FUNDAÇÃO OSWALDO CRUZ

CENTRO DE PESQUISAS GONÇALO MONIZ

FIOCRUZ

Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa

TESE DE DOUTORADO

AVALIAÇÃO DA ATIVIDADE ANTIMALÁRICA DE SUBSTÂNCIAS OBTIDAS DE ESPÉCIES VEGETAIS NATIVAS OU ENDÊMICAS DO SEMI-ÁRIDO BRASILEIRO E DERIVADOS SINTÉTICOS

MATHEUS SANTOS DE SÁ

Salvador – Brasil
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Tese apresentada ao Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa para a obtenção do grau de Doutor.

Salvador – Brasil
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À minha família, pelo amor e dedicação

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A virtude é o sublime impulso da alma imortal, já desperta, apontando à criatura a prática do bem

RESUMO

A malária é uma das mais importantes infecções parasitárias de seres humanos devido à alta morbidade e mortalidade atribuídas a esta doença, que constitui uma ameaça para mais de dois bilhões de pessoas vivendo nas áreas de alta incidência. O *Plasmodium falciparum*, um dos agentes causadores da malária, apresenta alta capacidade de adaptação por mutação e pode ser resistente a vários tipos de drogas antimaláricas já disponíveis, como a cloroquina, o que torna importante a busca de novos antimaláricos. A região do semi-árido brasileiro abrange cerca de 11,5% do território nacional, e possui o bioma menos estudado em relação à flora e fauna, e um dos que tem sofrido maior degradação pelo uso desordenado e predatório nos últimos 400 anos. Tendo em vista o potencial farmacológico dos produtos naturais, o objetivo desse trabalho foi avaliar a atividade antimalárica de substâncias puras extraídas de espécies vegetais nativas ou endêmicas do semi-árido brasileiro e derivados sintéticos. A partir de uma biblioteca de 160 substâncias triadas para atividade antimalárica, foram selecionadas duas classes de compostos para avaliações *in vitro* e *in vivo*: o ácido betulínico e derivados, bem como o lapachol e derivados. Foi selecionada ainda uma terceira classe de moléculas, as fisalinas, utilizando o método do *Similarity Ensemble Approach* (SEA), que previu a ação antimalárica dessas substâncias. Dentre os derivados do ácido betulínico testados, o acetato do ácido betulínico apresentou a maior potência farmacológica *in vitro* quando comparado com os outros derivados, e foi ativo *in vivo*. A atividade antimalárica das fisalinas foi confirmada em ensaios *in vitro*. Ao serem analisadas *in vivo*, as fisalinas F e D apresentaram resultados opostos (exacerbação e proteção contra a infecção, respectivamente), possivelmente devido à atividade imunossupressora da fisalina F e ausente na fisalina D. A análise do lapachol e seus derivados iniciou-se através de estudos in silico por *Quantitative Structure-Activity Relationship* (QSAR), que indicaram ser o isolacet o derivado com maior atividade, o que foi confirmado por ensaios *in vitro*. A atividade antimalárica do isolacet foi confirmada *in vivo*, sendo ainda realizados estudos de *Docking* desta molécula com a falcipaína 2 de *P. falciparum*, que indicaram ser esta cisteíno-protease um possível alvo do isolacet. Nossos resultados indicam o potencial antimalárico de compostos isolados a partir de plantas do semi-árido e demonstram a importância da associação de várias abordagens para entendimento dos mecanismos de ação de moléculas com atividade farmacológica.

Palavras - chave: Malária, acetato do ácido betulínico, isolacet, fisalinas e *Plasmodium falciparum.*

ABSTRACT

Malaria is one of the most important parasitic infections of humans due to the high morbidity and mortality attributed to this disease, which threatens to over two billion people living in areas with high incidence. *Plasmodium falciparum*, a causative agent of malaria, has a high capacity to adapt by mutation and may be resistant to various antimalarial drugs already available, such as chloroquine, which makes it important to search for new antimalarials. The Brazilian semi-arid region cover about 11.5% of the country, and the biome has been less studied in relation to flora and fauna, and one who has suffered further degradation and predation by the inordinate use in the last 400 years. Given the pharmacological potential of natural products, the aim of this study was to evaluate the antimalarial activity of pure compounds extracted from native or endemic plant species of arid and semi-synthetic derivatives. From a library of 160 substances screened for antimalarial activity, we selected two classes of compounds for evaluation *in vitro* and *in vivo*: The betulinic acid and derivatives, as well as lapachol and derivatives. It was also selected a third class of molecules, physalins using the method of Similarity Ensemble Approach (SEA), who predicted the antimalarial action of these substances. Among the tested derivatives of betulinic acid, betulinic acid acetate showed the highest pharmacological potency *in vitro* when compared with other derivatives, and was active *in vivo*. The antimalarial activity of physalins was confirmed *in vitro* assays. When analyzed *in vivo* the physalins F and D had the opposite results (exacerbation and protection against infection, respectively), possibly due to the immunosuppressive activity of physalin F and absent in physalin D. The analysis of lapachol and its derivatives was initiated through studies in silico by *Quantitative Structure-Activity Relationship* (QSAR), which indicated that the isolacet the derivative with greater activity, which was confirmed by *in vitro* assays. The antimalarial activity of isolacet was confirmed *in vivo*, and further studies of this molecule by *Docking* with falcipain 2 *P. falciparum*, which indicated that this cysteine protease is a possible isolacet target. Our results indicate the potential antimalarial compounds isolated from plants of the semi-arid and demonstrate the importance of the combination of various approaches to understanding the mechanisms of action of molecules with pharmacological activity.

Key words: Malaria, betulinic acid acetate, isolacet, phisalins and *Plasmodium falciparum*.

LISTA DE ABREVIATURAS

BA – Ácido betulínico

BAA – Acetato do ácido betulínico

BAME – Éster metílico do ácido betulínico

BAMEA – Acetato do éster metílico do ácido betulinico

BOA – Ácido betulônico

CPqGM – Centro de Pesquisas Gonçalo Moniz

DDT - Dicloro-difenil-tricloroetano

DMSO - Dimethyl sulfoxide

FIOCRUZ – Fundação Oswaldo Cruz

G6PD – Glicose 6 – fosfato desidrogenase

HA - Hydrogen bond acceptors

IC_{50} – Concentração inibitória para 50% da população exposta

ICAM-1 - Molécula de adesão intercelular-1

IPA – Incidência parasitária anual

ISOLACET – Acetil isolapachol

LC_{50} - Concentração letal para 50% da população exposta

LPS - Lipopolissacarídeo

MDM - Molegro data modeler

MHC - Complexo de histocompatibilidade principal

MR - Multiple regression

OMS - Organização mundial de saúde

OPAS - Organização pan-americana de saúde

PfEMP-1 - Proteína de Membrana 1

PIACM - Plano de intensificação das ações de controle da malária

PNCM - Programa nacional de controle da malária

QSAR – Quantitative structure–activity relationship

Rot - Rotatable bonds

SEA - Similarity ensemble approach

SI - Selectivity index

Tc - Coeficiente de Tanimoto

TCA - Terapias combinadas da artemisinina

TNF-α – Fator de necrose tumoral alfa

VCAM-1 - Molécula de adesão celular vascular-1

VS – Virtual Screening

WHO – World health organization

SUMÁRIO

1. INTRODUÇÃO

1.1 A malária

A malária é uma doença infecciosa, de evolução crônica, com manifestações episódicas de caráter agudo, transmitida por mosquitos do gênero *Anopheles*. Considerada uma doença negligenciada (Chirac, 2006), a malária talvez seja uma das mais antigas doenças conhecidas e ainda hoje causa grande impacto na história da humanidade. Por séculos, a malária interferiu no desenvolvimento econômico de diversos países e ainda continua sendo um enorme problema social, econômico e, principalmente, de saúde pública em países de clima tropical**.** Com o início da agricultura e com o crescimento populacional, bem como com a destruição dos ambientes naturais do mosquito, as populações de *Anopheles* aumentaram, elevando, assim, o risco de transmissão da doença.

Nos tempos antigos, a malária era relacionada aos vapores venenosos dos pântanos ou águas paradas. Devido a isso, o nome muito frequentemente utilizado para a doença foi mal´aria e depois malária ou Paludismo. O termo malária (mala de "mal" e aria de "ar") foi utilizado por italianos para a causa de febres intermitentes associadas com a exposição ao ar do pântano (Dias, 1901).

A malária humana é causada por parasitas classificados no filo Protozoa, classe Sporozoea, família Plasmodiidae, gênero *Plasmodium*, ao qual pertecem quatro espécies: *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae* e *Plasmodium ovale* (Mali, 2008). *Plasmodium knowlesi*, um parasita encontrado em macacos asiáticos, tem sido estudado com relação às infecções em humanos, incluindo algumas mortes no sudeste asiático (Figtree, 2010). Destas cinco espécies, o *P. vivax* é o mais amplamente distribuído pelas zonas tropicais e subtropicais do mundo (Lacerda, 2007). O *P. falciparum*, comparado às outras espécies, causa maior morbidade e mortalidade (Wright, 2010). E, sob o ponto de vista terapêutico, vem se tornando um problema cada vez maior devido ao aumento da resistência aos medicamentos antimaláricos (WHO, 2010).

1.1.2 O ciclo evolutivo do *Plasmodium sp*

O ciclo evoluvidos dos plasmódios inicia-se com a inoculação da forma infectante, esporozoítas, através da saliva da fêmea do mosquito introduzida no sangue durante o repasto sanguíneo. A seguir, os esporozoítas permanecem na corrente sanguínea durante alguns

minutos e rapidamente penetram nas células parenquimatosas do fígado, os hepatócitos, dando início ao ciclo pré-eritrocítico ou esquizogonia tecidual, que dura seis dias para o *P. falciparum*, oito dias para o *P. vivax* e semanas para o *P. malariae*. Os esquizontes teciduais, uma vez maduros, rompem-se e libertam formas evolutivas denominadas merozoítos, nos capilares intra-hepáticos (Marques, 2001). Nas infecções devidas ao *P. falciparum* e ao *P. malariae*, os esquizontes teciduais se rompem todos ao mesmo tempo e nenhum parasita persiste nos hepatócitos (Grimberg, 2008). No caso do *P. ovale* e do *P. vivax*, há o surgimento de algumas formas exoeritrocíticas, denominadas hipnozoítos, formas latentes aparentemente responsáveis pelas recaídas da doença meses ou anos após a infecção. Este estágio do parasita não ocorre no *P. falciparum* e no *P. malariae* (Baird, 2009).

Os merozoítos liberados nos capilares hepáticos invadem os eritrócitos por mecanismos variados e complexos. As hemácias contêm estruturas na sua superfície denominadas glicoforinas, as quais são compostas de ácido siálico e são subdividas em glicoforina A, B e C. Essas estruturas desenvolvem papel importante na invasão dos eritrócitos pelo *Plasmodium*, ou seja, podem ser receptoras para o *Plasmodium falciparum*. Entretanto, algumas pessoas com malária exibem invasão dos eritrócitos independente das glicoforinas (Spadafora, 2010). No caso do *Plasmodium vivax*, o fator Duffy, constitui o receptor específico necessário para a invasão dos eritrócitos (Cutbush, 1950 e Mercereau-Puijalon, 2003). Desse modo, a baixa incidência de malária por *P. vivax* em muitas regiões da África Tropical pode ser explicada pelo fato de que a maioria dos indivíduos residentes naquela região não possui esta estrutura nos eritrócitos (Escalante, 2005).

Uma vez no interior dos eritrócitos, os merozoítos transformam-se em trofozoítos jovens, conhecidos como forma em anel, que crescem e em determinado momento são convertidos em esquizontes, os quais originam um número variável de novos merozoítos, que iniciarão um novo ciclo (Marques, 2001). A fase eritrocítica assexuada do ciclo de vida do *Plasmodium falciparum* produz todos os sintomas clínicos e processos patológicos associados à malária (Spadafora, 2010). Esta fase é chamada de esquizogonia eritrocítica, onde os parasitas metabolizam a hemoglobina, originando um produto denominado ferriprotoporfirina IX, que é tóxico para o parasita. Por este motivo, o referido produto é metabolizado em um composto inerte chamado pigmento malárico ou hemozoína (Pasternack, 2010).

A periodicidade da esquizogonia sanguínea é variável, de acordo com a espécie de *Plasmodium*, sendo 48 horas para *P. vivax* e *P. ovale*, 72 horas para o *P. malariae* e 36 a 48 horas para o *P. falciparum*. Após três a quinze dias do início dos sintomas clínicos, alguns merozoítos se diferenciam em gametócitos femininos, também chamados de

macrogametócitos e masculinos ou microgametócitos**.** Quando a fêmea do *Anopheles* realiza o repasto sanguíneo em um indivíduo infectado, ocorre a ingestão dos gametócitos que irão iniciar o ciclo sexuado ou gametogônico no estômago do inseto. O microgametócito sofre exflagelação dando origem aos chamados microgametas, os quais podem se movimentar devido à ação dos flagelos. Os microgametas irão fecundar os macrogametas gerando o zigoto, os quais se tornam móveis e então chamados de oocinetos. Estas formas atravessam a parede do estômago transformando-se em corpúsculos esféricos denominados oocistos. Por último, os oocistos aumentam de tamanho de forma progressiva, produzindo um grande número de esporozoítos. Após a ruptura dos oocistos, os esporozoítos são liberados na cavidade celomática do inseto, migrando posteriormente para as glândulas salivares. Após essa última fase, as fêmeas do mosquito tornam-se infectantes e capazes inocular os esporozoítos em um novo repasto sanguíneo (Baker, 2010) (Figura 1).

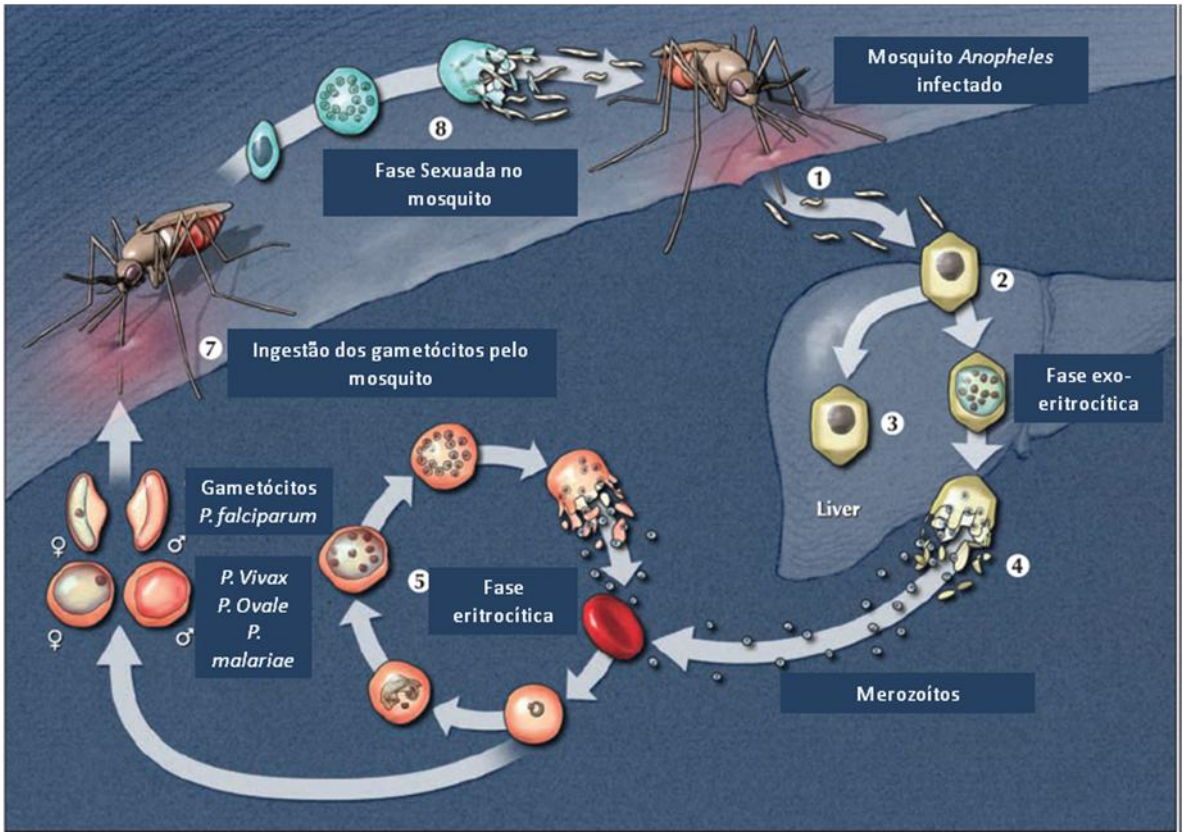

Figura 1. Ciclo evolutivo do *Plasmodium sp.* Fonte: Suh, K. e*t al*, 2004. Modificado por Sá, MS.

1.1.3 Epidemiologia da malária

Nos últimos anos, a doença vem adquirindo grande relevância, devido à extensa distribuição geográfica e por ser um fator limitante ao crescimento econômico em vastas áreas do mundo, particularmente nos países em desenvolvimento. A malária é uma das infecções parasitárias mais importantes, ocorrendo em mais de 100 países com uma estimativa de 3 bilhões de pessoas vivendo em áreas com risco de transmissão da doença (Pierce, 2009). Cerca de 200 a 400 milhões de pessoas adquirem pelo menos uma infecção por ano (Reiter, 2008) e no mundo morrem em média 1 a 2 milhões de pessoas devido a esta doença (Pierce, 2009). Casos de malária tem sido relatados em países da América Central e América do Sul, África, India, Sudeste Asiático e parte da Oceania (WHO, 2010). A figura 2 apresenta uma distribuição global da malária. Pelo fato de ser uma importante causa de morte em adultos e crianças, especialmente em países de clima tropical, o seu controle requer abordagens

integradas incluindo prevenção com a eliminação do vetor e tratamento com drogas antimaláricas efetivas.

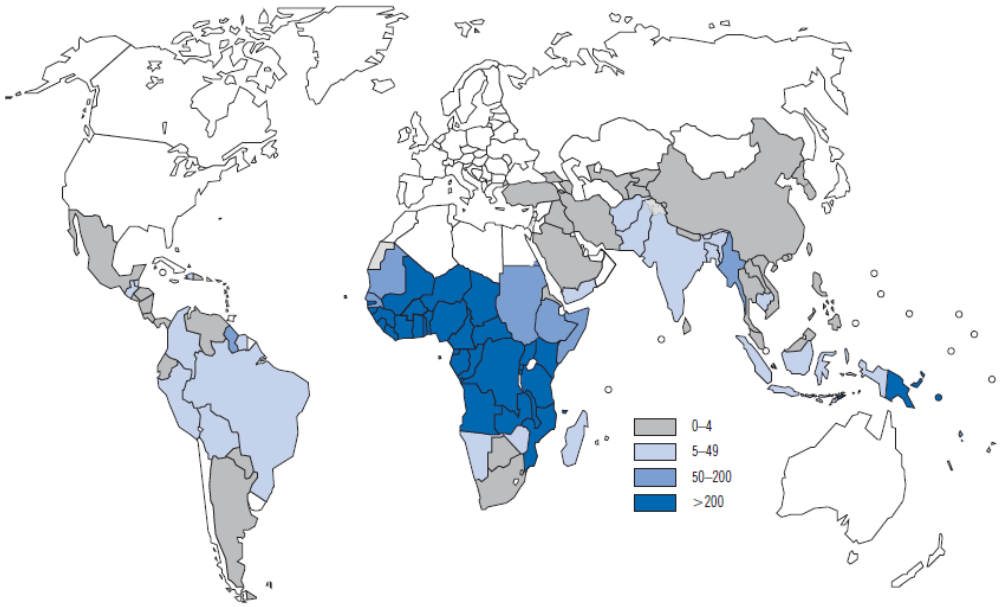

Figura 2. Distribuição da malária no mundo. Incidência estimada de número de casos por 1000 habitantes. Fonte: World Malaria Report 2008

No Brasil, a distribuição geográfica da malária é extensa. A área endêmica original, delimitada nos anos 50 por meio de estudos entomológicos e detecção de casos, abrangia cerca de 6,9 milhões de km^2 do território brasileiro. Na região amazônica verifica-se uma concentração crescente de casos de malária nos últimos anos. O número de casos ocorridos em 2009 foi superior a 300.000 pessoas em todo o país, sendo que, 99,9% foram transmitidos na Amazônia Legal, composta por nove estados: Acre, Amapá, Amazonas, Maranhão, Mato Grosso, Pará, Rondônia, Roraima e Tocantins. Esta região apresenta características geográficas e ecológicas favoráveis à interação do *Plasmodium* e do *Anopheles* com os fatores socio-econômicos, políticos e culturais, determinando um alto nível de endemicidade (Amazônia legal, 2010). Nos casos citados, a espécie causadora de quase 90% dos casos foi o *P. vivax*, talvez pelo fato de a transmissão do *P. falciparum* no Brasil ter diminuido nos últimos anos e o número de casos de infecção pelo *P. malariae* não seja significativo (Fontes, 2010). Segundo a Organização Mundial de Saúde, tal situação coloca o Brasil entre um dos 30 países com maior incidência de malária no mundo. Um fato relevante é que, embora a incidência de malária tenha diminuido nos últimos dois anos (Figura 3), houve um aumento na proporção de doentes entre mulheres e crianças abaixo de 10 anos (Oliveira-Ferreira, 2010).

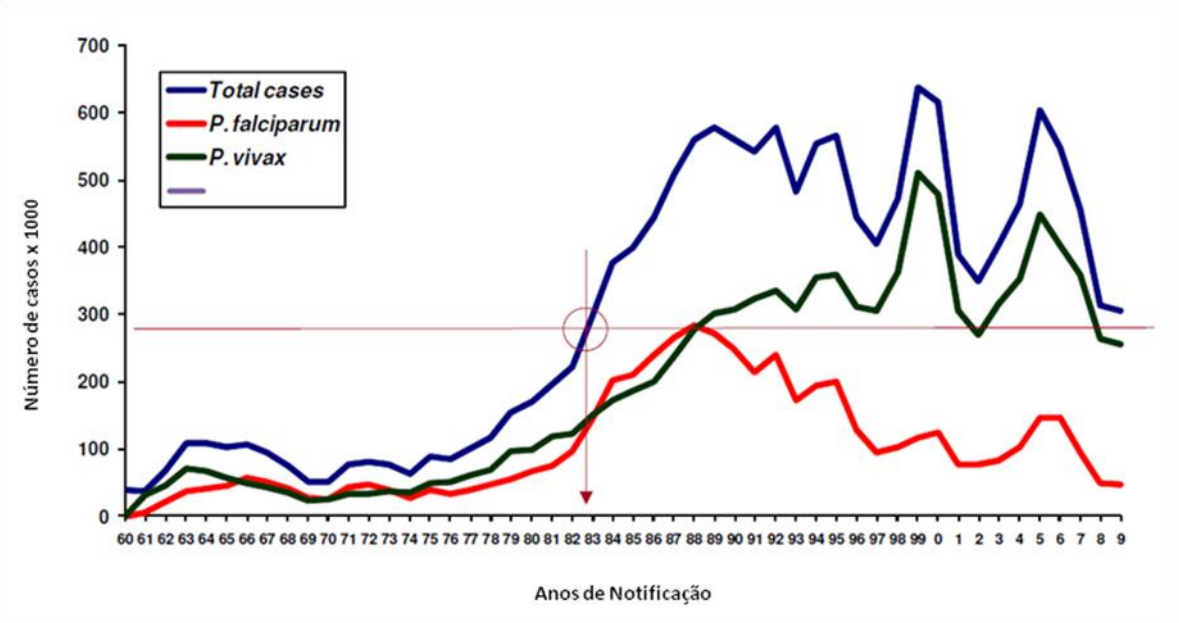

Figura 3. Número de casos de malária registrados na região Amazônica entre 1960 e 2009. Fonte: Oliveira-Ferreira, J. *et al*, 2010. Modificado por Sá, MS.

Na figura 4 pode-se observar a distribuição da malária no Brasil no ano de 2009 (Fontes, 2010).

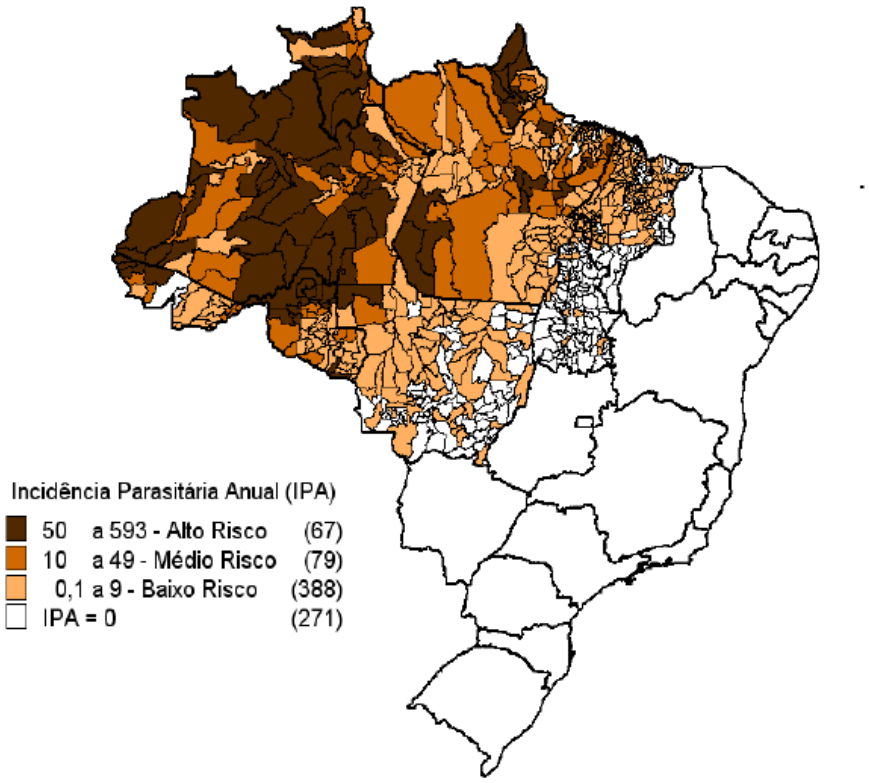

Figura 4. Destaque das áreas de risco para malária pelos diferentes níveis de incidência parasitária anual (IPA). Fonte: Fontes, 2010.

O IPA significa o número de exames positivos de malária, por mil habitantes, em determinado espaço geográfico, no ano considerado. Esse índice estima o risco de ocorrência de malária, numa determinada população em intervalo de tempo determinado, e a população exposta ao risco de adquirir a doença (Fontes, 2010).

1.1.4 Transmissão

A malária é o resultado da interação de fatores de natureza biológica, ambiental, socio-econômica e cultural. A transmissão é influenciada por características do ambiente, como temperatura e chuva; bem como pelos hábitos, condições de vida, moradia e trabalho das populações (Limongi, 2008). A transmissão da doença ocorre devido à picada do mosquito infectado, quando os esporozoítos são inoculados no hospedeiro vertebrado juntamente com proteínas salivares (Chertemp, 2010).

A transmissão da malária pode ocorrer também de forma acidental, devido à transfusão de sangue infectado (Temiz, 2008) ou através de contato com sangue infectado no âmbito ocupacional (Herwaldt, 2001). Apesar da dificuldade diagnóstica, a transmissão da malária pode ocorrer através da mistura do sangue materno com o fetal, ainda na fase intra-uterina (Marques, 1996), fato que vem sendo documentado de forma cada vez mais frequente (Lesi, 2010).

É conhecida hoje uma série de espécies de *Plasmodium* que infectam diversas espécies animais, dentre eles roedores, morcegos, macacos, entre outros (White, 2010). Acredita-se que nenhuma das espécies que infectam animais seja infectante para os humanos, exceto o *Plasmodium knowlesi*, que é encontrado em macacos asiáticos, mas pode estar relacionado a infecções em humanos (Figtree, 2010).

Existem características individuais dos humanos que podem conferir certa proteção contra a infecção pelo *Plasmodium*. Pode-se citar a ausência do antígeno Duffy nos eritrócitos, hemoglobinopatias e enzimopatias, como a deficiência de glicose 6-fosfato desidrogenase (G6PD). Essa característica hereditária acomete mais de 200 milhões de pessoas no mundo (Torres, 2005). A deficiência de G6PD aumenta o stress oxidativo nos eritrócitos e estes sofrem lise precocemente, gerando o que pode ser classificado como anemia hemolitica, a qual dificulta a invasão do eritrócito pelo *Plasmodium* (Leslie, 2010).

Os vetores da malária são os mosquitos do gênero *Anopheles*, cujas fêmeas são transmissoras naturais, devido à sua característica hematófaga (Bai, 2010). Existem cerca de

2500 espécies de mosquito conhecidas. No entanto, apenas 50 a 60 dessas espécies pertencentes ao gênero *Anopheles* são capazes de transmitir a malária. O *Anopheles gambiae* é a espécie mais comun na África (Barik, 2009). O *Anopheles darlingi* é o principal vetor de malária no Brasil. Vive em regiões tropicais e subtropicais, na América Central e do Sul em áreas de baixas altitudes, preferindo grandes corpos d'água onde tenha muito pouca ou nenhuma correnteza e florestas. Embora o *A. darlingi* esteja presente em 80% do país, a incidência de malária no Brasil ocorre quase que exclusivamente na região amazônica (Oliveira-Ferreira, 2010).

1.1.5 Patogênese da malária

A patogênese da malária é melhor compreendida quando a infecção é pelo *P. falciparum*, parasita responsável pela forma mais grave da doença. No caso da infecção por *P. falciparum*, alguns grupos de pacientes têm maior chance de desenvolver malária grave, dentre eles as crianças, as gestantes e os pacientes não imunes. A doença pode ser caracterizada por um paroxismo febril o qual se caracteriza por calafrios, acompanhados de mal estar, cefaléia, dores musculares e articulares generalizadas e manifestações digestivas. Os esporozoítas, ao serem inoculados no sangue do indivíduo, dão início ao ciclo evolutivo do *Plasmodium.* O ciclo exoeritrocítico no fígado e a presença dos gametócitos geram alterações fisiopatológicas mínimas, quando comparadas ao ciclo eritrocítico (Aikawa, 1990). Este apresenta fisiopatologia bastante complexa a qual está associada a uma série de eventos, como o bloqueio do fluxo sanguíneo capilar resultante do fenômeno de sequestro eritrocitário. Este fato contribui para oclusão microvascular, degeneração metabólica e acidose, a qual leva às manifestações da malária grave (Guinovart, 2006).

Em adição, uma resposta exacerbada, via produção de citocinas como o TNF-α, contra produtos liberados durante a ruptura dos esquizontes pode contribuir para os sinais clínicos da doença (Clark, 2009). Os níveis séricos de TNF-α estão bastante elevados em crianças e adultos com formas graves, incluindo as cerebrais, da infecção pelo *P. falciparum* (Maude, 2009). O TNF-α é capaz de produzir febre, hipoglicemia sequestro de leucócitos nos vasos pulmonares e potencializar o fenômeno de citoaderência das hemácias parasitadas ao endotélio vascular (Clark, 2010).

A sequestração de eritrócitos infectados na microvasculatura está associada à malária grave por *P. falciparum* como resultado da citoaderência das células vermelhas às células endoteliais (Suh, 2004). Estas células expressam uma série de receptores que servem de

ligantes para a adesão das células infectadas. Entre estes receptores, pode-se citar a trombospondina (Hegge, 2010), CD36 (Erdman, 2009), ICAM-1 (Molécula de adesão intercelular-1), VCAM-1 (Molécula de adesão celular vascular-1) (Cojean, 2008), selectinas P e E, integrinas, dentre outros.

A adesão das células parasitadas ao endotélio também depende de proteínas expressas na superfície das hemácias parasitadas em estruturas conhecidas como knobs, as quais, em adição, promovem a adesão das hemácias infectadas a eritrócitos não infectados (Chakravorty, 2008). Esta citoaderência constitui a base da patologia microvascular da forma grave da malária e é observada em diversos órgãos como o coração, intestino, pulmões e também o cérebro, gerando anóxia. A citoaderência é responsável por eventos como a ausência de esquizontes e merozoítos do *P. falciparum* no sangue periféfico, pois estes se encontram sequestrados no endotélio vascular (Suh, 2004).

A PfEMP-1 (proteína de membrana 1) é uma proteína transmembrana expressa no eritrócito infectado pelo *P. falciparum* e é responsável pela citoaderência das referidas células (Chakravorty, 2008). Os eritrócitos infectados são ainda capazes de gerar formações denominadas “rosetas” resultantes da adesão dos eritrócitos infectados com formas maduras do parasita a eritrócitos não infectados. Geralmente estas rosetas exibem a interação entre algumas hemácias não infectadas com uma ou duas hemácias parasitadas, embora este número possa ser maior, como pode-se observar na figura 5 (Lowe, 1998).

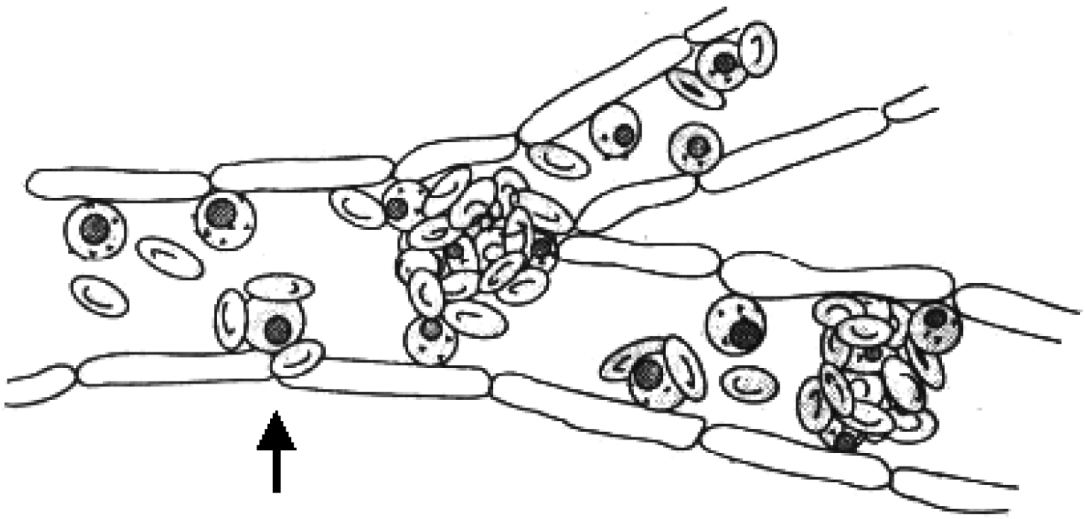

Figura 5. Representação esquemática das rosetas e a citoaderência levando a obstrução do vaso sanguíneo. Fonte: Kirchgatter, K. *et al*, 2005.

Na malária causada pelo *P. falciparum*, as rosetas parecem recrudescer a obstrução do fluxo sanguíneo, fato comum em pacientes com malária severa. As rosetas parecem proteger as células infectadas protegendo, desse modo, da fagocitose, um dos principais mecanismos da imunidade antiparasitária.

A maior parte do ciclo evolutivo do *Plasmodium*, no homem, ocorre no eritrócito. Esta célula não possui diferenças fenotípicas entre os estágios de maturação, pois não possui mecanismos de síntese, tráfego e expressão de proteínas de superfície como, por exemplo, as do MHC (Complexo de Histocompatibilidade Principal) (classe I ou II). Assim, o eritrócito é o ambiente ideal para a sobrevida do parasita (Esposito, 2010). No entanto, a síntese de proteínas do *Plasmodium* gera moléculas que atravessam a membrana plasmática do parasita e são exibidas na superfície da hemácia. Estas proteínas medeiam a adesão do eritrócito infectado ao endotélio vascular (Joergensen, 2010). Este fato impede que os eritrócitos infectados cheguem ao baço, órgão linfóide responsável pela destruição das células infectadas.

A infecção pelo *Plasmodium falciparum* causa uma série de sintomas clínicos que podem variar desde sinais de gripe moderada (especialmente em pacientes imunes vivendo em áreas endêmicas) até complicações raras de doença grave. Estes sintomas podem se manifestar com diferentes síndromes, como anemia grave, complicações respiratórias,

acidose, falência de múltiplos órgãos ou até mesmo a malária cerebral (WHO, 2010). De todas as complicações possíveis, a malária cerebral é a mais estudada. Segundo a Organização Mundial de Saúde, a malária cerebral pode ser definida como uma encefalopatia difusa causando um conjunto de problemas geradores de distúrbios neurológicos. Entretanto, esses distúrbios não são exclusivos da malária cerebral, uma vez que problemas cognitivos podem estar associados à anemia severa, ou até mesmo o choque e acidose podem estar associados à doença não complicada.

Os sintomas relacionados à malária cerebral variam desde confusão mental até o coma. Outro fato relevante é que a doença pode ser fatal mesmo quando o indivíduo é tratado ativamente com antimaláricos. A mortalidade na malária cerebral ainda permanece elevada, podendo atingir de 20 a 50% dos casos. Alguns fatores, como o nível do coma, hipoglicemia, elevados níveis de uréia, idade maior que dois anos e a predominância de formas maduras do parasita no sangue periférico são indicadores de maior letalidade desta complicação. No caso de o paciente sobreviver às complicações geradas pela malária cerebral, sequelas neurológicas como perda da fala, ataxia ou perda visual podem ocorrer.

A adesão das células parasitadas ao endotélio vascular é mediada por moléculas presentes na superfície das células infectadas. O sequestro parasitário dentro da microvasculatura cerebral pode ocorrer devido a eventos que causam redução de deformidade da célula. Esta redução de deformidade pode impedir o acesso das células infectadas ao parênquima cerebral. A adesão de outras células não infectadas, como plaquetas ou leucócitos, pode levar à formação de micro-agregados que diminuem o fluxo sanguíneo no cérebro.

1.1.6 Controle da malária

Durante a Segunda Guerra Mundial, utilizou-se em grande quantidade o DDT (dicloro-difenil-tricloroetano) contra o mosquito transmissor da malária. Este inseticida foi uma ferramenta relevante para o controle da malária naquela época. Entretanto, devido ao seu efeito tóxico à longo prazo, o DDT teve seu uso abolido (Rocha, 2006). Diversas estratégias foram elaboradas em nível mundial para o controle da malária. Em 1992, a OPAS (Organização Pan-Americana de Saúde) concentrou-se no combate aos mosquitos. Posteriormente, outras formas de controle foram propostas, como o diagnóstico precoce, tratamento rápido e eficiente, prevenção, dentre outros. No Brasil, em 1999, surgiu o PIACM (Plano de Intensificação das Ações de Controle da Malária). Subsequentemente, foi criado o

PNCM (Programa Nacional de Controle da Malária) com o objetivo de fornecer diretrizes para controle definitivo da doença, reduzindo a letalidade e gravidade dos casos, bem como deduzindo a incidência da doença, eliminando a transmissão em áreas urbanas, dentre outros (Ministério da Saúde, 2003).

1.1.6.1 Os medicamentos antimaláricos

O tratamento da malária objetiva atingir o *Plasmodium* em alguns pontos importantes do seu ciclo biológico. Os medicamentos antimaláricos podem interferir na esquizogonia sanguínea, fase responsável pelas manifestações clínicas da infecção; destruir as formas teciduais latentes (hipnozoítas das espécies *P. vivax* e *P. ovale*); e interromper a transmissão, impedindo o desenvolvimento das formas sexuadas (gametócitos). Desse modo, os antimaláricos são classificados como: fármacos utilizados para a profilaxia casual, fármacos utilizados para evitar recidivas, esquizonticidas sanguíneos utilizados para a cura clínica e gametocidas (Fontes, 2010).

Os antimaláricos utilizados para a profilaxia casual atuam nas formas tissulares primárias dos parasitas no fígado, que iniciariam o estágio eritrocítico em um prazo de, aproximadamente, 30 dias. Com a utilização desses medicamentos evita-se a invasão dos eritrócitos e a posterior transmissão da infecção. O proguanil é o protótipo desta classe. Devido ao desenvolvimento da resistência dos parasitas, este não oferece mais uma proteção se utilizado de forma isolada. Os medicamentos utilizados para evitar recidivas atuam nas formas teciduais latentes do *P. vivax* e do *P. ovale*. Estas drogas são úteis na cura das infecções recorrentes, cujos esquemas terapêuticos iniciam-se logo antes ou depois de a pessoa infectada deixar a área endêmica. A primaquina é o protótipo desta classe e deve ser administrada durante os longos períodos de infecção latente ou durante as crises, neste último caso, associada a outras drogas antimaláricas convenientes. As drogas esquizonticidas sanguíneas atuam no estágio eritrocítico assexuado do *Plasmodium*, impedindo a esquizogonia eritrocítica, responsável pela manifestação dos sintomas da doença. A maioria dos medicamentos antimaláricos apresenta atividade contra as formas eritrocíticas do parasita. A cloroquina, a quinina e seus derivados, bem como os derivados da artemisinina são exemplos de fármacos dessa classe. Já os gametocidas atuam contra as forma sexuadas do parasita, impedindo a transmissão, embora os antimaláricos não sejam utilizados apenas pela sua ação gametocida.

A quinina possui uso medicinal que data desde 350 anos atrás. Esta droga deriva da Cinchona, planta sul-americana também conhecida como Quina, Casca Jesuíta ou Cardeal (Amabeoku, 1991; Butler, 2010). Durante quase 200 anos a casca da Cinchona foi utilizada em forma de pó, extrato ou infusão. Somente em 1820 a quinina foi isolada da Cinchona e tem sido sintetizada. Esta droga apresenta ação esquizontocida, exercendo pouco efeito sobre esporozoítos ou as formas pré-eritrocíticas dos parasitas da malária (Butler, 2010; Newton, 2010). A quinina e a quinidina são utilizadas para o tratamento da malária grave por *P. falciparum* resistentes à cloroquina ou multirresistentes. A quinina concentra-se no vacúolo digestivo do parasita inibindo a polimerização de moléculas do heme e, como consequência, a formação da hemozoína. Este processo é realizado inicialmente com a ligação da droga ao heme, sendo que o complexo heme-fármaco resultante se liga e satura as cadeias poliméricas heme.

A cloroquina é 4-aminoquinolina com semelhança estrutural relacionada aos obsoletos antimaláricos 8-aminoquinolinas pamaquina e pentaquina (Henry, 2006), e foi sintetizada e estudada pela primeira vez antes da 2ª Guerra Mundial (em 1934). A amodiaquina é um congênere da cloroquina não mais recomendada para a terapia da malária causada pelo *P. falciparum*, devido à hepatotoxicidade gerada por esta droga (Gil, 2008). A hidroxicloroquina possui atividade antimalárica semelhante à cloroquina, mas é preferencialmente utilizada na terapia de doenças inflamatórias como a artrite reumatóide e o lúpus eritematoso. A cloroquina apresenta diversas vantagens em relação aos outros antimaláricos, como baixo custo e baixa toxicidade, sendo segura para crianças e mulheres grávidas, as vítimas mais vulneráveis da malária (Burgess, 2010).

Em relação à atividade antimalárica, a cloroquina é ativa contra formas eritrocíticas de *P. vivax*, *P. ovale*, *P. malariae* e *P. falciparum* sensíveis (Sasaki, 2010). Esta droga também apresenta ação gametocida contra todas as espécies citadas, exceto *P. falciparum*. Outra característica importante é ausência de ação contra as formas tissulares latentes de *P. vivax* e *P. ovale* (Yeshiwondim, 2010). Como dito anteriormente, os parasitas assexuados se proliferam nos eritrócitos do hospedeiro vertebrado digerindo a hemoglobina, gerando radicais livres e heme, ou ferri-protoporfirina IX. Sabe-se também que o heme livre se polimeriza formando o pigmento malárico ou hemozoína, produto não reativo insolúvel. Os quinolínicos, que são bases fracas, se concentram nos vacúolos digestivos dos parasitas sensíveis, elevando o pH do meio, inibindo assim a atividade peroxidativa do heme e interrompendo sua polimerização não-enzimática. Esse fato gera a morte dos parasitas por lesões oxidativas ou até ação de proteases digestivas (Solomon, 2009; Hommel, 2010).

O mecanismo de ação mais provável da cloroquina e outros quinolínicos antimaláricos é a inibição da polimerização do heme. Esta atividade está relacionada à prévia ligação da droga ao heme e posterior inibição da sua polimerização. Amodiaquina, quinacrina e quinina também apresentam mecanismo de ação semelhante. A primaquina, entretanto, não apresenta mecanismo semelhante às drogas citadas (Taylor, 2004).

A cloroquina é uma droga antimalárica muito útil e, ao mesmo tempo, tem seu uso diminuído em regiões onde há cepas de *P. falciparum* resistente à sua ação. A referida droga é mais potente e menos tóxica que a quinina, além de necessitar de reposição da dose uma vez por semana. A cloroquina apresenta supressão de crises agudas de malária causadas por *P. vivax* ou *P. ovale*. O fármaco controla os sintomas da malária rapidamente, com o paciente afebril em 24 a 48 horas, sendo que se os sintomas persistirem por um período superior pode-se suspeitar de *Plasmodium* resistente (Bagavan, 2010). Em doses terapêuticas, a cloroquina é bastante segura, mas quando administrada por via parenteral de forma rápida pode induzir aparecimento de toxicidade aguda, relacionada com o sistema cardiovascular e sistema nervoso central (Kwon, 2010).

Outro fármaco antimalárico digno de nota é a mefloquina. Este medicamento foi elucidado nos anos 1980 como substituto promissor da cloroquina. É um 4-quinolinometanol estruturalmente semelhante à quinina, potente esquizonticida sanguíneo, sem ação contra formas as hepáticas ou os gametócitos. O mecanismo de ação exato da mefloquina não é bem estabelecido, porém a atuação no vacúolo digestivo do parasita ocorre de forma semelhante à quinina e à cloroquina. A mefloquina pode atuar inibindo a polimerização do heme, ou formando complexos tóxicos com o heme livre. Segundo, a mefloquina deve ser reservada para prevenção e tratamento da malária causada por *P. falciparum* resistente à cloroquina e multirresistente. A mefloquina administrada por via oral em doses terapêuticas é geralmente bem tolerada. Efeitos colaterais, tais como náuseas, vômitos, dor abdominal, diarréia e vertigem, muitas vezes relacionados com a dose, são confundidos com os sintomas da doença. A mefloquina também é capaz de causar efeitos tóxicos a nível de sistema nervoso central (Egan, 2007; Toovey, 2009; Veiga, 2010; Nzila, 2010).

A primaquina foi desenvolvida durante a Segunda Guerra Mundial e, ao contrário de outros antimaláricos, age nos estágios tissulares do *P. vivax* e *P. ovale*. Na mesma época foram testadas outras drogas, como a pentaquina e isopentaquina. No entanto, apenas a primaquina é amplamente utilizada atualmente. Esta droga destrói os estágios hepáticos e as formas tissulares latentes de *P. vivax* e *P. ovale*, tendo relevante valor clínico na farmacoterapia da malária recidivante. Desse modo, a primaquina pode ser associada a outros

fármacos antimaláricos para a cura da malária causada por *P. vivax* e *P. ovale*. Doses terapêuticas ou tóxicas da primaquina, através de metabólitos oxidativos, podem causar hemólise e anemia hemolítica aguda em pessoas com deficiência de G6PD. Recomenda-se que os pacientes sejam monitorados com relação à deficiência de G6PD antes de receber primaquina (Oliver, 2008; WHO, 2010)**.**

O proguanil é um antimalárico atuante através do seu metabólito ativo, suprimindo as crises de malária por *P. vivax*. Como essa droga não apresenta ação contra as forma tissulares latentes da referida espécie, novas formas eritrocíticas aparecem logo após a interrupção do fármaco. O metabólito triazínico ativo do proguanil inibe a diidrofolato redutase dos parasitas susceptíveis, causando inibição da replicação (Jacquerioz, 2009). A atovaquona é um análogo da ubiquinona que apresenta ação muito potente contra a malária. Esta droga interfere no transporte de elétrons mitocondrial e nos processos relacionados, como a biossíntese de ATP e pirimidina nos parasitas sensíveis. Esta droga é utilizada com uma biguanina (como o proguanil) para obtenção de resultados clínicos ideais e evitar o desenvolvimento de cepas resistentes. O sinergismo entre o proguanil e atovaquona parece ser devido à capacidade do proguanil de aumentar a atividade de colapso da membrana gerado pela atovaquona (Osei-Akoto, 2005).

A artemisinina e seus derivados representam uma classe de medicamentos antimaláricos muito importante que vem sendo utilizada cada vez mais a cada dia. É uma lactona sesquiterpenóide com endoperóxido derivada da semente qing hao (*Artemisia annua*), também denominada absinto doce ou absinto anual. Os chineses atribuíram valor medicinal a essa planta há mais de 2000 anos (Meshnick, 2002). Além da artemisinina, foram sintetizados mais cinco derivados. São eles a diidroartemisinina, artemeter, arteeter, artesunato e 10-deoxoartemisinina (Krishna, 2004). Em quase todos os países onde a malária é endêmica, as terapias combinadas da artemisinina (TCA) são primeira escolha para a farmacoterapia da malária não complicada causada por *P. falciparum* (WHO, 2010). Quatro antimaláricos (lumefantrina, mefloquina, amodiaquina e sulfadoxina-pirimitamina) têm sido utilizados comumente para a TCA (Adam, 2010). Outras drogas têm sido incluídas na TCA, como a piperaquina e pironaridina (Smithuis, 2010). Esta mudança na política da terapia da malária surgiu quando se percebeu um forte aumento no número de cepas resistentes à cloroquina ou no crescimento das falhas terapêuticas relacionadas ao uso de sulfadoxina-pirimetamina (Dondorp, 2010). A molécula do endoperóxido é necessária para a atividade antimalárica dos compostos da artemisinina, enquanto as substituições no grupo carbonil da lactona aumentam acentuadamente sua potência (Meshnick, 2002). Esses compostos agem rapidamente nos

estágios eritrocíticos assexuados do *P. vivax* e das cepas de *P. falciparum* sensíveis ou resistentes à cloroquina, bem como multirresistentes. A artemisinina atua mais rapidamente que outros antimaláricos, tanto na morte dos parasitas quanto na inibição de processos metabólicos importantes. Esta atividade é dependente da produção de endoperóxidos (Krishna, 2004). Os peróxidos são uma fonte conhecida de espécies reativas de oxigênio tais como radicais hidroxil, superóxido, peróxido de hidrogênio e hidroperoxila, sendo que o radical hidroxil é um dos mais reativos, capaz de captar átomos de hidrogênio do grupo metileno de ácidos graxos, dando início a peroxidação lipídica e consequente lise da membrana celular (Campos, 2004). Esses radicais livres têm um papel importante no mecanismo de ação da artemisinina e seus derivados, pois a artemisinina é convertida em intermediários ativos após contato com o átomo de ferro (Reação de Fenton), o que é seguido por rearranjo molecular que produz radicais com carbonos centrais que formam aductos covalentes com proteínas específicas do parasita (Hartwig, 2008). Outro mecanismo de ação proposto para a artemisina é a inibição específica do Transportador de Ca^{++} do retículo sarcoplasmático ATPase (SERCA) do *Plasmodium falciparum* (PfATP6), mas não o transportador de Ca^{++} do hospedeiro (Eckstein, 2003).

1.1.6.2 A resistência aos medicamentos antimaláricos

A resistência dos parasitas às drogas antimaláricas é um fato que vem sendo estudado cada vez mais intensamente, especialmente no que diz respeito às cepas de *P. falciparum* e *P. vivax.* O desenvolvimento da resistência intrínseca do *P. falciparum* às quinolinas antimaláricas, especialmente à cloroquina, ocorreu de forma lenta, mas hoje é bastante comum ao redor do mundo e particularmente nos lugares onde há grande pressão das drogas antimaláricas. A resistência do *Plasmodium* sp pode se desenvolver através de diversos mecanismos, incluindo alterações na permeabilidade ou transporte e inativação da droga ou mudanças em alvos moleculares que geram diminuição da afinidade de ligação do inibidor. As alterações citadas anteriormente são geradas inicialmente através de mutações em genes ligados às moléculas-alvo dos medicamentos antimaláricos. O desenvolvimento de resistência às drogas antimaláricas envolve a interação de padrões de uso da droga, características da droga, fatores individuais do hospedeiro humano, características do parasita e do vetor, bem como por fatores ambientais (Winstanley, 2001).

Em relação às características das drogas, medicamentos com meia vida longa, como a mefloquina, podem exercer seleção residual em novas infecções após o tratamento do

indivíduo em uma infecção primária, pois nesse caso a droga pode persistir circulando no plasma do paciente em concentrações consideradas subterapêuticas (Gutman, 2009). Em locais onde a taxa de transmissão é intensa, essa questão é de extrema relevância. Outro fato importante é a imunocompetência do hospedeiro, onde uma resposta imune potente aumenta a eficácia da quimioterapia (Ballal, 2009).

A resistência do *P. falciparum* à cloroquina tem sido relatada há anos e está relacionada a polimorfismos em dois genes do parasita, o *pfcrt* (localizado no cromossomo 7 e que codifica uma proteína transportadora de membrana vacuolar) e o *pfmdr1*(localizado no cromossomo 5 e associado a um transportador ligado à resistência a múltiplas drogas), relacionado com a resistência não somente à cloroquina, mas também a outros antimaláricos como mefloquina, quinina e halofantrina (Pleeter, 2010). Muitos polimorfismos que estão associados à resistência à cloroquina têm sido identificados. Uma mutação em particular, relacionada a uma substituição de treonina por lisina no códon 76, tem mostrado relação absoluta com o *P. falciparum* em alguns países. O gene *pfmdr1* tem gerado interesse em relação à resistência à cloroquina e outras drogas antimaláricas. No entanto, ainda não há uma evidência conclusiva a respeito de qual mutação seria responsável pela resistência a múltiplas drogas, pois vários polimorfismos têm sido relacionados a essa característica, como Phe184, Cys1034, Asp1042, dentre outros (Pickard, 2003).

A resistência à sulfadoxina-pirimetamina foi percebida inicialmente na década de 1960, tornando-se um sério problema no Sudeste Asiático, Sul da China e Bacia Amazônica. Mutações específicas no *P. falciparum* que levam à resistência tanto à sulfadoxina, quanto à pirimetamina têm sido identificadas. Essas drogas são inibidoras da síntese de folato do parasita atuando de forma sinérgica através da inibição de duas enzimas relacionadas ao referido processo metabólico, a dihidrofolato sintetase e dihidrofolato redutase (Kone, 2010). Mutações pontuais em cinco códons do gene *dhps* estão relacionadas à resistência aos antimaláricos por diminuição da afinidade de ligação às enzimas (Lu, 2010).

Em relação à quinina, existem sugestões de que a resistência à esta droga esteja relacionada com mutações no gene *pfmdr1* (Pleeter, 2010). No Brasil, um estudo de mutações em *pfmdr1* em cepas cloroquina resistentes mostrou menor susceptibilidade destas cepas à quinina (Zalis, 1998; Vieira, 2001). A resistência do *Plasmodium falciparum* à mefloquina tem sido documentada desde 1980 em alguns lugares do mundo. Têm sido investigados marcadores moleculares de resistência à mefloquina associados ao gene *pfmdr1*(Preechapornkul, 2009). Alguns estudos mostram resistência à mefloquina (Rogers, 2009) e outros mostram um aumento da sensibilidade à droga relacionado ao gene *pfmdr1*,

sugerindo uma relação inversa entre sensibilidade à mefloquina e à cloroquina (Chaijaroenkul, 2009).

Em aproximadamente todos os países onde a malária é endêmica, a Organização Mundial de Saúde tem recomendado o uso de terapias combinadas com artemisinina (TCA) como primeira escolha para o tratamento da malária não complicada por *P. falciparum*, justamente devido ao aumento da resistência às drogas antimaláricas. No entanto, tem-se percebido o aparecimento de resistência à artemisinina desde 2004 (Butler, 2010). Não há clareza em relação à causa do fato citado anteriormente. Além disso, alguns autores consideram que, até o presente, TCA seja o único grupo de drogas antimaláricas onde a resistência do *P. falciparum* ainda não tenha se desenvolvido (Gemma, 2010). Por outro lado, em países como o Camboja, há uma redução da susceptibilidade do *P. falciparum* a artemisinina (Imwong, 2010). Vários fatores podem ter contribuído para o aparecimento da resistência à artemisinina no Camboja. Este foi um dos primeiros países a adotar a TCA como primeira linha de tratamento em 2001, mas a monoterapia com artesunato ou artemisinina tem sido realizada desde 1970. Atualmente, não existe nenhum grupo de drogas que possa substituir a TCA, pois nenhum medicamento antimalárico aprovado para uso na população apresenta a mesma segurança e eficácia da artemisinina (Kamat, 2010). Desse modo, deve-se atentar para o risco de aumento de resistência às drogas antimaláricas em outros países e adotar medidas para prolongar o máximo possível o aparecimento do referido processo.

1.1.7 Alvos moleculares do *Plasmodium falciparum*

Diante do crescimento da resistência do *Plasmodium* às drogas antimaláricas atualmente disponíveis, novos agentes terapêuticos com novos alvos moleculares são necessários. Após o sequenciamento completo do genoma do *P. falciparum*, foram identificados novos alvos moleculares dentro do parasita que podem sofrer interferência de drogas (Wegscheid-Gerlach, 2010). Dentre esses possíveis alvos, pode-se citar histonas deacetilases (Andrews, 2009), plasmepsinas (Gupta, 2010), aminopeptidases (Trenholme, 2010), diidroorotato desidrogenase (Phillips, 2010), falcipainas (Ettari, 2010), dentre outros. Esses possíveis alvos moleculares são enzimas que desenvolvem papéis importantes no metabolismo do parasita, como remoção de grupos acetila de resíduos de lisina durante a transcrição de genes do parasita (Chaal, 2010), digestão da hemoglobina no vacúolo digestivo ou degradação de proteínas do eritrócito (Hogg, 2006).

Estudos têm mostrado que a inibição de enzimas, como as citadas anteriormente, pode contribuir para interrupção de etapas do ciclo de vida do parasita dependentes dessas proteínas. No caso da falcipaína, um grupo de cisteínas proteases dividido em falcipaína 2, 3 e 2B, esta última sendo semelhante à falcipaína 2 (Aly, 2005; Blackman, 2008), a sua inibição pode causar morte do *Plasmodium* (Micale, 2009), tornando relevante o estudo de drogas inibidoras da falcipaína 2.

1.2 Os produtos naturais

O uso de produtos naturais com propriedades terapêuticas é tão antigo quanto a civilização humana e, por um longo tempo, produtos de plantas e animais foram as principais fontes de drogas. A revolução industrial e o desenvolvimento de produtos químicos orgânicos resultaram numa preferência por produtos sintéticos para o tratamento farmacológico. Entretanto, mesmo se somente considerarmos o impacto da descoberta da penicilina, obtida a partir de microrganismos, no desenvolvimento da terapia antiinfecção, a importância dos produtos naturais é claramente significativa: 25% dos medicamentos prescritos no mundo são originados de plantas. Das 252 drogas consideradas como básicas e essenciais pela Organização Mundial de Saúde (OMS), 11% são exclusivamente originadas de plantas e um significante número são drogas sintéticas obtidas a partir de precursores naturais (Rates, 2001).

Diante de um grande problema de saúde pública relacionado ao aumento progressivo da resistência dos parasitas aos antimaláricos disponíveis, principalmente no que se refere à artemisinina e seus derivados, torna-se necessário o desenvolvimento de novas alternativas de controle da doença. Uma opção viável neste sentido é o desenvolvimento de novos medicamentos antimaláricos a partir de produtos naturais. Dados históricos revelam que as plantas são fontes importantes de agentes químicos ativos contra a malária. Tanto a quinina quanto os derivados da artemisinina são exemplos desse fato (Ramazani, 2010).

O Brasil possui a maior biodiversidade do mundo, estimada em cerca de 20% do número total de espécies do planeta. Esse imenso patrimônio genético, já escasso nos países desenvolvidos, tem na atualidade valor econômico-estratégico inestimável em várias atividades, dentre as quais a de maior potencial é a de desenvolvimento de novos medicamentos. Esta afirmação é facilmente comprovada quando se analisa o número de medicamentos obtidos direta ou indiretamente a partir de produtos naturais, que podem ser

produzidos a partir de derivados das plantas superiores, toxinas animais e microrganismos (Calixto, 2003).

Para se ter uma noção do impacto dos medicamentos no mercado mundial, apenas as estatinas foram responsáveis por US$ 19 bilhões em 2002 (Downton, 2003). Neste mercado que atinge vários bilhões de dólares por ano, estima-se que 40% dos medicamentos disponíveis na farmacoterapia atual foram desenvolvidos a partir de fontes naturais (Gordon, 1997).

Estima-se também que 60 % das drogas anti-tumorais e anti-infecciosas já conhecidas ou sob triagem clínica são de origem natural (Shu, 1998). A grande maioria destas drogas não é sintetizada de maneira econômica e são ainda obtidas de plantas selvagens ou cultivadas. Compostos de origem natural podem dar origem a outros compostos, permitindo o desenho e planejamento racional de novas drogas e a descoberta de novas propriedades terapêuticas ainda não atribuídas a compostos conhecidos. O uso potencial de plantas superiores como fonte de novas drogas ainda é pequeno. Estima-se que existam cerca de 250 a 500 mil espécies de plantas conhecidas. Somente um pequeno percentual tem sido investigado quanto aos aspectos fitoquímicos e uma fração deste reduzido número é submetida a estudos de atividade biológica (Hamburguer, 1991).

No Brasil há a necessidade de se buscar alternativas para superar, no mercado de medicamentos, a dependência externa, principalmente quando se confrontam os altos preços praticados no país em comparação com os preços praticados nos países desenvolvidos. O panorama brasileiro nessa área mostra que 84% de todos os fármacos são importados e que 78% da produção brasileira são realizados por empresas multinacionais. Nesse quadro, confronta-se um hemisfério norte rico em tecnologia e menos favorecido em recursos genéticos, com um hemisfério sul deficiente em tecnologia, mas muito rico em diversidade biológica. Estima-se que um gene potencialmente útil originado na biodiversidade do hemisfério sul pode representar negócios de um bilhão de dólares no norte (Simões, 2003).

Neste contexto encontra-se o semi-árido brasileiro que abrange cerca de 975.000 km^2, correspondendo a 11,5% do território nacional, incluindo oito estados do nordeste e dois do sudeste. É uma terra marcada pela irregularidade das chuvas, determinando longos períodos de secas, com graves consequências sociais para seus 20 milhões de habitantes, os quais apresentam elevada dependência dos recursos naturais e os piores indicadores sociais do país. Do ponto de vista do meio ambiente, dois dos maiores problemas associados ao semi-árido são o elevado grau de degradação ambiental e o baixo conhecimento quantitativo e qualitativo de sua biodiversidade. O bioma do semi-árido é, provavelmente, o menos estudado em

relação à flora e fauna e um dos que têm sofrido maior degradação pelo uso desordenado e predatório nos últimos 400 anos (Asa Brasil, 2010). Várias causas podem ser apontadas para esta situação, dentre elas o distanciamento entre o conhecimento gerado no meio acadêmico e as populações locais.

Desse modo, algumas das linhas de pesquisa devem ser priorizadas para um melhor conhecimento da biodiversidade e seu uso pelas populações locais. Isso se justifica pelo fato do semi-árido apresentar uma das biotas mais particulares do mundo, em composição e adaptações às condições do meio. De um modo geral, o semi-árido tem sido encarado como um conjunto de problemas ambientais, sociais e desafios científicos, tecnológicos e de desenvolvimento. Portanto, são necessárias estratégias para a produção de conhecimentos que possam contribuir para sua superação. O isolamento, a identificação e a avaliação biológica de produtos naturais podem constituir os melhores caminhos para a descoberta de novos fármacos, considerando que a busca de moléculas ativas com estruturas moleculares complexas, por parte das indústrias farmacêuticas, é extremamente difícil de ser obtida por um processo sintético de custo racional.

2. OBJETIVOS

GERAL

Identificar a atividade antimalárica de substâncias puras extraídas de espécies vegetais nativas ou endêmicas do semi-árido brasileiro e derivados sintéticos.

ESPECÍFICOS

1 Avaliar a atividade anti-*Plasmodium in vitro* do ácido betulínico e seus derivados, nas formas eritrocíticas do *P. falciparum*, bem como investigar a atividade antimalárica *in vivo* do acetato do ácido betulínico em camundongos infectados por *Plasmodium berghei*.

2 Avaliar a atividade anti-*Plasmodium in vitro* das fisalinas e a atividade antimalárica *in vivo* das fisalinas D e F em camundongos infectados por *P. berghei*.

3 Avaliar a atividade anti-*Plasmodium in vitro* do lapachol e derivados, além da atividade antimalárica *in vivo* do acetato do isolapachol em camundongos infectados por *P. berghei*.

4 Determinar a toxicidade das substâncias puras *in vitro*, em sistema de células isoladas de camundongos.

5 Investigar o potencial mecanismo de ação antimalárica do lapachol utilizando modelos computacionais QSAR e Docking.

3. MANUSCRITO 1

O primeiro manuscrito intitulado **"Antimalarial activity of betulinic acid and derivatives *in vitro* against *Plasmodium falciparum* and *in vivo* in *P. berghei*-infected mice"** descreve a avaliação da atividade antimalárica *in vitro* e *in vivo* do ácido betulínico e seus derivados. Este trabalho foi publicado na **Parasitology Research** no ano de 2009 (105:275–279). DOI 10.1007/s00436-009-1394-0.

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ORIGINAL PAPER

Antimalarial activity of betulinic acid and derivatives in vitro against *Plasmodium falciparum* and in vivo in *P. berghei*-infected mice

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Abstract Malaria is one of the most important tropical diseases and mainly affects populations living in developing countries. Reduced sensitivity of *Plasmodium* sp. to formerly recommended antimalarial drugs places an increasing burden on malaria control programs as well as on national health systems in endemic countries. The present study aims to evaluate the antimalarial activity of betulinic acid and its derivative compounds, betulonic acid, betulinic acid acetate, betulinic acid methyl ester, and betulinic acid methyl ester acetate. These substances showed antiplasmodial activity against chloroquine-resistant *Plasmodium falciparum* parasites in vitro, with IC_{50} values of 9.89, 10.01, 5.99, 51.58, and 45.79 μM, respectively. Mice infected with *Plasmodium berghei* and treated with betulinic acid acetate had a dose-dependent reduction of parasitemia. Our results indicate that betulinic acid and its derivative compounds are candidates for the development of new antimalarial drugs.

Introduction

Malaria is one of the most important tropical diseases due to its high morbidity and mortality rates, affecting over two billion people in endemic countries. In a public health problem in countries where transmission occurs regularly, this disease mainly afflicts populations living in developing countries, constituting an obstacle to the efforts toward economic development (WHO 2005).

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The causative parasite of the malignant form of malaria, *Plasmodium falciparum*, frequently presents resistance to chloroquine, the cheapest and most utilized antimalarial drug (WHO 2008). Another problem concerning drug resistance in *P. falciparum* is the occurrence of cross-resistance among drugs belonging to the same chemical family (WHO 2001). Drug resistance plays an important role in the occurrence and severity of epidemic diseases in the world (Majori 2004). Moreover, there is a great disparity between the public health importance of tropical diseases, such as malaria, and the amount of resources invested in the development of new drugs to combat these diseases (WHO 2001).

As a consequence of drug resistance, drugs like quinine, chloroquine, primaquine, and mefloquine are ineffective in treating malaria in many endemic regions of the world (Schlitzer 2007). Thus, the development of new antimalarial pharmacotherapy is necessary. Many different classes of natural products, such as terpenes (Meshnick 2002), flavonoids (Auffret 2007; Froelich et al. 2007), and alkaloids (Chea et al. 2007), have been studied regarding antimalarial activity. This study aimed at evaluating the antimalarial activity of betulinic acid and its derivatives against chloroquine-resistant *P. falciparum* parasites in vitro and *Plasmodium berghei* parasites in vivo.

Materials and methods

Purification of betulinic acid and synthesis of derivatives

Betulinic acid was isolated from *Zizyphus joazeiro* Mart. (Rhamnaceae) (Barbosa-Filho et al. 1985). The derivatives were obtained by standard and straightforward chemical procedures already related in literature. The betulinic acid acetate was obtained by the acetylation reaction of betulinic acid with an acetic anhydride/pyridine mixture (Kim et al. 2001). Betulonic acid was obtained in the chromic acid oxidation from betulinic acid (Urban et al. 2007). The treatment of betulinic acid and the betulinic acid acetate with excess ethereal diazomethane solution rendered the corresponding methyl ester and derivatives (Urban et al. 2005; Uzenkova et al. 2005). All compounds were purified by column chromatography and showed ^{1}HNMR and ^{13}CNMR spectra in accordance with literature previously cited.

The structures of betulinic acid and its derivatives used in this study are shown in Fig. 1.

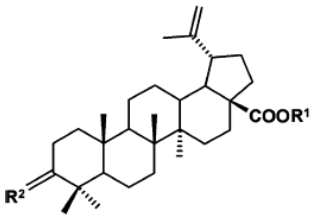
Cytotoxicity assay

To determine the cytotoxicity of betulinic acid (BA) and the derivative compounds betulonic acid (BOA), betulinic acid acetate (BAA), betulinic acid methyl ester (BAME), and betulinic acid methyl ester acetate (BAMEA), BALB/c mice splenocytes were cultured in 96-well plate (6×10^5 cells/well) in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (Cultilab, Campinas, São Paulo, Brazil) and 50 μg/ml of gentamycin (Novafarma, Anapolis, Goiás, Brazil). The pure substances were tested in five concentrations, in triplicates. One microCi/well [*methyl*-^{3}H]-thymidine (Amersham, Little Chalfont, England) was added to the cultures which were incubated during 24 h at 37°C and 5% CO_2. After this period, plates were harvested using a cell harvester (MPXRI 96TI, Brandel, Gaithersburg, MD, USA) to determine the ^{3}H-thymidine incorporation using a β-radiation counter (Multilabel Reader, Hidex, Turku, Finland). The viability of the cells was determined by ^{3}H-thymidine incorporation and the cytotoxicity was calculated in relation to the ^{3}H-thymidine incorporation of untreated cultures.

Anti-*P. falciparum* in vitro assay

Betulinic acid and the derivative compounds were tested for antimalarial activity in vitro using the *P. falciparum* W2 clone, which is chloroquine resistant and mefloquine sensitive (Junior 1999). All the parasites were maintained in continuous culture of human erythrocytes (blood group A^{+}) using Rapid Prototyping and Manufacturing Institute (RPMI) medium supplemented with 10% human plasma (Trager and Jensen 2005).

Parasites grown at 1% to 2% parasitemia and 2.5% hematocrit were incubated with the pure substances tested at various concentrations, diluted with 4% dimethyl sulfoxide (DMSO) in culture medium (RPMI 1640) without



	R¹	R²
(BA)	H	H, β-OH
(BOA)	H	O
(BAA)	H	H, β-OCOCH$_3$
(BAME)	CH$_3$	H, β-OH
(BAMEA)	CH$_3$	H, β-OCOCH$_3$

Fig. 1 Structures of betulinic acid and derivatives

hypoxanthine. Mefloquine was used in each experiment as a control for *P. falciparum* drug response. Cultures containing parasites were harvested using a cell harvester to evaluate the ^{3}[H]-hypoxanthine incorporation in a β-radiation counter. Inhibition of parasite growth was evaluated by comparison with ^{3}[H]-hypoxanthine uptake in drug-treated versus untreated wells. All the assays were performed in triplicates as described previously (Andrade-Neto et al. 2004; Zalis et al. 1998).

Assessment of antimalarial activity

The antimalarial activity of betulinic acid acetate was evaluated in mice infected with *P. berghei*, strain NK65 (Andrade et al. 2007). Parasites were maintained by weekly blood passage 10^{6} infected red blood cells per mouse by intraperitoneal (i.p.) route. The animals were randomly sorted into groups of five and treatment was administered daily, during four consecutive days, beginning on the first day of infection. BAA was suspended in saline solution plus 10% DMSO immediately before use in doses of 10, 50, and 100 mg/kg. Each mouse received 200 μl, by oral or by intraperitoneal route. Experiments included a control group treated with the standard antimalarial drug chloroquine at 50 mg/kg and a mock-treated group (10% DMSO in saline). The antimalarial activity was evaluated by counting parasitemia in blood smears at days 4 and 7 after parasite inoculation, by optical microscopy, after fixation with methanol and staining with fast panoptic (Laborclin, Pinhais, Brazil). Inhibition of parasite growth in drug-treated groups was calculated in relation to the control (mice treated with saline solution plus 10% DMSO) group. The results were expressed as the percentage of parasitemia reduction.

IC_{50} and LC_{50} calculations and statistical analyses

The lethal concentration of 50% of BALB/c mice splenocytes (LC_{50}) and the inhibitory concentration of 50% (IC_{50}) of *P. falciparum* were calculated based on a nonlinear regression (curve fit). The statistical analyses were made by one-way analysis of variance and Newman–Keuls multiple comparison tests using Graph Pad Prism version 4.0 (Graph Pad Software, San Diego, CA, USA). Differences were considered significant when p values were <0.05.

Calculation of the selectivity index

The selectivity index (SI) was defined by calculating the value of LC_{50} versus IC_{50} (Bézivin et al. 2003). The SI was considered as significant for values higher than three.

Results

Cytotoxicity of betulinic acid and derivatives

In order to determine the cytotoxic potential of the compounds studied, BA, BOA, BAA, BAME, and BAMEA were tested in five different concentrations. As shown in Table 1, all substances had lower cytotoxicity compared to mefloquine, used as the standard antimalarial drug. BOA was the compound with lower cytotoxicity (higher LC_{50} value) when compared to the other substances tested, while BAMEA was the most toxic.

In vitro antimalarial activity

To assess the antimalarial activity of the compounds, BA and its derivatives were first assayed in vitro against W2 strain of *P. falciparum*. BA and its derivative compounds BOA and BAA had antiplasmodial activity, with IC_{50} values below 10 μM, whereas BAME and BAMEA presented IC_{50} values above 45 μM (Table 1). Mefloquine had an IC_{50}=0.04 μM against this strain of *P. falciparum*, a result similar to that described before for an inoculum of 1% to 2% parasitemia and 2.5% hematocrit (Andrade-Neto

Table 1 Anti-*P. falciparum* activity and in vitro cytotoxicity of betulinic acid and derivative compounds

Substance	Yield (%)[a]	IC_{50} (μM)	LC_{50} (μM)	SI
BA	90.65	9.89±0.52	62.36±32.67*	6.3
BOA	93.28	10.01±1.29	96.10±8.52*	9.6
BAA	95.08	5.99±0.06	21.98±2.71	3.7
BAME	93.74	51.58±10.85*	83.21±6.46*	1.6
BAMEA	60.33	45.79±36.26*	15.42±19.30	0.3
Mefloquine	91.69	0.04±0.01	9.53±0.46	238

Values are mean ± standard deviation of three independent experiments

SI selectivity index

*$P<0.01$ compared to control

[a] The maximal concentration of BAA and derivatives tested was 100 μg/ml and of mefloquine was 0.25 μg/ml

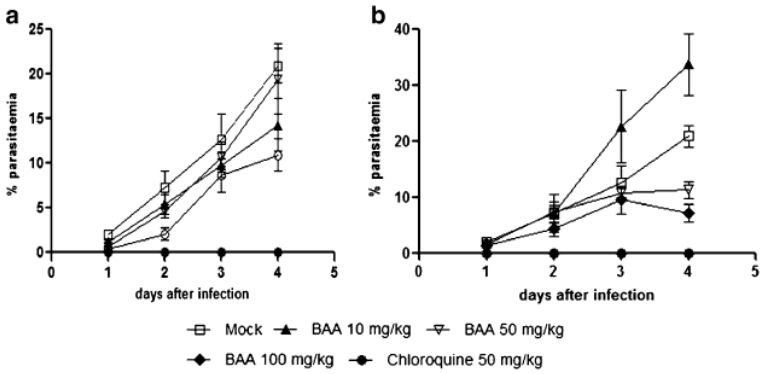


Fig. 2 Parasitemia of *P. berghei*-infected mice treated with 10% DMSO in saline (*mock*), BAA at 10, 50, and 100 mg/kg, or chloroquine at 50 mg/kg, by oral (*A*) or intraperitoneal (*B*) routes, daily, for four consecutive days. Blood parasitemia was determined on several days after infection. Values represent the means ± standard error of five mice per group in one experiment of two performed

et al. 2004). The analysis of the selectivity index (SI) showed that BAA was the compound with higher SI, whereas BAMEA had the lowest SI (Table 1). The SI index of mefloquine, however, was significantly higher than the one found for BAA.

In vivo antimalarial activity of BAA

Since BAA was the compound with best SI, we decided to test its antimalarial activity in vivo in *P. berghei*-infected mice. Treatment with BAA by oral route did not alter the levels of parasitemia in mice infected with *P. berghei* compared to mice treated with saline solution plus 10% DMSO (Fig. 2a). In contrast, the administration of BAA by i.p. route caused a dose-dependent reduction of parasitemia of at least 70% on the seventh day following infection (Fig. 2b). Mice treated with chloroquine, a standard antimalarial drug, had undetectable parasitemia (Fig. 2a, b).

Treatment with BAA i.p. increased the survival of *P. berghei*-infected mice at a dose of 100 mg/kg at least until day 21 postinfection. Treatment with BAA in doses of 10 and 50 mg/kg, however, did not affect the mortality rate of *P. berghei*-infected mice, which started to die by day 10, similar to control mice. All the mice in the chloroquine-treated group survived during the experiment (Fig. 3).

Discussion

In this study, we demonstrated that betulinic acid and the derivative compounds BOA and BAA have a significant antimalarial activity in vitro against *P. falciparum* chloroquine resistant (W2 strain). In addition, we showed that BAA, the most active anti-*Plasmodium* substance tested, has antimalarial activity in vivo.

The IC_{50} of mefloquine against *P. falciparum* is about 150 times lower than that of BAA, the most active betulinic acid derivative in vitro. On the other hand, this drug was more toxic than BAA, with LC_{50} two times lower than BAA. It is important to say that selectivity index is an important parameter to evaluate the potential of the drugs tested, and a SI value >3 indicates high selectivity (Prayong et al. 2008). BAA present a SI value of 3.7 which mefloquine SI was 238. Although mefloquine SI was 64 times higher than BAA SI, this drug may serve as basis for the development of new malaria pharmacotherapies able to control mefloquine-resistant parasites.

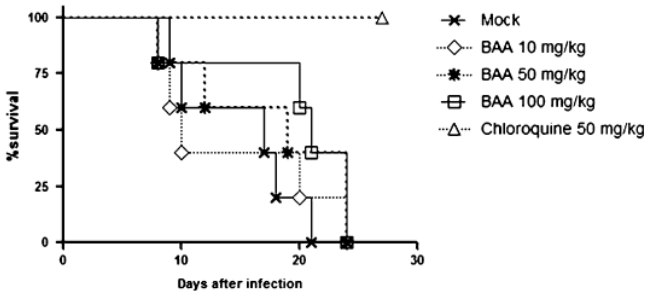


Fig. 3 Cumulative mortality rates of *P. berghei*-infected mice treated with 10% DMSO in saline (*mock*), BAA at 10, 50, and 100 mg/kg, or chloroquine at 50 mg/kg, by intraperitoneal route, daily, for four consecutive days. Mortality was evaluated several days after infection. Results are from one experiment done with five mice per group of two performed

The identification of new molecular targets in *Plasmodium* sp. may aid the design of compounds with selective activity during the process of drug development. The investigation of the mechanisms of action of BAA may help the identification of new molecular targets. With this regard, it has been suggested before that BA inhibits topoisomerases (Wada et al. 2005). Thus, BAA may also act by inhibiting topoisomerases from *Plasmodium* sp., a possibility that we are currently investigating.

BAA was also active in vivo when administrated to *P. berguei*-infected mice by intraperitoneal route, although it did not cause a reduction of parasitemia when used by oral route. This result may be attributed to a slow absorption of the compound, to the high metabolism rate of the mouse model, or to retention of the compound as lipid-bound complexes due to its lipophilic nature. These factors could lead to low bioavailability of the drug at the target site of action, the intracellular parasite.

A previous report has shown that treatment of *P. berghei*-infected mice with betulinic acid by intraperitoneal route did not cause a reduction of parasitemia (Steele et al. 1999). Our finding that the derivative betulinic acid acetate has in vivo activity suggests that the structural changes in the molecule significantly alter the anti-*Plasmodium* activity. Further studies to explore this compound as a prototype for an antimalarial drug should be carried out, especially aiming at the *P. falciparum* chloroquine-resistant parasites, which are rather frequent worldwide.

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4. MANUSCRITO 2

O segundo manuscrito intitulado **"Activity of physalins purified from *Physalis angulata* L. *in vitro* against *Plasmodium falciparum* and *in vivo* in *P. berghei*-infected mice"** descreve o uso da metodologia SEA (similarity ensemble approach) para prever a atividade antimalárica das fisalinas. Os dados previstos foram confirmados experimentalmente *in vitro* e *in vivo*.

Activity of physalins purified from *Physalis angulata* L. *in vitro* against *Plasmodium falciparum* and *in vivo* in *P. berghei*-infected mice

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We have previously shown that physalins B, F, and G, secosteroids isolated from *Physalis angulata* L., possess immunomodulatory and antileishmanial activities. In the present study we describe the antimalarial activity of physalins. *In silico* analysis performed using the similarity ensemble approach (SEA database) to identify likely activities of physalins B, D, F, and G based on structure similarities predicted antimalarial activity for the four physalins studied. The *in vitro* assay, carried out in red blood cell cultures infected with a *Plasmodium falciparum* clone, validated this finding, showing IC_{50} values of 2.76 ± 1.20, 55.01 ± 0.96, 2.23 ± 1.16, and 6.70 ± 0.37 μM for physalins B, D, F, and G, respectively. Treatment of *P. berghei*-infected mice with physalin F by intraperitoneal route increased the parasitemia levels and accelerated the mortality when compared to control mice. In contrast, treatment with physalin D caused a parasitemia reduction and a delay in mortality in *P. berghei*-infected mice. The worsening of *in vivo* infection by treatment with physalin F was probably due to its potent immunosuppressive activity, which is not present in physalin D. In conclusion, our results demonstrate, for the first time, that the secosteroids physalins present antiplasmodial activity *in vitro*.

Keywords: Physalins; *Plasmodium falciparum*; antimalarial activity; infection; *Plasmodium berghei*

Introduction

Malaria is a disease caused by parasites of *Plasmodium* genus and impacts 109 countries around the world. Infection with *P. falciparum*, if not promptly treated, may lead to death (WHO, 2011). Although antimalarial drugs play a central role in the control and elimination of the disease, their activity against *Plasmodium* falls due to selection and spreading of antimalarial resistant parasites, leading to an increase in treatment failure rates (WHO, 2011). This makes the development of new antimalarial pharmacotherapy a matter of great relevance to public health.

The search for alternative antimalarial molecules has stimulated the screening of potentially active substances from plant species (Camargo *et al.*, 2009). Several antimalarial drugs were developed based on natural substances isolated from plant species with ethnopharmacologic use, such as the quinoline derivatives (quinine from *Cinchona* spp.) and artemisinin (isolated from *Artemisia annua* L.).

Physalis angulata L. (Solanaceae) is an annual herb widely distributed throughout tropical and subtropical regions of the world (Soares *et al.*, 2003; Nagafuji *et al.*, 2004). This plant has been used in popular medicine as a treatment for a variety of illnesses (Abe *et al.*, 2006), including malaria (Ankrah *et al.*, 2003). We have previously shown that physalins, seco-steroids isolated from *Physalis* spp, have antileishmanial (Guimarães *et al.*, 2009), immunomodulatory (Soares *et al.*, 2003 and 2006; Brustolim *et al*, 2010) and antiinflammatory activities (Vieira *et al.*, 2005).

Here we used a tool named similarity ensemble approach (SEA), whose principle is based on idea that dissimilar molecules can have the equivalent types of interactions with a protein target and evaluates the similarity between the entry structure and a database of over 65.000 small molecules annotated for protein drug targets, where the majority of annotations contain hundreds of ligands (Keiser *et al.* 2007). Based on the SEA analysis, which predicted the antimalarial activity of physalins, we investigated here the activity of

four physalins purified from *P. angulata* against chloroquine-resistant *Plasmodium falciparum* parasites *in vitro* and *Plasmodium berghei* parasites *in vivo*.

Methods

Mice

Specific-pathogen-free, 5-week-old male Swiss mice were maintained at the animal facilities at the Gonçalo Moniz Research Center-FIOCRUZ, and provided with rodent diet and water ad libitum. Animals were handled according to the NIH guidelines for animal experimentation. All procedures described here had prior approval from the local animal ethics committee.

Drugs

Physalins B, D, F, and G (Figure 1) were obtained from plant specimens collected in Belém do Pará, Brazil, as described before[9]. Preparations of pure physalins were dissolved in dimethyl sulfoxide (DMSO) and diluted in culture medium or saline for use in the assays. Mefloquine and chloroquine (Farmanguinhos, Rio de Janeiro, RJ, Brazil) were used as control antimalarials in vitro and in vivo, respectively.

Similarity ensemble approach (SEA)

Three-dimensional structures for physalins were obtained from PubChem which is a public molecular information repository[11] (Figure 1). These files were downloaded in the sdf format for physalins B, D and F. Physalin G was built from the structure of physalin F using HyperChem software (Hypercube, Inc.) and MM+ molecular mechanics with the semi-empirical AM1 method[12]. These structures were converted to the SMILES format using HyperChem software and submitted to the SEA, in order to evaluate their potential activities. In the present work we used as cut-off for E-value 10^{-4}, values higher than this cut-off value were not considered in our analysis, since they indicate low statistical significance. The original proposal of the SEA application suggests also the use of the Tanimoto coefficient (Tc), with threshold of 0.57, being 1.0 the maximum similarity between two molecules. We adopted a threshold of 0.67 for Tc. Values lower than 0.67 were not considered in our analysis.

Cytotoxicity assay

To determine the cytotoxicity of physalins B, D, F, and G, BALB/c mice splenocytes were cultured in 96-well plate (6× 10^5 cells/well) in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (Cultilab, Campinas, São Paulo, Brazil) and 50 μg/ml of gentamycin (Novafarma, Anapolis, Goiás, Brazil). The substances were tested in five concentrations, in triplicates. One μCi/well [methyl-3H]-thymidine (Amersham, Little Chalfont, England) was added to the cultures which were incubated during 24 h at 37°C and 5% CO_2. After this period, plates were harvested using a cell harvester (MPXRI 96TI, Brandel, Gaithersburg, MD, USA) to determine the ^{3}H-thymidine incorporation using a β-radiation counter (Multilabel Reader, Hidex, Turku, Finland). The viability of the cells was determined by ^{3}H-thymidine incorporation and the cytotoxicity was calculated in relation to the ^{3}H-thymidine incorporation of untreated cultures.

Anti-Plasmodium falciparum *in vitro assay*

Physalin B, D, F, and G were tested for antimalarial activity in vitro using the *P. falciparum* W2 clone, which is chloroquine resistant and mefloquine sensitive[13]. All the parasites were maintained in continuous culture of human erythrocytes (blood group O^+) using RPMI-1640 medium supplemented with 10% human plasma[14].

Parasites grown at 2 to 5% parasitemia and 2.50% hematocrit were incubated with the pure substances tested at various concentrations, diluted with 4% dimethylsulfoxide (DMSO) in RPMI-1640 medium without hypoxanthine. Mefloquine was used in each experiment as a control for *P. falciparum* drug response. Cultures containing parasites were harvested using a cell harvester to evaluate the ^{3}[H]-hypoxanthine incorporation in a β-radiation counter. Inhibition of parasite growth was evaluated by comparison with ^{3}[H]-hypoxanthine uptake in drug-treated versus untreated wells. All the assays were performed in triplicates as described previously[15, 16].

Assessment of antimalarial activity

The antimalarial activities of physalins D and F were evaluated in mice infected with *Plasmodium berghei*, strain NK65[17]. Parasites were maintained by weekly blood passage 10^6 infected red blood cells per mouse by intraperitoneal route. The animals were randomly sorted into groups of five and treatment was administered daily, during four consecutive days, beginning on the first day of infection. The physalins were suspended in saline solution plus 10% DMSO immediately before use in doses of 50 and 100 mg/kg.

Each mouse received 200 µl, by intraperitoneal route. Experiments included a control group treated with the standard antimalarial drug chloroquine at 50 mg/kg and a mock-treated group (10% DMSO in saline). The antimalarial activity was evaluated by counting parasitaemia in blood smears at days 4-8 after parasite inoculation, by optical microscopy, after fixation with methanol and staining with fast panoptic (Laborclin, Pinhais, Brazil). Inhibition of parasite growth in drug-treated groups was calculated in relation to the control (vehicle-treated) group. The results were expressed as the percentage of parasitaemia reduction.

IC_{50}, LC_{50}, and SI calculations and statistical analyses

The lethal concentration of 50% of BALB/c mice splenocytes (LC_{50}) and the inhibitory concentration of 50% (IC_{50}) of *P. falciparum* were calculated based on a nonlinear regression (curve fit). The selectivity index (SI) was defined by calculating the value of LC_{50} versus IC_{50}[18]. The SI was considered as significant for values higher than three. The statistical analyses were made by one-way analysis of variance and Newman–Keuls multiple comparison tests using Graph Pad Prism version 4.0 (Graph Pad Software, San Diego, CA, USA). Differences were considered significant when p values were < 0.05.

Results and Discussion

The structures of physalins B, D, F, and G (Figure 1) were analyzed by similarity with compounds with known activity using the similarity ensemble database (SEA; Keiser *et al.*, 2007). The results from the SEA webserver indicate varied activities of one or more of the physalins studied, including antitumoural and antiinflammatory-related activities (Table 1). However, antimalarial activity was predicted to all four physalins, with E-values ranging from $8.55.10^{-6}$ (for physalin B) to $3.15.10^{-5}$ (for physalin F), as shown in table 1. Although none of the physalins presented the highest score predicted biological activity for antimalarial activity, this was the only activity common to all four physalins found by the SEA analysis. Furthermore, the Tanimoto coefficient was analyzed for all four physalins, and range from 0.67 (for physalin F) to 0.73 (for physalin B). These results also suggest that these molecules exhibit antimalarial activity, since the nearer to 1.0 for Tc, the greater

the similarity between the molecule analyzed with molecules that share a particular pharmacological activity.

We next sought to confirm the predictions of antimalarial activity based on SEA analysis in an *in vitro* assay against the *P. falciparum* W2 clone (Table 2). All four physalins had antiplasmodial activity *in vitro*. However, physalins B, F, and G presented IC_{50} values in low micromolar values, while physalin D was the least active (Table 2). The cytotoxic potential against mammalian cells of the compounds was also investigated. Physalins B, D, F, and G were tested in five different concentrations presented lower cytotoxicity compared to mefloquine, used as a standard antimalarial drug. Mefloquine had a low IC_{50} value, but high cytotoxicity. Physalin D presented the highest LC_{50} value, 60 times higher than of mefloquine (Table 2).

We first selected physalin F to test *in vivo* because this compound had the lowest IC_{50} value (Table 2). Treatment of *P. berguei* infected mice with physalin F (50 and 100 mg/kg) by intraperitoneal route did not decrease the parasitemia levels when compared to mock-treated controls (10% DMSO in saline). In fact, there was an increase in parasitemia levels upon treatment with physalin F. Mice treated with chloroquine, a standard antimalarial drug, had undetectable parasitemia (Figure 2A). In addition, no reduction of mortality was observed in physalin F-treated mice, which started to die by day 7, similar to control animals (Figure 2B). All the mice treated with chloroquine survived during the experiment. The worsening of infection after physalin F treatment may be explained by its potent immunosuppressive activity, demonstrated in our previous studies (Soares *et al.*, 2003 and 2006; Brustolim *et al*, 2010).

The suppression induced by physalin F may have inhibited the antimalarial immune response in infected animals, which is important for infection control (Hui & Hashimoto, 2008).Thus, although physalin D a lower pharmacological potency *in vitro* we decided to test its activity *in vivo* because this physalin dies not have immunosuppressive activity

(Soares *et al.*, 2003 and 2006). Physalin D treatment significantly decreased parasitemia of about 65% on the eighth day of infection (Figure 3A) and mortality of *P. berghei*-infected animals treated by 25% with a dose of 100 mg/kg, until day 24 after infection (Figure 3B).

In vitro assays are isolated systems that demonstrate the action of the drug only in contact with the parasite and host cell. Therefore, in addition to tests of antiparasitic activity *in vivo*, it is important to understand the effects of drug candidates on the immune system because this may influence their therapeutic effect *in vivo.* Another important aspect to be considered is the route of drug administration. In the case of physalin F, despite having a potent immunosuppressive action, treatment with this compound in *Leishmania amazonensis*-infected mice was beneficial topically but not orally (Guimarães *et al.*, 2009). While the beneficial effects of topical treatment with physalin F in case of cutaneous leishmaniasis caused by *L. amazonensis* infection may have a contribution from its action on the parasite, the local suppression of the immune response may also be beneficial since the skin lesion caused by this parasite has an inflammatory component that causes tissue destruction. In contrast, in the case of infection by *P. berghei*, systemic treatment seems not to be benefic, or even cause a worsening of infection.

In addition to the antimalarial activity, the SEA analysis also predicted other activities for the physalins studied. Some of the activities predicted for the physalins by this analysis, such as antitumoural, immunosuppressive, and antiinflammatory activities, have already been demonstrated (Magalhães et al, 2006; Chiang et al, 1992; Soares *et al.*, 2003 and 2006; Brustolim *et al*, 2010; Vieira *et al.*, 2005). Altogether, these results indicate the SEA analysis as an additional tool for the investigation of putative biological activities of chemical entities. In conclusion, our results demonstrate, for the first time, that the secosteroids physalins are endowed with antiplasmodial activity *in vitro* against *P. falciparum*, an activity suggested by an *in silico* analysis. Furthermore, we suggest that the

evaluation of the immunomodulatory activity of antiparasitic compounds should be taken into account when *in vivo* tests are carried out.

Table 1: Novel target selectivity predictions for physalins using SEA database

Query	Rank	Size	Activity class	E-value	Z-Score	Max Tc
Physalin B	1	15	Vaccine adjuvant	$7.23.10^{-9}$	19.10	0.59
	2	23	Aldosterone antagonist	$1.56.10^{-6}$	15	0.62
	3	436	Antimalarial	$8.55.10^{-6}$	13.60	0.73
	4	195	Agent for cystic fibrosis	$8.38.10^{+0}$	2.90	0.58
	5	388	Microtubule inhibitor	$4.58.10^{+1}$	1.50	0.58
	6	1082	Antimitotic	$6.39.10^{+1}$	1.20	0.58
	7	520	Agent for transplant rejection	$8.22.10^{+1}$	1	0.58
Physalin D	1	15	Vaccine adjuvant	$3.53.10^{-10}$	21.50	0.66
	2	14	Insulin derivative	$9.23.10^{-10}$	20.70	0.61
	3	436	Antimalarial	$1.87.10^{-5}$	13	0.69
	4	596	Squaleno synthetase inhibitor	$2.46.10^{-1}$	5.60	0.57
	5	248	Thrombolytic	$1.52.10^{+1}$	2.40	0.59
	6	936	Steroid 5-alpha reductase inhibitor	$4.22.10^{+1}$	1.60	0.61
	7	465	Agent for ARDS	$6.34.10^{+1}$	1.20	0.59
	8	474	Cell adhesion molecule antagonist	$6.57.10^{+1}$	1.20	0.59
	9	1152	Antineoplastic antibiotic	$6.63.10^{+1}$	1.20	0.62
	10	2803	Antifungal	$7.95.10^{+1}$	1.10	0.61
Physalin F	1	14	Insulin derivative	$2.47.10^{-9}$	20	0.59
	2	23	Aldosterone antagonist	$6.70.10^{-6}$	13.80	0.57
	3	436	Antimalarial	$3.15.10^{-5}$	12.60	0.67
	4	936	Steroid 5-alpha reductase inhibitor	$4.51.10^{+1}$	1.50	0.59
Physalin G	1	5	Sweetener	$8.17.10^{-20}$	38.80	0.57
	2	15	Vaccine adjuvant	$3.54.10^{-10}$	21.50	0.66
	3	14	Insulin derivative	$1.17.10^{-9}$	20.60	0.61
	4	436	Antimalarial	$2.22.10^{-5}$	12.90	0.68
	5	70	Chemopreventive	$1.04.10^{-1}$	6.30	0.57
	6	248	Thrombolytic	$1.48.10^{+1}$	2.40	0.59
	7	936	Steroid 5-alpha reductase inhibitor	$4.19.10^{+1}$	1.60	0.60
	8	465	Agent for ARDS	$6.26.10^{+1}$	1.30	0.59
	9	474	Cell adhesion molecule antagonist	$6.49.10^{+1}$	1.20	0.59
	10	1152	Antineoplastic antibiotic	$7.06.10^{+1}$	1.20	0.60

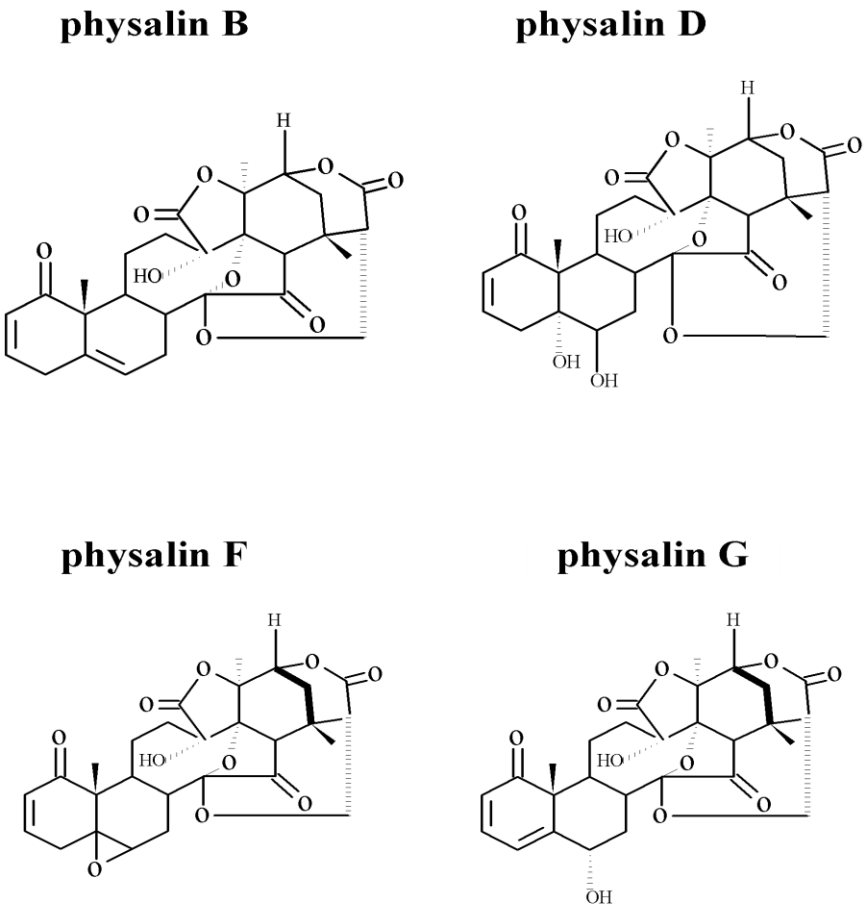

Figure 1. Chemical structures of physalins B, D, F, and G.

Table 2. Anti-*Plasmodium falciparum* activity and cytotoxicity in vitro

Drug	IC_{50} (µM)	LC_{50} (µM)	SI
Physalin B	2.76 ± 1.20	33.94 ± 9.60	12.30
Physalin D	55.01 ± 0.96*	570.40 ± 146.40	10.40
Physalin F	2.23 ± 1.16	13.25 ± 6.01	5.94
Physalin G	6.70 ± 0.37*	37.46 ± 7.10	5.60
Mefloquine	0.04 ± 0.01	9.53 ± 0.46	238

SI = Selectivity index. Values are mean ± SD of three independent experiments;

* P < 0.0001 compared to untreated control.

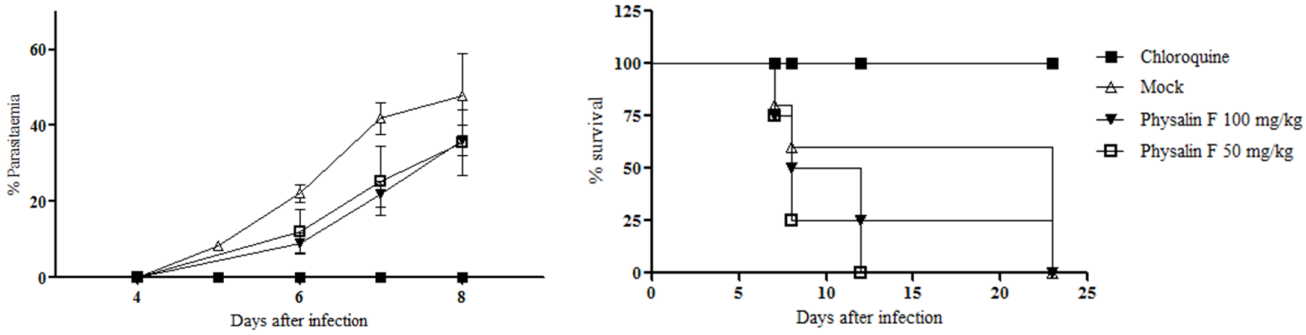

Figure 2. Treatment of *P. berghei*-infected mice with physalin F. Parasitemia of *P. berghei*-infected mice treated with 10% DMSO in saline (mock), physalin F at 50 and 100 mg/kg, or chloroquine at 50 mg/kg, by intraperitoneal route, daily, for four consecutive days. Blood parasitemia was determined on several days after infection. Values represent the means ± standard error of five mice per group in one experiment of two performed (A). In B, the results are from one experiment done with five mice per group.

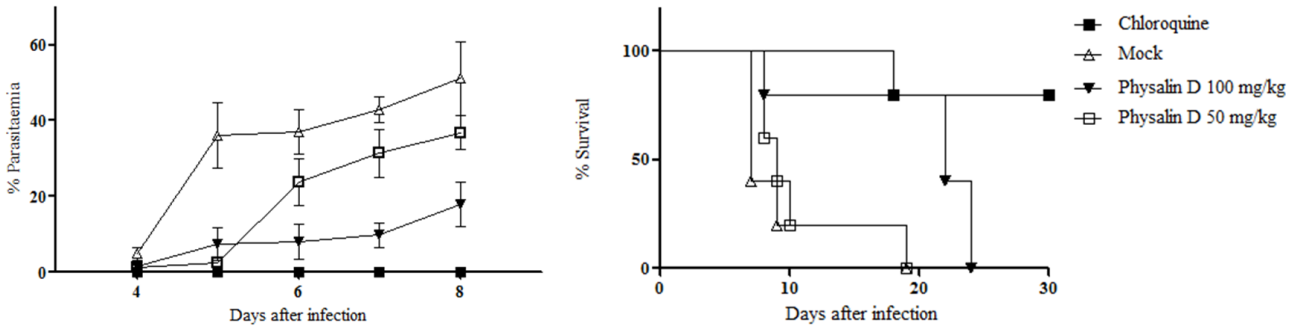

Figure 3. Treatment of *P. berghei*-infected mice with physalin D. Parasitemia of *P. berghei*-infected mice treated with 10% DMSO in saline (mock), physalin D at 50 and 100 mg/kg, or chloroquine at 50 mg/kg, by intraperitoneal route, daily, for four consecutive days. Blood parasitemia was determined on several days after infection. Values represent the means ± standard error of five mice per group in one experiment of two performed (A). In B, the results are from one experiment done with five mice per group of two performed.

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Transparency declarations

None to declare

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5. MANUSCRITO 3

No manuscrito intitulado **“Antimalarial activity of lapachol and derivatives against *P. falciparum in vitro* and *P. berghei in vivo*”** foi prevista a atividade antimalárica do lapachol e derivados, utilizando os modelos computacionais QSAR e Docking, com os resultados sendo confirmados *in vitro* e *in vivo* para o isolacet, um derivado do lapachol.

Antimalarial activity of naphthoquinones: in silico, in vitro and in vivo studies

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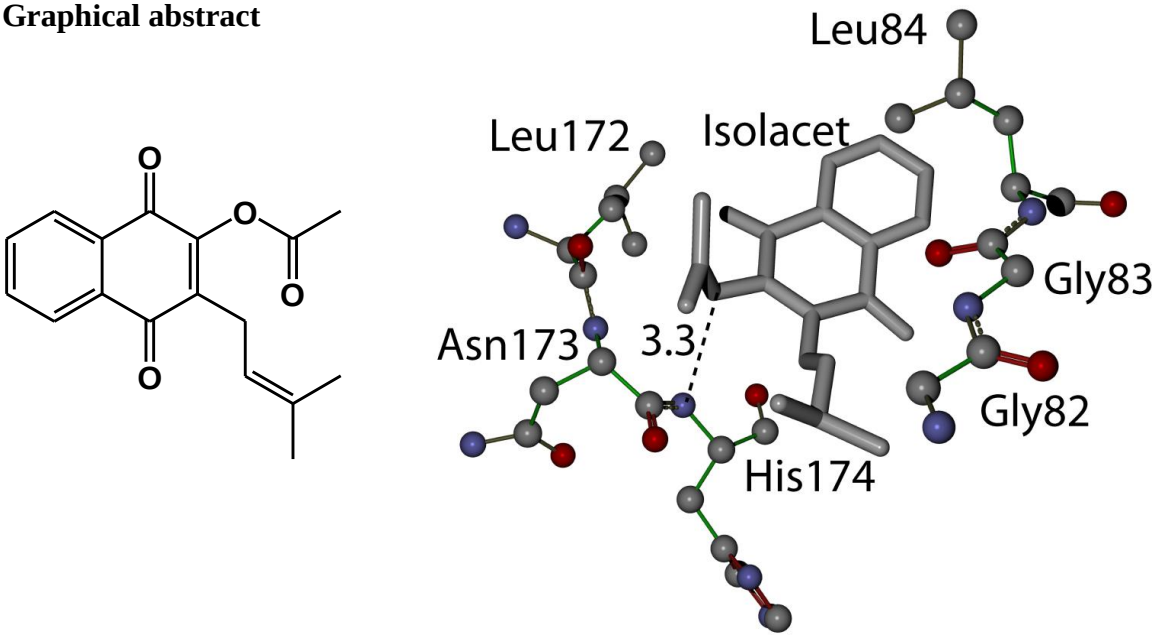
Abstract

The *Plasmodium falciparum* cysteine protease falcipain 2 is a potential target for antimalarial drugs. We report here a virtual screening on ZINC compound library against falcipain 2 and studies on in vitro anti *P. falciparum* activity evaluation. As a result of this integrative in silico and in vitro approach, the identification of naphthoquinones inhibiting the growth of *P. falciparum* parasites in culture was obtained. The most potent antimalarial among them, an acetyl 1,4-naphthoquinone derivative, was evaluated in a mouse model of infection, where a significant reduction of parasitemia was achieved in a dose of 25 mg/kg.

Keywords: Malaria, *Plasmodium faciparum*, naphthoquinones, lapachol, QSAR, docking**,** facipain-2.

Graphical abstract



The identification of new antimalarials based on naphthoquinones is described here.

Introduction

Malaria is a global disease caused by protozoan Apicomplexan parasites of the *Plasmodium* genus and is transmitted by *Anopheles* mosquito. This disease is one of the largest health problems in the world due to its high mortality and morbidity, and is a danger to more 2 billion people living in high incidence areas (WHO, 2010). Although many antimalarial drugs have been used for a long time, the use of these drugs today is severely limited by the emergence and spread of drug resistance (Travassos, 2009). *Plasmodium falciparum*, the causative agent of malaria malignant form, has a high capacity to adapt by mutation and is resistant to several antimalarial drugs (such as chloroquine), a serious problem for programs to combat of disease (O'neill, 2004; Mackinnon, 2010).

Among the molecular targets in *P. falciparum*, the papain-family cysteine protease falcipain-2 (FP-2) is known to catalyze the cleavage of host hemoglobin, a process that is ubiquitous for the development of erythrocytic parasites. Because of the important role of FP-2 as drug target, the search for FP-2 inhibitors is a task which has been receiving much attention.

Compounds from natural origin still represent a major source for the discovery and development of new antimalarials. From the plethora of antimalarial natural products (ANP), naphthoquinones have emerged as the most promising non-alkaloids ANP. Studies of Structure-Activity Relationships (SARs) in the synthetic analogues and metabolites of naphthoquinones have provided important structural insights into the active core. Given this promising outlook, the search for bioactive naphthoquinones has figured prominently in recent research and given rise to many successful outcomes.

Based on findings disclosed above, we decided to combine in silico and in vitro approaches to identify potential antimalarial naphthoquinones. Because of previous reports of naphthoquinones inhibiting different cysteine proteases, FP-2 was choosen as a

molecular target for the initial in silico screening. Further work on synthesis of a small chemical inhibitor library, their evaluation against *P. falciparum* parasites in culture, and subsequent selection of the most in vitro active compound to test in animal model of infection was achieved.

Materials and methods

Mice

Specific-pathogen-free, 5-week-old male Swiss mice were maintained at the animal facilities at the Gonçalo Moniz Research Center-FIOCRUZ, and provided with rodent diet and water *ad libitum*. Animals were handled according to the NIH guidelines for animal experimentation. All procedures described here had prior approval from the local animal ethics committee.

Drugs

Figure 2 has the molecular structures for all compounds used in this study. Natural lapachol (Câmara, 2001) was extracted from the bark of species of *Tabebuia* sp., by submitting wooden chips to an aqueous sodium carbonate extraction (10% w/v), followed by dilute hydrocloridric acid precipitation and then, diethyl ether crystallization of crude crystals, mp 137–9°C (lit. 140°C), in 1–2% yield from the bark and pure enough for the preparation of derivatives and tests. Beta-lapachone was synthesized in 76% yield by the use of sulfuric acid from natural lapachol derived from *Tabebuia avellanedae* (Bignoneaceae), following methodology adapted of Cavalcante, 2008. The compound was purified by silica-gel column chromatography with n-hexane-dichloromethane (8:2, v/v) eluent system. The product was characterized by usual spectroscopic methods, including 1HNMR and 13CNMR (Hydrogen/Carbon Nuclear Magnetic Resonance) and IR (infrared

spectroscopy), as a red solid with m.p. 152-4oC. The physico-chemical properties were evaluated chromatographically through comparison of the product profile against a pure sample and melting point. Under these conditions alpha-lapachone was obtained as a by-product during chromatographic procedure, as yellow crystals with m.p. 113-115oC, in 14 % yield. Nor-lapachol was obtained by Fieser's modification methodology of the original Hooker degradation procedure of lapachol (Barbosa, 2005) in 71% yield (mp 40.5–42 °C). The 3-Iodo-alpha-lapachone and 3-iodo-beta-lapachone were obtained following a patent procedure (Barbosa, 2006), by the reaction between lapachol dissolved in an aqueous potassium carbonate solution as and an aqueous solution of iodine/potassium iodide. The compounds were submitted to column chromatography with hexane/ethyl acetate in progressive increase of polarity, yielding 36 % of 3-iodo-alpha-lapachone (yellow crystals, m.p. 108,9-112oC) and 54% of 3-iodo-beta-lapachone (orange-reddish crystals, m.p. 137,1-138oC), characterized on the basis of the usual spectroscopic methods (1HNMR, 13CNMR, IR). The acetyl-isolapachol (isolacet) was obtained by reaction between isolapachol (2 g), anhydrous sodium acetate (2 g) and acetic anhydride (13 ml), under magnetic stirring, at 100 °C for 30 minutes. The monitoring of the reaction was carried out by chromatography on silica sheet. After cooling, the precipitate formed which was dried under reduced pressure with 54% yield and characterized on the basis of the usual spectroscopic methods (1HNMR).

Cytotoxicity assay

To determine the cytotoxicity of lapachol and derivatives BALB/c mice splenocytes were cultured in 96-well plate (6×10^5 cells/well) in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (Cultilab, Campinas, São Paulo, Brazil) and 50μg/ml of gentamycin (Novafarma, Anapolis, Goiás, Brazil). The substances were tested in five concentrations, in triplicates. 1μCi/well [methyl-^{3}H]-thymidine (Amersham, Little Chalfont, England) was added to the

cultures which were incubated during 24 h at 37°C and 5% CO_2. After this period, plates were harvested using a cell harvester (MPXRI 96TI, Brandel, Gaithersburg, MD, USA) to determine the ^{3}H-thymidine incorporation using a β-radiation counter (Multilabel Reader, Hidex, Turku, Finland). The viability of the cells was determined by ^{3}H-thymidine incorporation and the cytotoxicity was calculated in relation to the ^{3}H-thymidine incorporation of untreated cultures.

Anti-Plasmodium falciparum *in vitro assay*

Lapachol and derivatives were tested for antimalarial activity *in vitro* using the *P. falciparum* W2 clone, which is chloroquine resistant and mefloquine sensitive (Junior, 1999). All the parasites were maintained in continuous culture of human erythrocytes (blood group A^+) using RPMI-1640 medium supplemented with 10% human plasma (Trager and Jensen, 2005).

Parasites grown at 2 to 5% parasitemia and 2.5% hematocrit were incubated with the pure substances tested at various concentrations, diluted with 4% dimethylsulfoxide (DMSO) in RPMI-1640 medium without hypoxanthine. Mefloquine was used in each experiment as a control for *P. falciparum* drug response. Cultures containing parasites were harvested using a cell harvester to evaluate the ^{3}[H]-hypoxanthine incorporation in a β-radiation counter (Multilabel Reader, Hidex, Turku, Finland). Inhibition of parasite growth was evaluated by comparison with ^{3}[H]-hypoxanthine uptake in drug-treated versus untreated wells. All the assays were performed in triplicates as described previously (Zalis, 1998; Andrade-Neto, 2004).

Assessment of antimalarial activity

The antimalarial activity of acetyl-isolapachol was evaluated in mice infected with *Plasmodium berghei*, strain NK65 (Andrade, 2007). Parasites were maintained by weekly blood passage 10^6 infected red blood cells per mouse by intraperitoneal route. The animals were randomly sorted into groups of five and treatment was administered daily, during four consecutive days, beginning on the first day of infection. The acetyl-iaolapachol was suspended in saline solution plus 10% DMSO immediately before use in doses of 10, 25 and 50 mg/kg. Each mouse received 200 µl, by intraperitoneal route. Experiments included a control group treated with the standard antimalarial drug chloroquine at 50 mg/kg and a mock-treated group (10% DMSO in saline). The antimalarial activity was evaluated by counting parasitemia in blood smears at days 4-8 after parasite inoculation, by optical microscopy, after fixation with methanol and staining with fast panoptic (Laborclin, Pinhais, Brazil). Inhibition of parasite growth in drug-treated groups was calculated in relation to the control (vehicle-treated) group. The results were expressed as the percentage of parasitemia reduction.

IC_{50}, LC_{50}, and SI calculations and statistical analyses

The lethal concentration of 50% of BALB/c mice splenocytes (LC_{50}) and the inhibitory concentration of 50% (IC_{50}) of *P. falciparum* were calculated based on a nonlinear regression (curve fit). The selectivity index (SI) was defined by calculating the value of LC_{50} versus IC_{50} (Bézivin, 2003). The SI was considered as significant for values higher than three. The statistical analyses were made by one-way analysis of variance and Newman-Keuls multiple comparison tests using Graph Pad Prism version 4.0 (Graph Pad Software, San Diego, CA, USA). Differences were considered significant when p values were < 0.05.

QSAR modeling

In order to elaborate a quantitative structure–activity relationship (QSAR) model to predict antimalarial activity of lapachol and derivatives we submitted the available activity information to QSAR modeling. The quantitative structure-activity relationships are founded on the assumption that the compounds can be precisely codified as distributions of molecular descriptors [Michielan, 2010]. As a result, a statistical modeling approach is capable to achieve the correlation between the molecular descriptors and the defined target activity to estimate the equivalent activity of new compounds. This computational modeling has been shown to furnish insights in the design and development of novel drugs that are expected to achieve the improvements in their activity, as well as in their toxicity profile, and pharmacology, for recent reviews please see (Verma, 2010; Scior, 2009; Dearden, 2009). This methodology makes use of experimental information and relates it to the molecular structure via equations of the following form,

$$\log\left(\frac{1}{C}\right) = c_o + c_1 x_1 + .. + c_n x_x \qquad \text{(Eq. 1)}$$

where C is the concentration of a given compound required to produce a standard response in a given time, for instance, IC_{50}, the molar concentration of a compound that inhibits 50% of growth of the parasite population; log ($1/IC_{50}$) is the subsequent dependent variable that defines the biological parameter for the QSAR model. The x's terms in equation 1 are the descriptors and c's are the relative weights of each term, being c_o the regression constant (Hansch, 1967; Hansch, 1968). Here, we carried out all QSAR modeling using molegro data modeler 2.5 (MDM) program (Molegro, Molegro ApS C. F. Møllers Alle, Building 1110, DK-8000 Aarhus C Denmark), which is a cross-platform application for data mining, data modeling, and data visualization. Multiple regression (MR) analyses were applied to derive appropriate QSAR models using autoloaded descriptors available in the MDM program. The structures of lapachol and its derivatives were obtained from PubChem and saved as SDF files (Timmers, 2008) and used as input to MDM program. In

addition, the logarithm of molecule's partition coefficient between 1-octanol and water (log P) were calculated for all structure using the XLOGP3 program (Cheng, 2007).

In MR the model assumes that the dependent variable is a function of the independent variable (descriptors). To successfully apply MR the number of observations must be larger than the number of descriptors selected. There is a general rule-of-thumb that says that there should be at least four or five compounds for each descriptor that is included in the regression analysis (Scior, 2009). Once a MR equation is obtained we use the coefficient relevance for each descriptor, which indicates how relevant this descriptor is for model building with respect to modeling the target variable. The coefficient relevance score for MR is the product of the specific coefficient and the standard deviation of the corresponding numerical descriptor divided by the standard deviation of the target variable. In this work, the relevance scores were used to find the best descriptors for modeling the target variable.

Molecular structures

It has been proposed that quinone is an important pharmacophore for the development of antimalarial drugs (Ettari, 2010). The pharmacophore is an effort to capture the main structural aspects of the protein-ligand interaction. Therefore, it has to be specific enough to be functional for a particular target and, at the same time, general enough so that the information can be employed to find new molecules that are likely to bind the target. Based on this observation, we employed the quinone core to carry out a search in the ZINC database (Irwin, 2005) to build a small-molecule database, using the pharmacophoric fingerprints of quinone with a Tanimoto coefficient cutoff of 80 %. A total of 80 molecules were retrieved and used to build this database, which is also available for download at http://azevedolab.net (Timmers, 2008).

The crystallographic structure available for falcipain-2 in complex with epoxysuccinate E64 (PDB accession no. 3BPF) (Kerr, 2009) was used in all docking simulation. All ligands present in the quinone analogues database and the falcipain-2 crystallographic structure had hydrogen atoms added to their structure, when necessary, by the default protocol of MOLDOCK program (Thomsen, 2006). All water and ligands molecules present in the structure of falcipain-2 were removed for molecular docking simulations.

Docking methodology

Molecular docking is a simulation process that predicts the conformation of a receptor-ligand complex, in which the receptor can be either a protein or a nucleic acid, and the ligand is a small molecule. In order to further investigate the antimalarial activities of this series of lapachol analogues we employed molecular docking against falcipain-2. The lapachol is structurally related to some antimalarial drugs such as atovaquone and proguanil. Furthermore, falcipain-2 (EC 3.4.22.-) is the major cysteine protease of the human malaria parasite *Plasmodium falciparum*, is a hemoglobinase and promising drug target (Ettari, 2010). Structural analyses of the falcipain-2 in complex several different inhibitors indicate that this enzyme has the ability to accommodate different molecular moieties in its active site (Hogg, 2006; Wang, 2006; Kerr, 2009). In addition, quinone derivatives were found to be inhibitors for cysteine proteases (Valente, 2007; Bourguignon, 2010; Ettari, 2010). Therefore in the present study we investigate the potential inhibition of falcipain-2 by this series of quinone analogues.

We applied the evolutionary algorithms (De azevedo, 2010) available in the MOLDOCK program (Thomsen, 2006) to carry out virtual screening of the quinone database against falcipain-2. Docking applications can be classified by their search algorithm, which is defined by a set of rules and parameters applied to predict the

conformations. When we consider the flexibility of the ligand and/or the receptor docking, algorithms can be classified into two major groups: rigid-body and flexible docking (Dias, 2008). We applied the flexible docking protocol available in the program MOLDOCK, where the flexibility of the ligand is simulated. MOLDOCK is an implementation of a heuristic search algorithm that joins together differential evolution with a cavity calculation algorithm. In addition, MOLDOCK evaluates the best poses applying a docking scoring function, which uses piecewise linear potential, previously implemented in the program GEMDOCK (Yang, 2004). There two main options MOLDOCK score and re-ranking score functions. The re-ranking score function is computationally more expensive than the MOLDOCK scoring function used during the docking simulation but it is usually better than the MOLDOCK docking score function at determining the best pose among several poses originating from the same ligand or from library of small molecules with similar structures (De Azevedo, 2008). In the virtual screening we selected the best hits using re-ranking score function.

We used the default docking protocol of MOLDOCK (simplex evolution search algorithm) with center at coordinates x = -57.17, y = -1.06, z = -15.90 Å and docking sphere with radius of 15 Å. Figure 1 shows the docking sphere used in the docking simulations, which is centered at the active site of falcipain-2. The cavity search algorithm was set to identify up to 5 cavities in the structure. All simulations were performed in an iMac (Intel Processor Core 2 Duo de 2.66GHz, 2GB SDRAM DDR3 1066MHz). The dataset of molecules was submitted to docking against the active site of falcipain-2 (virtual screening).

Results

In vitro anti-*P. falciparum* activity

A set of naphthoquinones structurally congener were tested as putative anti-*P. falciparum* inhibitors (Table 1). It includes lapachol, α-lapachone, β-lapachone, *nor*-lapachol, 3-iodo-α-lapachone, 3-iodo-β-lapachone, and the acetyl-isolapachol (isolacet). Figure 2 shows their chemical structures.

Table 1. Anti-*Plasmodium falciparum* activity and cytotoxicity in vitro.

Compounds	IC_{50} (μM)	LC_{50}(μM)	SI
Lapachol	80.48	26.22	0.33
Alpha-lapachone	15.82	13.91	0.88
Beta-lapachone	20.50	3.02	0.15
Alpha-xiloidone	41.2	ND	ND
Nor-lapachol	25.10	15.86	0.63
3-iodo-alpha-lapachone	19.40	21.44	1.11
3-iodo-beta-lapachone	15.80	13.77	0.87
Acetyl-isolapachol	2.35	9.07	3.86
Mefloquine	0.04	5.24	131

SI = Selectivity index. ND = Not determined.

On one hand, we observed that α-lapachone, β-lapachone, and *nor*-lapachol, which are structural isomers, show similar, albeit modest, plasmodicidal activity. The halogen derivatives 3-iodo-α-lapachone and 3-iodo-β-lapachone show, on the other hand, weaker plasmodicidal property, having almost half of the potency when compared to non-halogenated analogues. We were also able to identify isolacet as the most potent antiplasmodial agent of this series. Isolacet did not show high cytotoxicity against splenocytes. In other words, it was a parasite inhibitor drug at concentrations that do not overly affect mammalian cells. Despite Isolacet was more effective than other

naphthoquinones, it proved to be less potent than Mefloquine, indicating that further optimization studies with a view to improving the antiplasmodial properties of this prototype should be performed.

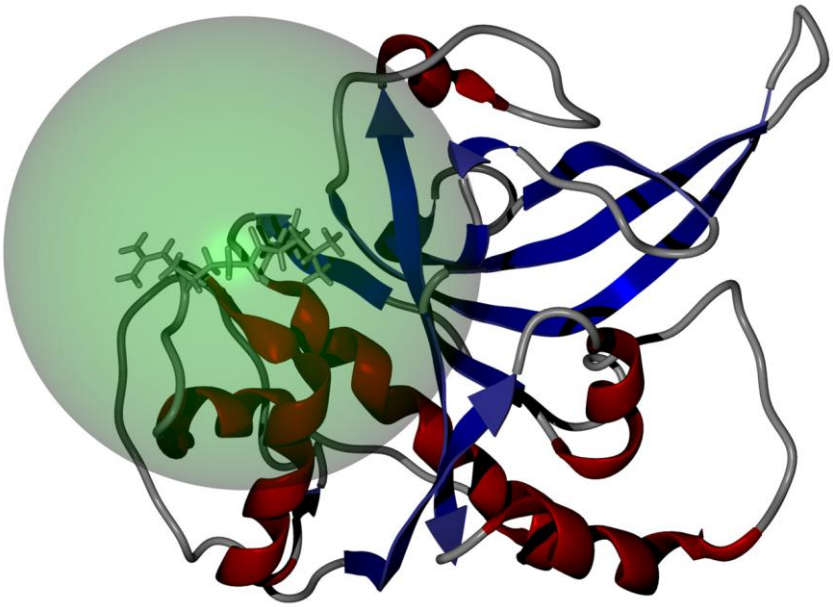


Figure 1 - Docking sphere (green) used in the docking simulations for the falcipain-2 structure (PDB accession no. 3BPF).

QSAR

A number of key steps are implicated in any QSAR modeling method, such as data collection, calculation of appropriate molecular descriptors from structures and selection, model generation (MR analysis), and lastly, validation (statistical analysis of the QSAR model). Data here came from IC_{50}, which were converted into log ($1/IC_{50}$) in molar concentration and given in Table 2. Figure 2 shows the structures of all compounds used in the QSAR modeling. The MDM program provides a total of 16 different molecular

descriptors all of them were tested and selected based on correlation coefficient (data not shown). In addition, log P was also used in the QSAR modeling. We used a QSAR model with 2 descriptors due to the limitation of experimental information. The two highest coefficient relevances were used to build a polynomial. All possible combination of pair of molecular descriptors was tested using MDM program (data not shown). The best QSAR model was obtained using the number of hydrogen bond acceptors (HA) and the number of rotatable bonds (rot). Both terms were squared and a QSAR, Eq. (2) was developed, as follows:

$$Log\left(\frac{1}{\mathrm{IC}_{50}}\right) = 0.50491 + 0.464598.\mathrm{HA}^2 - 0.144347.\mathrm{rot}^2 \quad \text{(Eq. 2)}$$

This QSAR model presents the following statistics: Pearson correlation squared of 0.91, Spearman rank correlation of 0.81, mean squared deviation of 0.1517, and root mean squared deviation of 0.1232. Since we have a relatively small number of molecules in this training set, this QSAR model presents adequate statistical quality [Scior et al., 2009. Inclusion of quadratic terms is not new in QSAR modeling, it has been proposed in the first QSAR studies in the late 1960s (Hansch, 1968).

Analysis of the QSAR model indicates coefficient relevances of 1.82 and 2.61 for rot^2 and HA^2, respectively. The positive coefficient and the higher coefficient relevance of HA^2 suggest that the presence of hydrogen bonds acceptors will be more favorable to the antimalarial activity. A comparison between observed and predicted values of log ($1/IC_{50}$) for these analogues used in the development of QSAR Eq. (2) is shown in Figure 3. The underlying principle for suggesting the dependence on HA^2 is that an increasing number of hydrogen bond acceptors increases solubility in water. In addition, increasing the number of rotatable bonds decreases the lead-likeness of drug, as has been suggested by Verber, 2002. Both descriptors seem to be enough to capture the essence of the drug-likeness features of this series of analogues. Nevertheless, analysis of the interaction between the

molecules and the protein target may give additional information to understand the structural basis for activity of these compounds, especially acetyl-isolapachol (isolacet). In addition to the 8 compounds used to build the QSAR model we employed the same equation to analyze the molecules present in the quinone database. A total of 80 molecules were analyzed, with log ($1/IC_{50}$) ranging from 3 to 5.6, being isolacet the compound with the highest activity.

Table 2. Biological (IC_{50}, mol L^{-1}), physicochemical, and structural parameters of used to derive the QSAR model.

Name	**$\log(1/IC_{50})_{obs}$**	**$\log(1/IC_{50})_{pred}$**	**HA^2**	**Rot^2**
Lapachol	4.09	4.11	9	4
Alpha-lapachone	4.80	4.69	9	0
Beta-lapachone	4.69	4.69	9	0
Alpha-xiloidone	4.39	4.69	9	0
Nor-lapachol	4.60	4.54	9	1
3-iodo-alpha-lapachone	4.71	4.69	9	0
3-iodo-beta-lapachone	4.80	4.69	9	0
Acetyl-isolapachol	5.63	5.63	16	16

HA: number of hydrogen bond acceptors. rot: number of rotatable bonds for non-hydrogen atoms.

Docking and virtual screening (VS)

The VS results were analyzed taking into account the piecewise linear potential, which is obtained from the MOLDOCK program. The small-molecule database employed in the present work was built using the quinone core as inspiration. The analysis of the 80 docked molecular structures indicated a docking re-ranking score ranging from -77.88 to 10.28, being the lowest considered the highest affinity ligand. Figure 4 shows the best 10 hits obtained from VS. Analysis of these hits indicates that they are tethered to the main chains of falcipain-2 through a glycine residue that is highly conserved in the S3 subsite of cysteine proteases (Gly83 in falcipain-2). Identification of acetyl-isolapachol as the best hit

is in agreement with IC_{50} values determined for the series of 8 analogues previously described.

Figure 5 shows acetyl-isolapachol docked to the active site of falcipain-2. Isolacet displays binding mode with falcipain-2 similar to those found the crystallographic structures of falcipain-2 and 3 complexed with inhibitors (Kerr, 2006), with acetyl-isolapachol mainly targeting the canonical S2 and S3 subsites of this cysteine protease. Furthermore, the acetyl-isolapachol which shows the lowest IC_{50} among the compounds tested here is involved in a number of hydrophobic interactions. These interactions involve residues Gly 82, Gly 83, Leu 84, Leu 172. In the structure of falcipain-2-acetyl-isolapachol, this compound forms only one intermolecular hydrogen bond, involving residue His 174 (main chain NH). In addition, QSAR model has predicted that hydrogen bond acceptors play a pivotal role in antimalarial activity. Analysis of the complex falcipain-2-isolacet suggests that further inhibition improvement may be obtained with a derivative with additional hydrogen bond acceptor, especially in the position near to Gly 82 and 83.

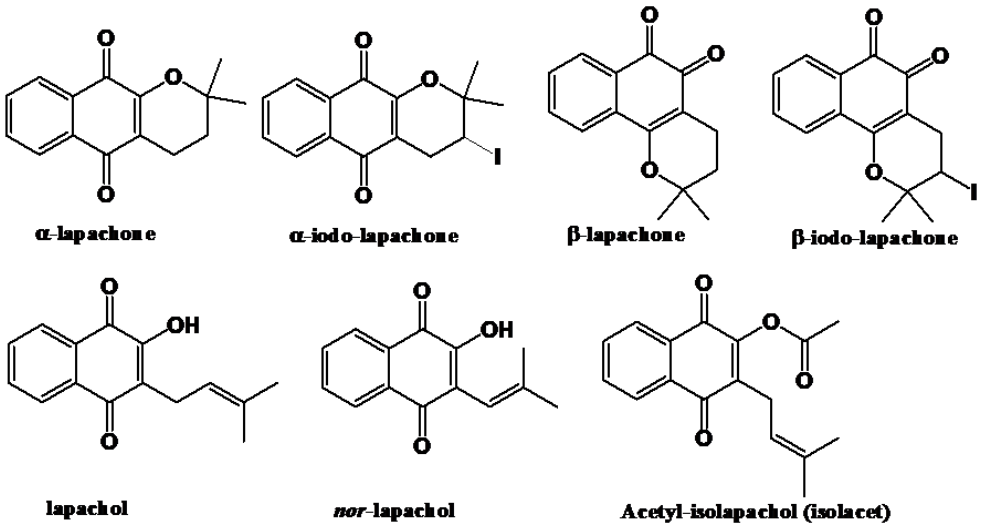


Figure 2 - Molecular structures for all compounds used in the QSAR modeling.

In vivo anti-P.berghei assay

We evaluated the antimalarial activity in vivo of isolacet, the molecule presenting the highest anti-*P. falciparum* activity (Table 1), in *P. berghei*- infected mice. Isolacet was tested in three different doses (10, 25 and 50 mg/kg). The dose of 25 mg/kg showed an inhibition of parasitemia higher than 10 and 50 mg/kg and ensured the survival of the animals for 39 days. While the doses of 10 and 50 mg/kg, the animals survival was of 24 and 32 days respectively (Figure 6).

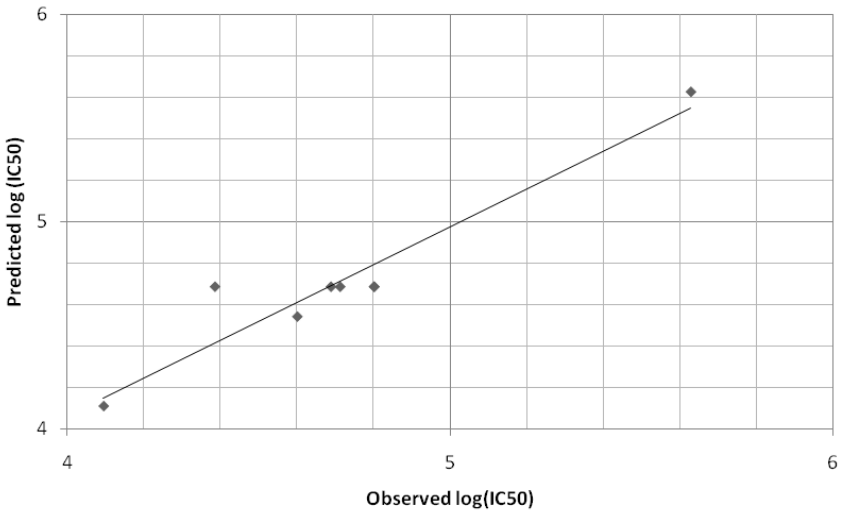


Figure 3 - Observed and predicted values of log ($1/IC_{50}$) for analogues used in the development of QSAR model. The least square fit equation is y=0.42126+0.910634x.

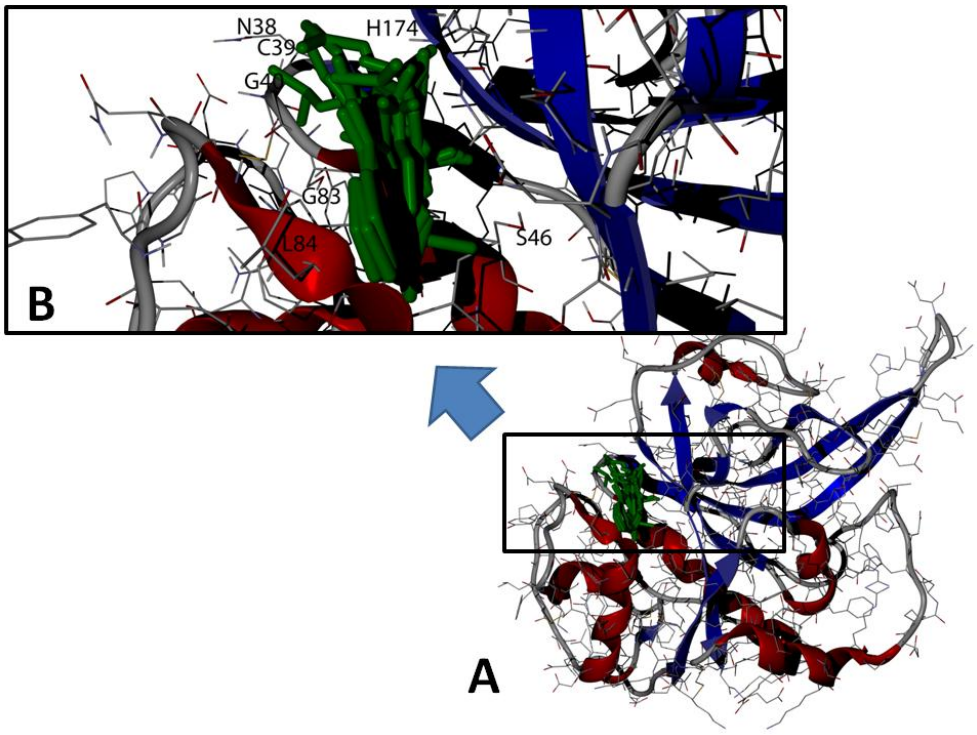


Figure 4 - **A.** 10 best hits (green) obtained in molecular docking simulations against falcipain-2 structure. **B.** Zoom of the active site, where the main residues involved in intermolecular interaction are shown (Asn38, Cys39, Gly40, Ser46, Gly83, Leu84, and His174). In the figure labels are indicated with one-letter code for the amino acids.

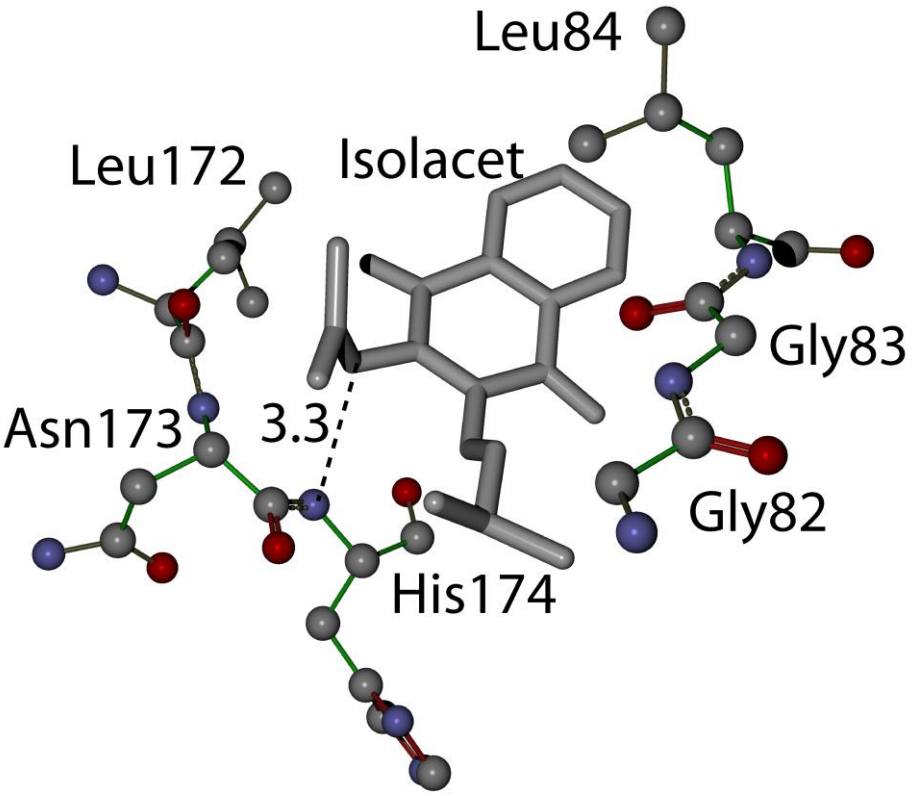


Figure 5 - Intemolecular interactions observed in the complex involving acetyl-isolapachol and falcipain-2. Dashed lines indicated hydrogen bond, distance is in Å.

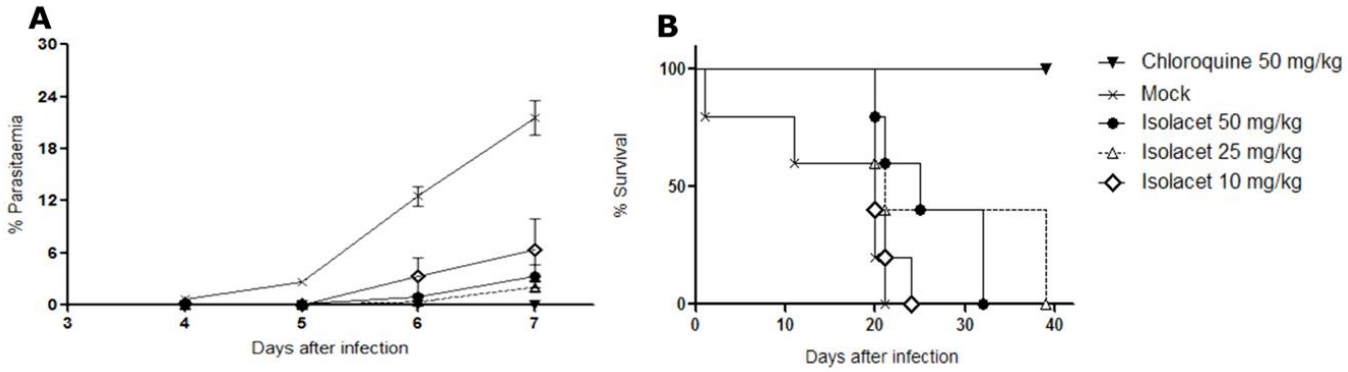


Figure 6 - Treatment of *P. berghei*-infected mice with acetyl-isolapachol. In **A**, parasitemia and in **B**, cumulative mortality rates of *P. berghei*-infected mice treated with 10% DMSO in saline (mock), acetyl-isolapachol at 10, 25 and 50 mg/kg, or chloroquine at 50 mg/kg, by intraperitoneal route, daily, for four consecutive days. Blood parasitemia was determined on several days after infection. Values represent the means ± standard error of 10 mice in two experiments performed (**A**). In **B**, the results are from one experiment done with five mice per group.

Conclusion

In the present work we analyzed a series of 80 quinone analogues employing QSAR and molecular docking approaches. Both computational methodologies returned as the best hit the compound isolacet. An analysis of the QSAR model revealed a number of interesting points. The most important of these are related to HA and rot descriptors. The QSAR model suggests that the antimalarial activity of a derivative might be improved by the presence of higher number of hydrogen bond acceptors and a reduction in the number of rotatable bonds. In addition, activity tests indicated that isolacet presents antimalarial activity in μM range. Analysis of the complex between isolacet and falcipain-2 obtained from docking simulation indicated that most of the intermolecular interaction came from hydrophobic contacts, with only one intermolecular hydrogen bond. These results suggest that further improvement of antimalarial activity may be obtained addition hydrogen bond acceptors in the region of isolacet close to Gly 82 and 83.

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6. DISCUSSÃO

Neste trabalho foram avaliadas diversas substâncias isoladas de plantas nativas ou endêmicas do semi-árido brasileiro, bem como alguns derivados dessas obtidos por síntese química. Estes compostos foram selecionados com base em uma triagem prévia de 160 substâncias puras que foram avaliadas quanto à atividade antimalárica. No caso das fisalinas, estas foram selecionadas com base no SEA. No screening inicial, 35 substâncias apresentaram atividade antimalárica. Determinamos os valores de IC_{50} e LC_{50} para três classes de compostos, o ácido betulínico e derivados, o lapachol e derivados, bem como as fisalinas. Destas três classes, algumas moléculas foram analisadas em experimentos *in vivo*, por apresentarem atividade *in vitro* e por estarem disponíveis em quantidade suficiente para utilização em ensaios *in vivo*. No caso do lapachol e seus derivados, foram aplicados modelos computacionais no sentindo de se sugerir o mecanismo de ação dessas drogas.

Dentre as moléculas investigadas com atividade anti-*Plasmodium in vitro*, encontramos o ácido betulínico, que foi analisado em comparação com quatro derivados. O ácido betulínico foi isolado de uma planta nativa da Caatinga *Zizyphus joazeiro* (Cartaxo, 2010), a qual já foi estudada quanto às atividades antipirética (Nunes, 1987), antibacteriana (Schühly, 1999) e antifúngica (Cruz, 2006). Em um estudo prévio, foi relatada a atividade anti-*Plasmodium in vitro* do ácido betulínico, com IC_{50}= 5.1 µM, porém esta molécula não apresentou atividade antimalárica *in vivo* (Lenta, 2007). Em nosso trabalho, o ácido betulínico também apresentou uma significativa atividade antimalárica *in vitro* contra uma cepa de *P. falciparum* cloroquina-resistente. No entanto, em comparação com os quatro derivados testados, o acetato do ácido betulínico (BAA) apresentou a melhor atividade anti-*Plasmodium*, com valor de IC_{50} mais baixo, demonstrando a maior potência farmacológica de um derivado e reforçando a importância dos estudos químicos no sentido de se triar novas moléculas com maior atividade biológica e menor toxicidade, derivadas de compostos com atividade conhecida.

Outro dado relevante utilizado foi o cálculo do Índice de Seletividade (IS) para todas as substâncias testadas. Com o IS apresentando valor maior que três, entende-se que há alta seletividade da molécula da droga para o *Plasmodium*. O BAA apresentou IS acima de três e IC_{50} menor que os outros derivados testados, demonstrando maior potência e seletividade. Desse modo, selecionamos esta substância para testes *in vivo* em

camundongos infectados por *P. berghei*. Embora o BAA não tenha demonstrado atividade *in vivo* por via oral, por via intraperitoneal a redução da parasitemia em 70% pôde ser considerada significativa, visto que compostos que reduzem a parasitemia em 30% ou mais são considerados ativos (Carvalho, 1991). O fato de o BAA não ter funcionado por via oral pode ser atribuído a uma baixa absorção por esta via, que pode ser aumentada através da elaboração de sistemas que permitam uma melhor biodisponibilidade da molécula, como por exemplo, sistemas de entrega de drogas autoemulsificadas (Xi, 2009). Outros derivados do ácido betulínico (BAME e BAMEA) apresentaram IS abaixo de 3, demonstrando baixa seletividade. Além disso, o valor de IC_{50}, no caso do BAMEA foi maior que o valor de LC_{50}, demonstrando que esse derivado é mais tóxico para as células de mamíferos que para o parasita. Estudos adicionais poderão ser realizados para o BOA, pois este apresentou IS superior a todos os outros derivados do BA, mesmo com IC_{50} quase duas vezes maior que o BAA.

A *Physalis angulata* L. tem sido utilizada popularmente devido aos seus efeitos antiinflamatórios (Bastos, 2008) e até mesmo antimaláricos (Ruiz, 2010). Para as fisalinas, uma ferramenta adicional utilizada foi o SEA, cujo princípio é baseado na similaridade entre as moléculas analisadas e uma atividade biológica. No caso das fisalinas, o SEA forneceu informações sugerindo que todas as fisalinas apresentam atividade antimalárica prevista com valores de E iguais a 8.55×10^{-6} para a fisalina B e 3.15×10^{-5} para a fisalina F, sendo que valores de E acima de 1.0 são considerados insignificantes (Keiser, 2007). A atividade antimalárica foi a única prevista para todas as fisalinas no modelo do SEA, sugerindo que as fisalinas poderiam apresentar alguma atividade anti-*Plasmodium*, o que foi confirmado nos experimentos *in vitro* com as fisalinas B, D, F e G, bem como *in vivo* para a fisalina D.

Além do exposto, foi calculado o coeficiente de Tanimoto (Tc) que, no caso da fisalina F foi 0.67 e da fisalina B foi 0.73, sugerindo que essas moléculas apresentam atividade antimalárica, pois quanto mais próximo de 1.0 for o Tc, maior será a similaridade entre a molécula analisada com moléculas que apresentem uma determinada atividade farmacológica. A atividade antimalárica sugerida, pela primeira vez, pelo modelo citado anteriormente foi confirmada pelo menos para algumas das moléculas testadas *in vitro* e *in vivo*. As fisalinas B, F e G apresentaram valores de IC_{50} de 2.76, 2.23 e 6.70 µM, respectivamente, enquanto que a fisalina D foi a menos ativa *in vitro* apresentando IC_{50}=55 µM.

O tratamento de camundongos infectados por *P. berghei* com a fisalina F não causou uma redução da parasitemia e muito menos reduziu a mortalidade. Quando analisamos a fisalina D, cujo valor de IC_{50} foi cerca de 24 vezes maior que o da fisalina F, demonstrando menor potência farmacológica *in vitro,* observamos que a fisalina D diminuiu a parasitemia e aumentou a sobrevida dos animais tratados. Esses resultados podem ser justificados pelo fato da fisalina D não apresentar atividade imunossupressora, como pôde ser observado para a fisalina F, em outros trabalhos (Soares, 2003 e 2006). A supressão induzida pela fisalina F pode ter inibido a resposta imune antimalárica nos animais infectados, importante para o controle da infecção.

Ensaios *in vitro* são sistemas isolados que demonstram a ação da droga apenas em contato com o parasita e célula hospedeira. Portanto, além dos testes de atividade antiparasitária *in vivo*, é importante entender os seus efeitos sobre o sistema imune, pois isto pode influenciar no seu efeito terapêutico *in vivo*. Outro aspecto importante é a via de administração da droga. No caso da fisalina F, mesmo possuindo ação imunosupressora, o tratamento com este composto de camundongos infectados com *Leishmania amazonensis* foi benéfico por via tópica, mas não por via oral (Guimarães, 2009). A ação benéfica do tratamento com fisalina F no caso da infecção por *L. amazonensis* pode ter a contribuição tanto do seu efeito no parasita, quanto no sistema imune local, uma vez que a lesão cutânea causada por este parasita apresenta um componente inflamatório causador de destruição tecidual. Neste caso, assim como no caso da infecção por *P. berghei*, o tratamento sistêmico parece não ser benéfico, ou até causar uma piora da infecção.

Modelos computacionais foram utilizados no terceiro trabalho da tese, onde foi avaliada a atividade antimalárica (*in vitro* e *in vivo*) do lapachol e seus derivados, com o objetivo de entender a interação das moléculas do lapachol e derivados com alvos moleculares no parasita, como a falcipaína 2, enzima essencial para a continuidade do ciclo de vida e sobrevivência do *Plasmodium* (Micale, 2009). O lapachol foi isolado de *Tabebuia avellanedae*, planta que ocorre do Maranhão até a região Sul do Brasil (Souza, 2004). Naftoquinonas bioativas isoladas de espécies do gênero *Tabebuia* têm sido avaliadas quanto às atividades antitumoral (Yamashita, 2009) e anti-*T. cruzi* (Pinto, 2000). A análise dos derivados do lapachol, como alfa-lapachona, beta-lapachona, nor-lapachol, alfa-iodo-lapachona, beta-iodo-lapachona e acetil-isolapachol (isolacet), foi motivada pelo fato de moléculas semelhantes, as naftoquinonas, apresentarem atividade antimalárica já demonstrada. O lapachol, também já considerado um agente antimalárico (De Andrade-Neto, 2004), quando comparado com os seus derivados, apresenta modesta ação

antimalárica (IC_{50}=80,5 μM), sugerindo que alterações moleculares mínimas podem alterar também a atividade biológica desta molécula.

Dentre os derivados do lapachol analisados *in vitro*, o isolacet apresentou maior potência farmacológica em relação às outras moléculas analisadas. Outros derivados do lapachol (alfa-lapachona, beta-lapachona, nor-lapachol, alfa-iodo-lapachona e beta-iodo-lapachona) também apresentaram potência farmacológica pelo menos 4 vezes maior que o lapachol, porém nenhuma delas apresentou valor de IS acima de 3, exceto o isolacet. Esse perfil farmacológico do isolacet, com baixo valor de IC_{50} associado ao maior valor de IS, determinou a escolha desta molécula para os experimentos *in vivo*.

Quando testado *in vivo* na dose de 25 mg/kg, o isolacet causou uma redução da parasitemia 3 vezes maior que na dose mais baixa testada. Além disso, os animais sobreviveram por quase 40 dias. Mesmo diante dos dados expostos, pode-se sugerir que talvez o tempo de tratamento (4 dias) seja muito curto, embora tenha sido utilizado por diversos grupos de pesquisa de antimaláricos no Brasil e no mundo (De Pilla Varotti, 2008). É possível que uma farmacoterapia mais prolongada cause uma redução maior da parasitemia, bem como um aumento da sobrevida dos animais testados.

Com a utilização do modelo de QSAR, foi possível identificar diversos descritores moleculares diferentes, os quais foram testados e selecionados baseados em coeficiente de relação. Como os dados preliminares utilizados foram apenas os valores de IC_{50}, uma limitação para o uso do método QSAR, utilizamos apenas dois descritores, com um melhor modelo de QSAR obtido com HA (número de aceitadores de hidrogênio) e ROT (número de rotações na ligação). Com os resultados obtidos a partir da utilização dos descritores citados, pode-se sugerir que a presença de HA é favorável à atividade antimalárica. Ao observar os resultados obtidos para o isolacet por docking, percebemos que a maioria das interações isolacet-falcipaína 2 era hidrofóbica, com apenas uma ligação de hidrogênio intermolecular (His 174). Esses dados demonstram a importância da ligação de hidrogênio para a atividade da molécula, sugerindo que mudanças moleculares que possibilitem maiores interações de hidrogênio entre o isolacet e a falcipaína 2 possam aumentar a potência farmacológica da droga, já que apenas uma ligação de hidrogênio no complexo isolacet-falcipaína 2 favoreceu uma maior potência dessa substância em relação aos outros derivados do lapachol. É possível ainda que uma maior interação entre o ligante e o receptor favoreça uma menor toxicidade, visto que a molécula torna-se mais seletiva. No caso do isolacet já há uma seletividade grande quando comparado com os outros derivados do lapachol, demonstrada pelos valores de IS observados para esta molécula.

A partir do *virtual screening* realizado com 80 moléculas apresentando estrutura similar à do lapachol foram obtidas pontuações de classificação variando entre -77,88 e 10,28, sendo o valor mais negativo considerado o de menor energia, e quanto menor a energia maior pode ser a facilidade de interação entre duas moléculas e mais fácil uma se ligará a outra. A análise realizada por nós sugere uma maior facilidade de ligação entre alguns dos derivados testados e a falcipaína 2. Os resultados com o isolacet sugerem que este apresenta maior facilidade de se ligar à falcipaína 2 devido à menor energia de ligação mostrada por esta molécula, quando comparada com as outras analisadas no *virtual screening*. Além disso, o isolacet apresentou um perfil de ligação semelhante ao encontrado em outros inibidores da falcipaína 2, demonstrando que este pode ser um possível mecanismo de ação do isolacet, já que a inibição da falcipaína 2 prejudicaria significativamente o metabolismo do parasita (Dahl, 2005; Ettari, 2010) . No entanto, para que se possa confirmar essa sugestão, são necessários testes de inibição enzimática utilizando a falcipaína 2 purificada. Adicionalmente, o valor de IC_{50} do isolacet foi menor do que a de outras moléculas estudadas neste trabalho, reforçando os nossos resultados experimentalmente.

Todas as moléculas analisadas quanto à atividade anti-*Plasmodium*, foram comparadas com a mefloquina, droga utilizada no tratamento da malária humana, à qual a cepa W2 (cloroquina resistente) é sensível (Junior, 1999). Os valores de IC_{50} apresentados pela mefloquina, em todos os trabalhos citados, foram muito inferiores aos apresentados pelas moléculas testadas. No entanto, quando analisamos a toxicidade da droga padrão utilizada, percebemos que em todos os casos a mefloquina foi mais tóxica que as drogas analisadas em nosso trabalho, exceto a beta-lapachona que, além de ser 10 vezes menos potente que o isolacet, foi cerca de 2 vezes mais tóxica que a mefloquina. Mais especificamente, quando observamos a LC_{50} da fisalina D observamos valor 60 vezes maior que o da mefloquina demonstrando muito menor toxicidade da fisalina D. Quando comparamos os resultados obtidos nos experimentos *in vivo* com a cloroquina, o isolacet demonstrou potência antimalárica bem próxima da droga padrão utilizada, com parasitemia de 2% até o sétimo de dia após a infecção.

No curso de nossas pesquisas para o desenvolvimento de novos fármacos, nós analisamos três classes de drogas quanto à atividade antimalárica utilizando diversas ferramentas, como SEA, QSAR e Docking, além dos ensaios *in vitro* e *in vivo*. O uso dessas ferramentas revelou importantes informações a respeito da atividade biológica das substâncias e também interações químicas que podem contribuir para se entender os

prováveis mecanismos de ação das referidas drogas. A relevância do nosso trabalho pode ser ratificada quando observamos resultados obtidos em outras pesquisas de novos medicamentos, como o desenvolvimento do MEFAS, uma combinação entre mefloquina e artesunato (De Pilla Varotti, 2008), que está sendo desenvolvido pelo Instituto de Tecnologia em Fármacos (Farmanguinhos) da Fundação Oswaldo Cruz (Fiocruz) (Fiocruz, 2010).

No presente trabalho foram apresentados diversos dados de atividade antimalárica relacionados a substâncias puras obtidas a partir de plantas endêmicas ou nativas do semi-árido brasileiro. Entretanto, muitos fatores limitam o desenvolvimento de novas drogas como alto custo, resistência, baixa eficácia e segurança limitada. Esses fatores são, inclusive, limitações das drogas antimaláricas disponíveis atualmente. A descoberta de compostos *lead* com potencial para elucidação de drogas viáveis é essencial para o melhoramento do controle da malária. Com relação às drogas analisadas neste trabalho, o BAA e o isolacet, com inibição da parasitemia de 90% e 98% respectivamente, são moléculas com potencial para se tornarem compostos *lead*, visto que inibição da parasitemia acima de 90% em uma dose máxima de 50 mg/kg/dia é considerada suficiente para um composto lead (Nwaka, 2006).

Outros estudos são necessários para a elucidação de moléculas candidatas a medicamentos antimaláricos que possam, em um futuro próximo, substituir as drogas existentes no arsenal terapêutico atual. São necessários testes de toxicidade *in vivo* para todas as substâncias analisadas no nosso trabalho, objetivando traçar o perfil de toxicidade aguda e crônica dessas drogas, utilizando modelos experimentais pré-estabelecidos para diversas espécies de animais norteando, dessa forma, os estudos clínicos que podem vir a se realizar.

7. CONCLUSÕES/SUMÁRIO DE RESULTADOS

1. O ácido betulínico e seus derivados apresentam atividade antimalárica *in vitro* contra *P. falciparum* (cepa W2) resistente à cloroquina, e o acetato do ácido betulínico (BAA), mais ativo *in vitro*, apresenta atividade antimalárica *in vivo* por via intraperitoneal.
2. A aplicação da metodologia SEA (*Similarity Ensemble Approach*), para as fisalinas foi capaz de prever a atividade antimalárica destas moléculas.
3. A fisalina F é a mais ativa das quatro fisalinas testadas *in vitro*, mas não apresenta atividade antimalárica *in vivo*, possivelmente pela sua atividade imunossupressora.
4. A fisalina D apresentou atividade antimalárica *in vivo*.
5. Os modelos computacionais empregados indicaram que o isolacet é o mais ativo dentre os derivados do lapachol testados.
6. Os resultados experimentais comprovaram os dados previstos computacionalmente, com o isolacet apresentando atividade *in vitro* e *in vivo*.

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9. ANEXOS

1. Antiparasitic and Immunomodulatory Activities of 1,1-bis(4-Hydroxyphenyl)-2-Phenyl-but-1-ene and Its Protected and Free 2-Ferrocenyl Derivatives
2. Structure–activity relationships of mononuclear metal–thiosemicarbazone complexes endowed with potent antiplasmodial and antiamoebic activities
3. Early Toxicity Screening and Selection of Lead Compounds for Parasitic Diseases

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Research Article

Antiparasitic and Immunomodulatory Activities of 1,1-bis(4-Hydroxyphenyl)-2-Phenyl-but-1-ene and Its Protected and Free 2-Ferrocenyl Derivatives

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Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT The ferrocenyl diphenol 1 [1,1-bis(4-hydroxyphenyl)-2-ferrocenyl-but-1-ene] displays strong cytotoxic effects against a variety of cancer cells. In the present study we have evaluated the immunomodulatory and antiparasitic activities of compound 1 and its protected dipalmitate analogue 2. We have furthermore compared the antiparasitic results of 1 and 2 with the organic analogue, 3 [1,1-bis(4-hydroxyphenyl)-2-phenyl-but-1-ene], where the ferrocenyl group has been replaced by a phenyl ring. When assayed against normal (noncancerous) splenocytes, all compounds were considered nontoxic. Compound 1 inhibited NO production by macrophages, inhibited concanavalin A-induced lymphoproliferation, and was active against *Leishmania amazonensis* and *Trypanosoma cruzi*. Compound 2 had lower activity in all assays performed. Surprisingly, compounds 1 and 2 exhibited similar and significant activity against *Plasmodium falciparum*, with IC_{50} values of 3.50 and 1.33 μM, respectively. Compound 3 showed an inverse activity profile, being active against *T. cruzi* but far less active against *P. falciparum*. Drug Dev Res 2009. © 2009 Wiley-Liss, Inc.

Key words: ferrocene; malaria; bioorganometallic chemistry; immunomodulatory; nitric oxide; Chagas disease; *Plasmodium falciparum*; *Trypanosoma cruzi*; *Leishmania amazonensis*

INTRODUCTION

The discovery of the antiproliferative effects of simple ferricenium salts on Ehrlich ascites tumors in 1984 [Köpf-Maier et al., 1984a,b] inspired an enormous body of work on the anti-cancer properties of ferrocene-containing compounds [Hillard et al., 2007a; Metzler-Nolte and Salmain, 2008, and references therein]. Ferrocenyl phenols in particular demonstrated strong antiproliferative activity [Hillard et al., 2007a,b;

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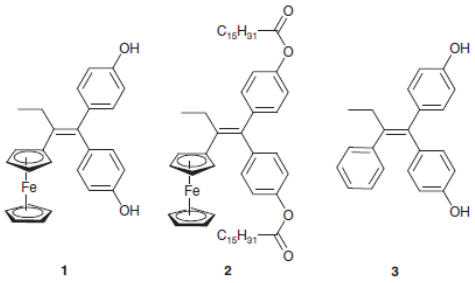

Fig. 1. Compounds studied in this report.

Pigeon et al., 2009; Plażuk et al., 2009; Vessières et al., 2005]. One of the most efficacious compounds, [1,1-bis(4-hydroxyphenyl)-2-ferrocenyl-but-1-ene] **1** (Fig. 1), is cytotoxic against hormone-dependent MCF-7 and hormone-independent MDA-MB-231 breast cancer cells, with an IC_{50} value for the latter of ~0.5 μM [Vessières et al., 2005]. Compound **1** is also toxic against 9L gliosarcoma transfected in rat flank and brain, when encapsulated into lipid nanocapsule delivery devices [Allard et al., 2008, 2009]; it displays activity against malignant melanoma cells in vitro [Michard et al., 2008]. We have shown that the anticancer properties of such compounds may arise from the oxidation chemistry of the ferrocene moiety, ultimately leading to a quinoid species [Hillard et al., 2006, 2008]. We believe this to be a key active metabolite, particularly due to the observation that quinones play a major role as reactive oxygen species (ROS) enhancers, redox catalysts, and electrophiles [Asche, 2005; Bolton et al., 2000; De Abreu et al., 2002; Goulart et al., 2003, 2004; Hillard et al., 2008; Monks and Jones, 2002; Ferreira et al., 2009], and for this reason are commonly encountered in chemotherapeutic agents.

Redox-active and/or organometallic moieties are furthermore important actors in antiparasitic compounds. For example, ferrocenyl derivatives of penicillin and cephalosporin have shown an improved therapeutic spectrum against drug-resistant organisms [Edwards et al., 1975, 1976]. Ferroquine, the ferrocenyl analogue of the antimalarial chloroquine, is extremely toxic against several strains of chloroquine-resistant *Plasmodium falciparum*, and is now in phase II clinical trials [Biot et al., 1997, 2009; Dive and Biot, 2008]. Organometallic ruthenium derivatives of chloroquine also show activity against chloroquine-resistant strains of *P. falciparum* [Sánchez-Delgado et al., 1996; Rajapakse et al., 2009] and Chagas disease [Sánchez-Delgado et al., 1993], a debilitating disease caused by the flagellate protozoan *Trypanosoma cruzi*. Quinone compounds likewise have found applications in Chagas disease [Goulart et al., 1997], likely due to the unique sensitivity of *T. cruzi* to the action of intracellular generators of H_2O_2. *T. cruzi* possesses a redox defense system based upon trypanothione and trypanothione reductase, which regenerates trypanothione from its oxidized (disulfide) form. It lacks catalase and glutathione peroxidase and is therefore more sensitive to H_2O_2-induced oxidative stress than its biological hosts. This mechanism is also operative in *Leishmania amazonensis* [Castro-Pinto et al., 2008; Lima et al., 2004], the parasite responsible for cutaneous and visceral leishmaniasis.

Immunopathologies such as autoimmune and allergic processes are diseases with high incidence and in which currently available drugs, although efficacious in most instances, produce undesirable side effects, resulting in complications to patients, especially after long-term use. The search for new bioactive molecules is of great interest, due to the need of new medicines for more effective treatment of pathologies with lower toxic effects. In the context of antiparasitic compounds, it is especially desirable to find a drug candidate that kills the parasite in the body while dampening the immune response of the body.

We now report the results of our investigation of the free ferrocenyl phenol **1** and its dipalmitate analogue **2** (Fig. 1), in terms of immunomodulatory activity (inhibition of lymphoproliferation and activated macrophage nitric oxide production), as well as their activity against *L. amazonensis*, *T. cruzi*, and *P. falciparum*. The role of the ferrocene moiety in cytotoxicity and antiprotozoal effects was evaluated by comparing the antiprotozoal results with those of the organic analogue of the free phenol, **3** (Fig. 1).

MATERIALS AND METHODS

Preparation of Compounds

Compounds **1** [Vessières et al., 2005], **2** [Allard et al., 2009], and **3** [Yu and Forman, 2003] were synthesized as previously reported. Samples were purified by semi-preparative HPLC or silica gel chromatography before use.

Animals

Male and female 4–6 weeks old BALB/c mice were used as spleen cell donors and for macrophage isolation. They were housed in standard environmental conditions and maintained at the animal facilities at Gonçalo Moniz Research Centre, FIOCRUZ (Salvador, Brazil). Mice were provided rodent diet and water ad libitum. All mice were handled and sacrificed according to the National Institutes of Health

(NIH) guidelines for laboratory animals, and protocols were approved by the Ethics Committee for Animal Use of FIOCRUZ.

Cytotoxicity Assay

Splenocytes from BALB/c mice (6×10^5 cells/well) were cultured in 96-well plate in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Cultilab, Campinas, SP, Brazil) and 50 µg/ml of gentamycin (Novafarma, Anápolis, GO, Brazil). Each substance was evaluated in five concentrations starting at 100 µg/ml, in triplicate. Cells were incubated for 24 h at 37°C and 5% CO_2. After this period, cultures were harvested using a cell harvester (MPXRI 96TI, Brandel, Gaithersburg, MD) to determine [^{3}H]-thymidine incorporation using a beta radiation counter (Plate Chameleon V Microplate Reader, Hidex, Turku, Finland). The viability of the cells was determined by the thymidine incorporation; cytotoxicity was calculated in relation to the [^{3}H]-thymidine incorporation of untreated cultures.

Nitric Oxide Assay

Peritoneal exudate cells were obtained by washing the peritoneal cavity of BALB/c mice with cold Hank's balanced salt solution (HBSS; Life Technologies, GIBCO-BRL, Gaithersburg, MD) 5 days after injection of 3% thioglycolate in saline (1.5 ml per mouse). Peritoneal cells were washed twice with HBSS and resuspended in RPMI medium (GIBCO-BRL) supplemented with 10% FCS (Cultilab), L-glutamine (2 mM), RPMI 1640 vitamins solution (1%) (Sigma), sodium pyruvate (1 mM), HEPES (10 mM), 2-mercaptoethanol (50 µM), and gentamycin (50 µg/ml) (Sigma). Cells were plated (2×10^5 cells/well) in 96-well plates. After 1 h incubation at 37°C, nonadherent cells were removed by washing with HBSS. Cultures were then stimulated with 500 ng/ml lipopolysaccharide (LPS from *Escherichia coli* serotype 0111:B4, Sigma) in combination with 5 ng/ml interferon-γ (IFN-γ; PharMingen, San Diego, CA) and treated with various concentrations starting at 100 µg/ml of the pure substances, in a final volume of 200 µl. Cell-free supernatants were collected at 24 h of culture for determination of nitrite concentration, using the Griess method, as previously described [Ding et al., 1988].

Lymphoproliferation Assay

BALB/c mice splenocytes suspensions were prepared in complete RPMI medium and cultured in 96-well plates at 6×10^5 cells/well in 200 µl, in triplicate, in the presence of 2 µg/ml concanavalin A (Con A; Sigma), alone or in various concentrations starting at 100 µg/ml of analyzed compounds, as described in Figure 2. After 48 h, plates were pulsed with 1 µCi of [methyl-^{3}H] thymidine for 12 h, and proliferation was assessed by measurement of ^{3}H-thymidine uptake. The percentage of inhibition of lymphocyte proliferation by the compounds was determined in relation to the concanavalin A stimulated control [Costa et al., 2008].

Anti-*Leishmania amazonensis* Assay

L. amazonensis (MHOM/BR88/BA-125 Leila strain) promastigotes were cultured in liver infusion tryptose (LIT) medium supplemented with 10% FBS, 5% sterile human urine, and 50 µg/ml gentamycin, pH 7.2, at 26°C until logarithmic phase. Parasites were cultured in 96-well plates at 5×10^6 cells/well in 200 µl, in triplicate wells, alone or in the presence of the samples analyzed in five different concentrations (100, 33, 11, 3, and 1 µg/ml). After 5 days, a direct counting of viable parasites was carried out in a Neubauer chamber, using a phase-contrast microscope [Costa et al., 2008]. Amphoterycin B was used as the reference drug.

Anti-*Trypanosoma cruzi* Assay

T. cruzi (Y strain) epimastigotes were cultured in liver infusion tryptose (LIT) medium supplemented with 10% FBS and 50 µg/ml gentamicin, pH 7.2, at 26°C, until logarithmic phase. Parasites were cultured in 96-well plates at 10^7 cells/well in 200 µl, in triplicate, alone or in the presence of the samples analyzed in five concentrations (100, 33.3, 11.1, 3.7, and 1.2 µg/ml). After 11 days, direct counting of viable parasites was carried out using a Neubauer chamber and phase-contrast microscopy [Leite et al., 2006]. Amphoterycin B was used as a reference drug.

Anti-*Plasmodium falciparum* In Vitro Assay

Compounds were tested for antimalarial activity in vitro using the *P. falciparum* W2 clone, which is chloroquine resistant and mefloquine sensitive [Junior et al., 1999]. All parasites were maintained in continuous culture of human erythrocytes (blood group A^+) using RPMI medium supplemented with 10% human plasma [Trager and Jensen, 2005]. Parasites grown at 1–2% parasitemia and 2.5% hematocrit were incubated with the pure substances tested at five concentrations, diluted with 4% DMSO in RPMI culture medium without hypoxanthine. Mefloquine was used in each experiment as a control for the *P. falciparum* drug response. Cultures containing parasites were harvested using a cell harvester to evaluate the [^{3}H]-hypoxanthine incorporation in a β-radiation counter. Inhibition of parasite growth was evaluated by comparison with [^{3}H]-hypoxanthine uptake in drug-treated versus

untreated wells. All the assays were performed in triplicate, as described previously [Andrade-Neto et al., 2004; Zalis et al., 1998]. Mefloquine was used as a reference.

IC_{50} Calculations

The inhibitory concentration for 50% (IC_{50}) of parasite growth was calculated based on a nonlinear regression (curve fit).

RESULTS

Cytotoxicity

The cytotoxicity of compounds **1**–**3** was tested against BALB/c mice splenocytes at five concentrations, starting at 100 µg/ml, as described above. IC_{50} values (24-h incubation) for compounds **1** and **2** could not be determined due to the low cytotoxicity at the concentrations tested. In the case of compound **3**, an IC_{50} value of 36 µg/ml (114 µM) was determined. The variable dose results are shown in Figure 2.

Immunomodulatory Activity Assays

The activity of compounds **1** and **2** on cultures of activated macrophages was evaluated. At 100 µg/ml, compound **1** almost completely inhibited nitric oxide production by macrophages activated by LPS and IFN-γ (Fig. 2), while compound **2** did not have a significant inhibitory activity on NO production, with an inhibition of only 18% at 100 µg/ml.

Likewise, compound **1** exhibited strong inhibitory activity in the proliferation of Con A-activated lymphocytes (Fig. 2), with an IC_{50} value of 9 µM, while compound **2** showed weak activity. The inhibitory activity of compound **1** on lymphocytes was not due to a cytotoxic effect, since the cytotoxicity levels were below the levels of immunomodulatory activities (Fig. 2).

Antiprotozoal Activity

The antiprotozoal activity of compounds **1** and **2** was investigated using two trypanosomatid parasites: *T. cruzi* and *L. amazonensis*, and chloroquine-resistant *P. falciparum*. Compound **1**, but not **2**, was active against *T. cruzi* and *L. amazonensis*. In contrast, both compounds were highly active against *P. falciparum*, with lower IC_{50} values compared with those for *T. cruzi* and *L. amazonensis* (Table 1). Interestingly, compound **3** displayed an opposite profile, being more active

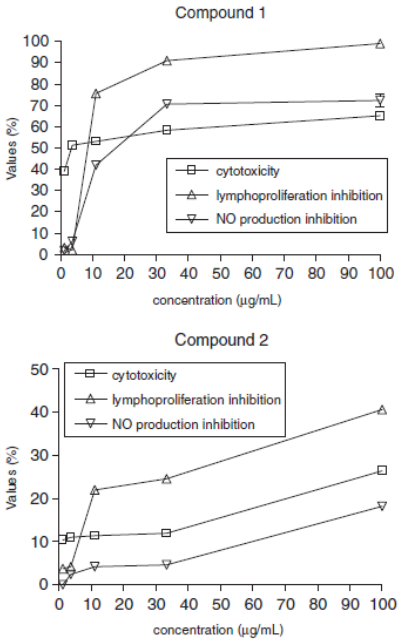

Fig. 2. Cytotoxicity and immunomodulatory activity of compounds **1** and **2**. Mouse splenocytes were cultured during 48 h in the presence or absence of different concentrations of the compounds tested and concanavalin A. Inhibition was calculated based on values of untreated, Con A-stimulated cultures, as described in Materials and Methods. NO production inhibition was determined in cultures of peritoneal murine macrophages stimulated with LPS+ IFN-γ and treated with compound **1**, at different concentrations, measuring nitrite concentrations in culture supernatants by the Griess method, as described in Materials and Methods. For cytotoxicity assays, splenocytes of BALB/c mice were cultured during 24 h in the presence or absence of compounds and [^{3}H]-thymidine. The percentage of viability was calculated comparing values of [^{3}H]-thymidine incorporation of cultures treated with compounds to those of untreated cultures. Values represent the means ± SD of values obtained from three individual experiments.

TABLE 1. Antiparasitic Activity of Compounds 1–3

Compound	IC_{50} *L. amazonensis* µg/ml (µM)	IC_{50} *T. cruzi* µg/ml (µM)	IC_{50} *P. falciparum* µg/ml (µM)
1	30.3 (71.4)	26.38 (62.2)	1.5 (3.50)
2	Not active	Not active	1.2 (1.33)
3	15.31 (48.4)	2.62 (8.3)	28.66 (90.6)
Amphotericin B	0.061 (0.068)	0.138 (0.149)	—
Mefloquine	—	—	0.018 (0.043)

against *T. cruzi* and *L. amazonensis*, but was less active against *P. falciparum*.

DISCUSSION

Interest in the use of organometallic compounds in medicine has grown exponentially, ever since the modern definition of bioorganometallic chemistry by Jaouen in 1985 [Jaouen, 2006]. Although the preponderance of studies focuses on anti-cancer compounds, there have been several studies on the application of such compounds as antiprotozoals, particularly ferrocene containing compounds [Barameе et al., 2006; Biot et al., 2000; Guillon et al., 2008; Itoh et al., 2000]. Notably, the organometallic compound ferroquine displays IC_{50} values in the nanomolar range against chloroquine-resistant *P. falciparum* strains; it is currently the most advanced anti-malaria project at Sanofi-Aventis [Biot et al., 2009]. In the present work we describe, for the first time, the antiparasitic and immunomodulatory activity of the ferrocenyl phenol **1**, previously shown to have strong antiproliferative effects against a variety of cancer cells lines. To test whether the pharmacological properties could be enhanced by increasing the lipophilicity of the molecule, the analogue **2**, where the phenol groups have been converted to long-chain esters, was also tested. Finally, to evaluate role of the ferrocene group, the organic analogue of **1** (**3**) was also studied.

All the compounds displayed low cytotoxicity against spleen cells, and an LC_{50} value could be calculated only for the organic compound **3** which was more toxic. Values for **1** and **2** could not be determined because the results obtained did not permit the calculation of the lethal doses, indicating that LC_{50} values for the ferrocenyl compounds are higher than 100 μg/ml. A low cytotoxicity for **1** on normal brain cells has previously been observed in vitro [Allard et al., 2008], with an IC_{50} value of 50 μM found for newborn rat astrocyte primary cultures. This value is approximately 100 times higher than the value determined for MDA-MB-231 cancer cells or 9L-glioma cells. Indeed, in the current study, we observe that the purely organic compound **3** is more toxic to normal cells than those containing ferrocene.

Compound **1** displayed an elevated immunomodulatory activity, being able to inhibit the activation of lymphocytes and macrophages. These cells are important effectors of immune responses; therefore, the immunomodulatory potential of this compound in models of immune-mediated diseases, as well as its mechanism of action, should be investigated. Although some cytotoxic activity was seen (data not shown), the inhibitory activity of compound **1** in mammalian cells was not due to a toxic effect, since the levels of cytotoxicity were below the values of inhibition of immune responses.

Both ferrocenyl compounds were active against chloroquine-resistant *P. falciparum*, with IC_{50} values within the range of 1.3–3.5 μM. In addition, **1** was active against epimastigote forms of *T. cruzi* and promastigote forms of *L. amazonensis*. Compound **2**, however did not show activity against these protozoa. This mirrors the trend observed for compounds **1** and **2** against 9L glioma cells. While compound **1** and its acetate analogue showed strong and similar antiproliferative activity against such cells (IC_{50} around 0.5 μM), the palmitate ester **2** was much less active ($IC_{50} = 20$ μM) [Allard et al., 2009]. The authors attributed the poor activity of **2** to the lack of hydrolysis of the palmitate ester in situ. This interpretation suggests that the free phenol function may be necessary for the activity of **1** against *T. cruzi* and *L. amazonensis*. Consistent with this interpretation, the organic compound **3** was found to be more active than the ferrocenyl **1**, suggesting that ferrocene does not impart any particular benefit against these protozoa. To our knowledge, such triphenyl ethylene compounds have never been studied in the context of Chagas disease (American trypanosomiasis). Because of its limited toxicity against human cells [Lubczyk et al., 2002] and its high toxicity against *T. cruzi* (Table 1) compound **3** could be an attractive lead compound in this context.

It is clear, however, that the ferrocenyl group is a key to the antiproliferative effects of compounds **1** and **2** against *P. falciparum* when compared with **3**, which shows only weak effects [Molyneaux et al., 2005]. Although the mechanism of ferroquine is not fully understood, it has been shown that the ferrocene moiety can generate reactive oxygen species (ROS) under oxidizing conditions, such as in the food vacuole of the *P. falciparum* [Biot et al., 2009]. The ability of compounds such a **1** to produce ROS in a variety of cell types is currently under study. The enhanced lipophilicity of **1** ($P_{o/w} = 5.0$) and **2**, compared with **3** ($P_{o/w} = 4.4$), could also play a role. Recent studies suggest that the activity of ferroquine results, at least in part, from its superior lipophilicity compared with chloroquine, and possibly from its localization in the lipidic sites of heme crystallization [Biot et al., 2009]. Because the lipophilicity of such compounds can be easily and systematically modified by the choice of protecting groups on the phenol moiety, a structure–activity relationship study is feasible.

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Structure–activity relationships of mononuclear metal–thiosemicarbazone complexes endowed with potent antiplasmodial and antiamoebic activities

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ABSTRACT

A useful concept for the rational design of antiparasitic drug candidates is the complexation of bioactive ligands with transition metals. In view of this, an investigation was conducted into a new set of metal complexes as potential antiplasmodium and antiamoebic agents, in order to examine the importance of metallic atoms, as well as the kind of sphere of co-ordination, in these biological properties. Four functionalized furyl-thiosemicarbazones (**NT1–4**) treated with divalent metals (Cu, Co, Pt, and Pd) to form the mononuclear metallic complexes of formula $[M(L)_2Cl_2]$ or $[M(L)Cl_2]$ were examined. The pharmacological characterization, including assays against *Plasmodium falciparum* and *Entamoeba histolytica*, cytotoxicity to mammalian cells, and interaction with pBR 322 plasmid DNA was performed. Structure–activity relationship data revealed that the metallic complexation plays an essential role in antiprotozoal activity, rather than the simple presence of the ligand or metal alone. Important steps towards identification of novel antiplasmodium (**NT1Cu**, IC_{50} of 4.6 μM) and antiamoebic (**NT2Pd**, IC_{50} of 0.6 μM) drug prototypes were achieved. Of particular relevance to this work, these prototypes were able to reduce the proliferation of these parasites at concentrations that are not cytotoxic to mammalian cells.

1. Introduction

According to the World Health Organization (WHO), there are 300–500 million clinical cases of malaria each year, resulting in an alarming rate of about 1.5–2.0 million deaths annually.[1] However, it is estimated that this death rate is likely to increase further because of the high level of drug resistance to most of the clinically used antimalarials.[2] Given the evidence of the global spread of drug resistance,[3] there is a need for the identification of new antiplasmodial drugs. After malaria, amebiasis (caused by *Entamoeba histolytica*) is the second leading cause of death from a protozoan parasite.[4] Metronidazole, the only WHO-recommended drug for treating amebiasis, is toxic and of questionable effectiveness in eliminating the parasite.[5] New safe and affordable amoebicidal drugs are therefore also urgently needed.

The elucidation of metabolic pathways of fundamental importance (e.g., fatty acid biosynthesis[6] and heme detoxification[7]) and of valid molecular targets, such as the falcipain-2,[8] CDK,[9] purine nucleoside phosphorylases,[10] and protein serine/threonine phosphatases[11] of *Plasmodium falciparum* have contributed to more rational design of drug candidates. In combination with this knowledge, the employment of modern concepts of medicinal chemistry, such as bioisosterism,[12] molecular hybridization,[13] bioinspired design in potent hit-compounds,[14] and the metallic complexation of plasmodicidal compounds[15] have accelerated the discovery of antiplasmodium drug candidates.

A significant number of transition metal-containing compounds have been either recently launched on the pharmaceutical market or entered into clinical trials, as exemplified by Ferrocifen, NAMI-A, Picoplatin, Ferroquine, and AMD3100.[16] In the specific case of antiplasmodium agents, in addition to Ferroquine that has recently entered in clinical trial, other metallic structures (complexes of co-ordination and organometallics) have shown promising in vitro and in vivo properties (Fig. 1).[17] It can thus be concluded that one successful drug development strategy is the complexation of transition metals with plasmodicidal agents, as it is possible to enhance the pharmacological and chemical properties (such as potency, selectivity, chemical stability, and lipophilicity) of the antiplasmodium agent employed.

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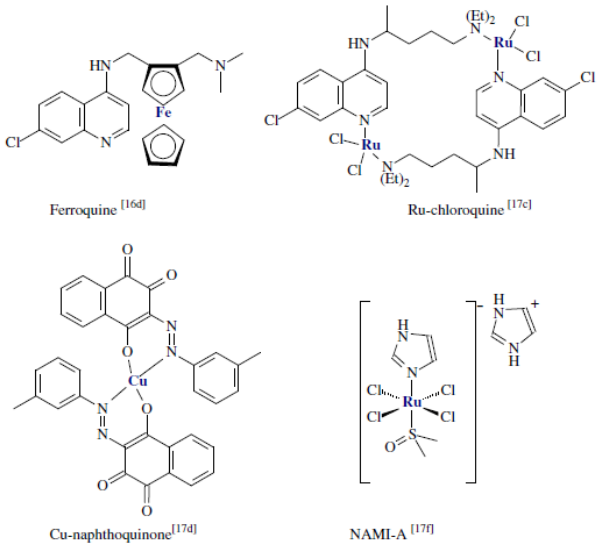


Figure 1. Examples of metallic compounds endowed with potent anti-*Plasmodium falciparum* activity.

Studies of thiosemicarbazone-based libraries have proved that they are useful ligands for the building of metal complexes with a wide variety of biological targets, including protozoan parasites.[8,18] Such ligands constitute an ideal source of bioactive ligands because they are endowed with the unique capacity of metallic co-ordination, semi-labile, chemically stables, and are synthetically treatable-features which make them suitable for performing structure–activity relationships (SAR) studies.[19] It is thus reasonable to believe that thiosemicarbazones are authentic privileged structures.[20]

According to this line of reasoning, metal–thiosemicarbazone complexes previously developed by us were found to improve anti-*E. histolytica* activity against the HK-9 and HM1:1MSS strains, when compared to the metal-free thiosemicarbazones.[21] On the one hand, we demonstrated that the cyclic bioisosters of thiosemicarbazones (thiocarboxamide-2-pyrazolines) are less effective against *E. histolytica* than thiosemicarbazones, suggesting that the replacement of a flexible backbone by a more rigid backbone results in a distinct interaction with the protozoan targets.[22] Moreover, insertion of bulk groups at the 4-position of thiosemicarbazones helps to improve the chemical stability of metal complexes and their lipophilicity, resulting in more potent antiamoebic complexes (Fig. 2).[23] Although there is a large number of thiosemicarbazones endowed with antiplasmodium activity,[24] investigations of the antiplasmodium activities for complexes of co-ordination containing thiosemicarbazones are scarce.[25]

In light of these findings, we decided to investigate the antiprotozoal properties of a new set of mononuclear metal–thiosemicarbazone complexes. In our design, the furfuryl ring was explored, in view of previous observation of its antiparasitic properties.[26] We have excluded attachment of the nitro group, as it is well-known that this induces toxicity, which is a drawback of the medicinal chemistry of antiparasitic drugs.[26c] Later, we selected platinum, palladium, cobalt, and copper, in search of complexes bearing one ($MLCl_2$) or two (ML_2Cl_2) ligands on each co-ordination sphere, thereby aiming to investigate how the ligand sphere contributes to antiprotozoal activity. Therefore, the main achievement of this study was to have gathered, for the first time, valuable SAR data on antiprotozoal metal–thiosemicarbazone complexes.

2. Results

2.1. Synthesis and general remarks of structural elucidation

The synthesis of ligands **NT1–4** was straightforward and proceeded moderate to good yields (41–90%). **NT1–4** were further used as chelating ligands to complex with $[Pd(DMSO)_2Cl_2]$, $[Pt(DMSO)_2Cl_2]$, $CoCl_2{\cdot}6H_2O$ or $CuCl_2{\cdot}2H_2O$ to generate the respective mononuclear metal–thiosemicarbazone complexes. Pt (**NT1Pt**, **NT2Pt**) and Pd (**NT1Pd–NT4Pd**) complexes were obtained as monomers by heating the ligand and appropriate metallic precursor under reflux, while the Co (**NT1Co–NT4Co**) and Cu

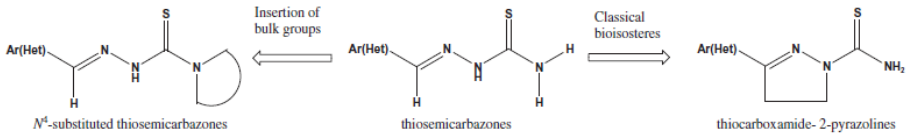


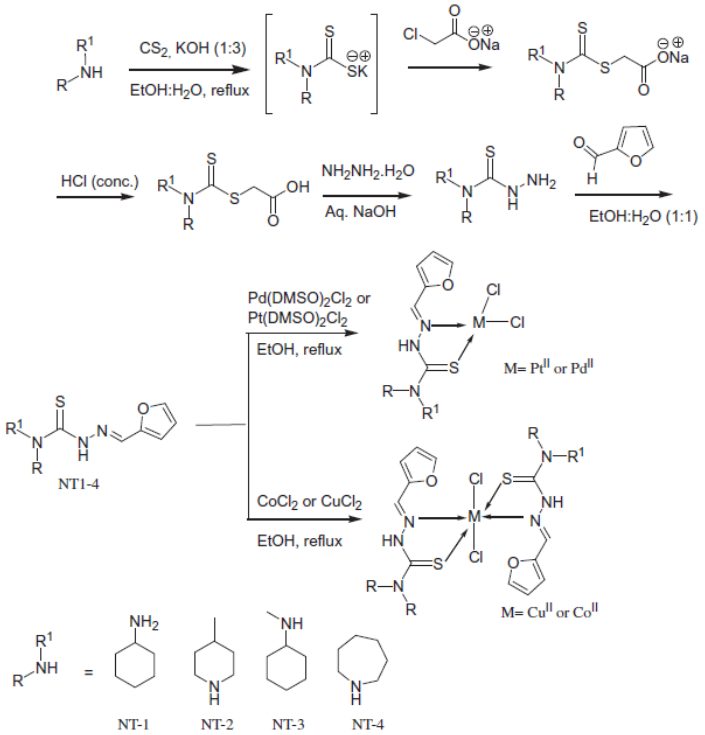
Figure 2. Our design concepts for thiosemicarbazones, their bioisosters classical (thiocarboxamide-2-pyrazolines) and their analogs containing bulk groups. Ar(Het) means either aromatic or heterocyclic rings.

(**NT1Cu–NT4Cu**) were obtained as bis-chelated complexes under similar conditions (Scheme 1). Elemental analysis data (C, H, N, and Cl) confirmed this metal–ligand ratio and accorded well with the calculated values.

The IR spectral signatures of **NT1–NT4** thiosemicarbazones show bands in the region at 1059–1109 cm^{-1} due to (νC=S), while no absorption appeared due to (νC–SH) in the region 2500–2600 cm^{-1}, suggesting that these **NT1–NT4** remain in its thione tautomeric form. For the metal complexes, although no shift was observed in the vibrations attributed to (νC–O–C) of furan ring, absorptions attributed to (νC=S) at 1059–1109 cm^{-1} were shifted to lower frequency (12–30 cm^{-1}) after the metallic complexation, indicating that thionic sulfur does participate in the sphere of co-ordination. More notably, strong bands at 1576–1603 cm^{-1} were observed in all the complexes, which can be attributed to chelation of (νC=N) with the metal.[19a,26b] By contrast, the (νCH=N) bands for the metal-free ligands are generally of higher frequency (1613–1615 cm^{-1}) and are of weaker intensity than those observed in the metal complexes. Bands observed in the region of 3100 cm^{-1} (νN–H) were also slightly shifted in the complexes, probably by any kind of adjustment of current around the thioamide group. Taking into account previous assignments for similar metal–thiosemicarbazone complexes, bands corresponding to metal-to-ligand stretching modes were tentatively assigned.[21] Thus, bands in the region of low wave-number (472–522 and 347–435 cm^{-1}) were assigned to νM–N and νM–S, respectively.

The ^{1}H NMR spectra of the ligands and their complexes were consistent with their corresponding protons both in terms of chemical shifts and the number of hydrogen atoms, in accordance with the proposed structure, allowing the assignment of each proton for the complexes. Regarding the ligands, it is worth reporting that even in DMSO-d_6 they were obtained in the thione form, which was confirmed by the absence of signal at ca. 4.0 ppm (–SH proton). Regarding the metallic complexes, the CH=N protons resonated at 6.6–7.3 ppm (singlet), owing to a deshielding effect observed for those protons that are in close proximity to the co-ordinating atoms (azomethine nitrogen), while, on free ligands, CH=N protons resonated at 7.8–8.0 ppm. On the basis of these comparisons, we suggest that an *N,S*-bidentate co-ordination is involved in these complexes, as proposed in Scheme 1.

Attempts to obtain single crystals of these metallic complexes suitable for X-ray diffraction were unsuccessful. To overcome this, additional analyzes were recorded and carefully studied. The mass spectra of the ligands and their metal complexes have showed the expected fragment ions, confirming the respective molecular weights. The overall splitting pathways of selected metal complexes are summarized in Supplementary data (Scheme S1). According to the electronic spectra, the **NT1–4** ligands exhibited three bands in the region at 220–207, 276–263, and 348–330 nm. The most probable assignments for these bands are the $n\rightarrow\pi^*$ (thiosemicarbazone), $\pi\rightarrow\pi^*$ (thiophene), and $\Phi\rightarrow\Phi^*$ (thiophene) transitions, respectively. A careful comparison of these bands with the electronic spectra of metallic complexes showed that there was an increase in intensity and a decrease in frequency due to the extended conjugation of ligands after the complexation. The observation of strong bands between 660 and 560 nm for the Pt (**NT1Pt**, **NT2Pt**) and Pd



Scheme 1. Preparation of ligands (**NT1–4**) and their metal complexes.

(**NT1Pd–NT4Pd**) complexes were assigned to $^1A_1g \rightarrow 1A_{2g}$ that are typically of a square planar geometry. For these complexes, the ground state is ^1A_1g and the excited states are ^1A_2g, ^1B_1g, and ^1Eg in order of increasing energy. Intense bands were also observed at 433–470 nm and assignable to a combination of S→Pd^{II} and $^1A_{1g} \rightarrow {}^1B_{1g}$ transitions.[27] The Pt (**NT1Pt**, **NT2Pt**) and Pd (**NT1Pd–NT4Pd**) complexes were diamagnetic, as expected for a kind of complex that assumes the planar geometry.

For the Co (**NT1Co–NT4Co**) and Cu (**NT1Cu–NT4Cu**), two-spin was observed to allowed transitions at 575–550 nm and at 475–450 nm and attributed to $^4T_{1g}(F) \rightarrow {}^4T_{2g}(F)$ (ν_1) and $^4T_{1g}(F) \rightarrow 4T_{1g}(P)$ (ν_3). These transitions are suggestive of an octahedral geometry. Likewise, the ^2E_g and $^2T_{2g}$ microstates of the octahedral Cu^{II} ion (of configuration d^9) split under the influence of the tetragonal distortion and such distortion causes the transitions $^2B_{1g} \rightarrow {}^2B_{2g}$ and $^2B_{1g} \rightarrow {}^2A_{1g}$, although this remains unresolved in the electronic spectra.[28] These transitions lie within the single broad envelope centered on the range mentioned above. But these assignments accorded well with the general observation that transitions of the Cu^{II} d–d kind are often very similar in terms of energy.[29] The N–Cu^{II} and S–Cu^{II} ligand-to-metal charge transfer transitions were also observed at 460 nm (Table S1, supplementary data). The Cu and Co complexes exhibited room temperature magnetic moments (μ_{eff}) values in the range of 1.92–1.97 and 5.05–5.12 B.M., respectively (Table 1). These values are expected for magnetically diluted Cu^{II} and Co^{II} complexes having unpaired electrons in the d-orbital.[30]

The EPR spectra of the Cu^{II} and Co^{II} complexes were measured and the parameters are summed in Table 1. The general trend $g_{\parallel} > g_{\perp} > 2.0$ for the Cu complexes suggests that the unpaired electron is located on the $d(x^2 - y^2)$ ground state orbitals on the Cu^{II}[31] and can also be taken as evidence of an octahedral environment.[32] Likewise, the Co complexes exhibiting the g value between 2.05 and 2.08, which also suggests an octahedral environment around the metal.[33] To sum up, a square planar geometry is suggested for both the Pt^{II} (**NT1Pt**, **NT2Pt**) and Pd^{II} (**NT1Pd–NT4Pd**) complexes, while Cu^{II} complexes (**NT1Cu–NT4Cu**) and Co^{II} complexes (**NT1Co–NT4Co**) probably are of octahedral geometry.

These complexes are insoluble in water, sparingly soluble in methanol and ethanol, but soluble in DMF and DMSO, producing intense reddish, greenish or brownish solutions. The ^1H NMR spectra of metal complexes in DMSO-d_6 remained unchanged at room temperature for several days, showing no evidence of displacement of the thiosemicarbazones by the solvent. The solid-state geometry of dichloropalladium (**NT1Pd–NT4Pd**) and dichloroplatinum (**NT1Pt**, **NT2Pt**) complexes is not retained in DMSO solution, taking place an fast transformation of [$MLCl_2$] to [ML(DMSO)Cl] complexes. All complexes were suspended in DMSO, thereby the biological activity reflects the presence of (DMSO)chlorometal complexes for the Pt and Pd ones.[34]

2.2. Pharmacological and physicochemical assays

All the ligands and complexes were tested against the erythrocytic stage (bloodstream form of clinical relevance) of *P. falciparum* (W2 clone, Chloroquine-resistant and Mefloquine-sensitive), HM-1:IMSS strain of *E. histolytica* trophozoites and also for cytotoxicity using BALB/c mouse splenocytes. The antiprotozoal properties were expressed in terms of the IC_{50} (μM) values, the cytotoxicity being expressed as the highest concentration tested that was non-cytotoxic for the splenocytes (Table 2). Mefloquine (**MQF**) and Metronidazole (**MNZ**) were used as reference drugs.

Recently, Sanchez-Delgado and co-workers have disclosed a number of correlations between physicochemical parameters and the antiplasmodium activity for a number of ruthenium–chloroquine complexes.[17c,35] From these studies, important insights into the antiplasmodium action mechanism were provided. Therefore, the saline/*n*-octanol partition coefficient (log *P*), studies with the pBR 322 plasmid DNA, and the inhibitory effects on the β-hematin formation for our most potent antiplasmodium metal–thiosemicarbazone complexes were also recorded (Table 3, Figs. 3 and 4).

2.3. Structure–activity relationships (SAR)

The first analysis of the pharmacological results showed that the **NT1–4** ligands were only weak *P. falciparum* inhibitors. Likewise, they were only modestly active against *E. histolytica*, the most potent being **NT2**, which is still four times less potent than **MNZ**. Although it was not possible to draw SAR data from these results, they displayed generally low cytotoxicity against mammalian cells, which suggests an attractive application for the building of metallic complexes.

As Cu complexes (**NT1Cu–NT4Cu**) are isoelectronic and isostructural with the Co complexes (**NT1Co–NT4Co**), while a square planar geometry is adopted by both Pt (**NT1Pt**, **NT2Pt**) and Pd (**NT1Pd–NT4Pd**) complexes, we concluded that the most appropriate discussion of the biological results is through the comparison between the similar metals, along the comparative analysis of the complex and the respective metal-free ligand.

Table 1
Analytical and physical data for the metal complexes

Compd	Color	Yield[a] (%)	Mp (°C)	(μ_{eff})[b]	EPR parameters[c]		
					$g_{\parallel}$	$g_{\perp}$	g
NT1Pt	Yellowish solid	34	260	0	—	—	—
NT2Pt	Pale yellow	43	200	0	—	—	—
NT1Pd	Pale orange	56	268	0	—	—	—
NT2Pd	Brick red	30	257	0	—	—	—
NT3Pd	Pale yellow	30	260	0	—	—	—
NT4Pd	Pale orange	55	220	0	—	—	—
NT1Co	Brownish solid	42	262	5.10	—	—	2.08
NT2Co	Wine red	35	165–170	5.05	—	—	2.05
NT3Co	Wine red	33	265	5.12	—	—	2.05
NT4Co	Chocolate brown	45	240	5.07	—	—	2.06
NT1Cu	Greenish solid	54	158	1.92	2.30	2.07	—
NT2Cu	Pale green	51	154	1.97	2.28	2.05	—
NT3Cu	Greenish solid	32	160	1.92	2.10	2.05	—
NT4Cu	Pale orange	40	164	1.95	2.20	2.06	—

[a] Isolated products.
[b] Effective magnetic moment expressed as Bohr Magneton (B.M.) and recorded at room temperature.
[c] From frozen solutions (77 K) in DMSO.

Table 2
Biological results of ligands and their metal complexes

Compd complexes	*P. falciparum* W2 strain IC_{50} (μM) after 24 h[a]	*E. histolytica* HM-1:IMSS strain IC_{50} (μM) after 72 h[a]	Cytotoxicity to mammalian cells (μg mL^{-1})[b]
NT1	20.3 ± 0.2	10.12 ± 00.5	33
NT2	36.2 ± 0.06	8.02 ± 00.1	100
NT3	21.8 ± 0.4	9.07 ± 00.4	>100
NT4	35.8 ± 0.9	12.08 ± 00.3	>100
NT1Pt	37.0 ± 0.1	3.47 ± 00.1	33
NT2Pt	42.3± 0.6	1.44 ± 00.3	>100
NT1Pd	10.0 ± 0.08	0.99 ± 00.3	3.3 (7.7)
NT2Pd	10.9 ± 0.1	0.6 ± 00.5	33
NT3Pd	18.3 ± 0.3	2.42 ± 00.5	33
NT4Pd	20.5 ± 0.01	1.66 ± 00.1	33
NT1Co	Nd	2.28 ± 00.1	33
NT2Co	41.0 ± 0.4	3.40 ± 00.2	33
NT3Co	21.4 ± 0.2	7.50 ± 00.5	33
NT4Co	58.7 ± 1.7	2.00 ± 00.3	100
NT1Cu	4.6 ± 0.1	1.11 ± 00.2	33
NT2Cu	5.2 ± 0.1	4.05 ± 00.1	11
NT3Cu	7.8 ± 2.0	1.80 ± 00.2	33
NT4Cu	4.6 ± 0.1	1.06 ± 00.4	3.3 (5.1)
MQN[c]	0.039 ± 0.01	–	N.d.
MNZ[c]	–	1.80 ± 00.3	N.d.

N.d., not determined at concentrations tested.
[a] Calculated from five concentrations using data obtained from at least three independent experiments. Values are mean ± standard deviation of three determinations.
[b] Expressed as the highest non-cytotoxic concentration for BALB/c mouse splenocytes. Values given in parentheses are expressed in μM.
[c] MQN is Mefloquine and MNZ is Metronidazole.

Table 3
Inhibitory effects on the β-hematin formation and partition coefficient (log *P*) for the copper complexes

Compd complexes	IC_{50} (μg mL^{-1})[a]	log *P*[b]
NT1Cu	>50	−1.73
NT2Cu	48	−1.54
NT3Cu	43	−1.49
NT4Cu	>50	−0.89
CP[c]	1.3	–

[a] Calculated from six concentrations using data obtained from two independent experiments. Values mean ±1.0.
[b] Performed as described in Section 4.
[c] CP is chloroquine diphosphate.

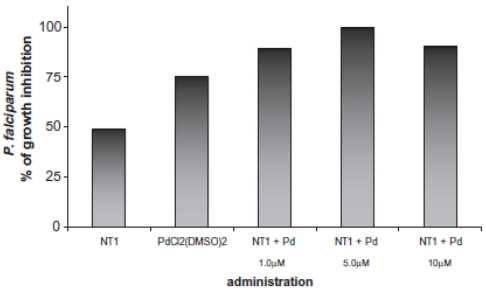


Figure 3. In vitro inhibition of *P. falciparum* growth at alone or concomitant addition of ligand (**NT1**) plus metal [$PdCl_2(DMSO)_2$, represented as Pd]. **NT1** was added at fix concentration of 20 μM, while Pd was used at 10 μM (alone) or at increasing concentrations (in concomitant with **NT1**). Data were measured in triplicate after 24 h of incubation, and the percentages of inhibitions were established in comparison to non-treated cells (control).

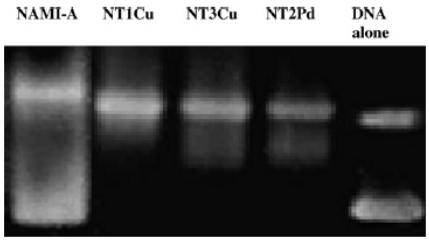


Figure 4. Effect of complexes **NAMI-A**, **NT1Cu**, **NT3Cu**, and **NT2Pd** (50 μM) on the mobility of pBR 322 plasmid DNA. DNA was incubated in 20 μM phosphate buffer (pH 7.5) to a final volume of 20 μL, and incubated in the absence or presence of tested complexes at 37 °C in the dark for 24 h.

We first analyzed the Pt and Pd series. Although the investigation of Pt complexes was limited by synthetic considerations and only two complexes (**NT1Pt** and **NT2Pt**) were prepared, these showed near IC_{50} values against *P. falciparum*. On the one hand, the Pt complexes showed improved potency against *P. falciparum* compared with than the metal-free ligands (**NT1** and **NT2**). On the other hand, the replacement of Pt by Pd led to only a slight increase in potency against *P. falciparum* (IC_{50} of 10.0–20.5 μM), far lower than the potency of **MQN**, which is active in nanomolar concentrations. Apart from **NT1Pd**, which was cytotoxic at low doses, the other Pt and Pd complexes retained their low cytotoxicity in mammalian cells. Noticeably, most of Pt and Pd complexes were highly potent in inhibiting the growth of *E. histolytica*, exhibiting the same range of potency as the reference drug, **MNZ**. A comparison of the inhibitory activity of ligand **NT2** and the **NT2Pd** complex, the latter proved to be 13 times more potent in inhibiting the proliferation of *E. histolytica*. By contrast, the replacement of ligand **NT2** by **NT3** resulted in the **NT3Pd** complex, which was only three times more potent than the free-ligand in inhibiting the growth of *E. histolytica*.

Regarding the antiplasmodial activity, the screening of Co and Cu complexes demonstrated more promising results than the Pt and Pd complexes. Analysis of these Co and Cu complexes bearing two molecules of ligands gave rise to interesting SAR observations. The complexation of **NT1–4** ligands with Co led a new set of

complexes that were (at least) one order of magnitude less active against *P. falciparum* (or in some cases virtually inactive, e.g., for **NT1Co** and **NT4Co**) when compared with the metal-free ligands. Conversely, the complexation with Co resulted in antiamoebic complexes more potent than the corresponding metal-free ligands.

Regarding the metal-free thiosemicarbazones (IC_{50} of 20.3–36.2 μM), their Cu complexes were proven to be good antiplasmodial agents (IC_{50} of 4.6–7.8 μM), exhibiting the following order of potency: **NT1Cu** = **NT4Cu** > **NT2Cu** > **NT3Cu**. Altering the structure of the cyclohexyl ring on the ligands of *C*-methyl (**NT2Cu**) to *N*-methyl (**NT3Cu**) led to a slight reduction in potency (IC_{50} = 5.2 vs 7.8 μM). Moreover, it was observed that, although the ring expansion of cyclohexyl (**NT1Cu**) by cycloheptane (**NT4Cu**) retains the antiplasmodial activity, **NT4Cu** was cytotoxic against mammalian cells.

2.4. Advanced biological experiments

On the basis of the SAR data presented in Table 2, it seems that the antiplasmodial activity of these complexes is dependent on the substituents (R) on the structures of thiosemicarbazones (**NT1–4**). In general, the lipophilicity of metallic structures is a relevant descriptor for explaining the cell uptake, and for explaining the difference in potency between the metal complex and its metal-free ligand. In the case of anti-malarial complexes, such as Ferroquine[16d] and ruthenium–chloroquine,[17c,35] the antiplasmodium activity is governed by the influence of lipophilicity. The lipophilicity (log *P*) of our most potent antiplasmodium complexes (**NT1Cu–NT4Cu**) was thus measured, in order to check any kind of correlation with the biological activity. Comparing the experimental values of log *P* (Table 3) with the IC_{50} values against the W2 strain *P. falciparum*, a correlation between the potency and lipophilicity is not observed. However, comparing the lipophilicity of **NT1** (log *P* = 2.16) ligand and its copper complex (**NT1Cu**), it is possible to conclude that the copper complexes are in fact more hydrophilic than the free thiosemicarbazones.

To ascertain whether complexation with a ligand is an essential factor for antiprotozoal activity, the same culture of W2 strain *P. falciparum* was simultaneously treated with stoichiometric quantities (20 μM) of metal-free ligand **NT1** and its metallic precursor $[PdCl_2(DMSO)_2]$, with the percentage of cell growth inhibition being measured after 24 h of treatment. In this condition, only 56% of the *P. falciparum* proliferation was inhibited, approximately twice lower than that of the corresponding **PdNT1** complex (98% of inhibition at 20 μM). Since the bioactivity of simultaneously treatment (ligand plus metallic precursor) and its corresponding metallic complex are distinct, the antiplasmodium activity of these complexes is not governed by synergistic effects alone. Cumulatively, it is supposed that the formation of a metal complex, rather than the simple presence of the ligand or transition metal alone, plays a crucial role in determining its antiprotozoal properties.

Regarding the lability of thiosemicarbazones, Bernhardt and coworkers have proposed that thiosemicarbazones from metallic complexes are displaced (dissociated) under physiological conditions or, in other words, the metals act as lipophilic vehicles, facilitating the intracellular delivery of the metal-free thiosemicarbazones into cellular compartments.[19b,c] In light of these findings, it is supposed that the processes of precomplexation and dissociation play a pivotal role in determining the pharmacological potency for the metallic complexes described here. To gather additional evidences, additional biological assays were performed with palladium, which is kinetically more labile than Cu, Co, or Pt.

The same culture of W2 strain *P. falciparum* was simultaneously treated with metal-free ligand **NT1** (20 μM) and its metallic precursor $[PdCl_2(DMSO)_2]$ at increasing concentrations (1.0, 5.0, and 10 μM), with the percentage of cell growth inhibition being measured after 24 h of treatment. From the data on Figure 3, it is possible to suggest that the increasing addition of Pd enhances the antiplasmodium activity of **NT1** ligand at certain point. However, at high concentrations of Pd (more than 10 μM), no differences of inhibitions are observed, may because of metal toxicity (data not shown). Although preliminary, the role of metal facilitating the intracellular delivery of thiosemicarbazones into cellular compartments may occur in the complexes described here, providing a means for drug activation or formation of reactive species that could eventually interact with the protozoan targets.

As previously cited, there are various examples of drug targets of *P. falciparum*. Following the findings provided by Sanchez-Delgado and co-workers, the DNA and hematin were considered as potential drug targets.[35] Therefore, studies of DNA interaction in cell-free media was conducted, employing pBR 322 plasmid DNA (composed of the supercoiled form of higher mobility and an open circular relaxed form) and CT-DNA (denatured form). We chose three of the most potent and non-cytotoxic complexes to be tested, **NT1Cu**, **NT3Cu**, and **NT2Pd**. These complexes are insoluble in pure water, and DMSO was thus used as a co-solvent in concentrations that do not affect the CT-DNA. The addition of the metal complexes to the CT-DNA solution resulted in small changes in the UV–vis absorption spectra. The observed changes were almost identical in the presence of each of these three complexes, suggesting that the complexes interact with CT-DNA in the same way (data not shown).

More valuable informations were gathered by way of agarose gel electrophoresis assays using pBR 322 plasmid DNA. In this assay, the NAMI-A (an imidazole–ruthenium^{II} complex, Fig. 1) of recognized in vitro antiplasmodium property[17e] was used, so as to establish a point of reference for the metal entity. At concentrations below 50 μM, the complexes (**NT1Cu**, **NT3Cu**, and **NT2Pd**) did not alter either the migration of plasmid DNA bands or other interaction processes (cleavage, for instance) to a significant degree, while the NAMI-A showed interactions as soon as a concentration of 10 μM was reached. However, at 50 μM, a new pattern was observed after the incubation of these complexes with plasmid DNA. Figure 4 suggests that the DNA interaction of these complexes occurs through the conversion of the supercoiled form, probably to the relaxed form, while the NAMI-A-treated DNA behaved quite differently, exploiting the cleavage process.

Inhibition of β-hematin formation was also measured for our more potent copper complexes (Table 3). All the tested complexes did not inhibit the β-hematin formation to a significant extent, while chloroquine did. This suggests these complexes use a different action mechanism than quinoline-based antimalarial drugs. In view of the current problem of resistance to anti-malarial drugs,[3] it is especially important to identify novel anti-malarial drug candidates not based on quinolines.

As a result of these assays, the plasmodicidal action mechanism must, in part, be related to action on the DNA structure. As for the antiamoebic properties of these metal complexes, it is not clear whether the antiamoebic action mechanism involves their interaction with DNA. Although additional experimental data is not available at present, a very recent survey of the literature indicates that the sequestration of Fe via either chelator compounds or exchange of endogenous Fe by exogenous metal (transmetalation) affects *E. histolytica* viability.[36] In light of these studies, it is reasonable to propose that, given the recognized ability of thiosemicarbazones to act as Fe chelators, as well as the feasibility of the complexes described here to participate in the processes of dissociation, they may use this action mechanism to inhibit the growth of *E. histolytica*.

3. Conclusions

We were able to identify **NT2Pd** as the most potent antiamoebic agent tested in this study, being twice as effective as **MNZ**. **NT2Pd**

did not show cytotoxicity against splenocytes at a concentration 10 times higher than that capable of inhibiting *E. histolytica* growth. In other words, it was effective at concentrations that do not overtly affect mammalian cells. This represents the kind of profile, that is, generally required for antiparasitic agents, with selective toxicity against parasites, although future investigations in animal models are necessary.

Our most potent antiplasmodial complex, **NT1Cu**, is far less potent than **MNQ**. However, the copper complexes, particularly **NT1Cu** and **NT2Cu**, represent good starting points for further medicinal chemistry programs aiming to discover antimalarial drug candidates based on metallic structures. Our future goals are to study Pd and Cu complexes bearing mixed-ligands to further validate the hypothesis that the process of dissociation may occur and could be exploited to produce complexes more active complexes against the aforementioned parasites.

4. Experimental section

4.1. Chemistry

General remarks: reactions were monitored by TLC analysis using Merck pre-coated aluminum plate silica gel $60F_{254}$ thin layer plates. All the chemicals were purchased from Aldrich Chemical Company (USA). Elemental analysis (C, H, N) was carried out by Central Drug Research Institute, Lucknow (India). Chlorine was estimated by decomposing the complexes with Na_2O_2/NaOH and precipitating as AgCl with $AgNO_3$ after dissolving in diluted HNO_3. Melting points were recorded on KSW melting point apparatus and are uncorrected. Electronic spectra were recorded in methanol on a Shimadzu UV-1601 PC UV-visible spectrophotometer. IR spectra on KBr disks were recorded on a Perkin Elmer model 1620 FT-IR spectrophotometer. ^{1}H NMR spectra were obtained at ambient temperature using a Brucker spectroscopin DPX-300 MHz spectrophotometer in DMSO-d_6 using TMS as an internal standard. Splitting patterns are designated as follows: s, singlet, d, doublet, m, multiplet. The FAB mass spectra of all the complexes were recorded on a JEOL SX 102/DA-6000 Mass Spectroscopy/Data System, using argon/xenon (6 kV, 10 mA) as the FAB gas and *m*-nitrobenzyl alcohol (NBA) as the matrix. Room temperature magnetic susceptibility was measured at 298 K by a Vibrating sample Magnetometer 155, E-112 ESR Spectrometer, Varian, USA using nickel as standard and such values were expressed as effective magnetic moment (μ_{eff}) in Bohr Magneton (B.M.). The electron paramagnetic resonance (EPR) spectra were recorded with a Bruker ESP 300E X-band spectrometer.

4.1.1. Synthesis of ligands

The thioglycolic acids were prepared as outlined in reference.[37] The thiosemicarbazones were synthesized by heating at reflux an aqueous solution of respective aminothiocarbonylhydrazines (0.003 mol in 10 mL) with ethanolic solution of furan-2-carboxaldehyde (0.228 g, 0.003 mol in 10 mL) for 3 h with continuous stirring. After cooling, the precipitated compound was filtered and recrystallized from appropriate solvent.

4.1.2. Synthesis of Pd^{II} and Pt^{II} complexes of thiosemicarbazones

The synthesis of Pd^{II} and Pt^{II} complexes $[M(DMSO)_2Cl_2$, where M is Pd or Pt] were prepared in accordance the procedure outlined in the literature.[38] A solution of $[Pd(DMSO)_2Cl_2]$/$[Pt(DMSO)_2Cl_2]$ (2 mmol) dissolved in methanol (5 mL) was added to a solution of respective ligand (2 mmol) previously dissolved in a minimum quantity of methanol and the reaction mixture was heated under reflux for 1–3 h. After keeping the solution at 0 °C overnight, the colored solid was filtered out. This was washed with hot water followed by a small quantity of methanol and dried in a vacuum desiccator over anhydrous silica gel to give amorphous solids.

4.1.3. Synthesis of Cu^{II} and Co^{II} complexes of thiosemicarbazones

A stirred solution of hydrated metal chloride (2 mmol) dissolved in minimal quantity of methanol was added to a stirring hot solution of ligand (4 mmol) in methanol (20 mL) and the reaction mixture was heated under reflux for 1–3 h. This solution was kept to room temperature overnight, when the precipitate was filtered out, washed with hot water followed by small quantity of methanol, and dried. Recrystallization was carried out from methanol.

4.2. Pharmacological procedures

4.2.1. Cytotoxicity to mammalian cells

The cytotoxicity of the compounds and metal complexes was determined using BALB/c mouse splenocytes (5×10^6 cells well^{-1}) cultured in 96-well plates in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% of fetal calf serum (FCS; Cultilab, Campinas, SP, Brazil) and 50 μg mL^{-1} of gentamycin (Novafarma, Anápolis, GO, Brazil). Each compound was evaluated in five concentrations (1.1, 3.3, 11, 33, and 100 μg mL^{-1}), in triplicate. Cultures were incubated in the presence of ^{3}H-thymidine (1 μCi well^{-1}) for 24 h at 37 °C and 5% CO_2. After this period, the content of the plate was harvested to determine the ^{3}H-thymidine incorporation using a β-radiation counter (Multilabel Reader, Hidex, Turku, Finland). The cytotoxicity of the compounds was determined comparing the percentage of ^{3}H-thymidine incorporation (as indicator of viability cell) of drug-treated wells in relation to untreated wells. Non-cytotoxic concentrations were defined as those causing a reduction of ^{3}H-thymidine incorporation below 10% in relation to untreated controls. *Note*: BALB/c mice were handled according to the NIH guidelines for animal experimentation. All procedures described here had prior approval from the animal ethics committee of FIOCRUZ (Brazil).

4.2.2. Antimalarial activity

It was performed using the [^{3}H]-hypoxanthine incorporation assay, as previously described.[39] Briefly, parasites were maintained in continuous culture of human erythrocytes (blood group O^+) using RPMI 1640 medium supplemented with 10% human plasma. Parasites grown at 1–2% parasitemia and 2.5% hematocrit were incubated with the pure substances tested at five different concentrations, diluted with 4% DMSO in culture medium (RPMI 1640) without hypoxanthine. Cultures containing parasites were harvested using a cell harvester to evaluate the [^{3}H]-hypoxanthine incorporation in a β-radiation counter. Inhibition of parasite growth was evaluated by comparison with [^{3}H]-hypoxanthine uptake in drug treated versus untreated wells after 24 h of incubation with the tested compounds. IC_{50} values were calculated triplicates, comparing with the Mefloquine (**MQN**) as standard drug.

4.2.3. Antiamoebic activity

Thiosemicarbazones and their metal complexes were screened against the HM-1:IMSS strain of *E. histolytica* by using the microplate method.[40] All the experiments were carried out in triplicates at each concentration level and repeated thrice. *E. histolytica* trophozoites were cultured in TYI-S-33 growth medium in wells of 96 well microtiter plates.[41] DMSO (40 μL) was added to all the samples (1 mg) followed by enough culture medium to obtain concentration of 1 mg/mL. The maximum concentration of DMSO in the test did not exceeded 0.1%, and at this level no inhibition of amoebal growth has occurred. Compounds were further diluted with medium to a concentration of 0.1 mg mL^{-1}. Twofold serial

dilutions were made in the wells of 96-well microtiter plate. Each test included Metronidazole (MNZ) as the standard amoebicidal drug, control (culture medium plus parasite) and a blank (culture medium only). The cell suspension was then diluted to 10^5 organism/mL by adding fresh medium and 170 μL of this suspension was added to the test and control well in the plate. Plate was sealed and gassed for 10 min with nitrogen before incubation at 37 °C for 72 h. After incubation, the growth of amoebae in the plate was checked with a low power microscope and the optical density of the solution in each well was determined at 490 nm with a microplate reader. The% inhibition of amoebal growth was calculated from the optical densities of the control and test wells and plotted against the logarithm of the dose of the drug tested. Linear regression analysis was used to determine the best-fitted straight line from which the IC_{50} value was found.

Acknowledgements

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Supplementary data

Supplementary data (compound characterization of the compounds outlined in Scheme 1. Pharmacological protocols conducted can also be found in the Supporting Information) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.07.039.

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Early Toxicity Screening and Selection of Lead Compounds for Parasitic Diseases

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Abstract: Despite many advances made in disease mechanisms knowledge and drug discovery and development processes, the election of promising lead compounds continues to be a challenge. Efficient techniques are required for lead selection of hit compounds selected through *in vitro* pharmacological studies, in order to generate precise low cost throughput data with minimal amount of compound to support the right decision making. In this context, the selection of lead compounds with physicochemical parameters that will benefit orally bioavailable drugs are crucial for patients compliance and cost effectiveness, as well as for successful pharmacology. A concept based in Lipinski's rules point out the importance of analyzing these informations in early stages. A hepatocyte screening system may provide data on many processes such as drug-drug interaction, metabolite formation, drug toxicity and ADME profile of a hit. Drug-induced liver injury is the most frequent reason for the withdrawal of an approved drug from the market and hepatocytes have a central role in the metabolism of xenobiotics. Cytotoxicity screening assays can also give some information about toxicity early drug discovery process. A set of goals in lead compound selection must be shared between all areas involved so the chances of success can be improved in translational research.

Key Words: Toxicity screening, lead compound, parasitic diseases, drug discovery.

INTRODUCTION

The search for new and improved treatments for diseases endemic of developing countries is increasing. Bacterial, protozoan and helminth infectious diseases such as tuberculosis, malaria, African sleeping sickness, leishmaniasis, Chagas' disease, onchocerciasis, lymphatic filariasis and schistosomiasis are among them. Although there is a need for new drugs to reduce mortality and morbidity caused by the mentioned infections [1], high cost, poor compliance, drug resistance, low efficacy and poor safety are limiting factors for drug development in resource-poor settings where these diseases occur.

Public-private partnerships have proved that they can move compounds quickly through the R&D process [2]. The Medicines for Malaria Venture is an example of public-private partnerships working on diseases for the developing world [3]. Gaps between basic research and clinical development can be transposed by joining expertise from academia, public sector and pharmaceutical industry.

R&D costs increase substantially as compounds move through each successive phase (Fig. (**1**)). Directing the focus of R&D program to a fewer lead candidates would represent costs reduction and maximization of efforts towards a more promising drug candidate [4]. In this context, basic research *(in vitro* and *in vivo* pharmacology and toxicology assays) needs to share the same goals of pharmaceutical industry in order to supply leads for translational research.

A hit compound identified by the *in vitro* activity screening of drug design, natural products or chemical libraries has to be efficacious in disease animal models with no evident toxicity at efficacious doses in order to be called a lead compound. Effective networks help to establish a library that can be constructed based on pharmacologic, toxicological and pharmacokinetic properties of a lead compound in order to draw a drug candidate, which is an optimized lead compound that can be compared to drug standard activity with acceptable pharmacokinetic and toxicity profile [1].

Nowadays a hit is selected by biological activity and later on the properties related to its "drugability", i.e., ADME/toxicity, are investigated [5]. Cytotoxicity should also be evaluated in the beginning of a drug screening program, in order to identify among the highly active compounds those excessively cytotoxic and thus incapable to be classified as lead compounds [6].

Safety evaluation in the early stage of drug discovery is crucial for the selection of a drug candidate among many compounds. Safety was defined by White [7] as sufficient selectivity for the target receptor so that an adequate dose range exists in which the intended pharmacological action is essentially the only physiological effect of the compound. The ideal system would generate "go/no-go" decision employing only a small amount of the compounds [7]. Pharmaceutical industry is challenged on how to prioritize scale up

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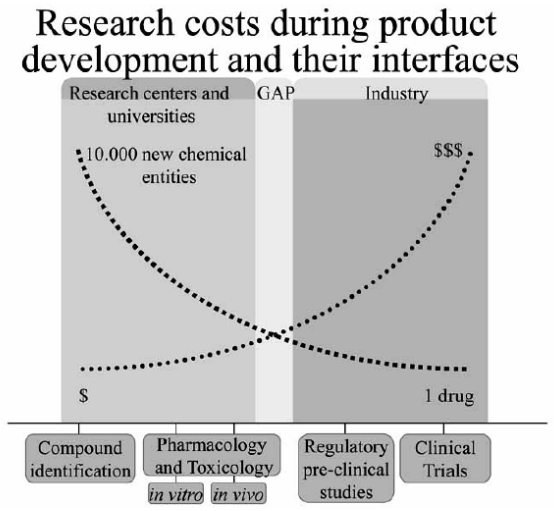

Fig. (1). Public-private-partnerships and their interfaces in drug development process.

of potential leads, considering the quantity (grams) required for secondary testing and safety assessment and the difficulty in obtaining large amounts of compound in early stages [6]. Hence, each step has to be planned to obtain the data required with minimum effort.

In 1997 a report was published pointing out the main reasons for failures in drug development, which were attributed to poor pharmacokinetics (39%) and animal toxicity (11%) [8]. Pharmacokinetic prediction is a hard task once absorption, distribution, metabolism and excretion (ADME) experimental screens are multi-mechanisms and the accumulation of data turns it into a complex work. In long term the advent of a single mechanism ADME assay and biology systems may modify the drug discovery process with its application being prior to target identification [5,9].

Because the majority of discovery projects are focused on the development of orally bioavailable drugs, lead compound selection needs to correlate *in vitro* data to *in vivo* absorption. Multiple assays and approaches can be applied for decision making. After the hit identification by pharmacological *in vitro* studies, lead-like physicochemical properties, cytotoxicity, hepatocyte screening system assays are the next step followed by *in vivo* pharmacological studies. The synergistic combination of all this information generated is crucial for the selection of lead compounds.

1. LEAD-LIKE PHYSICOCHEMICAL PROPERTIES

The investigation of chemical properties can be used to predict *in vivo* performance of a compound or even to optimize a lead. A successful drug is an intricate balance of biological activity and drug-like properties. Absorption and first-pass metabolism in the liver or gut wall are generally responsible for the amount of compound orally administered found in systemic circulation. Solubility and lipophilicity together determine the extent and rate of absorption and have a direct influence in oral bioavailability, defined as the range of the oral dose that reaches systemic circulation [10].

Over the last years many scientists reported physicochemical properties related to the "drugability" of a compound and that a correlation of partition coefficient (log P), molecular weight, the number of hydrogen bonding groups and bioavailability of a compound could be established [11, 12].

By computational analyses of the World Drug Index and previous knowledge of chemical properties, researchers from Pfizer proposed that simple chemical rules could be used prospectively to compare and prioritize groups of compounds and different chemical series with greater potential to become orally active compounds. A high percentage of compounds that entered clinical trials, with the desired drug-like properties, was found to have: hydrogen bond donors ≤ 5; hydrogen bond acceptors ≤ 10; relative molecular weight ≤ 500; and calculated octanol-water partition coefficient (prediction of the ability of a molecule to cross biological membranes) ≤ 5 [5]. Those rules were called Lipinski's "rule-of-five" and became a guide to identify and select small molecules intended to be orally administered.

Although it raised the awareness that drug-like compounds exhibit physicochemical properties that are important for successful drug development, the need to go further Lip-

inski's rules in recent years brought extended versions with evolution of concepts with the aim to provide quality leads [13]. Lipinski's "rule-of-five" is certainly necessary but not sufficient to create an oral drug-like molecule [14]. Researchers from GlaxoSmithKline, after oral bioavailability measurements in rats for over 1100 drug candidates, suggested that compounds with 10 or fewer rotatable bonds (as a measure of reduced molecular flexibility), and 12 or fewer H-bond donors and acceptors (as a measure of low polar surface area) will have a high probability of good oral bioavailability in the rat [15]. The main limitations of these rules are false positives (compliant compounds are not automatically good drugs) and restricted application (applies only to compounds administered orally and absorbed by passive mechanisms). Besides, there are important exceptions, such as natural products that, over the evolution, might have been optimized by nature to take advantage of active transport or have developed special conformational features that are beneficial for passive transport [13]. Natural products structures present different properties when compared to libraries of synthetic and combinatorial compounds, such as high chemical diversity and biochemical specificity [16].

With emerging valuable tools, it is up to drug development scientists to combine multiple assays and approaches that will permit the selection of lead compounds with physicochemical parameters that will benefit orally bioavailable drugs.

1.1. Solubility

The aqueous solubility of a substance is crucial information that has to be readily defined in the very beginning of drug development. Poor solubility and permeability lead to poor bioavailability and lack of *in vivo* activity [17]. Solubility at a given pH is influenced by the basic or acidic functional groups of a compound. The solubility of a compound in aqueous media is greater when it is in ionized state [18]. Lipinski estimated the required level of compound solubility to minimize poor absorption. It was found that, for an orally active drug, a compound with medium intestinal permeability and human potency of 1 mg/kg needs a minimum thermodynamic aqueous solubility of 52 μg/ml (at pH 6.5 or 7.0) [5]. The level of solubility needed for oral absorption is related to the potency and permeability, although oral activity can still be achieved with lower solubility when a compound has great potency and is highly permeable. On the other hand, solubility needs to be higher in cases when a compound is not very potent and permeable [17].

1.2. Permeability

The two most important determinants of *in vivo* intestinal absorption are solubility and intestinal permeability [7]. Permeability and solubility are independent and its determinant molecular components are molecular size and hydrogen bonding capacity. An increase in molecular weight and lipophilicity will increase permeability and decrease solubility, whereas the increase hydrogen-bonding capacity and charge will increase solubility and decrease permeability. The orally absorbable compound is a resultant of a balance between the different physicochemical properties. Because the change of one molecular component will affect the other, when a decision needs to be taken, preference should be given to more permeable compounds since it is possible to improve solubility using adequate pharmaceutical technology [17].

During the last few years an increased use of *in vitro* absorption model, such as Caco-2, a human colon epithelial cancer cell line, was observed in many research fields, such as pharmaceutical sciences for the studies of absorption, permeability and transepithelial transport of drugs [19]. This approach measures rates of compound diffusion down a concentration gradient across cultured Caco-2 cell monolayers. Apparent permeability coefficients can be calculated by the measurement of the net flux of a compound over this cellular barrier followed by LC/MS/MS (liquid/chromatography/tandem mass) assay with cells cultured in semi-permeable plastic supports. These coefficients represent rates of permeability for each compound tested [20,21]. This permeability experiment can be automated, allowing rates of a few hundreds of compounds to be performed. Although this system cannot be considered high-throughput screening, it is better than manual cell culture methods [7].

One of the drawbacks of the Caco-2 cell lines is that, when compared to normal intestinal enterocytes, they present an over-expression of CYP1A1 and a down-regulation of CYP3A4, resulting in differences in metabolic competence [22]. This can be restored by the use of a genetically engineered Caco-2 variant expressing high levels of CYP3A4, allowing assessment of permeability and gut wall metabolism and improving predictability [19].

1.3. Lipophilicity

Lipophilicity can be expressed as a partition coefficient between octanol and aqueous phases. When dividing the bioavailability of drugs into absorption and metabolism it is clear that pharmacokinetic parameters are closely related to lipophilicity: high lipophilicity increases metabolic clearance and limits solubility, causing poor absorption [22]. It significantly impacts ADME properties and can be improved by increasing molecular size and decreasing hydrogen-bonding capacity of a compound [17]. The partitioning of a compound is dependent on the pH of the solution that directly affects its ionization state [18]. They can be estimated very early by simple calculations and guide the choice or design of molecules with acceptable pharmacokinetic profiles.

2. *IN VITRO* CELL SYSTEMS

Cell system models can be designed to scan a wide range of molecules more rapidly and cost effectively than *in vivo* studies. In a drug discovery process, two initial tests must be applied for predictive toxicology profiling: cytotoxicity and hepatotoxicity. These assays require minimal amounts of compounds, are not time-consuming and, in combination the information generated from these well-established systems, may be a valuable tool for decision-making. Detection and removal of compounds presenting hepatotoxicity and cytotoxicity before getting into the more costly phase should be the goal during discovery phases. Hepatotoxicity can be predicted by a combination of computational and *in vitro* systems, depending on the availability of time and information needed at a given stage.

2.1. Cytotoxicity

Cytotoxicity is an important parameter in drug development and *in vitro* determinations are well established in the pharmaceutical and biotechnology industries. It has been used previously for predicting acute toxicity. There are different methodologies for evaluating cell toxicity and assays using 3T3 fibroblast cell line and those based on neutral red uptake are now accepted by authorities from European Union and OECD for regulatory purposes. Biokinetic properties can be predicted by results of *in vitro* methods and high or medium throughput screening (HTS/MTS) can guide preclinical studies [20]. Regardless of the chosen methodology, a cytotoxicity assay aims to guide the prediction of *in vivo* toxicity and is a tool for lead compound selection.

A drug candidate should have selective toxicity. Many promising compounds active against protozoans already tested *in vitro* have no cell cytotoxicity determined. Molecules against intracellular parasites should be able to cross the cell membrane and act specifically on the microorganism. The use of cytotoxicity determination is very well established, and aims to identify hits among the compounds screened in *in vitro* assays. The purpose of this determination is to ensure that an inhibition in a cell-based assay is not due to inhibited cell viability. Information about adsorption, distribution, metabolism and excretion associated to the cytotoxicity evaluation can reveal an early preclinical profile of the compound [20].

V79, L929, Ehrlich, 3T3 fibroblasts, HepG2, Vero, Caco-2 and murine splenocytes are examples of very common cells used in cytotoxicity determination assays [23-27]. Nowadays, the reduction in the number of animals in research is stimulated by international, and the substitution of freshly isolated cells by cell lines is an option [28]. The readouts of the viability assessment assays vary, and can be based on diverse assay methodologies, such as fluorimetry, luminescence, scintillation, colorimetry, cell staining/dye exclusion, among others.

There are diverse methodologies based on fluorimetry for cytotoxicity assessment. In general they are based on the measurement of signaling molecules liberated by non viable cell or on staining of nucleic acids, such as calcein/ethidium homodimer method, which allows to evaluate cell viability/cytotoxicity [29]; the glucose-6-phosphate released by damaged and dying cells, which can be detected by an enzymatic process including the reduction of resazurin into red-fluorescent resorufin [29]; and propidium iodide (PI), a molecule able to bind to DNA between the bases, which only penetrates in dead cells [30].

PI is a toxic molecule, which can induce mutation and should be handled with care. This characteristic does not invalidate the method because, if handled with care, PI is absolutely secure and does not generate radioactive residues. Cytotoxicity analysis using PI associated with flow cytometry is a sensitive and precise method and can be employed in analysis of normal or neoplastic murine or human cells, since PI to nucleic acid in not viable cells. The existence of automated high-speed processing for this method should be considered, which facilitates its use, as reported by Nunez [31]. Assays based in fluorimetry analyzed by flow cytometry permit high-content screening reflecting some of recent efforts to advance its automation and more efficient application to the drug discovery process [32]. When a compound has a natural color the evaluation of cytotoxicity by colorimetric assays may be difficult due to its influence on the spectrophotometric analysis. In the PI viability assessment, however, there is no interference of the compound color, since the technique is based on emitted fluorescence resultant of the PI binding to the nucleic acid.

Regarding methods based on luminescence, the ATP/luciferase can be cited as an example of method for detection of viable cells, since the luminescence production by luciferase requires ATP [29]. Cell lines transfected with the luciferase gene can be used in this luminescence-based cell viability assays.

Among methods based on scintillation count, ^{3}H-thymidine uptake is largely used. Thymidine is a nucleoside that can be incorporated by viable cells in their nucleic acids metabolism in non-stimulated and stimulated cell cultures. When cell proliferation is stimulated, it induces the production of nucleic acid, which requires nucleosides. When ^{3}H-thymidine is added to the culture medium, the labeled nucleotide competes with endogenously available thymidine for incorporation into newly synthesized nucleic acid [33, 34]. Other viable eukaryotic cells can do the same, by the described mechanism, including neoplastic lineages. In non-stimulated cells there is a basal t-RNA metabolism also that requires thymidine [33,34]. Thus, splenocytes can incorporate thymidine in a lowest proportion, but enough to quantify if compared with cultures with non-viable cells. The incorporation of ^{3}H-thymidine can be measured in a scintillation counter, expressing proportional scintillation value to the cell viability. Quantification can be accessed by detecting radiolabeled nucleic acid, using glass-fiber filter mats or plates, which trap genomic molecules along with some cell fragments but spare oligonucleotides or single bases [33]. The method based on ^{3}H-thymidine uptake is very practical because it is possible to perform the assay in 96-well plates, allowing the screening of a large quantity of compounds/plate. In addition to the advantages cited above, this method also eliminates the color interference in when a colorful compound is being analyzed. This method has a disadvantage, which is the generation of radioactive solid and liquid waste. Manipulation should be carried out by trained personnel and care should be adopted aiming to protect the laboratory area, other researchers and the environment.

Common colorimetric assays are based in cell metabolism of one substrate producing a colorful product, like formazan, using lactate dehydrogenase (LDH) or MTT methods. LDH, an enzyme released from damaged cells, oxidizes lactate to pyruvate which reacts with tetrazolium salt producing formazan. MTT (4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) is a tetrazolium salt that active mitochondria can cleave [35]. This is an assay useful, secure, simple and fast to measure cell viability in culture. The MTT cleavage is used for cell viability evaluation or cell proliferation [36], and can be used to determine small alterations in the cell redox status and changes in mitochondrial activity. Since the introduction of MTT for cell viability evaluation in 1983, many other tetrazolium salts have been used, such as

XTT, MTS, and WST [35]. Among the many methods for cell viability that evaluate the cell oxidative capacity, the MTT method has been the most used because of its sensitivity and reliability [37]. Although the cell that does not metabolize MTT may only be presenting some deficiency in the oxidative metabolism, without being necessarily dead, the quantity of formazan produced is usually considered as directly proportional to the total number of active mitochondria by cell in culture [38]. MTT method has some advantages in comparison to other methods. It does not produce radioactive residues, is not an expensive method and adherent and non-adherent cells can be used in high-throughput screening processes. Disadvantages of the method include the difficulty of analysis of heterogeneous cell populations due to differences in the cell metabolism individual capacity and interference of colorful compounds in absorbance values and, therefore, in the cytotoxicity results.

Cell viability assessment by trypan blue (TB) exclusion is a very useful, practice and cheap method. The cytotoxicity is determined counting cells manually, using TB solution, which is a dye used to evaluate cellular death. Viable cells, when observed through optical microscope, stay uncolored, while dead cells appear stained in blue. Cell membrane of non-viable cells allows the absorption of TB. Some disadvantages of this method can be listed, such as the quantification is made by manual counts, requiring longer time and allowing procedure errors, or the need to remove adherent cells (e.g. using trypsin) for counting. Considering this, the use of this technique characterizes a low-throughput screening process.

2.2. Selectivity Index

The selectivity index (SI) is a parameter that can be used to determine drug specificity for any activity. The SI is calculated dividing the LC_{50} by IC_{50} values. This index can be related to any biological activity like antiprotozoal, immunomodulatory or antineoplastic activity. The SI is considered significant when its value is higher than three [24] and the more elevated is the drug selectivity index, the higher is the drug more selectivity. One example of a highly selective drug is nifurtimox (an anti-*Trypanosoma cruzi* agent), which presents an SI equal to 154 [39]. Another example is chloroquine, an antimalarial agent showing a SI equal to 200 for a chloroquine-sensitive *Plasmodium* strain while the same drug shows a low SI (20) for a chloroquine-resistant strain, as reported by Desoubzdanne [40].

2.3. Hepatocyte Screening System

Drug-induced liver injury is the most frequent reason for the withdrawal of an approved drug from the market and inclusion of black box warning [41]. Between 1979 and 1999, 45 previously approved drugs received one or more black box warning (10 due to hepatotoxicity) and 16 were withdrawal from the market because of safety reasons (5 due to hepatotoxicity) [42]. Currently the investigation of drug metabolism and toxicity is done much earlier in the discovery process. The central role of hepatocytes in the metabolism of xenobiotics with the generation of intermediate or final metabolites with potential toxic effect for the organism places the liver into a central context.

During the discovery lead optimization process a hepatocyte screening system should provide data on (Fig. (**2**)):

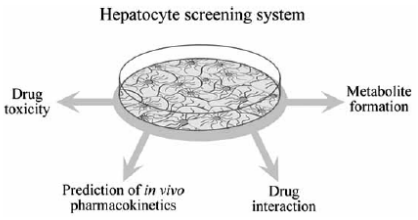

Fig. (2). *In vitro* methods performed with hepatocytes, microssomes and CYP cultures for prediction of drug toxicity, metabolite formation, drug-drug interaction and pharmacokinetics *in vivo*.

- Drug toxicity - provides the opportunity to study in detail mechanisms of hepatotoxicity in comparison with *in vivo* conditions.
- Drug-drug interaction – can be predicted by the detection of substrates inhibitors or inducers of the enzyme involved in the metabolic pathway of a compound.
- Metabolite formation - the formation of reactive metabolites can be investigated by the use of primary hepatocytes.
- Prediction of *in vivo* pharmacokinetics - preliminary prediction of the *in vivo* behavior of a compound with potential to become a drug.

Drug metabolism is a crucial determinant of drug clearance and interindividual pharmacokinetic differences, leading to efficacy or failure of a given drug candidate during clinical test [43]. Gene polymorphisms can be the reason for interindividual differences concerning drug response once there is no standard profile for CYP expression in man [44,45].

Among the whole universe of CYP isoforms found in human liver (Fig. (**3**)), attention must be paid to the ones known to have the highest impact on drug metabolism. CYP3A4 is the most abundant enzyme in the liver and is involved in the metabolism of over 50% of drugs [46]. Because of its high content in the liver and history of well conserved genes this enzyme represents a good choice for selecting a high affinity lead candidate in a drug screening program. However, there are other factors, such as sex, age, use of ethanol and other drugs underlying diseases that can modulate gene expression, resulting in susceptibility for adverse reaction. Those events are often unpredictable and will be detected in late stages during clinical trials of drug development [47].

On the other hand, a drug design avoiding compounds that present high affinity substrates for CYP2D6 is desired. Although present in low amounts in human liver (Fig. (**3**)), this enzyme metabolizes 20-25% of drugs used clinically and its high polymorphism represents huge differences in the clinical response that goes from reduced pharmacologic effect (for the ultra-rapid metabolisers) to toxicity (for the poor

metabolisers) [45]. In addition, clinically relevant polymorphisms have been identified for de CYP 2C9 and 2C19 and this has a profound effect in drug efficacy and toxicity [48,49].

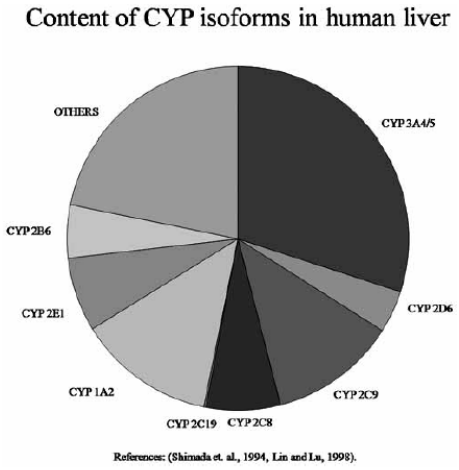

Fig. (3). The estimated content of CYP isoforms in human liver according to key references.

2.3.1. Drug Toxicity

It is hard to detect hepatotoxicity occurring at low incidence with the number of animals used in toxicology studies. Considering that it is time consuming and costly to perform *in vivo* studies with massive number of drugs, robust *in vitro* screening assays are being developed for prediction of human hepatotoxicity caused by drugs [49-51].

Drug toxicity is the most common cause of acute liver failure. It is a serious health problem that impacts society and represents a major impediment in drug development [52]. Hepatotoxicity can be caused by the direct action of a drug or, more often, a reactive metabolite of a drug against hepatocytes or by drug-induced immune-mediated liver injury [53].

One of the predominant forms of drug-induced liver injury is acute hepatitis, defined as a marked increase in aminotransferases coinciding with hepatocellular necrosis [53]. Hepatic necrosis can be detected in nearly 50% of cases reported [54]. Apoptosis can also be detected in hepatocyte models and is an important parameter used in the toxicity analysis of xenobiotics [55].

A cytotoxicity assay specifically developed to predict clinical acute hepatotoxicity (acute liver failure) based on immortalized cell lines representing an exaggerated case of metabolizing enzyme has been used early in discover process. The CYP450 enzyme isoforms and the 3A4, 2C9, 2C19 and 2D6 cell lines are the basis of an assay that determines IC_{50} values and can be used to evaluate the potential for hepatotoxicity across candidate compounds for lead optimization ranking [49].

2.3.2. Prediction of In Vivo Pharmacokinetics

Metabolism in animals differs from that in humans, and thus the prediction of human pharmacokinetics from animal data might not be straightforward, being crucial to determine previously the specific CYP involved in the drug metabolism in order to select an appropriate *in vivo* system. As reviewed by Zuber [46], for CYP1A mediated pathways, with the exception of the dog, all the experimental models commonly used are appropriate, whereas for drugs metabolized by CYP2D the dog seems to be the most suitable. The CYP3A seems to be well modeled in pigs, whereas a good alternative for CYP2C may be a system based on monkeys (*Maccacus rhesus*) [46].

A major challenge in this phase is to find a model that provides high throughput data in order to screen a large number of compounds. As reviewed by Yu and Adedoyin [56], integration of experimental and computational technologies can be used to characterize ADME profile of a compound. The assay applied will depend on the stage of the process. An early discovery stage demands the evaluation of hundreds or thousands of compounds quickly and does not require the details and depth of data necessary in the next stages. *In silico* ADME models may soon serve as a filter for a large number of compounds before *in vitro* ADME toxicological tests [57]. Each therapeutic class has its own pharmacokinetics requirements. Therefore, the range of ADME must be very comprehensively and carefully studied in each phase of the program [58].

2.3.3. Drug Interaction

Reduced pharmacological effect or increased toxicity of a drug (by production of reactive intermediates) may be observed when the drug tested is co-administrated with another drug that causes increased activity of one or more enzymes [44,59]. A competitive or noncompetitive inhibition of a CYP 450 enzyme responsible for the metabolism of a co-administered drug, leading to its accumulation, can also be observed. In case the drug has inherent toxicity, adverse effects may be observed [49]. This phenomenon is called drug-drug interactions and has serious clinical consequences.

It is important to identify the metabolic pathways of candidate compounds by conducting *in vitro* evaluation of changes in CYP 450 enzyme mRNA level and monitoring enzyme activity [49,60]. The identification of drug metabolizing enzymes helps to predict the drug-drug interaction that may occur in man [58]. Most of the drug clearance is mediated by five CYP isoforms, 3A4, 2D6, 2C9, 1A2, and 2C19 [44], and therefore these should be the ones tested.

Inhibition of cytochrome P450 is a principal mechanism for metabolism-based drug-drug interactions. The development of high-throughput CYP-mediated cocktail assay with quantification by liquid chromatography/tandem mass spectrometry (LC/MS-MS) has a potential demonstrated using drug library compounds, which showed that this assay has application in early-stage drug discovery [59,61].

Drug-drug interaction is not a direct measurement of in *vitro* CYP450 inhibition assay. It occurs when two co-administered drugs compete for the same CYP enzyme and when the metabolic reaction catalyzed by this enzyme is the major elimination pathway [62]. Hence, all the data generated must be carefully analyzed.

2.3.4. Metabolite Formation

The rate of metabolism of a drug can be determined using *in vitro* cell systems by the measurement of the drug disappearance over time, as well as metabolite formation [44]. Active metabolites of several marketed drugs have been developed as drugs with better efficacy and ADME profile [59]. Characterization of metabolites is critical for optimizing lead compounds with improved metabolic stability and toxicological profile [44,62]. Otherwise, compounds that form toxic metabolites that could cause adverse effects can be eliminated [63].

Metabolites can be generated with the use of animal and human recombinant expressed enzymes, human liver and hepatocytes and pre-clinical animal models [58,63]. The metabolite structure elucidation can be determined by the combined use of accurate mass liquid chromatography/tandem mass spectrometry (LC/MS/MS). This methodology integrates the parent disappearance, metabolite identification, and the formation of the metabolites along the time course using a single rapid LC/MS/MS analysis [64,65].

Throughput studies for drug metabolite identification have increased significantly in the last years. Although it is still too labor-intensive in the drug discovery setting, recent publications anticipate that the combination of LC/MS/MS and LC/NMR will be crucial for structure metabolite elucidation and feasible to apply in early drug discovery [66].

CONCLUDING REMARKS

ADME tests begin with the identification of a hit and continue during lead optimization, selection and development stages [58]. Unsuitable ADME is, together with animal toxicity, responsible for half of all failures in drug development [57]. At the lead compound selection phase, there is a need for high throughput methods to screen many hits. Although there is no need to go depth into the information, early data related to hepatotoxicity, ADME profile and cytotoxicity are crucial. All of these parameters are interrelated and need to be considered in parallel. There is a need for new technologies to be explored as a way of reducing drug attrition rates and either in academia or industry these key questions (toxicity and ADME profile) must be answered. It is important to point out that the methods discussed here are predictions of *in vivo* behavior of a compound. The safety of new compounds is multimechanism determination dependent on each individual's response, and therefore it cannot be known with certainty until a drug has been on the market for many years.

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ABBREVIATIONS

ADME	=	Absorption, distribution, metabolism and excretion
ATP	=	Adenosine triphosphate
CYP	=	Cytocrome P450
DL_{50}	=	Lethal dose for 50%
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
G6P	=	Glucose-6-phosphate
IC_{50}	=	Inhibitory concentration for 50%
LC/MS/MS	=	Liquid/chromatography/tandem mass
LC_{50}	=	Lethal concentration for 50%
MTT	=	4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide
NADPH	=	Reduced nicotinamide adenine dinucleotide phosphate
OECD	=	Organisation for economic co-operation and development
PBS	=	Phosfate buffered saline
pH	=	Potential hydrogenionic
PI	=	Propidium iodide
RD	=	Research and development
RNA	=	Ribonucleic acid
SI	=	Selectivity index
TB	=	Trypan blue
t-RNA	=	Transporter ribonucleic acid
WST or XTT	=	2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5 caroxanilide

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