 4c, 4g, 4m and 4o totally inhibit the oocyst formation.

PQTZs were more active to block oocyst formation when the *P. berghei* model was used, as compared with results observed with the *P. gallinaceum*. Only in the former some compounds (4b, 4g, 4m and 4o) completely blocked transmission to the mosquito. One possibility to explain the results is a different metabolism of the PQTZs between the mammal and avian hosts; nevertheless in both models PQ caused 100% inhibition of sporogony. Importantly one PQ-TZ derivative also inhibited the development of *P. berghei* liver stages, but whether they will eliminate the hypnozoites of *P. vivax*, like PQ does, has yet to be determined.

In conclusion, the mains findings were: (i) unlike PQ, all the 15 PQ-TZs tested were not toxic *in vitro* to mammalian cells, nor to mice treated with high doses of the drugs; (ii) only PQTZs caused no hemolysis to normal human erythrocytes (RBC) or to RBC deficient in the enzyme G6PD; (iii) most PQTZs displayed a significantly lower toxicity than PQ to the mammalian cell lines, human hepatoma, kidney from green monkey and mice hepatocytes; (iv) some PQTZs (4c, 4g, 4m and 4o) administered to the blood donor host inhibited 50-90% of the infections by *P. gallinaceum* in the *Aedes* mosquitoes blood fed 6 h after drug treatment, as compared to mosquitoes blood-fed from the blood donor prior to the drug treatment (considered 100% of sporogony) and reduced the number of oocysts in the test mosquitoes more than 80%; one compound (4m) also reduced sporogony at a lower dose (25 mg/kg); (v) the four compounds totally block the malaria transmission in *P. berghei* model

using *A. stephensi* mosquitoes; (vi) one compound (4m) reduced de number of EEF *in vitro* and *in vivo*, as well caused a delay of 3 days in the pre patent period, making some PQTZs promising drugs to help malaria control, after further pharmacological studies.

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Figures and legends

Figure 1. General structures of (a) PQ-imidazolidinones and (b) PQ-thiazolidinones (PQ-TZ).

Figure 2: Dispersion of *P. gallinaceum* oocyst number in the midintestines of *A. fluviatilis* fed on the same infected chicken at time zero (0h) or 6h after the treatment with compound 4m, administered orally. The various drug doses significantly ($p<0.001$) reduce the oocyst number.

Figure 3: Number of EEF 42 hours after infection with sporozoites by intravenous route, measured by real time PCR in mice treated with PQ (A) and 4m (B). *$p< 0.05$ by Mann-Whitney test, and parasites reduction, in comparison with non treated control, represented in %.

Figure 4: Parasitemia of malaria in mice with *P. berghei* induced by sporozoite infection, after oral treatment with 4M, PQ and in non-treated mice (A). The mice survival was also observed until day 30 (B).

Figure 5: Number (A) and size (B) of the exoerythrocytic forms of *P. berghei* (EEF) per well in cultures of sporozoites in the mouse hepatocytes (HEPA1-6) after treatment with the compound 4M in 3 different concentrations (0.1, 1 and 10μM), in comparison with non-treated cells similarly infected with the *P. berghei* sporozoite. The EEF development was registered in control HEPA 1-6 cells in a drug-free culture medium (C) and in the cells treated with various concentrations of 4M [10 μM] (D). **$p< 0.05$ by Mann-Whitney test.

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Figure 1

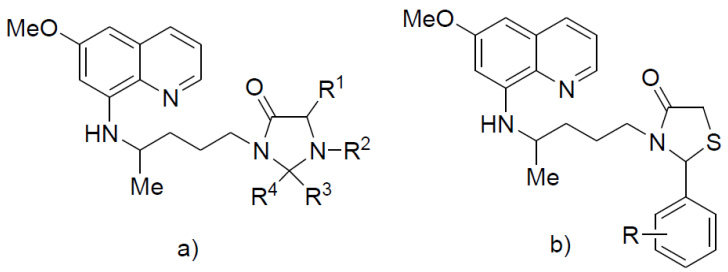

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Figure 2

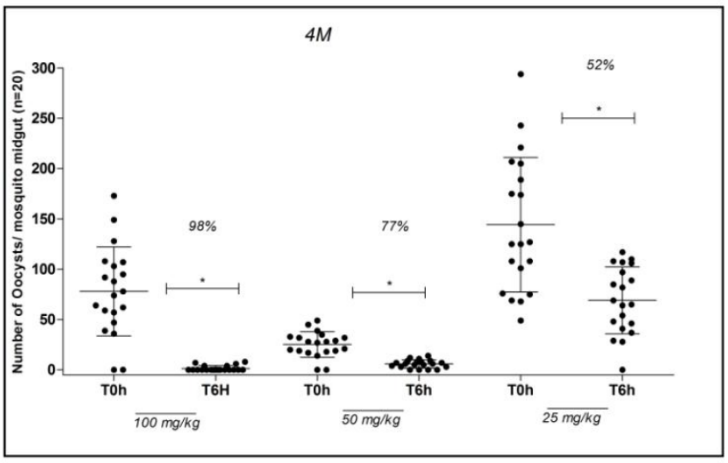

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Figure 3

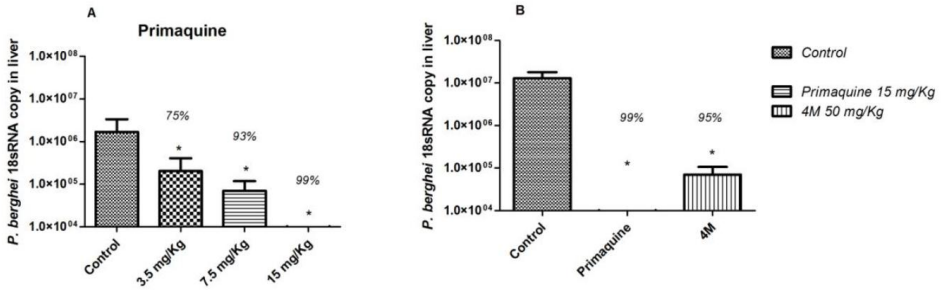

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Figure 4

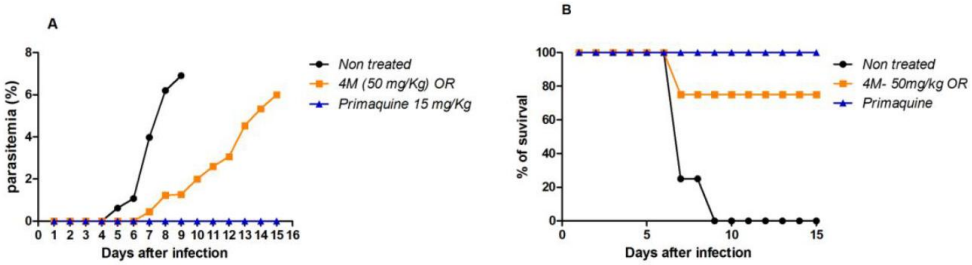

Figure 4: Parasitemia of malaria in mice with *P. berghei* induced by sporozoite infection, after oral treatment with 4M, PQ and in non-treated mice (A). The mice survival was also observed until day 30 (B).

Figure 5

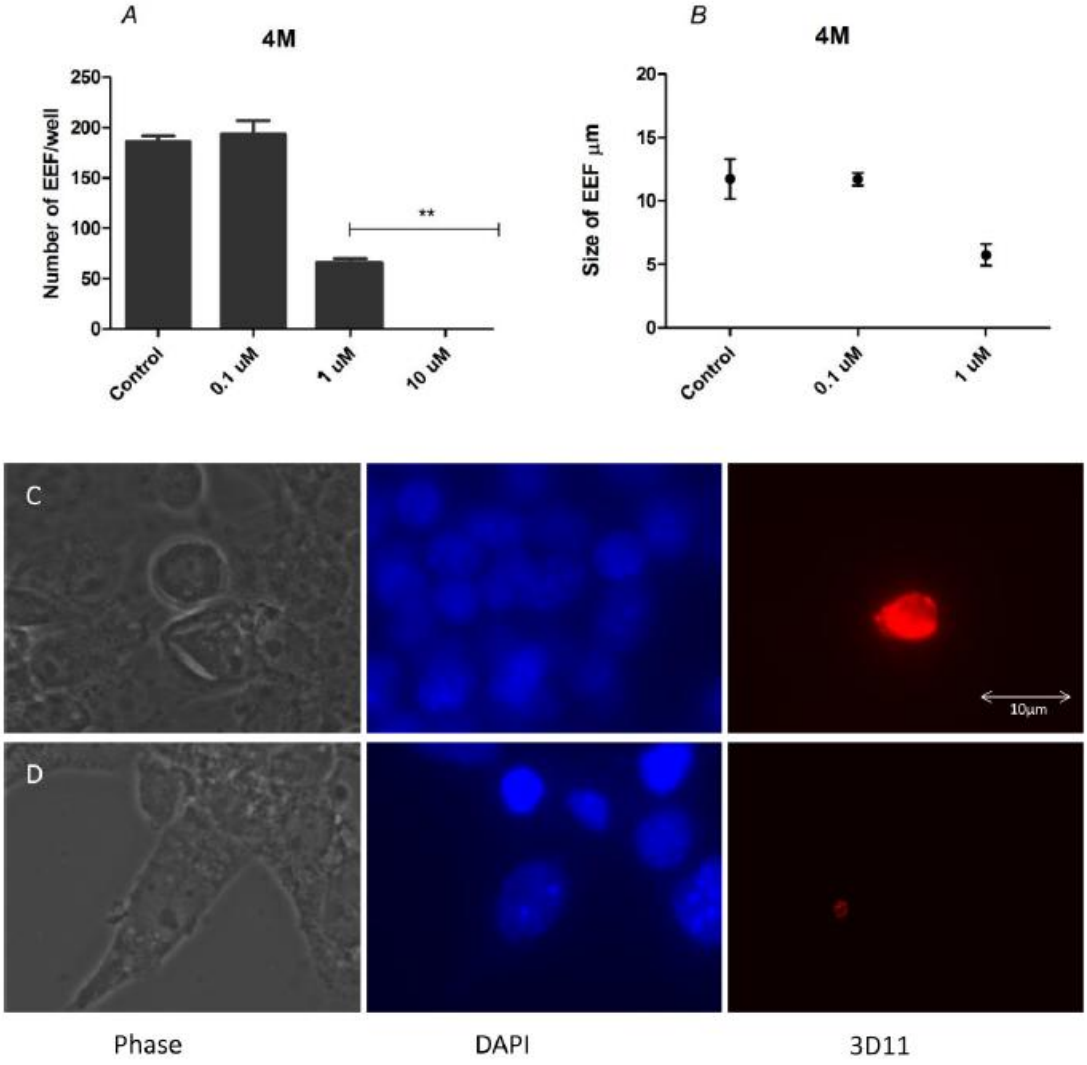

Figure 5: Number (A) and size (B) of the exoerythrocytic forms of P. berghei (EEF) per well in cultures of sporozoites in the mouse hepatocytes (HEPA1-6) after treatment with the compound 4M in 3 different concentrations (0.1, 1 and 10µM), in comparison with non-treated cells similarly infected with the *P. berghei* sporozoite. The EEF development was registered in control HEPA 1-6 cells in a drug-free culture medium (C) and in the cells treated with various concentrations of 4M [10 µM] (D). **p< 0.05 by Mann-Whitney test

Supplementary Date

Table S1- Inhibition of *P. gallinaceum* sporogony by PQ-TZ derivatives in *Aedes* mosquitoes blood fed 6h after oral treatment (6h) with one compound dose in relation to control mosquitoes fed in the same chicken before treatment (0h).

Chicken treated with (50mg/kg)	**% *Aedes* infected[a] (% reduction)**		**Average oocyst number ± SD (% reduction)**		**Inhibition of sporogony**
	0h	**6h**	**0h**	**6h**	
4b	90	80 (11%)	16 ± 10	3 ± 3 (81%)	Partial
4c	90	10 (89%)	18 ± 8	2 ± 1 (89%)	Partial
4d	85	80 (6%)	21 ± 4	26 ± 14 (0%)	No
4e	89	89 (0%)	42 ± 29	38 ± 25 (10%)	No
4f	100	95 (5%)	75 ± 16	60 ± 19 (20%)	No
4g	90	45 (50%)	35 ± 15	2 ± 1 (81%)	Partial
4h	80	90 (0%)	44 ± 44	30 ± 22 (32%)	No
4i	80	70 (13%)	33 ± 26	34 ± 26 (0%)	No
4j	60	75 (0%)	42 ± 38	38 ± 30 (10%)	No
4k	100	95 (5%)	92 ± 13	91 ± 20 (2%)	No
4l	94	84 (16%)	29 ± 14	23 ± 22 (21%)	No
4m	100	45 (55%)	47 ± 15	5 ± 2 (90%)	Partial
4n	95	95 (0%)	111 ± 72	83 ± 19 (25%)	No
4o	100	15 (85%)	49 ± 5	4 ± 5 (96%)	Partial
4p	80	75 (6%)	16 ± 11	13 ± 9 (25%)	No
Primaquine (15mg/kg)[b]	90	0 (100%)	105 ± 70	0 ± 0 (100%)	Yes

[a] The number of oocysts and percent of mosquitos with oocysts blood-fed at time 0 h was considered to be 100% infected. [b]Dose of PQ chosen based on the fact that 150 mg/kg kills all the mice.

Table S2- Inhibition of *P. berghei* sporogony by PQ-TZ derivatives in *Anopheles* mosquitoes blood fed 2h after oral treatment (2h) with one dose of each compound in relation to mosquitoes blood fed in the mouse before drug treatment (0h).

Mice treated with (50mg/kg)	**% *Anopheles* infected[a]**		**Average oocyst number ± SD (% reduction)**		**Inhibition of sporogony**
	0h	**2h**	**0h**	**2h**	
4b	70	0 (100%)	22 ± 15	0 ± 0 (100%)	Yes
4c	86	0 (100%)	64 ± 114	0 ± 0 (100%)	Yes
4d	90	86 (4%)	38 ± 12	42 ± 31 (0%)	No
4g	80	0 (100%)	75 ± 109	0 ± 0 (100%)	Yes
4h	86	86 (0%)	108 ± 87	146 ± 89 (0%)	No
4m	86	0 (100%)	49 ± 42	0± 0 (100%)	Yes
4n	80	71 (30%)	49 ± 84	27 ± 37 (45%)	No
4o	86	0 (100%)	36 ± 39	0 ± 0 (100%)	Yes
4p	80	86 (0%)	48 ± 23	35 ± 18 (27%)	No
Primaquine (15mg/kg)[b]	90	0 (100%)	128 ± 98	0 ± 0 (100%)	Yes

[a] The percent of mosquitos with oocysts was calculated in relation to mosquitoes blood-fed in the same mouse, immediately before drug treatment (time 0 h), considered to be 100% infected. [b]Dose chosen based on the fact that primaquine is toxic, 150 mg/kg kills 80% of mice in two days. Compounds 4e, 4f, 4i, 4j, 4k, and 4l were not tested.

Table S3- Activity of PQ-TZs against *P. falciparum* evaluated using [^{3}H] hypoxanthine and anti-HRPII (IC_{50}) assays, cytotoxicity (MDL_{50}) to human hepatoma cells (HepG2) and therapeutic or selectivity indexes (SI).

Compound	Drug activity *in vitro* (IC_{50} μM)			SI (MDL_{50} /IC_{50})	
	A ^{3}H Hypoxantine	B Anti-HRPII	HepG2 MDL_{50}	A	B
	IC_{50}				
4b	30 ± 0	16 ± 3.4	[b]	-	-
4c	32 ± 6	18 ± 4.6	1302 ±108	41	72
4d	27 ± 4.5	16	≥ 6831	253	427
4e	4 ± 2	2.2	≥ 2193	548	996
4f	37 ± 11	33 ± 4	641 ± 31	17	19
4g	20 ± 0	26 ± 6.5	3984 ±116	199	151
4h	43 ± 10.7	19 ± 6	1463 ± 1 05	34	162
4i	26 ± 1	17 ± 2	536 ± 17	21	67
4j	26 ± 2	19 ± 2	≥ 6651	256	111
4k	18 ± 2	7 ± 2	≥ 6833	380	739
4l	18 ± 5	9 ± 1	≥ 6651	370	1662
4m	27 ± 1	11 ± 2	≥ 6644	246	1328
4n	18 ± 3	10 ± 2	≥ 2242	125	560
4o	20 ± 3	25 ± 2	≥ 6726	336	611
4p	41 ± 8	30 ± 4	≥ 6651	162	512
Primaquine	1.9[a]	1.5	233 ± 5	122	117

[a] Activity based on the IC_{50} levels <20 μM active; >20 and < 50 μM partially active; > 50 μM inactive. [b] Cytotoxicity not determined due to insufficient amount of the compounds.

Table S4- Overall results of the PQ-TZ activity as blood schizonticidal activity *in vitro* and in mice and as inhibitors of the *P. gallinaceum(Pg)* sporogony in *Aedes* and *Anopheles* mosquitoes infected with *Pg* and *P. berghei*, respectivelly.

Compound	*P. falciparum* Selectivity Index (anti-HRPII test)	*P. berghei* reduction of parasitemia*	%Inhibition of sporogony in *Aedes* and *Anopheles***	
4b	NT	NT	81	100
4c	72	42%	89	100
4d	142	NT	0	0
4e	925	NT	10	NT
4f	19	47%	20	NT
4g	83	NT	81	100
4h	76	58%	32	NT
4i	31	0%	0	NT
4j	111	27%	10	NT
4k	333	NT	2	NT
4l	250	NT	21	NT
4m	200	20%	90	100
4n	250	10%	25	45
4o	91	NT	96	100
4p	77	36%	25	0
Primaquine	117	100%	100	100

*Reduction of parasitemia on day five of malaria infection, after 3 days of treatment with 50mg/kg by oral rout per day. NT= Not tested . ** *Aeds* in chickens with *P. gallinaceum* and *Anopheles* fed in mice with *P. berghei*, after drug treatment by gave.

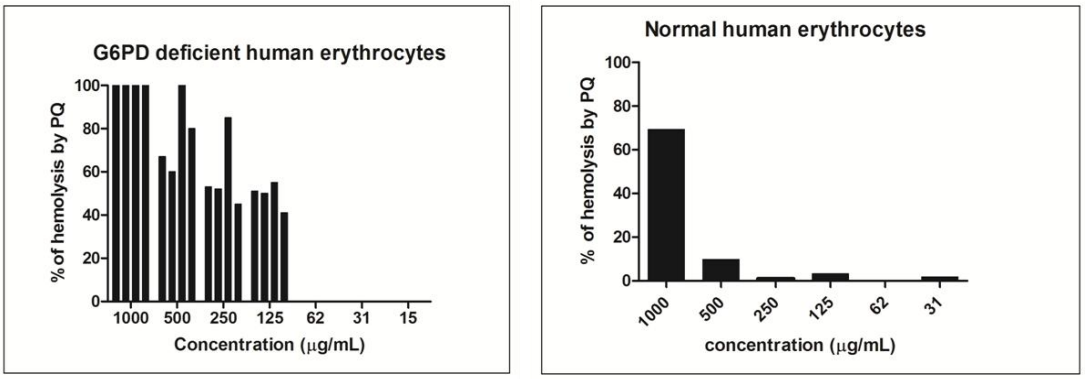

Figure S1- Percentage of hemolysis *in vitro* caused by primaquine to human erythrocytes obtained from four individuals with G6PD enzyme deficiency (left figure) or to normal human erythrocytes (right figure). Unlike PQ, none of the 15 PQ-TZ caused hemolysis even with the higher doses.

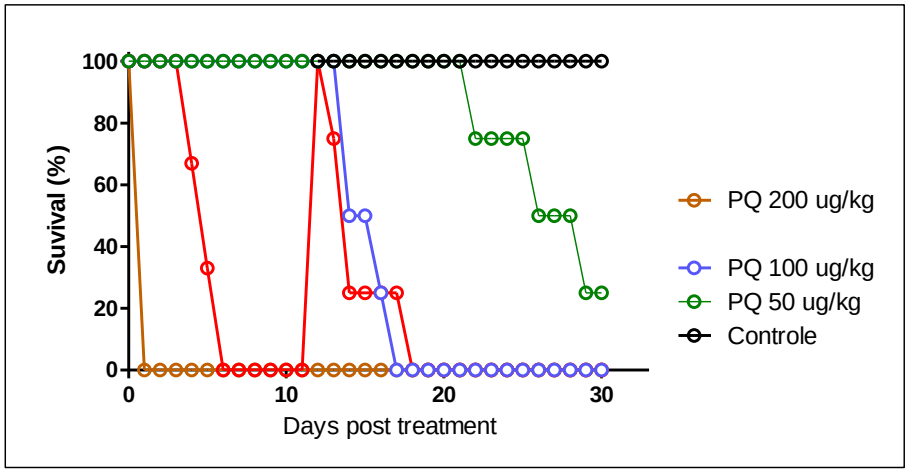

Figure S2- Percentage of mice survival treated with different cumulative doses of primaquine.

ANEXO IV

TERMO DE CONSENTIMENTO

Prezado Sr./Sra.

Neste momento, você está sendo convidado (a) a participar desta pesquisa por estar com malária provocada por *Plasmodium vivax ou P. falciparum* e este texto explica o que será realizado. Você receberá todas as informações que desejar, por favor, sinta-se a vontade em fazer qualquer pergunta.

O objetivo desse estudo é avaliar a atividade de fármacos, o que pode contribuir, no futuro, para o desenvolvimento de novos antimaláricos contra essa doença. Antes de assinar esse documento você deverá tomar conhecimento de:

(1) O seu sangue será coletado (5ml);
(2) O sangue coletado será usado para os ensaios de atividade bem como para obtenção de DNA, o mesmo é o material genético dos parasitos da malária que se encontram no seu sangue e que são necessários aos estudos relacionados a esse projeto de malária;
(3) Você pode recusar participar do estudo;
(4) Confidencialidade: A sua identidade será mantida em sigilo em qualquer publicação futura resultante desse estudo;
(5) Custos á você: Sua participação neste estudo não ocasionará em custos adicionais para você. Você não receberá nenhum pagamento para participar.
(6) Riscos: Os riscos deste estudo são, basicamente aqueles que fazem parte dos procedimentos médicos como, por exemplo, o incômodo durante a coleta do sangue. Esses riscos são muito pequenos e podem incluir tonturas, dor, manchas arroxeadas ou infecção. Você pode esperar uma pequena dor de picada que pode durar por alguns segundos quando furar o dedo ou o braço para coletar sangue. Precisamos de um total de 10 ml de sangue (cerca de meia colher de sopa) para o estudo completo. Esta quantidade de sangue não é suficiente para causar anemia.
(7) Benefícios: Este projeto não trará nenhum beneficio imediato a você, no entanto futuramente novos medicamentos mais efetivos contra a doença poderão ser desenvolvidos.

Contato para qualquer tipo de esclarecimento:

Pesquisadores responsáveis:

Antoniana U Krettli: (031) 33497823

Dhelio Batista Pereira: (69) 3216-5442

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CEP: Comitê de ética e pesquisa

Endereço e telefone institucional do Comitê de Ética - CPqRR: Av. Augusto de Lima, 1715 – Barro Preto - Belo Horizonte (Cep: 30.190-002)

Secretária: Bernadete Patrícia Santos –TeleFax: (31) 3349- 7825 e-mail: bernadet@cpqrr.fiocruz.br

Eu declaro que concordo com este consentimento por escrito e que decidi participar desse projeto de pesquisa após ter sido completamente esclarecido sobre o mesmo. Eu estou ciente que receberei uma cópia desse consentimento escrito e que uma segunda cópia será registrada nos arquivos do Centro de Pesquisas René Rachou.

Nome:

Endereço:

Assinatura:

Investigador:

Assinatura:

Data e Local:

ARTIGOS PUBLICADOS EM COLABORAÇÃO

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Short communication

Synthesis and antimalarial activity of thioetherhydroxyethylsulfonamides, potential aspartyl protease inhibitors, Part 3

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ABSTRACT

A series of novel thioetherhydroxyethylsulfonamide derivatives has been synthesized from the coupling of intermediates 3-amino-4-phenyl-1-thioetherazine-butan-2-oles **6,7** with arenesulfonyl chlorides in good yields. Characterizations of products were achieved by NMR techniques and specifically for compound **8e** by X-ray crystallography. Preliminary results of antimalarial activity *in vitro* against the *Plasmodium falciparum* W2 clone (chloroquine resistant and mefloquine sensitive) showed moderate activity for hydroxyethylsulfonamide **8f**. In addition, none of the compounds tested showed cytotoxicity at high concentration tested against HepG2 and BGM cell lines.

Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. *107*(7): 831-845, November 2012 831

New approaches in antimalarial drug discovery and development - A Review

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Malaria remains a major world health problem following the emergence and spread of Plasmodium falciparum *that is resistant to the majority of antimalarial drugs. This problem has since been aggravated by a decreased sensitivity of* Plasmodium vivax *to chloroquine. This review discusses strategies for evaluating the antimalarial activity of new compounds in vitro and in animal models ranging from conventional tests to the latest high-throughput screening technologies. Antimalarial discovery approaches include the following: the discovery of antimalarials from natural sources, chemical modifications of existing antimalarials, the development of hybrid compounds, testing of commercially available drugs that have been approved for human use for other diseases and molecular modelling using virtual screening technology and docking. Using these approaches, thousands of new drugs with known molecular specificity and active against* P. falciparum *have been selected. The inhibition of haemozoin formation in vitro, an indirect test that does not require* P. falciparum *cultures, has been described and this test is believed to improve antimalarial drug discovery. Clinical trials conducted with new funds from international agencies and the participation of several industries committed to the eradication of malaria should accelerate the discovery of drugs that are as effective as artemisinin derivatives, thus providing new hope for the control of malaria.*

Key words: human malaria - *Plasmodium falciparum* - antimalarials - drug screening - chloroquine - artemisinin - drug resistance

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PLOS ONE

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

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PLOS ONE

Evaluation of Antimalarial Activity and Toxicity of a New Primaquine Prodrug

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Chierrito et al. Malaria Journal 2014, 13:142
http://www.malariajournal.com/content/13/1/142

MALARIA JOURNAL

RESEARCH Open Access

Anti-malarial activity of indole alkaloids isolated from *Aspidosperma olivaceum*

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Abstract

Background: Several species of *Aspidosperma* (Apocynaceae) are used as treatments for human diseases in the tropics. *Aspidosperma olivaceum*, which is used to treat fevers in some regions of Brazil, contains the monoterpenoid indole alkaloids (MIAs) aspidoscarpine, uleine, apparicine, and N-methyl-tetrahydrolivacine. Using bio-guided fractionation and cytotoxicity testing in a human hepatoma cell line, several plant fractions and compounds purified from the bark and leaves of the plant were characterized for specific therapeutic activity (and selectivity index, SI) *in vitro* against the blood forms of *Plasmodium falciparum*.

Methods: The activity of *A. olivaceum* extracts, fractions, and isolated compounds was evaluated against chloroquine (CQ)-resistant *P. falciparum* blood parasites by *in vitro* testing with radiolabelled [^{3}H]-hypoxanthine and a monoclonal anti-histidine-rich protein (HRPII) antibody. The cytotoxicity of these fractions and compounds was evaluated in a human hepatoma cell line using a 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay, and the SI was calculated as the ratio between the toxicity and activity. Two leaf fractions were tested in mice with *Plasmodium berghei*.

Results: All six fractions from the bark and leaf extracts were active *in vitro* at low doses ($IC_{50} < 5.0$ μg/mL) using the anti-HRPII test, and only two (the neutral and basic bark fractions) were toxic to a human cell line (HepG2). The most promising fractions were the crude leaf extract and its basic residue, which had SIs above 50. Among the four pure compounds evaluated, aspidoscarpine in the bark and leaf extracts showed the highest SI at 5.6; this compound, therefore, represents a possible anti-malarial drug that requires further study. The acidic leaf fraction administered by gavage to mice with blood-induced malaria was also active.

Conclusion: Using a bio-monitoring approach, it was possible to attribute the anti-*P. falciparum* activity of *A. olivaceum* to aspidoscarpine and, to a lesser extent, *N*-methyl-tetrahydrolivacine; other isolated MIA molecules were active but had lower SIs due to their higher toxicities. These results stood in contrast to previous work in which the anti-malarial activity of other *Aspidosperma* species was attributed to uleine.

Keywords: *Aspidosperma olivaceum*, Indole alkaloids, Aspidoscarpine, Medicinal plants, Antimalarial, *Plasmodium falciparum*

Chem Biol Drug Des 2014; **84:** 325–332
Research Article

New Compounds Hybrids 1*H*-1,2,3-Triazole-Quinoline Against *Plasmodium falciparum*

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Hindawi

Research Article

Antimalarial Activity of *Cocos nucifera* Husk Fibre: Further Studies

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In this study, the antimalarial and toxicity potentials of husk fibre extracts of five Nigerian varieties of *Cocos nucifera* were evaluated *in vitro*. The only active extract fraction, West African Tall (WAT) ethyl acetate extract fraction, was then evaluated for its phytochemical constituents, antimalarial and toxicity potentials at varying doses (31.25–500 mg/kg body weight) using various organ function indices. The results revealed that WAT ethyl acetate extract fraction (WATEAEF) contained alkaloids, tannins, and flavonoids and was active against *Plasmodium falciparum* W2 strain maintained in continuous culture, with a selectivity index of 30.3. The same extract fraction was active *in vivo* against *Plasmodium berghei* NK65, causing more than 50% reduction in parasitaemia on days 4 and 6 after inoculation at various doses administered. WATEAEF did not significantly alter ($P > 0.05$) function indices of the liver and cardiovascular system at all doses administered but significantly increased ($P < 0.05$) plasma creatinine concentration at 250 and 500 mg/Kg body weight compared to controls. The results of this study suggest that WATEAEF possesses antimalarial activity and may not adversely affect normal liver function nor predispose subjects to cardiovascular diseases but may impair normal kidney function at higher doses. Further studies are underway to isolate the active principles.

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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

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Article

New Trifluoromethyl Triazolopyrimidines as Anti-*Plasmodium falciparum* Agents

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ARTICLE

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Novel 1,2,3-Triazole Derivatives for Use against *Mycobacterium tuberculosis* H37Rv (ATCC 27294) Strain

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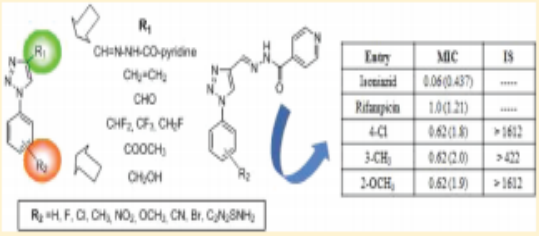
ABSTRACT: The purpose of this study was to prepare various 4-substituted *N*-phenyl-1,2,3-triazole derivatives using click chemistry. The derivatives were screened in vitro for antimicrobial activity against *Mycobacterium tuberculosis* strain H37Rv (ATCC 27294) using the Alamar Blue susceptibility test. The activity was expressed as the minimum inhibitory concentration (MIC) in μg/mL (μM). Derivatives of isoniazid (INH), (*E*)-*N*′-[(1-aryl)-1*H*-1,2,3-triazole-4-yl)methylene] isonicotinoyl hydrazides, exhibited significant activity with MIC values ranging from 2.5 to 0.62 μg/mL. In addition, they displayed low cytotoxicity against liver cells (hepatoma HepG2) and kidney cells (BGM), thereby providing a high therapeutic index. The results demonstrated the potential and importance of developing new INH derivatives to treat mycobacterial infections.



Entry	MIC	IS
Isoniazid	0.06 (0.437)	.....
Rifampicin	1.0 (1.21)	.....
4-Cl	0.62 (1.8)	>1612
3-CH_3	0.62 (2.0)	>422
2-OCH_3	0.62 (1.9)	>1612

TEXTOS EM JORNAIS DE NOTÍCIAS/REVISTAS

Antoniana Ursine Krettli[1,2] Nicolli Bellotti de Souza[2], Anna Caroline Campos Aguiar[1], Katia Maria Chaves[3] e Pedro Luiz Tauil[4] . 2013. **Casos fatais de malária fora da área endêmica - evitar é possível** Ciência Hoje.