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AVALIAÇÃO FENOTÍPICA *IN VITRO* DE NOVAS AMIDINAS AROMÁTICAS SOBRE *Trypanosoma cruzi*

MARIANNE ROCHA SIMÕES SILVA

Rio de Janeiro
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Avaliação fenotípica *in vitro* de novas amidinas aromáticas sobre *Trypanosoma cruzi*

Dissertação apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Mestre em Biologia Celular e Molecular

Orientadora: Prof. Drª. Maria de Nazaré Correia Soeiro

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“Amadores se sentam e esperam pela inspiração. O resto de nós apenas se levanta e vai trabalhar.” Stephen King

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AVALIAÇÃO FENOTÍPICA *IN VITRO* DE NOVAS AMIDINAS AROMÁTICAS SOBRE *Trypanosoma cruzi*

RESUMO

DISSERTAÇÃO DE MESTRADO EM BIOLOGIA CELULAR E MOLECULAR

Marianne Rocha Simões Silva

A doença de Chagas (DC), causada pelo protozoário *Trypanosoma cruzi*, é uma doença negligenciada endêmica em diferentes regiões empobrecidas da América Latina. O tratamento, baseado em dois nitroderivados, Nifurtimox e Benzonidazol (Bz), é insatisfatório, demandando a busca de novos fármacos com ação tripanocida que sejam mais seletivos e eficazes. Nesse âmbito, o presente trabalho busca a identificação de novos agentes antiparasitários para a DC, explorando a avaliação fenotípica de novas amidinas aromáticas sintéticas *in vitro* incluindo ensaios de combinação entre estes compostos. Dez novas amidinas foram testadas sobre tripomastigotas sanguíneos e amastigotas de diferentes cepas do *T. cruzi* (Y e Tulahuen) e também sobre células de mamífero hospedeiras (linhagem L929 e células cardíacas) para determinar seu perfil eficácia e de toxicidade, respectivamente. Dentre as moléculas testadas (apresentando um ou dois grupamentos catiônicos terminais), cinco foram mais ativas sobre tripomastigotas sanguíneos que o fármaco de referência (Bz), sendo uma delas, a DB2267 (molécula dicatiônica) a mais eficaz, exibindo EC_{50} de 0,23 µM e um índice de seletividade (IS) de 417. Esta diamidina foi 28 vezes mais ativa e cerca de três vezes mais seletiva que Bz. Para determinar se a combinação de duas amidinas teria um efeito tripanocida superior ao seu uso em monoterapia, tripomastigotas sanguíneos foram incubados com DB2267 e DB2236 em proporções fixas e os resultados mostraram apenas um efeito aditivo com $\Sigma FIC<4$. Interessantemente, quando formas intracelulares foram expostas à DB2267, sua atividade foi relacionada à cepa do parasito, sendo eficaz (EC_{50} = 0.87 ± 0.05 µM) contra DTU II (cepa Y), mas não contra um representante da DTU VI (Tulahuen), mesmo quando utilizamos veículo diferente do DMSO (β-ciclodextrina). Esta divergência pode estar relacionada a diferenças inerentes aos alvos e/ou rotas metabólicas das cepas do parasito e que merecem ser mais investigadas visando conhecer melhor a relação entre as características estruturais e ação universal das moléculas sobre DTUs e formas do *T. cruzi* para o desenho de compostos mais promissores. Devido à fluorescência intrínseca apresentada pelas amidinas, a captação de duas delas foi avaliada. Os dados obtidos com a amidina não ativa (DB2236) e com a outra ativa (DB2267) sobre amastigotas da cepa Y revelaram que ambas foram localizadas intracelularmente, mas em compartimentos distintos: DB2236 no citoplasma, enquanto DB2267 no núcleo. Nossos dados são encorajadores a respeito da atividade antiparasitária das amidinas aromáticas, no tocante a futuras investigações de novos agentes promissores para o tratamento da DC.

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PHENOTYPIC SCREENING *IN VITRO* OF NOVEL AROMATIC AMIDINES ON *Trypanosoma cruzi*

ABSTRACT

MASTER DISSERTATION IN BIOLOGIA CELULAR E MOLECULAR

Marianne Rocha Simões Silva

Chagas disease (CD), caused by the protozoan *Trypanosoma cruzi,* is a neglected disease endemic in different poor areas of Latin America. The treatment, based on two nitroderivatives, Nifurtimox and Benznidazole (Bz), is unsatisfactory, demanding the screening of new potential trypanocidal drugs more selective and potent. In this scope, the present work deals with the identification of new anti-parasitic agents for CD, exploring the phenotypic screening of novel synthetic aromatic amidines *in vitro* and also combination assays between these compounds. The novel ten amidines were tested against bloodstream trypomastigotes (BT) and amastigote forms of different *T.cruzi* strains (Y and Tulahuen) and were also evaluated on mammalian host cells (L929 cells and cardiac cells) to check their toxicity profile. Among these molecules, five were more active against BT than the reference drug (Bz), being one of them, the DB2267 (a dicationic molecule) the most potent, exhibiting an EC_{50} value of 0.23 µM and a selectivity index (SI) of 417. This diamidine was 28-fold more active and about 3 times more selective than Bz. To ascertain if the combination of two amidines could improve their trypanocidal activity, BT were incubated with DB2267 and DB2236 in fixed-ratio proportions and the data showed only an additive effect with ΣFIC<4. Interestingly, when intracellular forms were exposed to DB2267, its activity was related to the parasite strain, being effective (EC_{50} = 0.87 ± 0.05 µM) against DTU II (Y strain) but not against one representative of DTU VI (Tulahuen) even using different vehicles (β-cyclodextrins and DMSO). This discrepancy might be related to differences concerning the targets and/or metabolic pathways of the parasite that deserve to be more investigated aiming to better understand the relationship between the structural characteristics and universal action of the molecules against DTUs and forms of the *T.cruzi* for the design of more promising compounds. Due to the intrinsic fluorescence presented by the amidines, the uptake of two of them was assessed. The data obtained with the non-active amidine (DB2236) and the other active one (DB2267) against amastigotes of the Y strain showed that both were localized intracellularly, but in distinct compartments: DB2236 in the cytoplasm, while DB2267 in the nucleus. Our results are encouraging regarding the antiparasitic activity of the aromatic amidines, referring to future investigations of new promising agents for the treatment of CD.

ÍNDICE

RESUMO **X**

ABSTRACT **IXI**

1 INTRODUÇÃO **14**

1.1 As doenças negligenciadas **15**

1.2 A doença de Chagas **14**

1.2.1 Transmissão e ciclo 16

1.2.1 Aspectos clínicos 17

1.2.1 Tratamento etiológico 17

1.3 Quimioterapias antiparasitárias experimentais **20**

1.3.1 As amidinas aromáticas 21

1.3.2 Associação de fármacos 24

2 OBJETIVOS **26**

2.1 Objetivo Geral **26**

2.2 Objetivos Específicos **26**

3 ARTIGO SUBMETIDO **27**

4 DISCUSSÃO **51**

5 PERSPECTIVAS **59**

6 CONCLUSÕES **60**

9 REFERÊNCIAS BIBLIOGRÁFICAS **61**

APÊNDICE A - INFORMAÇÕES TÉCNICAS ADICIONAIS **70**

APÊNDICE B - COLABORAÇÕES EM TRABALHOS **71**

ÍNDICE DE FIGURAS

Figura 1 Esquema ilustrativo das etapas do ciclo de vida do *Trypanosoma cruzi* (adaptado de CDC – Center for Disease Control and Prevention, 2015)

Figura 2 Estruturas químicas do benzonidazol (A) e do nifurtimox (B), fármacos utilizados para o tratamento da doença de Chagas

Figura 3 Estrutura química da pentamidina e exemplares de duas classes de derivados: uma diamidina aromática (furamidina - DB75) e uma arilimidamida (DB766)

LISTA DE TABELAS

Tabela 1 Perfil do produto alvo do DNDi (adaptado de DNDi, 2015)

Tabela 2 Exemplos de doenças infecciosas cujos tratamentos recomendados pela OMS são realizados por combinação de fármacos

LISTA DE SIGLAS E ABREVIATURAS

AT	Adenosina e Timina
Bz	Benzonidazol
CC	Células cardíacas
CDC	Center for Disease Control and Prevention
CYP51	C-14α-demetilase
DC	Doença de Chagas
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
DNDi	Drugs for Neglected Diseases Initiative
DTU	Discrete Typing Unit
CE_{50}	Concentração mínima de 50% de eficácia
Ex	Exemplo
h	Hora
IS	Índice de seletividade
kDNA	DNA do cinetoplasto
LAFEPE	Laboratório Farmacêutico do Estado de Pernambuco
LC_{50}	Limiar de toxicidade
Nf	Nifurtimox
OMS	Organização Mundial de Saúde
ONG	Organização Não Governamental
OPAS	Organização Pan-Americana da Saúde
PCR	Reação em cadeia da polimerase
pKa	Constante de ionização de ácidos
Ps	Posaconazol
Pt	Pentamidina
RNA	Ácido ribonucleico
TPP	Target Product Profile

1 INTRODUÇÃO

1.1 As doenças negligenciadas

As doenças negligenciadas acometem mais de um bilhão de pessoas, principalmente populações pobres que vivem em regiões de clima tropical e subtropical. Mais de 70% dos países e territórios que as notificam são subdesenvolvidos, e de um modo geral, as crianças são as maiores vítimas. A prevalência das doenças negligenciadas é favorecida pelas desigualdades sociais, resultando em baixa oferta ou ausência de infraestrutura e educação sanitária adequadas, ocorrendo, em muitos casos, a infecção simultânea por mais de um agente etiológico (WHO, 2015).

Doença de Chagas (DC), tripanossomíase africana, leishmanioses, tuberculose, dengue, tracoma, hanseníase e esquistossomose são algumas das 17 patologias infecciosas denominadas “negligenciadas”. Na visão de saúde pública preventiva, a condução de medidas profiláticas representa uma maneira eficaz de controlá-las, quando associada ao diagnóstico precoce e tratamento adequado (WHO, 2013). Entretanto, devido ao predomínio em regiões de baixo poder aquisitivo, os portadores “negligenciados” não atraem os interesses das grandes indústrias farmacêuticas (Coura & De Castro, 2002; Chatelain, 2015).

Há alguns anos, contudo, iniciativas público-privadas voltaram seu olhar para tais endemias e a corrida por novos fármacos mais potentes e seletivos foi intensificada. Adicionalmente, o avanço das tecnologias possibilitou melhorar as metodologias, permitindo a realização de uma pesquisa racional por novos fármacos (Chatelein, 2015).

1.2 A doença de Chagas

A DC é uma enfermidade, portanto, negligenciada e endêmica em 21 países da América Latina. De acordo com a Organização Mundial de Saúde (OMS), existem cerca de oito milhões de pessoas infectadas no mundo, aproximadamente 100 milhões sob risco de infecção, 55 mil novos casos e 12 mil mortes por ano (WHO, 2015; DNDi, 2015). Esta parasitose foi descoberta pelo pesquisador brasileiro Carlos Chagas (Chagas, 1909) e tem como agente etiológico o protozoário flagelado

Trypanosoma cruzi, que em condições naturais é capaz de infectar mais de 150 espécies de mamíferos, incluindo seres humanos, animais domésticos e silvestres.

1.2.1 Transmissão e ciclo

As vias de transmissão são diversas, incluindo a transmissão vetorial por insetos triatomíneos hematófagos da família Reduviidae (popularmente denominados “barbeiros”), a transfusão de sangue ou transplante de órgãos infectados, a transmissão congênita e a contaminação oral (Coura & Viñas, 2010; Bern, 2011). No ciclo enzoótico da doença (**Figura 1**), o inseto vetor ingere as formas tripomastigotas sanguíneas de *T. cruzi* no momento do repasto sanguíneo em um indivíduo infectado. No intestino do barbeiro, os tripomastigotas sanguíneos se diferenciam nas formas epimastigotas que se multiplicam e então, induzidos por diversos fatores bioquímicos e fisiológicos se transformam nos tripomastigotas metacíclicos. Ao se alimentar novamente, o triatomíneo libera estas formas infectantes nas fezes e urina, que são então capazes de invadir o tecido não integro ou regiões de mucosa, originando os sinais de porta de entrada, o chagoma de inoculação (pele) ou o sinal de Romaña (bipalpebral unilateral). Uma vez no organismo, os tripomastigotas metacíclicos invadem células e se diferenciam nas formas amastigotas. Após a multiplicação, estas formas se diferenciam em tripomastigotas sanguíneos e pela ruptura da membrana plasmática das células hospedeiras, ocorre liberação dos parasitas que podem atingir a circulação sanguínea e linfática do hospedeiro, bem como infectar células vizinhas e/ou distantes, ou ainda serem captadas pelo inseto vetor no ato do repasto sanguíneo, fechando o ciclo (Lima et al., 2010).

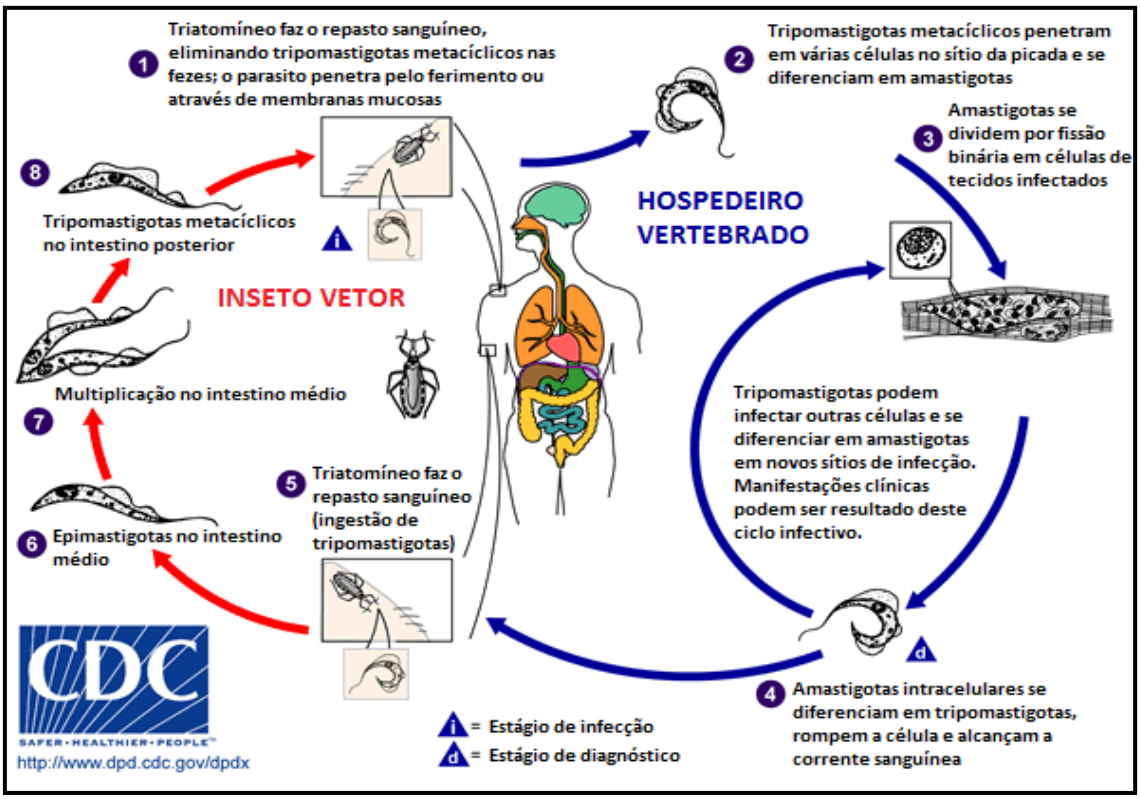

Figura 1: Esquema ilustrativo das etapas do ciclo de vida do *T. cruzi* (adaptado de CDC – Center for Disease Control and Prevention, 2015).

1.2.2 Aspectos clínicos

A DC pode ser caracterizada por duas fases, aguda e crônica. A fase aguda tem início logo após a infecção e os pacientes podem apresentar-se assintomáticos ou oligossintomáticos, dificultando o diagnóstico diferencial precoce (Prata, 2001). A seguir, após cerca de quatro semanas, inicia-se a fase crônica. Nela a sintomatologia em quase todos os pacientes desaparece independentemente da realização do tratamento etiológico. Aproximadamente 60-70% dos indivíduos infectados desenvolvem a forma crônica indeterminada da doença, em que não há sintomas clínicos ou evidências de alterações radiológicas e/ou eletrocardiográficas, mas constata-se a presença de anticorpos anti-*T. cruzi* no soro. Cerca de 30-40% dos pacientes desenvolve, em 10 a 30 anos da doença, a forma determinada cardíaca, digestiva ou mista, caracterizadas principalmente pela síndrome dos megas (coração, cólon e esôfago) (Coura & Dias, 2009).

1.2.3 Tratamento etiológico

A terapia da DC é baseada em dois medicamentos nitroderivados, o Nifurtimox (Nf) e o Benzonidazol (Bz) (**Figura 2**), introduzidos na clínica há mais de 40 anos (Soeiro & De Castro, 2011). Os mecanismos de ação destes compostos estão relacionados à geração de radicais livres e/ou metabólicos eletrofílicos. O grupo nitro de ambos compostos é reduzido a um grupo amino através da ação de nitroredutases do *T. cruzi*, levando à formação de vários radicais e de metabólitos eletrofílicos que se associam a lipídeos, proteínas e DNA (Stoppani, 1999; Maya et al., 2007). Dados *in vitro* sugerem que o principal mecanismo de ação do Nf é via nitroredução, com geração de radical nitroaniônico que em presença de oxigênio resulta na produção de íon superóxido (O_2^-.) e peróxido de hidrogênio (H_2O_2) que são tóxicos para o *T. cruzi* (Maya et al., 2006). O parasita, diferentemente das células de mamíferos, é parcialmente deficiente nos mecanismos de detoxificação, tornando-se mais susceptível que células de mamíferos aos produtos de redução do oxigênio gerados pelo Nf, particularmente o peróxido de hidrogênio e o radical hidroxila. Já o efeito do Bz não parece ter relação com geração de radicais livres, sendo mais provável que os metabólitos eletrofílicos gerados a partir deste composto estejam envolvidos na morte do *T. cruzi* através de sua ligação covalente a macromoléculas dos parasitas e/ou associação ao DNA, lipídeos e proteínas (Stoppani, 1999; Murta et al., 2001; Maya et al., 2003; Croft, Barrett e Urbina, 2005; Maya et al., 2007).

Hipóteses descrevem a associação do estresse oxidativo com a possível deficiência do parasite em metabolizar ROS (Boveris et al., 1980; Turrens, 2004). Subsequentemente, diversas enzimas do parasita contendo FAD incluindo a tripanotiona redutase, lipoamida desidrogenase e citocromo P450 redutase mostraram-se capaz de reduzir Nf (Krauth-Siegel et al., 1987; Blumenstiel et al., 1999). Outra hipótese apresentada por Wilkinson e colaboradores é baseada na redução de dois elétrons do grupo nitro por uma nitrorredutase tipo I insensível a oxigênio, produzindo aminas através de intermediários nitro e hidroxilamina (Hall & Wilkinson, 2012; Wilkinson & Kelly, 2009). Ambos intermediários podem reagir com biomoléculas (DNA, proteínas), resultando no dano celular (Whitmore & Varghese, 1986).

Nf foi largamente utilizado na clínica, porém seu uso foi suspenso em alguns países da América Latina, principalmente no Brasil, na década de 1980. A indústria farmacêutica alemã Bayer descontinuou sua produção em 1997, mas no ano de 2000 a reiniciou em El Salvador com o uso do medicamento combinado a eflornitina para o tratamento da tripanossomíase africana causada por *T. brucei gambiense*

(Coura & De Castro, 2002; Jannin & Villa, 2007; Nussbaum et al., 2010). A distribuição gratuita de Nf tem sido realizada pela OMS/OPAS.

O Bz era produzido pela empresa suíça Roche, que transferiu sua tecnologia de fabricação e doou o princípio ativo do medicamento para o Laboratório Farmacêutico do Estado de Pernambuco (LAFEPE), em 2003 (Jannin & Villa, 2007). Entretanto, com a carência de matéria-prima pela ausência de produção pela LAFEPE, a produção foi interrompida, sendo retomada em 2012, por uma iniciativa público-privada argentina. Até 2011, o produto era preparado com o princípio ativo doado pela Roche, quando ocorreu a interrupção do seu fornecimento (Manne et al., 2012). Recentemente, a Argentina iniciou a produção de Bz comercializado como Abarax® pelo Laboratório Elea, em parceria com o Ministério da Saúde da Argentina e a ONG Fundação Mundo Sano, com produção da formulação para adultos e crianças (DNDi, 2014). Desde 2008, a Drugs for Neglected Diseases Initiative (DNDi) em parceria com o LAFEPE já haviam disponibilizado a formulação pediátrica do Bz, diminuindo os riscos de administrações incorretas do medicamento às crianças acometidas por DC.

Figura 2: Estruturas químicas do benzonidazol (A) e do nifurtimox (B), fármacos utilizados para o tratamento da doença de Chagas.

A administração de Nf e Bz provoca importantes efeitos colaterais nos pacientes, como hipersensibilidade cutânea, polineurites e anorexia. Além disso, ambos apresentam eficácia terapêutica limitada e requerem longos períodos de utilização. Para o Bz, observa-se entre 70 e 100% de cura nas fases aguda e crônica recente; entretanto, durante a fase crônica tardia esse percentual cai para menos de 30% (Clayton, 2010). Existem ainda evidências da ocorrência de cepas naturalmente resistentes aos medicamentos, fora a resistência cruzada, que impede a administração de um fármaco em lugar do outro nos casos de falha terapêutica (Murta et al., 1998).

Estudos acadêmicos são fundamentais para a identificação de novos agentes quimioterápicos mais seletivos e eficazes, e que encorajem a continuidade do tratamento a partir da maior adesão dos pacientes.

1.3 Quimioterapias antiparasitárias experimentais

Com base no que se conhece da patologia e da biologia do parasita, a OMS e o DNDi sugerem parâmetros para pesquisa de novos agentes quimioterápicos que possam ser considerados candidatos ideais a novo fármaco para DC. Tais substâncias devem: i) apresentar cura parasitológica nas fases aguda e crônica; ii) ser eficaz em uma ou poucas doses; iii) ser acessível aos pacientes a baixo custo; iv) não ter efeitos colaterais ou teratogênicos; v) não induzir resistência (Coura & De Castro, 2002). Além disso, é essencial que o novo fármaco seja aplicável às diferentes cepas do parasito (incluindo as DTU I, II, V e VI relevantes para infecção humana), principalmente as resistentes ao tratamento convencional, garantindo sua atividade em portadores da DC nas diversas regiões endêmicas, e que ocorra baixa ou nenhuma interação com as enzimas do complexo do citocromo P450, de modo a evitar interações com outros medicamentos que os pacientes possam eventualmente utilizar, como anticoagulantes ou antiarrítmicos (Buckner & Navabi, 2010).

No site do DNDi encontra-se o perfil ideal e o aceitável para novo medicamento da DC, o chamado TPP ("Target Product Profile") (**Tabela 1**), ou seja, o perfil do produto alvo, que apresenta os elementos que caracterizam o medicamento ideal. Estas informações corroboram com os requisitos da OMS, e permitem direcionar as pesquisas na busca de novos agentes quimioterápicos anti-*T. cruzi*, visando atender de modo mais objetivo as necessidades dos pacientes (Ribeiro et al., 2009).

Tabela 1: Perfil do produto alvo do DNDi (adaptado de DNDi, 2015)

	Aceitável	Ideal
Fase alvo	Crônica recente/ Indeterminada	Aceitável + Reativações
Cepas	TcI, TcII, TcV eTcVI	Todas
Distribuição	Todas as áreas	Todas as áreas
Adultos/crianças	Adultos	Adultos e crianças
Eficácia clínica	>Bz em áreas endêmicas (cura parasitológica)	> 70% em pacientes crônicos > 95% em pacientes reativados (cura parasitológica e sorológica)
Segurança	>Bz / 3 avaliações clínicas e dois testes laboratoriais padrões durante o tratamento	>Bz / Sem necessidade de monitoramento durante o tratamento
Atividade contra cepas resistentes	Sem necessidade	Ativo contra cepas resistentes ao Bz e ao Nf
Contraindicações	Gravidez / lactação	Nenhuma
Precauções	Ausência de genotoxicidade; ausência de potencial pró-arrítmico	Ausência de genotoxicidade; ausência de teratogenicidade; ausência de efeitos inotrópicos negativos; potencial pró-arrítmico significativo
Interações	Ausência de interações clínicas significativas com medicamentos anti-hipertensivos, antiarrítmicos e anticoagulantes	Nenhuma
Apresentação	Oral	Oral
Estabilidade	3 anos em zona climática IV	5 anos em zona climática IV
Regime terapêutico	≈ Antifúngicos	1x dia / 30 dias

1.3.1 As amidinas aromáticas

A Pentamidina (Pt) é uma amidina aromática utilizada para o tratamento da tripanossomíase africana e da leishmaniose cutânea (DNDi, 2015). Na década de 1930, a atividade sobre protozoários despertou o interesse em derivados deste fármaco (King et al., 1937) cuja atividade estaria relacionada à sua associação à fenda menor do DNA do patógeno nas regiões ricas em bases AT, por meio de interações não covalentes e não intercalantes (Wilson et al., 2008). Tais compostos têm sido investigados, portanto, para diversos outros patógenos, principalmente o *T. cruzi* (Soeiro et al., 2013). As amidinas aromáticas e seus análogos (**Figura 3**) apresentam expressiva atividade contra diversos microorganismos incluindo *Leishmania sp*. (Stephens et al., 2003; Wang et al., 2010; Zhu et al., 2012),

Neospora caninum (Debache et al., 2011; Schorer et al., 2012), *Besnoitia besnoiti* (Cortes et al., 2011) e *Echinococcus multilocularis* (Stadelmann et al., 2011) e *T.cruzi* (Soeiro et al., 2013).

Alguns de seus análogos sintetizados pelo grupo do Dr. David Boykin (Georgia State University, Atlanta, GA, EUA), como por exemplo, as arilimidamidas, e estudados pelo nosso grupo têm demonstrado resultados muito promissores sobre infecção por *T.cruzi* em modelos *in vitro* e *in vivo* (Batista et al., 2010; Da Silva et al., 2012; De Araújo et al., 2014).

As arilimidamidas diferem das diamidinas clássicas como a Pt ou furamidina (DB75, derivada da Pt), por apresentarem a amidina ligada ao núcleo aromático da molécula, através de um átomo de nitrogênio, ao invés de estar mediado por um átomo de carbono. Com valores menores de pKa (~7) e maior lipofilicidade em comparação com diamidinas clássicas, as arilimidamidas apresentam atividade superior (>200x) contra formas intracelulares de *Leishmania* (Silva et al., 2007) devido ao aumento da permeabilidade sobre membranas biológicas (Rosypal et al., 2008; Wang et al., 2010) e de *T. cruzi* (Silva et al., 2007).

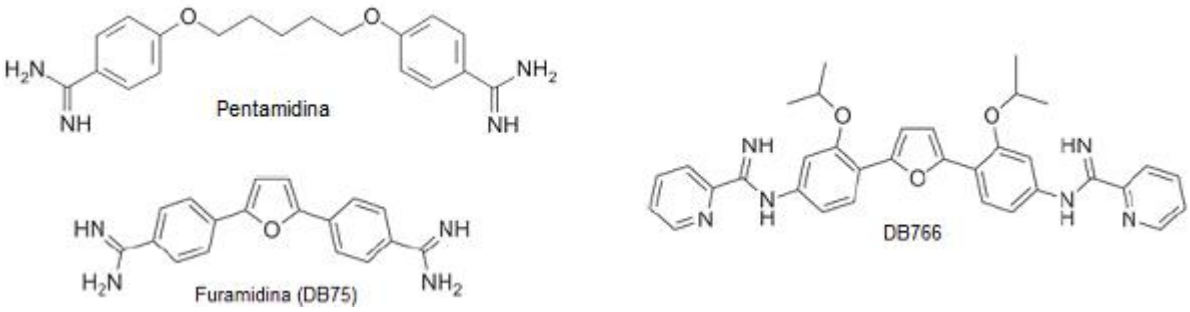

Figura 3: Estrutura química da pentamidina e exemplares de duas classes de derivados: uma diamidina aromática (furamidina - DB75) e uma arilimidamida (DB766).

Dados de correlação entre estrutura e atividade de amidinas sobre *T. cruzi* revelaram que grande número de moléculas que apresentam dois grupos catiônicos terminais (denominadas diamidinas) são mais efetivas que as monoamidinas, como a DB824 (Pacheco et al., 2009). Dados semelhantes foram observados também com bis-arilimidamidas em relação às moléculas que apresentam somente um grupamento terminal (De Araújo et al., 2014).

Outro importante aspecto observado na relação estrutura-atividade das amidinas é que moléculas curvas (de tamanho semelhante ou maiores, ex. DB1645 e DB1651, respectivamente) e/ou de mesmo tamanho (inferior a DB75), exibem atividade e seletividade superiores sobre as formas amastigotas e tripomastigotas

sanguíneas de *T. cruzi*, quando comparadas a moléculas análogas lineares (Batista et al., 2010). Os dados, então, sugerem que a maior eficácia das moléculas dicatiônicas de determinada curvatura estaria relacionada à sua maior capacidade de associação à fenda menor do kDNA do parasito; contudo, Daliry et al. (2011) demonstraram através de ensaios termodinâmicos que algumas das arilimidamidas mais ativas não apresentavam forte ligação ao kDNA, embora sejam capazes de induzir graves alterações na topologia do DNA nuclear e do kDNA de *T. cruzi*.

De fato, vários estudos *in vitro* e *in vivo* têm demonstrado a excelente atividade de amidinas aromáticas e análogos sobre *T. cruzi*. Nosso grupo demonstrou a eficácia tripanocida, dose e tempo-dependente de vários representantes desta classe na faixa de atividade biológica submicromolar e nanomolar, exercendo ainda baixa toxicidade para células de mamífero (Soeiro et al., 2013). Diversas amidinas apresentam (i) excelente atividade a 4ºC na presença de sangue sugerindo potencial uso em bancos de sangue; (ii) algumas foram identificadas em acidocalcissomas, núcleo e cinetoplasto do *T.cruzi*, sendo esta última estrutura um importante alvo (primário/secundário) de dano ultraestrutural (De Souza et al., 2004, 2006a; Silva et al., 2007b; Daliry et al., 2009; Da Silva et al., 2010); (iii) são ativas sobre diferentes cepas do parasito incluindo aquelas naturalmente resistentes ao Bz (Batista et al., 2010), e (iv) são capazes de reduzir a carga parasitária cardíaca *in vivo* (em níveis semelhantes ao tratamento com Bz), resultando em menor dano tissular e aumento de sobrevida dos animais tratados em relação ao grupo não tratado (Batista et al., 2010). Estes compostos conferem também proteção contra lesões cardíacas e alterações eletrocardiográficas evidenciadas na fase aguda final e crônica da infecção murina experimental pelo *T.cruzi* (De Souza et al., 2006b, 2007; Batista et al., 2010; Da Silva et al., 2008).

Ademais ao padrão de baixa toxicidade que as amidinas aromáticas apresentam, Timm et al. (2014a) e De Araújo et al. (2014) demonstram também seu rápido perfil de ação sobre formas tripomastigotas sanguíneas, característica desejável em novos compostos para que possam ser utilizados em situações agudas, que ocorrem, por exemplo, quando da reativação da infecção em condições de imunossupressão.

Entretanto, apesar dos excelentes atributos dessa classe de moléculas, principalmente considerando as arilimidamidas, observa-se que algumas diamidinas não possuem atividade sobre formas intracelulares do *T. cruzi* superior ao Bz, o que pode estar relacionado à ocorrência de um diferente mecanismo de ação, distintos

alvos celulares de acordo com a forma evolutiva do parasito, ou ainda variações no mecanismo de captação/extrusão das moléculas pelas células mamíferas hospedeiras ou parasitas (Timm et al., 2014a). Essas divergências no comportamento dos compostos podem ser compreendidas pela existência de potenciais eletrostáticos particulares, que influenciam diretamente na afinidade de ligação das amidinas em relação à fenda menor do DNA e as sequências ricas em AT, descritas como seus alvos primários para a indução de dano celular (Chai et al., 2014).

Essas informações direcionam os estudos de novas moléculas para a associação de compostos, em que a finalidade é abordar diferentes alvos celulares, com entidades químicas de uma mesma ou distintas classes (Soeiro et al., 2011; Bustamante & Tarleton, 2014).

1.3.2 Associação de fármacos

A resistência a diferentes fármacos tem sido relatada no curso do tratamento de diversas doenças como leishmaniose e malária. A estratégia atual de combinar medicamentos existentes e/ou a novas entidades farmacológicas representa uma importante e atual abordagem (Soeiro et al., 2011), tendo em vista que o sinergismo entre compostos pode ser determinante para a cura ou melhoria absoluta dos pacientes. Adicionalmente, o reposicionamento e associação de fármacos já utilizados na clínica médica para outras patologias representam uma promissora estratégia na descoberta de novas terapias para doenças negligenciadas tendo em vista a redução de custos e tempo no fluxograma relativo à translação de estudos pré-clínicos em ensaios clínicos.

Maldonado et al. (1993) e Araújo et al. (2000) demonstraram no modelo murino que a associação de Bz a outros antifúngicos, como o Itraconazol e o Cetoconazol, leva melhores resultados terapêuticos para a DC. Mais recentemente, demonstrou-se que a associação de arilimidamidas como a DB766, com azóis como o Posaconazol (Ps), resultou no efeito sinérgico importante sobre a infecção *in vitro* de *L. donovani* (Pandharkar et al., 2014). Nossos dados de associação de DB766 com Bz também mostraram aumento dos índices de cura parasitológica em modelos de infecção experimental pelo *T.cruzi* (Batista et al., 2011). Neste estudo, o uso de Bz associado a amidina DB289, um pró-fármaco da DB75, também resultou em redução de parasitemia e aumento de sobrevida dos animais infectados (Batista et al., 2011).

A administração de fármacos já patenteados em combinação e por curtos períodos de tempo pode ser capaz de induzir efeitos curativos em camundongos, como demonstrado por diversos autores (Cencig et al.,2012; Moreira da Silva et al., 2012; Bustamante et al.,2014). A associação de Bz com Ps ou Nf leva a cura parasitológica camundongos com infecção aguda ou crônica por cepas sensíveis ao medicamento referência (Cencig et al., 2012). Outros autores descreveram ainda a ocorrência de sinergismo entre Bz e Ps, e um tratamento reduzido de apenas dez dias sobre a cepa Brasil (sensível ao Bz) permitiu que após os ciclos de imunossupressão não fossem observados parasitos circulantes (Bustamante et al., 2014), apesar de este esquema terapêutico ter sido completamente ineficaz sobre cepas resistentes de *T. cruzi*, como a Colombiana.

A OMS preconiza o tratamento com associação de fármacos para diversas doenças infecciosas, incluindo algumas parasitárias (**Tabela 2**), com o objetivo de evitar ou transpor os casos de microorganismos resistentes, combater formas latentes, e minimizar a severidade de uma morbidade nos casos de coinfecções.

Tabela 2: Exemplos de doenças infecciosas cujos tratamentos recomendados pela OMS são realizados por combinação de fármacos

Doença infecciosa	Tratamento por combinação de fármacos
Tripanossomíase africana (Doença do sono)	Nifurtimox-eflornitina (administração sequencial)
HIV pediátrico	- Primeira escolha: Zidovudina-lamivudina ou lopinavir-ritonavir - Localidades com limitações de recursos: Combinação de doses fixas de estavudina-lamivudina-nevirapina
Tuberculose	- Isoniazida-rifampicina-etambutol-pirazinamida - Isoniazida-rifampicina
Malária	Combinações com artemisina (ACT) Incluem: lumefantrino, mefloquina, amodiaquina, sulfadoxina/pirimetamina, piperaquina ou clorproguanil/dapsona, além de derivados da própria artemisina, como diidroartemisina, artesunato ou arteméter

2 OBJETIVOS

2.1 Objetivo Geral

Investigar a atividade tripanocida (monoterapia e terapia combinada) de novos compostos sintéticos da classe das amidinas aromáticas *in vitro* e que apresentem eficácia e seletividade igual ou superior ao benzonidazol, de modo a identificar possíveis futuras alternativas terapêuticas ao tratamento da doença de Chagas.

2.2 Objetivos Específicos

Para determinar a atividade biológica *in vitro*, dez amidinas aromáticas foram selecionadas e os seguintes objetivos específicos foram projetados:

a) Avaliar a ação dos compostos sobre as formas relevantes para infecção humana: formas tripomastigotas sanguíneas e amastigotas, avaliando diferentes cepas do *Trypanosoma cruzi* representantes dos DTUs II e VI;
b) Buscar o perfil de toxicidade dos compostos (cinética de exposição) sobre culturas de células de mamíferos: cultivo primário (células cardíacas) e de linhagem celular (L929);
c) Correlacionar a estrutura química e a atividade biológica das moléculas testadas que apresentam dois ou somente um grupamento catiônico terminal;
d) Determinar a janela terapêutica dos compostos testados a partir do cálculo do índice de seletividade (IS) visando definir os mais promissores para futuros estudos *in vivo*;
e) Explorar o perfil de captação e localização intracelular dos compostos estudados em parasitos e células hospedeiras;
f) Investigar o potencial perfil sinérgico *in vitro* de associação de compostos amidínicos sobre a infecção pelo *T. cruzi*

3 ARTIGO SUBMETIDO

O trabalho a seguir intitulado “Phenotypic screening *in vitro* of novel aromaticamidines on *Trypanosoma cruzil*” foi desenvolvido durante o mestrado em concordância com os objetivos da presente dissertação. Nele são investigadas a ação tripanocida de dez amidinas aromáticas selecionadas (DB226, DB2259, DB2260, DB2261, DB2262, DB2263, DB2266, DB2267, DB2268 e DB2269), dentre elas compostos mono ou dicatiônicos, sobre as formas evolutivas do parasito mais relevantes para a infecção humana, a amastigota intracelular e a tripomastigota sanguínea, utilizando cepas representantes de duas diferentes DTUs (II e VI). As dez diamidinas foram também avaliadas quanto ao seu perfil de toxicidade celular sobre células de mamífero hospedeiras, além de serem analisadas correlacionando sua estrutura química com a atividade biológica. No artigo também foram determinados a janela terapêutica dos compostos, a partir do cálculo do índice de seletividade e o perfil de captação e localização intracelular dos compostos, tanto para células do *T. cruzi*, quanto para as células hospedeiras. Finalmente foi realizado estudo *in vitro* sobre o potencial sinergismo entre duas das amidinas testadas sobre o parasito.

O artigo foi recentemente submetido na revista Antimicrobial Agents and Chemotherapy para publicação.

3 ARTIGO SUBMETIDO

Phenotypic Screening *In Vitro* of Novel Aromatic Amidines Against *Trypanosoma cruzi*

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Running Title: *In vitro* trypanocidal effect of novel amidines

Key Words: Chagas disease, experimental chemotherapy, *Trypanosoma cruzi*, amidines

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Abstract

Chagas disease (CD), caused by the protozoan *Trypanosoma cruzi,* is a neglected disease endemic in different poor areas of Latin America. The current treatment, based on two nitroderivatives, Nifurtimox and Benznidazole (Bz), is unsatisfactory, demonstrating the urgent need for screening of new potential trypanocidal drugs. The present work addresses this need by the phenotypic screening of novel mono- and di-amidines *in vitro* for effectiveness against CD and by performing drug interaction assays among selected test compounds. Ten novel amidines were tested against bloodstream trypomastigotes (BT) andamastigote forms of different *T.cruzi* strains (Y and Tulahuen) and additionally their toxicity profiles were evaluated on mammalian host cells (L929 cells and cardiac cells).Among these molecules, seven of ten were more active against BT than the reference drug (Bz). Five of the six monoamidines were more active than Bz, however the most active compound was the diamidine DB2267, which exhibited an EC_{50} value of 0.23 µM and a selectivity index (SI) of 417. The diamidine was 28-fold more active and about 3 times more selective than Bz. To ascertain if the combination of two amidines could improve their trypanocidal activity, BT were incubated with DB2267 and DB2236 in fixed-ratio proportions, however the data showed only an additive effect with ΣFIC<4. Interestingly, when intracellular forms were exposed to DB2267, its activity was dependent on the parasite strain, being effective (EC_{50} = 0.87 ± 0.05 µM) against DTU II (Y strain) but not against one representative of DTU VI (Tulahuen) even using different vehicles (β-cyclodextrins and DMSO).The intrinsic fluorescent properties of several diamidines, allowed their uptake to be studied using one inactive diamidine (DB2236) and the most effective one (DB2267), against Y strain amastigotes. Both compounds were localized intracellularly but in different compartments: DB2236 in the cytoplasm and DB2267 in the nuclei. Our data present encouraging results regarding the anti-parasitic activity of amidines and provides information which will be helpful for the future identification of novel promising agents for the therapy of Chagas disease.

Introduction

Chagas disease (CD) is a neglected disorder endemic in 21 countries of Latin America. According to the World Health Organization, there are 8 million people infected worldwide, approximately 10 million others are at risk of infection, 14,000 new cases and 12,000 deaths a year occur (WHO, 2013). This pathology was discovered by the Brazilian researcher Carlos Chagas (Chagas, 1909) and has as the etiological agent, the flagellate protozoan *Trypanosoma cruzi*. There are several transmission routes, including vector transmission by the triatomine bugs from the Reduviidae family, blood transfusion or organ transplantation, congenital transmission and oral contamination (Coura & Albajar-Viñas, 2010; Bern, 2011). The disease is characterized by two distinct phases, acute and chronic. During the acute phase, patients can be asymptomatic or mildly symptomatic, which likely impairs an early and differential diagnosis. After parasitemia is controlled, a chronic phase is established, which is represented by subpatent parasitism and positive serology. Approximately 60-70% of the patients in this phase present an indeterminate form, while 30-40% develop a cardiac and/or digestive form of the disease, years or even decades after the infection (Coura & Dias, 2009).

CD therapy is based on two medicines: Nifurtimox and Benznidazole (Bz). Both require long term treatment and produce severe adverse effects, while yielding only low cure rates for the late chronic phase (Coura & De Castro, 2002; Soeiro et al., 2010; Soeiro & De Castro, 2011). New studies are essential for the identification of more selective and efficient chemotherapeutic agents, with reduced toxic effects, to avoid the therapy which is often abandoned by patients. In this context, the potency and efficacy of hit compounds on strains representative of the different *T.cruzi* DTUs relevant for the human infection are essential to provide a new drug usable in the different endemic areas for CD (Zingales et al., 2014).

Drug discovery programs are increasingly employing combination strategies in large part due to the seemingly constant problem of drug resistance but also due to the potential of discovery of synergism. Recent successes with this approach have been achieved with several parasitic diseases, including leishmaniasis, malaria and CD (Coura, 2010; Diniz et al., 2014; Chatelein, 2015). It seems important to routinely include this strategy in drug discovery efforts for treatment of CD.

Aromatic diamidines such as pentamidine are in clinical use for different pathologies such as leishmaniasis, systemic fungi infections, stage one HAT as well as for several veterinary purposes (Soeiro *et al*., 2013). Recently, a new class of diamidines, arylimidamides has been found effective against several pathogens including *T.cruzi*. The excellent trypanocidal activity, *in vitro* and *in vivo,* of this new class of amidines encourages further exploration of the amidine family of compounds (Batista et al., 2010; Da Silva et al., 2012; De Araújo et al., 2014).

Thus, the goal of this work is the screening of new heterocyclic amidines, aiming to identify new compounds with efficacy and selectivity equal or superior to Bz that could be considered promising candidates for treatment of CD.

Materials and Methods

Compounds: The diamidines (DB2236, DB2259, DB2267, DB2268, DB2269) were synthesized using methodology previously reported (Ismail et al., 2004; Farahat et al., 2011; Hu et al., 2013) and synthesis of the monoamidines (DB2261, DB2262, DB2263, DB2266, DB2268, DB2269) has been described (Green, 2014). All compounds have been fully characterized by spectral methods (NMR, MS) and by satisfactory C,H,N analysis. Stock solutions were prepared in dimethyl sulfoxide (DMSO) with the final concentration of the solvent never exceeding 0.6%, which did not exert any toxicity towards the parasite or mammalian host cells (data not shown) (Timm et al., 2014a), or β-cyclodextrin prepared in a 1:1 proportion with the tested compound and vortexed in dry ice, for 10 min. Benznidazole (Bz) (N-benzyl-2-nitroimidazole-1-acetamide, Laboratório Farmacêutico do Estado de Pernambuco [LAFEPE], Brazil) was used as reference drug.

Parasites: Bloodstream trypomastigotes (BT) of Y strain were obtained from Swiss Webster mice, at the parasitemia peak of infection (Meirelles et al., 1986; Batista et al., 2010). Trypomastigotes of Tulahuen strain expressing the β-galactosidase gene from *Escherichia coli* (Buckner et al., 1996) were collected from the supernatant of infected cell cultures (L929 culture) as reported (Romanha et al., 2010).

Mammalian cells: For the toxicity assays, primary cultures of mammalian cardiac cells (CC) obtained from mice embryos were plated onto coverslips in 96 or 24-well plates previously coated with 0.01% gelatin (Meirelles et al., 1986). For the analysis of the effect on intracellular parasites, monolayers of mouse L929 fibroblasts were cultivated (4 X 10^3 cell/well into 96-well microplates) at 37° C in RPMI-1640 medium (pH 7.2-7.4) without

phenol red (Gibco BRL) supplemented with 10% foetal bovine serum and 2 mM glutamine (RPMIS), as reported (Romanha et al., 2010).

Trypanocidal activity: a)Bloodstream trypomastigotes (BT) of the Y strain (5 X 10^6 per mL) were incubated for 2 and 24 h at 37° C in RPMI in the presence or absence of serial dilutions of the compounds (0 to 32 µM). After compound incubation, the parasite death rates were determined by light microscopy through the direct quantification of the number of live parasites using a Neubauer chamber, and the EC_{50} (compound concentration that reduces 50% of the number of parasites) was then calculated (Timm et al., 2014a). b) For the assay on intracellular forms, *T.cruzi* infected-cell cultures (Tulahuen strain expressing β-galactosidase) were incubated for 96 h at 37°C with each compound at 10 µM diluted in RPMIS. After this period, 500 µM chlorophenol red glycoside in 0.5% Nonidet P40 was added to each well; the plate was incubated for 18 h at 37°C, and then, the absorbance was measured at 570 nm. Controls with uninfected cells and infected cells both treated only with vehicle and/or with the reference drug (Bz) were run in parallel. Those compounds with activity similar to Bz were further assayed using decreasing concentrations to determine the EC_{50} values as above described. The results were then expressed as percentage of *T. cruzi* growth inhibition by comparing the optical density data of the tested compound with those obtained from those infected cell cultures exposed only to vehicle cell cultures (Romanha et al., 2010). Next, the most promising compounds (activity ≥ Bz) were then evaluated for 48 h with the infection of CC using Y strain, as standardized (Batista et al., 2010). After 24 h of infection, the cell cultures were treated with non-toxic concentrations of the compounds and after 48 h of treatment, the CC were rinsed with saline, fixed with Bouin (5 min), stained with Giemsa and examined by light microscopy. The percentage of infected host cells was determined, as well as the number of parasites per cell and the infection index (II) calculated, which represents the product of the multiplication between the percentage of infection and the number of parasites per cell. Then, the EC_{50} values were determined as reported (Batista et al., 2010).

Cytotoxicity assays: CC were incubated for 24 and 48 h at 37° C with different concentrations of each compound (up to 96 µM) diluted in RPMIS and then their morphology and spontaneous contractibility evaluated by light microscopy and their cellular viability determined by the PrestoBlue test as reported (Timm et al., 2014a). The results were expressed as percent difference in reduction between compound treated and vehicle treated cells by following the manufacturer instructions and the value of LC_{50}, which

corresponds to the concentration that reduces in 50 % the cellular viability. Selectivity index (SI) is expressed by the ratio between the values obtained for LC_{50} over the host cells and the EC_{50} obtained over the parasites or infected cell cultures.

Cell internalization of diamidines: CC were infected with Y strain BT and incubated for 48 h at 37°C. Then, the infected cultures were incubated for 2 h with each compound (10 µg/mL) at 37°C, fixed with paraformaldehyde at 4% and analyzed through fluorescence microscopy (Batista et al., 2010).

Determination of drug interactions against BT of *T. cruzi*: *In vitro* drug interactions were assessed using a fixed-ratio method (Fivelman *et al.*, 2004) combining two previously screened diamidines, DB2267 and DB2236, which exhibited the best activity against two different *T.cruzi* DTUs: BT (Y strain) and intracellular forms (Tulahuen strain), respectively. In these assays, pre-determined EC_{50} values were used to determine the top concentrations of the individual drugs to ensure that EC_{50} fell near the midpoint of a six-point two fold dilutions series. Top concentrations used were 0.96 µM for DB2267 and 16 µM for DB2236 in a 24 h assay. The top concentrations were used to prepare fixed-ratio solutions at ratio of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5 of DB2267 and DB2236 as reported (Diniz et al., 2013).

Determination of fractional inhibitory concentrations (FICs) index, isobologram construction and classification of the nature of interaction: FICs and the sum of FICs (ΣFICs) were calculated as follows: FIC of DB2267 = EC_{50} of DB2267 in combination/ EC_{50} of DB2267 alone. The same equation was applied to the partner drug (DB2236). ΣFICs = FIC DB2267 + FIC DB2236. An overall mean ΣFIC was calculated for each combination and used to classify the nature of interaction. Isobolograms were constructed plotting the EC_{50} of 2267 against the 2236.

All the assays were conducted at least in duplicate and result on the average of at least three independent experiments and the statistics were done using the ANOVA test.

Results

The compounds DB2236, DB2259, DB2260 and DB2267 were selected to explore the effect of diamidine dimensions on *T. cruzi* activity. A small set (DB2261, DB2262, DB2266, DB2268 and DB2269) of monoamidines with tethered potential intercalation units were studied totest such hybrid molecules for activity against *T.cruzi*. DB2263 represents a monoamidine control compound without a tethered unit. The biological analysis was started with a time to kill assay against Y strain BT. After 2 and 24 h of incubation, most of the tested

compounds (7 of 10) showed higher activity than Bz (Table 2). After the shorter period of incubation, DB2266 and DB2269 displayed EC_{50} values of ≤ 4.2 μM, being at least 24-fold more active than the reference drug. After 24h, three of the monoamidines (DB2262, DB2266 and DB2269) showed EC_{50} values below 3.0μM; however the most effective compound was the diamidine DB2267 which gave an EC_{50} value of 0.23 μM which is 28-fold more efficient than Bz.

Next, we evaluated the *in vitro* toxicity of these compounds to mammalian host cells (non-infected primary cultures of cardiac cells) incubated for 24 and 48 h with the different molecules using light microscopy and through a colorimetric assay with PrestoBlue. After 24h of incubation, all amidines, except for DB2236, did not reach LC_{50} values up to 96 μM, demonstrating their low toxicity profile. DB2236, DB2260 and DB2263 only caused mild toxicity when used in the higher concentration after 48 h of compound exposure. The selectivity determination demonstrated once again that DB2267 was the most promising compound with a SI value of approximately 417, hence almost 2.8-fold more selective than Bz (Table 2).

The third part of the *in vitro* assays involved evaluation of these compounds using intracellular forms of the parasite with the treatment of L929 cell cultures infected with Tulahuen strain of *T. cruzi,* using in a first step, a fixed concentration of 10 μM, which is equivalent to EC_{90} of Bz (Timm et al., 2014a). In this assay, only DB2236 consistently showed comparable activity to the reference drug and was then selected to determine its EC_{50} value against these intracellular parasites. Also, as DB2267 was the most active against BT forms, it was subjected to this assay (Table 3). The results demonstrated that DB2236 presented a trypanocidal activity in the same range as Bz, with an EC_{50} value = 5.4± 1.7 μM after 96 h of incubation (Table 3). On the other hand, despite being the most active compound against Y strain BT, DB2267 was not effective against the intracellular forms of Tulahuen strain (Table 3). The lack of trypanocidal activity of DB2267 on intracellular forms of this parasite strain persisted even when another vehicle (β-cyclodextrin) was employed (data not shown).

It is unclear if the lack of activity of DB2267 against intracellular forms of Tulahuen strain is due to strain (Y vs Tulahuen) or form (BT vs intracellular) differences. To clarify this matter we evaluated DB2267 and Bz concurrently against Y strain amastigotes (Table 4). The EC_{50} value found for DB2267 against the intracellular forms of Y strain was 0.87 μM; which suggests that the differences in activity noted are due to strain not form differences.

The intrinsic fluorescent properties of the diamidines allowed cell uptake studies to be undertaken and we selected two representative diamidines with different molecule sizes, a small (DB2236) and a large (DB2267) one, which were investigated using Y strain amastigotes. Both compounds were localized intracellularly but in different compartments: DB2267 in the DNA rich nuclei sites (Figure 2 A-B) and DB2236 (Figure 2 C-D) was dispersed in the cytoplasm.

Finally, since combination therapeutic strategies are often used in attempt to reduce toxicity and improve activity, *in vitro* interactions of DB2267 and DB2236 were evaluated at their respective EC_{50} levels. Mean ∑FICs are presented in Table 5 and representative isobolograms are in Figure 3. Interactions were categorized according to Seifert et al (2006); being classified as synergistic with mean ∑FICs of ≤0.5, as antagonistic with mean >4.0, and as indifferent (or additive) with mean ∑FICs between >0.5 and ≤4.0. The interaction of DB2267 and DB2236 was classified as indifferent with mean of ∑FICs 1.13 to 1.66 at the EC_{50} level (Table 5 and Figure 3).

Discussion

Previous *in vitro* and *in vivo* studies demonstrated the excellent activity of many diamidines against *T. cruzi:* these molecules exhibited dose and time dependent trypanocidal effect in a submicromolar and nanomolar ranges, presented low toxicity for mammalian cells, were able to reduce cardiac parasitic load *in vivo*, provided lower tissue damage and higher survival rates of the treated animals, in comparison to those untreated (Batista et al., 2010; Soeiro et al., 2013).Among the diamidines studied, arylimidamides have been shown to be highly effective against *T.cruzi* and several different species of *Leishmania* as well as other intracellular pathogens (Soeiro et al., 2013; De Araújo et al., 2014). In this study, ten amidino molecules were tested *in vitro* following a well-established workflow and methodology, resulting in the determination of the EC_{50}, LC_{50} and selectivity index (SI) (Romanha et al., 2010). Among the ten, seven molecules presented an EC_{50} lower than 4 µM after 24 h of incubation. In fact, six out of ten amidines presented an EC_{50} value < 6 µM after only two hours of incubation with BT, being much more effective than the reference drug and corroborating previous data showing the fast action of diamidines, which is a very important characteristic, considering acute situations in cases of reactivation in immunossupressed conditions (Timm et al., 2014a).

The monoamidines (DB2261, DB2262, DB2266, DB2268 and DB2269) with tethered potential intercalation units gave EC_{50} values between 2.14 and 3.37 μM against Y strain BT. As this is a new class of potential anti-*T.cruzi* agents this constitutes an important finding. The set is too limited to merits SAR discussion nor does the moderate *in vitro* activity merit *in vivo* studies, nevertheless this new class of compounds does merit hit-to-lead investigations. DB2263, a control monoamidine of this class minus the tethered unit, exhibits an EC_{50} value of only 12.34 μM which strongly suggests an important role for the tethered unit in the anti-*T. cruzi* activity of this series.

Recent findings for aromatic diamidines, especially for the arylimidamide class, showed that many of them were highly active against different strains of the parasite, including those naturally resistant to Bz, and also against both parasite forms relevant to human infection: BT and amastigotes (Batista et al., 2010). DB2236 was moderately active against both Y strain (BT) and Tulahuen (intracellular), showing EC_{50} values of 3.97 and 5.4 μM, respectively. However, the most active compound against Y strain BT, DB2267, was inactive against intracellular forms of Tulahuen strain but retained efficacy against amastigotes of the Y strain. Hence, our data shows that the activity of DB2267 is strain-dependent, which is not a desirable feature for novel CD drug candidates (Chatelein, 2015). It can be concluded that structural modifications of the diamidines may impact their mechanism of action and/or cellular targets and compound metabolization and/or uptake/extrusion with different parasite strains deserves to be further investigated.

Fluorescence microscopy studies conducted with molecules such as furamidine took advantage of the intrinsic fluorescent properties of diamidines to identify intracellular targets (as the kDNA) of trypanosomatids. For *T.cruzi*, previous studies identified diamidines located in different intracellular compartments including the nucleus, kinetoplast and acidocalcisomes suggesting site(s) of cellular insult (primarily/secondarily) and/or site of compound storage (De Souza et al., 2004, 2006a; Silva et al., 2007b; Daliry et al., 2009; Da Silva et al., 2010). Therefore, we performed a fluorescence analysis to ascertain the uptake of two representative molecules with different sizes: a small diamidine (DB2236) and a larger one (DB2267). Both were internalized by the parasite; DB2236 was diffusely found in the host cell and parasite cytoplasm, whereas the larger diamidine was localized in DNA-rich compartments such as the nuclei and kDNA as previously reported for other diamidines (Soeiro et al., 2013). The different localization may reflect distinct cellular targets that merit further studies.

The strategy of targeting different molecular sites has been recently evaluated by several groups (Cencig et al., 2012; Moreira da Silva et al., 2012; Bustamante et al., 2014) in order to try to avoid strain resistance and enhance biological activity during drug therapy for several neglected diseases including CD. In this work when the two diamindines DB2236 and DB2267 were combined in a serially diluted fashion (Fivelman et al., 2004) and evaluated against Y strain BT forms, the combination showed no difference in activity compared to monotherapy.

These results corroborate the general anti-*T.cruzi* activity of aromatic diamidines, however the discovery of strain dependency for DB2267 underscores the importance of complete screening of strains and forms. The discovery of a new class of amidines (monoamidines with tethered units) with *in vitro* activity worthy of future hit-to-lead studies is a significant finding. Despite the lack of improvement of activity by the combination pair studied here it remains prudent to include such studies with future analogues. Thus, taken as a whole, our data encourages further analysis of novel amidino analogues, seeking ones which display safer and more potent anti-parasitic phenotypic effects that may be helpful for the future identification of novel promising agents for the therapy of Chagas disease.

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Legend of Figures

Figure 1: Fluorescence microscopy (A and C) and their corresponding DIC (B and D) images of cardiac cells infected with *T. cruzi* (Y strain) after 2 h exposure to 10 µg/mL of DB2267 (A-B) and DB2236 (C-D), showing the internalization of both compounds with their intracellular localization in the nuclei (A) and cytoplasm (C) of the mammalian host cells and the parasites (arrows).

Figure 2: Light microscopy images of untreated (A) and DB2267-treated cardiac cells (1.18 µM for 48 h/37°C) infected with *T. cruzi* (Y strain) showing a strong decline (60%) in the number of parasites (arrows) present in the treated cell cultures when compared to untreated samples.

Figure 3: Representative isobologram. *In vitro* interaction using DB2267 and DB2236 combined against bloodstream trypomastigotes of *T. cruzi* (Y strain). The EC_{50} of DB2236 was plotted in the abscissa and the DB2267 was plotted in the ordinate. The plotted points are EC_{50} of each fixed ratio correspond to the proportion of 5:0. 4:1. 3:2. 1:4 and 0:5 of DB2267 and DB2236. ∑FICs<0.5 = synergism. ∑FICs>0.5 and <4.0 = additivity. ∑FICs>4.0=antagonism.

Table 1: Structures of the diamidines and monoamidines assayed in this work.

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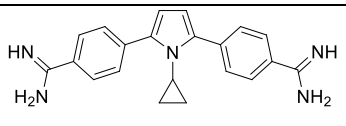
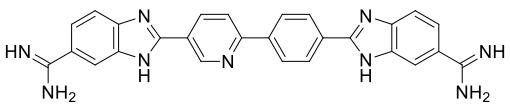
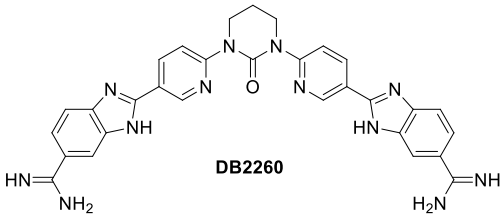
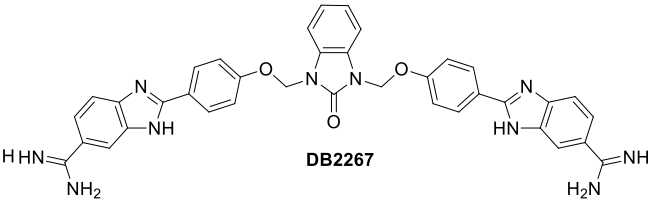
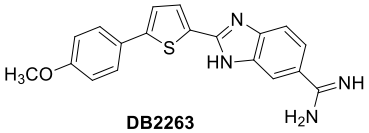
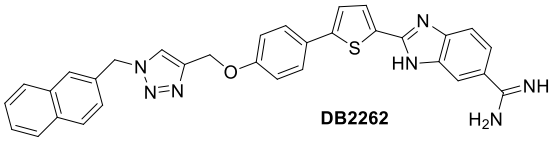
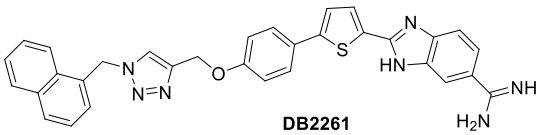
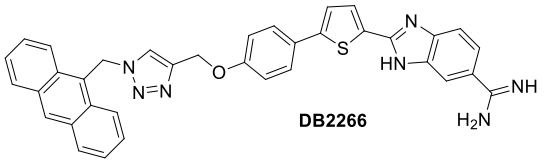
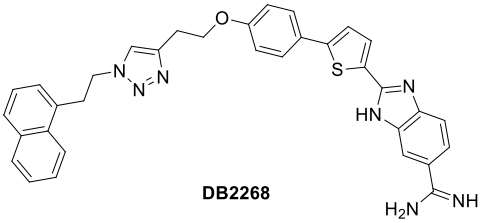
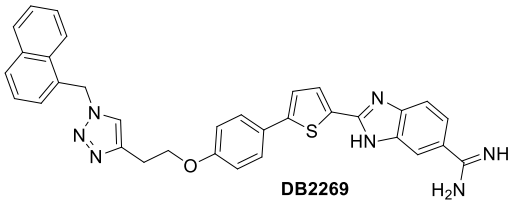
 DB2236
 DB2259
 DB2260
 DB2267
 DB2263
 DB2262
 DB2261
 DB2266
 DB2268
 DB2269

Table 2: *In vitro* activity of the amidines and benznidazole on bloodstream trypomastigotes of the Y strain: EC_{50} and EC_{90} values after two and 24 hours of incubation at 37°C and the corresponding selectivity index

Compound	EC_{50} (mean ± SD) µM		EC_{90} (mean ± SD) µM at 37°C		
	2 h	24 h	2 h	24 h	SI* 24 h
Bz	>100	6.65 ± 1.8	>100	18.04 ± 7.6	>149
DB2236	>32	3.97 ± 1.8	>32	10.64 ± 2.6	>24
DB2259	>32	20.00 ± 1.0	>32	32 ± 0	>4.8
DB2260	24.35 ± 6.8	7.26 ± 1.2	>32	10.4 ± 0.7	>13
DB2261	11.75 ± 4.4	3.57 ± 1.2	>32	7.37± 7.7	>27
DB2262	5.57 ± 2.1	2.76 ± 0.8	14.93 ± 9.5	6.80 ± 2.5	>34
DB2263	>32	12.34 ± 3.6	>32	28.75 ± 0.2	>5
DB2266	3.88 ± 1.1	2.46 ± 0.9	9.42 ± 0.5	3.33 ± 0.8	>38
DB2267	5.37 ± 1.6	0.23 ± 0.02	>32	1.00 ± 0.06	>417
DB2268	5.96 ± 1.9	3.07 ± 1.0	16.72 ± 5.8	7.78 ± 1.9	>31
DB2269	4.22 ± 1.9	2.14 ± 0.1	8.62 ± 1.8	5.78 ± 2.4	>46

*based on the EC_{50}

Table 3: Activity of DB2236 and DB2267 over L929 cell cultures infected with Tulahuen strain of *Trypanosoma cruzi* after 96 hours of interaction

Compound	EC_{50} (mean ± SD) µM 96h: Tulahuen amastigotes/L929
Bz	2.8 ± 1.3
DB2236	5.4 ± 1.7
DB2267	>32

Table 4: Activity of DB2267 and benznidazole on cardiac cell cultures infected with Y strain of *Trypanosoma cruzi* after 24 hours of interaction

Compound	EC_{50} (mean ± SD) µM	EC_{90} (mean ± SD) µM
Bz	3.6	11.0
DB2267	0.87 ± 0.05	>1.18

Table 5: Mean of ∑FICs of interaction between **DB2267 and DB2236** towards bloodstream trypomastigotes of *Trypanosoma cruzi* of Y strain

Compounds in combination	Bloodstream trypomastigotes		
	FICs 2267	FICs 2236	∑FIC
4+1	1.05	0.08	1.13
3+2	1.05	0.22	1.27
2+3	1.13	0.53	1.66
1+4	0.64	0.80	1.44
Mean FICs in combination	0.97	0.41	1.37

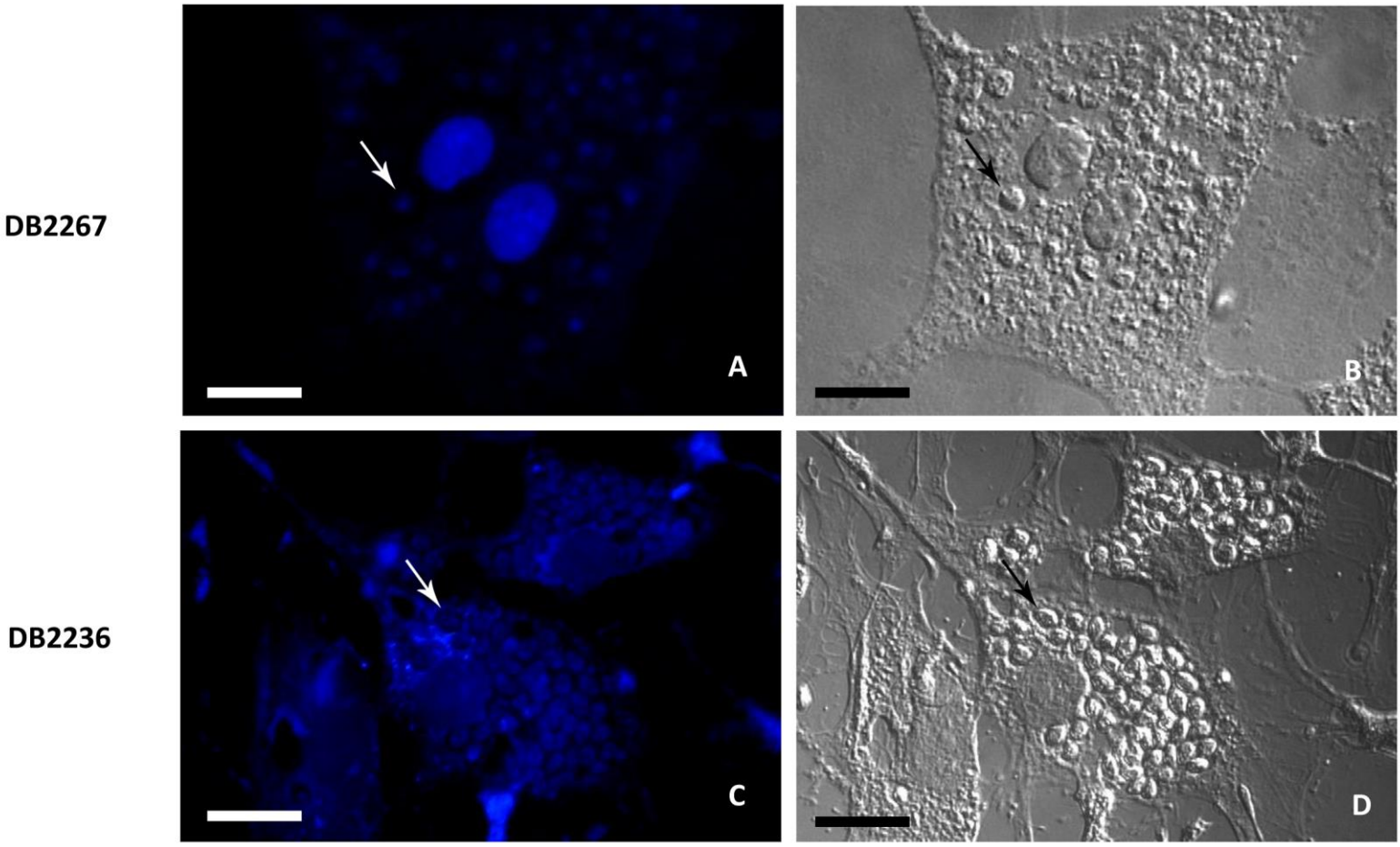


Figure 1

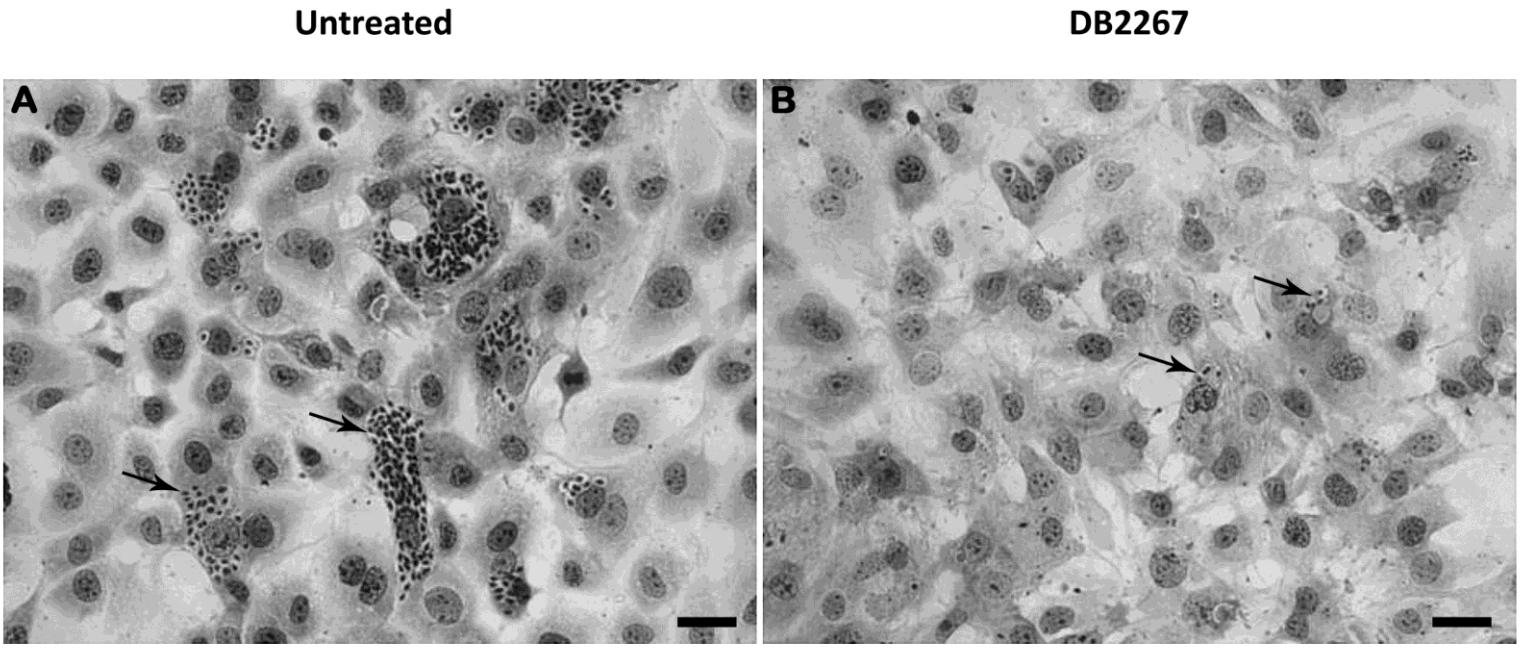


Figure 2

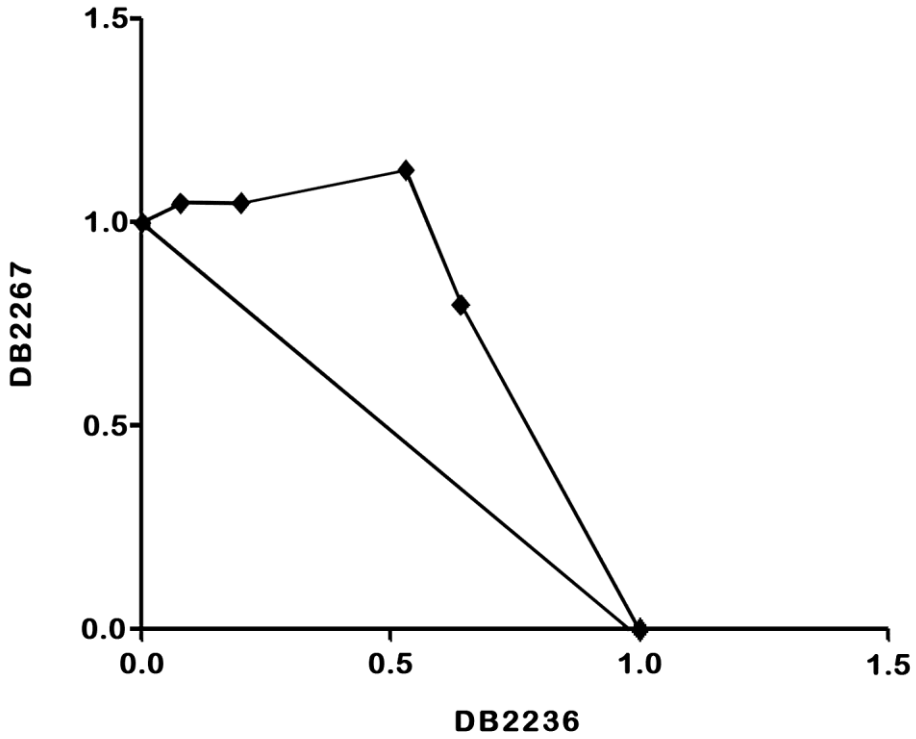


Figure 3

4 DISCUSSÃO

A busca de alternativas terapêuticas para a DC foi, por muito tempo, pouco explorada devido às divergentes correntes de pensamento acerca da gênese das manifestações crônicas (em especial a cardiomiopatia), incluindo a teoria de que após a infecção ocorreria um quadro autoimune, e que a presença do parasito não seria necessária para produzir a sintomatologia relacionada às formas clínicas recorrentes (Bonney & Engman, 2015). Com o passar dos anos, a associação da persistência do parasito no coração e/ou sistema digestivo com o conseguinte desbalanceamento da resposta imune no hospedeiro estimulou a investigação de novas entidades farmacológicas que pudessem tratar a moléstia.

A convicção de que o fenômeno da autoimunidade seria o fator principal relacionado ao dano do miocárdio durante a fase crônica (Cunha-Neto et al., 1995; Kalil & Cunha-Neto, 1996), levou a controvérsias quanto ao tratamento etiológico na fase crônica. Entretanto, esta hipótese foi contestada a partir de estudos experimentais (Tarleton, 2003; Garcia et al., 2005; Hyland et al., 2007; Bustamante et al., 2008) e clínicos (Andrade et al., 1996; Viotti & Vigliano 2007; Viotti et al., 2006), que revelaram por técnicas mais refinadas (ex. PCR) a presença do parasito mesmo em quantidades subpatentes mas suficiente para manter estimulo antigênico para recrutamento do repertório inflamatório cardíaco (Higuchi et al., 2009) além da demonstração da eficácia do tratamento com o Bz em reduzir a carga parasitária e prevenir a progressão clínica e eletrocardiográfica da doença.

Bz e Nf foram identificados de modo empírico como agentes tripanocidas e introduzidos na clínica há mais de quatro décadas (Filardi & Brener, 1982). Ambos são compostos nitroderivados que eram anteriormente utilizados como agentes antifúngicos sistêmicos. Entretanto, apesar do sucesso terapêutico de até 100% em casos agudos e 70% em crônicos recentes (Coura & Dias, 2009), esses agentes apresentam limitações, como ineficácia na fase crônica tardia, existência de cepas do *T. cruzi* naturalmente resistentes, resistência cruzada para os dois fármacos, severos efeitos adversos (dermatite alérgica, polineuropatia periférica, febre, intolerância gastrointestinal) e a necessidade de longos períodos de administração (Prata, 2001; Coura & Dias, 2009). Estes fatores dificultam a adesão ao tratamento pelos pacientes, e somado a isso, a limitada obtenção e produção de Bz (com boas práticas de fabricação, como é o caso atual do LAFEPE) pelos sistemas de saúde pública dos países acometidos faz com que hoje menos de 10% da população portadora da infecção receba a terapia adequada, permanecendo como

reservatórios da doença e representando um grande fardo com perdas de vidas e impacto negativo para o quadro socioeconômico do país (Dias et al., 2014; Tarleton et al., 2014).

Nos últimos dez anos, o número de grupos acadêmicos e agências de fomento interessados no estudo de doenças negligenciadas, como a DC, aumentou consideravelmente. Antes disso, os recursos tecnológicos eram escassos, as metodologias não eram padronizadas e as famílias de compostos testadas eram limitadas, destacando-se as naftoquinonas, os nitroimidazólicos e seus derivados, as diamidinas e os complexos com rutênio (Chatelain, 2015; Keenan & Chaplin, 2015). Anteriormente os alvos eram principalmente vias metabólicas, como a biossíntese do ergosterol, ou enzimas dessas vias, como a cisteína protease e a tripanotiona redutase (Chatelain, 2015; Keenan & Chaplin, 2015).

Diversos consórcios foram estabelecidos no campo das doenças negligenciadas entre 2013 e 2014, como KINDRED e PDE4NPD, com o intuito de buscar novos candidatos terapêuticos para essas morbidades, inclusive a DC. As indústrias farmacêuticas, como a GSK e a Novartis, passaram a integrar esses grupos financiados por fundações internacionais filantrópicas como “Bill e Melinda Gates Foundation” e “Welcome Trust”, sendo sua participação determinada pela disponibilização de suas bibliotecas de compostos, o que permitiu à comunidade acadêmica a condução de testes químicos com suas moléculas (Bustamante & Tarleton, 2014). A partir de então, a busca por novos agentes tripanocidas deixou de se basear apenas em enzimas e vias metabólicas, dirigindo-se para uma abordagem fenotípica. Em paralelo, padronizações das técnicas permitiram garantir a confiança e a qualidade nos resultados, considerando a reprodutibilidade das metodologias (Bustamante & Tarleton, 2014; Chatelain, 2015; Keenan & Chaplin, 2015).

Isso essencialmente conferiu segurança aos pesquisadores para propor e realizar ensaios clínicos com o próprio medicamento referência e duas outras entidades farmacológicas, o Posaconazol (Ps) (também um antifúngico sistêmico) e o E1224 (pró-fármaco do Ravuconazol - Rav, outro inibidor da CYP51). O primeiro ensaio clínico na última década foi o BENEFIT, ensaio clínico de fase 3 de longo prazo iniciado em 2005, mas ainda não completamente finalizado, que visa avaliar o potencial clínico do Bz no tratamento da cardiomiopatia chagásica pela prevenção da progressão da doença e morte (os resultados são esperados para o corrente ano) (Bustamante & Tarleton, 2014; Chatelain, 2015; Keenan & Chaplin, 2015). O E1224 em ensaio clínico apresentou-se seguro e eficaz, mas pouco ou incapaz de

sustentar o efeito tripanocida após um ano de tratamento, o que não ocorreu novamente para o medicamento referência (Bustamante & Tarleton, 2014; Chatelain, 2015). Já o estudo CHAGASAZOL trabalhou com o tratamento de duas doses por 60 dias de Bz e Ps separadamente, e foi demonstrada a falha terapêutica do segundo (PCR do sangue dos portadores), que confirmou a não sustentação do efeito terapêutico após o fim da administração, enquanto o Bz mostrou-se capaz no tempo de acompanhamento (Molina et al., 2014).

A falha do Ps é discutida a partir de três pontos principais: a população de estudo não era representativa no tocante às diferentes cepas do *T. cruzi* existentes; a quantidade de fármaco oferecida foi cerca de 5 a 10 vezes inferior em relação à administrada em camundongos, mesmo sendo aproximadamente 1000 vezes superior ao EC_{50} do composto; e o tempo de tratamento relativamente curto (60 dias). Isso tudo deve ser confrontado com o TPP para DC (**Tabela 1**), e assim entender o que realmente deve ser considerado e reavaliado não apenas para o Ps, que é o composto atualmente mais promissor, mas também para os outros ensaios clínicos mencionados ou que estão por vir.

De todos os ensaios é possível ainda perceber que os azóis não são eficazes em monoterapia para a DC na forma indeterminada e o Bz é a alternativa atual mais eficaz pela sustentação prolongada do efeito da resposta terapêutica. Deve-se considerar que os azóis são inibidores da enzima CYP51, que é fundamental para a biossíntese do ergosterol. Esta via metabólica, entretanto, é muito importante para as formas ativamente replicativas do parasito, sendo pouco ativo contra as formas não replicativas, os tripomastigotas sanguíneos. Dessa forma, observa-se na literatura a descrição de um efeito mais supressor que curativo por esta família de compostos, em modelos *in vivo*, e assim o questionamento da sua posição como compostos líderes na busca por novos fármacos para a DC.

Considerando o número de pacientes cujo diagnóstico não é confirmado por PCR, apenas por sorologia, e a falta de conhecimento da relação entre a existência de parasitos circulantes e o quadro clínico na DC (*i.e.*, a interrupção da progressão da doença), Bz continua sendo o medicamento de escolha (Bustamante & Tarleton, 2014; Chatelain, 2015). Porém a necessidade da triagem de novas entidades farmacológicas é real, pois além da premência de superar os obstáculos representados pelas limitações acima descritas, a falta de eficácia observada principalmente após menos de quatro anos do tratamento de populações amazônicas brasileiras (Pinto et al., 2013) é factual. Outro nitroderivado em teste é o

Fexinidazol, já administrado com sucesso na doença do sono (tripanossomíase africana, estágio III) e sob ensaio também para leishmanioses (Bahia et al., 2012, 2014; DNDi, 2015).

Dentre os compostos atualmente em teste, os inibidores da CYP51 mais promissores além do Ps e do Rav vêm em destaque, citando VNI e VFV, nitroderivados obtidos pela Drª. Galina Lepesheva (Vanderbilt University, Nashville, TN, EUA) a partir de uma biblioteca da Novartis. Estudos recentes demonstraram sua potência comparável ao Ps, entretanto, tais moléculas apresentam vantagens, como a não indução de resistência e a maior seletividade por enzimas de *T. cruzi* em detrimento das enzimas humanas e mesmo de fungos.

A partir da premissa do reposicionamento de antifúngicos, realiza-se também a busca por compostos contra o *T.cruzi* dentre outros agentes farmacológicos, e destacando-se a Pentamidina (Pt) e seus derivados da classe das amidinas aromáticas. Esta classe possui diversas aplicações, inclusive a atividade tripanocida em baixas concentrações e poucas doses. Contudo, a via de administração intramuscular (que muitas vezes demanda internações) e o perfil de toxicidade conduziram os pesquisadores para a estratégia do desenvolvimento racional de compostos, utilizando do desenho de novas estruturas moleculares, direcionadas para o tratamento de doenças causadas por tripanossomatídeos, como o *T.cruzi*. Sendo assim, espera-se que os novos derivados apresentem um perfil de segurança que permita a administração por via oral e a manutenção da eficácia inerente à sua classe, que em muitas entidades pode ser comparada à do Bz.

Estudos anteriores *in vitro* e *in vivo* demonstraram a excelente atividade de muitas amidinas contra *T. cruzi*: estas moléculas exibem efeito tripanocida tempo-dependente em faixas submicromolares e nanomolares, apresentando também baixa toxicidade para células mamíferas, sendo capazes de reduzir a carga parasitária *in vivo*, resultando em menor dano tecidual e maiores taxas de sobrevivência dos animais tratados em comparação aos não tratados (Batista et al., 2010, Soeiro et al., 2013). Entre as amidinas estudadas, as arilimidamidas demonstraram-se mais efetivas sobre *T.cruzi* e diferentes espécies de *Leishmania*, bem como outros patógenos intracelulares (Soeiro et al., 2013; De Araújo et al., 2014). No presente trabalho, dez moléculas aromáticas foram testadas *in vitro* de acordo com a metodologia estabelecida na literatura, possibilitando a determinação de EC_{50}, LC_{50} e do índice de seletividade (IS) (Romanha et al., 2010). Dentre as dez, sete apresentaram EC_{50} abaixo de 4 µM após 24 h de incubação. De fato, cinco das

dez moléculas exibiram valor de EC_{50} menor que 6 µM depois de apenas 2 h de incubação com tripomastigotas sanguíneos, sendo muito mais eficazes que o fármaco de referência. Dessa forma, os resultados corroboram com dados prévios que mostraram o rápido perfil de ação de algumas amidinas aromáticas, característica muito importante quando se considera situações de infecção aguda grave (ex. surto contaminação oral, como em Mérida/Venezuela, em junho de 2015) em casos de reativação da doença em condições de imunossupressão (Timm et al., 2014a).

Os compostos aromáticos aqui testados encerram em si menores ou maiores diferenças estruturais que podem refletir distintos padrões de atividade tripanocida e toxicidade para as células mamíferas hospedeiras. De acordo com Chai et al. (2013), a diversidade da estrutura molecular destes compostos viabiliza potenciais eletrostáticos particulares que influenciam diretamente na sua afinidade de ligação à fenda menor do DNA e nas sequências AT. As monoamidinas (DB2261, DB2262, DB2266, DB2268 e DB2269) cujas cadeias apresentam unidades intercalantes apresentaram valor de EC_{50} entre 2,14 e 3,37µM sobre tripomastigotas sanguíneos da cepa Y. Sendo esta uma nova classe de promissores agentes anti-*T.cruzi*, isso constitui um importante achado. O conjunto de moléculas ora estudadas é limitado para que se possa discutir com profundidade a relação estrutura-atividade; porém, a moderada atividade *in vitro* destes elementos não favorece a continuidade para ensaios *in vivo*. Sobremaneira, esta nova classe de compostos merece ser investigada visando a otimização de compostos líderes dentro da descoberta racional de novos fármacos anti-*T.cruzi*. DB2263, uma monoamidina serviu de controle dos compostos avaliados na dissertação por não apresentar unidade intercalante da cadeia. Esta amidina apresentou valor de EC_{50} de apenas 12,34 µM, o que sugere fortemente um importante papel dessa unidade intercalante para a atividade anti-*T.cruzi* desta série de entidades químicas.

Dados recentes sobre amidinas aromáticas, especialmente da classe das arilimidamidas, mostraram que muitas delas foram potentes contra diferentes cepas do parasita, incluindo aquelas naturalmente resistentes ao Bz, como também a ambas as formas relevantes para a infecção humana: os tripomastigotas sanguíneos e as amastigotas (Batista et al., 2010). DB2236 foi moderadamente ativa contra as cepas Y (formas sanguíneas) e Tulahuen (formas intracelulares), com valores de EC_{50} aproximados de 3,97 e 5,4 µM, respectivamente. Entretanto, o composto mais ativo contra os tripomastigotas da cepa Y, DB2267, foi inativo contra as amastigotas

da cepa Tulahuen, retendo, contudo, a eficácia sobre as formas intracelulares da cepa Y. Assim, nossos resultados sugerem que a atividade de DB2267 ocorra de modo cepa-dependente, atributo que infelizmente não é desejável para novos candidatos terapêuticos para a DC (Chatelain, 2015; Keenan & Chaplin, 2015). Estas informações deixam claro que sutis modificações na estrutura química de tais moléculas podem impactar (i) no seu mecanismo de ação e alvos celulares, (ii) na sua metabolização e/ou captação/extrusão em relação a diferentes cepas do parasita, o que merece investigações futuras. De fato, o perfil diferencial de cepas do parasito já é conhecido para vários agentes incluindo os nitroderivados hoje utilizados na clínica médica, o Bz e Nf (Soeiro & De Castro, 2011).

Adicionalmente, para avaliar se um melhor efeito dos compostos poderia ser obtido com o uso de outro veículo além do DMSO, considerando a novidade farmacológica representada pela utilização de nanopolímeros naturais, ensaios de atividade tripanocida foram conduzidos empregando β-ciclodextrina como solvente, o que poderia resultar em maior solubilidade e melhor permeabilidade através das membranas biológicas das células mamíferas hospedeiras e do parasita (Katageri-Akshay & Sheikh-Mohsin, 2012). As ciclodextrinas são carboidratos complexos compostos de unidades de glicose (sendo sete as unidades das β-ciclodextrinas) que formam complexos de inclusão com estruturas semelhantes a um tronco de cone (Cunha-Filho & Sá-Barreto, 2007). Esses complexos, portanto, são moléculas biocompatíveis que atuam como veículos de solubilização em água. São aplicadas na indústria farmacêutica para diferentes finalidades, sendo a principal como agente carreador de princípios ativos, permitindo sua liberação controlada dentro de um organismo. Admitem uma grande variedade de moléculas hóspedes, portanto sua formulação não pressupõe uma técnica geral de preparo, mas sim metodologias variáveis de acordo com a natureza do composto de inclusão (Cunha-Filho & Sá-Barreto, 2007, Katageri-Akshay & Sheikh-Mohsin, 2012). Todavia, observamos que não houve diferenças em relação aos compostos não inclusos no polímero, o que merece ser melhor investigado no futuro quanto à produção da formulação e a natureza das interações das moléculas com o polímero.

Considerando a captação de compostos, estudos prévios utilizando microscopia de fluorescência foram conduzidos com moléculas da classe das amidinas que possuem fluorescência intrínseca, como a furamidina (DB75), e permitiram a identificação dos alvos celulares (como o kDNA) em tripanossomatídeos (Daliry et al., 2010). Em *T. cruzi*, investigações anteriores

mostraram a presença das amidinas em diferentes compartimentos intracelulares como o núcleo, o cinetoplasto e acidocalcissomos, sugerindo os sítios de dano celular (primários e secundários) e/ou os locais de armazenamento de tais compostos (De Souza et al., 2004, 2006a; Silva et al., 2007b; Daliry et al., 2009; Da Silva et al., 2010). Portanto, realizamos análises de fluorescência para verificar a captação de duas moléculas representativas dentre os compostos aqui ensaiados com diferentes tamanhos moleculares: uma pequena (DB2236) e uma maior (DB2267). Ambas foram internalizadas pelo parasita, mas enquanto DB2236 foi encontrada dispersa de maneira difusa pelo citoplasma, a amidina maior foi localizada em compartimentos ricos em DNA (núcleo e kDNA), corroborando os dados previamente apresentados para outras amidinas (Soeiro et al., 2013). As diferentes colocações possivelmente refletem distintos alvos celulares que devem ser determinados em estudos posteriores.

Outra abordagem para diversas patologias, principalmente as negligenciadas, que pode também ser relacionada ao reposicionamento de medicamentos trata da associação ou coadministração de fármacos. Além de direcionar a terapia para diferentes alvos celulares, reduzindo a manifestação de resistência, essa estratégia viabiliza a redução de custos no processo de testagem dos compostos, uma vez que pode partir de entidades já licenciadas e comercializadas, não apenas as recém descobertas; a melhoria da posologia, estabelecendo menor número de doses; e o encurtamento do tempo de ensaio até a chegada às prateleiras, dado que podem ser utilizados fármacos já disponíveis à população, mas que são utilizados para outras finalidades. Essa otimização na busca de novas alternativas terapêuticas para as doenças negligenciadas representa também uma diminuição nos custos do processo da produção, além de poupar esforços que podem ser melhor aproveitados na dinâmica da academia. Casos de sucesso no reposicionamento e/ou associação de medicamentos são apresentados para tripanossomíase africana, HIV pediátrico, tuberculose e malária na **Tabela 2** da introdução.

A estratégia de direcionar o tratamento para diferentes alvos celulares tem sido avaliada para a DC por diversos grupos, nos últimos anos (Cencig et al., 2012; Moreira da Silva et al., 2012; Bustamante et al., 2014), com o objetivo de tentar evitar a resistência de cepas e melhorar a atividade biológica, no tocante à terapia de doenças negligenciadas, incluindo a DC. DB2236 e DB2267 foram combinadas proporcionalmente e diluídas de modo seriado (Fivelman et al., 2004), e a seguir incubadas com tripomastigotas sanguíneos da cepa Y. A interação não exibiu

atividade diferente daquela observada para os compostos primeiramente utilizados em monoterapia, contudo, o efeito tampouco foi antagônico, o que incentiva a condução de ensaios *in vivo* para avaliar o comportamento farmacodinâmico da combinação.

Deve-se considerar a associação de compostos com o foco nas melhores características de duas moléculas, assim ambicionando resultados superiores, a partir da adequação das propriedades farmacodinâmicas e redução dos possíveis efeitos colaterais para os pacientes.

Finalmente, o conjunto de resultados obtidos corrobora com dados anteriores da atividade tripanocida para essa classe de compostos, as amidinas aromáticas, encorajando o seguimento das metodologias utilizando tais entidades químicas, permitindo assim a continuidade da geração de conhecimento e contribuição para identificação de novas alternativas terapêuticas para esta silenciosa e negligenciada enfermidade que resulta em altos níveis de morbidade e mortalidade anual de cerca de 15 mil portadores. Assim, os milhões de portadores e suas famílias, seguem aguardando novos medicamentos.

5 PERSPECTIVAS

Nossos dados mostram que a molécula mais ativa e seletiva para formas sanguíneas de *T. cruzi* (DB2267) é composta por dois grupamentos catiônicos terminais. Ensaios conduzidos sobre formas intracelulares revelaram uma importante divergência quanto à atividade fenotípica de modo cepa-dependente, sendo mais potente que o medicamento de referência para cepa Y (DTU II), mas ineficaz sobre a cepa Tulahuen (DTU VI), o que representa uma característica indesejável para um novo fármaco voltado para esta patologia, e, portanto, a desclassifica para progressão em modelos de eficácia *in vivo*. Porém, esta diamidina que se localiza em compartimentos ricos em ácido nucleicos, à semelhança de outras moléculas já anteriormente estudadas, possivelmente atua sobre alvos celulares primários e/ou rotas metabólicas diferenciadas entre as distintas cepas do parasito. Assim, sobre o ponto de vista da biologia celular e mecanismo de ação desta classe de compostos, sugerimos como perspectiva um aprofundamento de suas vias de atuação sobre o *T. cruzi* através de estudos ultraestruturais e mesmo termodinâmicos (estruturas alvo como DNA, kDNA, topoisomerases) visando desenho de moléculas com ação biológica universal sobre diferentes formas e cepas do parasito.

A respeito da combinação de DB2267 e DB2236, mesmo que não tenha sido observado efeito sinérgico entre elas, sugerimos estudos futuros entre as entidades amidínicas e outros agentes com reconhecida ação tripanocida (ex. inibidores de via de biossíntese de ergosterol) visando promover eficácia e segurança pela atuação sobre diferentes alvos celulares, aprimoramento da posologia, e redução da possibilidade de resistência a fármacos.

6 CONCLUSÕES

- A maioria das amidinas aromáticas presentemente estudadas apresenta ação tripanocida *in vitro* sobre *T. cruzi*, destacando-se a molécula dicatiônica, a DB2267 com ação submicromolar, e superior atividade em relação às moléculas que apresentam apenas um grupamento catiônico terminal em sua estrutura;
- Cinco amidinas dentre as selecionadas (DB2262, DB2266, DB2267, DB2268 e DB2269) tiveram perfil de ação rápida, com EC_{50} menor que 6µM após somente duas horas de incubação com tripomastigotas sanguíneos o que representa efeito desejável para uso em casos de reativação ou de infecção por contaminação oral nas quais uma rápida intervenção se faz necessária; DB2267 demonstrou atividade sobre tripomastigotas sanguíneos e amastigotas de cepa Y (DTU II), mas não foi efetiva sobre a cepa Tulahuen (formas intracelulares) denotando um possível mecanismo de ação cepa-dependente o que inviabilizou sua análise sobre modelos de infecção experimental *in vivo*;
- O ensaio de fluorescência com duas diamidinas selecionadas (DB2236 e DB2267) de diferente perfil de atividade biológica permitiu identificar sua localização intracelular, tanto nas células hospedeiras, quanto no parasito; revelando alvos e/ou sítios de estoque (citoplasma, núcleo e kDNA) diferenciados a depender de pequenas mudanças estruturais nas moléculas desta classe de compostos;
- A metodologia da combinação de compostos foi padronizada e a associação de DB2236 e DB2267 em tratamento combinado demonstrou efeito aditivo sobre formas sanguíneas da cepa Y;
- Nossos dados representam resultados encorajadores a respeito da atividade antiparasitária das amidinas aromáticas, no tocante a futuras investigações de novos agentes promissores para o tratamento da DC.

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8 APÊNDICE A – INFORMAÇÕES TÉCNICAS ADICIONAIS

As estruturas químicas das moléculas foram desenhadas no *Chem 3D® Ultra* Molecular Modeling and Analysis, Version 8.0, © 1985-2003 CambridgeSoft Corporation.

Os ensaios colorimétricos foram analisados com SoftMax® Pro software, v 5.4, © 1999-2009 MDS Analytical Technologies (US), no espectrofotômetro SpectraMax Plus 384 Microplate Reader / Molecular Devices.

As imagens foram adquiridas utilizando o microscópio Carl Zeiss Axio Observer.A1, com a câmera AxioCam MRc5.

Os valores de IC-50 foram obtidos através do *software* CalcuSyn, que utiliza o plot de efeito médio ("median effect plot"). Para classificar a natureza da interação entre os compostos DB2267 e DB2236 *in vitro,* foi utilizada a fração de concentração inibitória (FIC) e análise do isobolograma. FICs e a soma dos FICs (∑FICs) foram calculadas da seguinte forma: FIC DB2267= IC50 na combinação/IC50 DB2267 em monoterapia. A mesma equação foi aplicada a DB2236. Esse cálculo foi feito para os valores obtidos para cada uma das diluições. ∑FICs= FIC DB2267+ FIC DB2236 A média do ∑FICs calculada foi usada para classificar a natureza da interação. Se <0,5 indicará sinergismo; 0,5<∑FIC<4, aditividade e se >4 indicará antagonismo. Os isobologramas foram construídos plotando-se a FIC DB2267 *versus* FIC DB2236.

9 APÊNDICE B – COLABORAÇÃO EM TRABALHOS

Durante o mestrado, foi publicado um artigo referente a uma parte das atividades desenvolvidas durante o período de iniciação científica na Universidade Federal de Ouro Preto, entitulado “Sesquiterpene lactone in nanostructured parenteral dosage form is efficacious in experimental Chagas disease” na revista Antimicrobial Agents and Chemotherapy.

Além desse, foi recentemente submetido um artigo na mesma revista, fruto de colaboração dentro do meu próprio grupo de trabalho atual, entitulado “Different therapeutic outcomes of benznidazole and VNI treatment in distinct genders of mouse experimental models of *Trypanosoma cruzi* infection”.

Antimicrobial Agents and Chemotherapy

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Sesquiterpene Lactone in Nanostructured Parenteral Dosage Form Is Efficacious in Experimental Chagas Disease

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The drugs available for Chagas disease treatment are toxic and ineffective. We studied the *in vivo* activity of a new drug, lychnopholide (LYC). LYC was loaded in nanocapsules (NC), and its effects were compared to free LYC and benznidazole against *Trypanosoma cruzi*. Infected mice were treated in the acute phase at 2.0 mg/kg/day with free LYC, LYC-poly-ε-caprolactone NC (LYC-PCL), and LYC-poly(lactic acid)-co-polyethylene glycol NC (LYC-PLA-PEG) or at 50 mg/kg/day with benznidazole solution by the intravenous route. Animals infected with the CL strain, treated 24 h after infection for 10 days, evaluated by hemoculture, PCR, and enzyme-linked immunosorbent assay exhibited a 50% parasitological cure when treated with LYC-PCL NC and 100% cure when treated with benznidazole, but 100% of the animals treated during the prepatent period for 20 days with these formulations or LYC-PLA-PEG NC were cured. In animals with the Y strain treated 24 h after infection for 10 days, only mice treated by LYC-PCL NC were cured, but animals treated in the prepatent period for 20 days exhibited 100, 75, and 62.5% cure when treated with LYC-PLA-PEG NC, benznidazole, and LYC-PCL NC, respectively. Free LYC reduced the parasitemia and improved mice survival, but no mice were cured. LYC-loaded NC showed higher cure rates, reduced parasitemia, and increased survival when used in doses 2five times lower than those used for benznidazole. This study confirms that LYC is a potential new treatment for Chagas disease. Furthermore, the long-circulating property of PLA-PEG NC and its ability to improve LYC efficacy showed that this formulation is more effective in reaching the parasite *in vivo*.

Chagas disease (CD) is recognized by the World Health Organization (1) as one among 13 of the world's most neglected tropical diseases. This disease remains a relevant social and economic problem in Latin America, where eight million people are infected with the causative intracellular parasite, *Trypanosoma cruzi*. More than 25 million people are at risk of infection, and 12,500 deaths are attributed to American trypanosomiasis (Chagas disease). Furthermore, the majority of people affected do not receive effective treatment for several reasons (1). As a consequence of the intense migration of individuals from areas of endemicity in Latin America to North America, Europe, and Asia, CD is now considered a global disease and represents a critical public health problem in several countries of the Northern Hemisphere (2, 3). Similarly to many other neglected diseases, CD chemotherapy research suffers from limited economic potential, because it is not the main focus of interest of the pharmaceutical industry (4). *T. cruzi* infection involves different morphological forms along its life cycle. This protozoan proliferates alternatively between hematophagous triatomines insect and vertebrate hosts. The disease presents two successive phases: a short acute phase characterized by patent parasitemia, and a long and chronic phase that may progress after several years or decades to cardiomyopathy and/or digestive megasyndromes in 30 to 40% of the infected individuals (1, 5).

Although CD was discovered more than 100 years ago, no efficient chemotherapy is available to treat this disease in either the acute or chronic phases of infection. Nitroheterocyclics, benznidazole (BZ), and nifurtimox (NF) are far from ideal medicines, particularly due to their long periods of treatment, the frequency of serious side effects, and their poor activity in the late chronic phase (6, 7). Moreover, differences in the susceptibility and natural resistance of different *T. cruzi* isolates to both nitroderivatives have also been reported (8, 9).

The main challenge of CD pharmacotherapy is the lack of ability of anti-*T. cruzi* agents with the suitable selectivity to reach infected cells and attain the intracellular parasites, because the plasma membrane and complex microenvironment of the host cells prevent the selective and massive delivery of drugs to the intracellular amastigote nests (10). Thus, a drug or a drug delivery system that provides a high volume of distribution, long plasmatic half-lives, and high efficacy in both the acute and the chronic phases of the infection is especially desirable (11).

Lychnopholide (LYC) (Fig. 1) is a sesquiterpene lactone (SL) that was isolated from *Lychnophora trichocarpha* (12, 13). The anti-*T. cruzi* activity of LYC *in vitro* was first reported by Oliveira et al. (14). This substance presents many other activities (15–17), including clastogenic and cytotoxic effects (18). Despite its pharmacological potential, the therapeutic application of LYC is limited due to its physicochemical properties that hamper oral administration, such as poor aqueous solubility, high lipophilicity ($log\,P = 5.03$), and potential chemical instability in alkaline media (13). Recently, our group developed a pharmaceutical formulation of LYC loaded in polymeric nanocapsules (NC) (19). These

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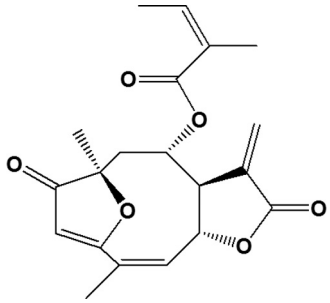

FIG 1 Chemical structure of lychnopholide, a sesquiterpene lactone extracted from *Lychonophora trichocarpha*.

NC can be easily dispersed in water and are therefore suitable for administration by oral and parenteral routes. These LYC-NC also possess controlled release properties (13, 19). Furthermore, the validated methods were also developed to quantify this formulation in this new dosage form, which are necessary for preclinical evaluation *in vivo* (13).

The advantages of associating a CD drug with a nanocarrier has already been reviewed (20). Polymeric NC are formed by an oil droplet surrounded by a polymeric membrane stabilized by surfactants (21) and have been used successfully for the following purposes: to increase the dispersibility of poorly water-soluble drugs, to protect drugs against inactivation (22), to reduce drug toxicity (23), to control drug release (13), and to prolong blood circulation time after intravenous (i.v.) administration (24).

Thus, the aim of the study was to evaluate the efficacy of LYC-NC *in vivo* during the acute phase of infection in mice experimentally infected with *T. cruzi* strains with different susceptibility patterns to BZ. Two types of polymeric NC were used: one conventional form that is rapidly cleared from blood circulation by phagocytes of the mononuclear phagocyte system (PCL NC) and the other (PLA-PEG NC) that circulates longer in blood due to its polymeric steric stabilization which reduces uptake by phagocytes (25). Thus, the probability of LYC to target sites of inflammation induced by the presence of the parasite, particularly during the acute phase of infection, is expected to be increased due to increased plasma circulation time of PEG sterically stabilized NC (20, 24, 25, 26).

MATERIALS AND METHODS

Plant Collection, isolation and identification of lychnopholide. *L. trichocarpha* Spreng was collected at Ouro Preto city in Minas Gerais state, Brazil, in August 2006. A voucher specimen (no. 20635) was deposited in the Herbarium of the Instituto de Ciências Exatas e Biológicas, Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil. All ethanol extract preparation, isolation, and identification of LYC were performed as described by Saúde et al. (12) and Branquinho et al. (13). LYC characterization and isolation from *L. trichocarpha* were performed as described previously by Saúde et al. (12) and Branquinho et al. (13), and its chemical structure is shown in Fig. 1.

Chemicals, adjuvants, and solvents. Benznidazole (BZ) (*N*-benzyl-2-nitro-1-imidazole-acetamide) as Rochagan tablets obtained from LAFEPE (Brazil) was used. The analytical standard BZ (97.0%) was purchased from Sigma-Aldrich (St. Louis, MO). Epikuron 170 (soy lecithin with ~70% phosphatidylcholine) was purchased from Lucas Meyer (Le Blanc Mesnil, France). Poly-ε-caprolactone (PCL) with average Mn of 42,500 g/mol and Poloxamer 188 (nonionic surfactant) were provided by Sigma-Aldrich. PLA-PEG [poly(D,L-lactide)-co-polyethylenoglycol average Mn of 49,000 g/mol] with a PEG block of Mn of 5,000 g/mol was obtained from Alkermes (Cambridge, MA) and used without further purification. PLA [poly(D,L-lactide)] Resomer 203R 203H (molecular mass, 18,000 g/mol) was purchased from Boehringer Ingelheim (Germany). Miglyol 810N (capric/caprilic triglyceride) was purchased from Hulls (Germany). Polyethylene glycol 300 (PEG 300), dimethylacetamide (DMA), ethyl acetate (AcOEt), methanol (MeOH), and acetone, all analytical grade, were purchased from Vetec (Rio de Janeiro, Brazil). The analytical-grade revelators were *N*-(1-naphthyl)-ethylenediamine-dihydrochloride and tin(II) chloride and were obtained from Merck (Germany). Silica gel 60 GF_{254} was purchased from Merck (Darmstadt, Germany) and was used for thin-layer chromatography (TLC). Milli-Q water was purified using a Symplicity System (Millipore, Bedford, MA) and used throughout the experiments.

Preparation of lychnopholide solution and lychnopholide-loaded NC. To administer LYC i.v. solution (2 mg/ml), LYC was dissolved in a DMA-PEG 300 mixture at 40:60 (vol/vol) as described by Leite et al. (23) and further diluted in 5% (wt/vol) glucose to reach the correct dose of LYC of 2.0 mg/kg/day to be administered i.v. Both formulations were filtered in 0.45-μm-pore-size sterile filter before injection. Conventional NC (PCL NC) were loaded with LYC according to the method of Branquinho et al. (13). These NC were prepared with 0.8% (wt/vol) polymer (PCL) dissolved in acetone solution containing 0.4% (wt/vol) Epikuron 170, 2.5% (vol/vol) Miglyol 810N, and LYC to obtain a 2-mg/ml final concentration. This organic solution was poured into the external aqueous phase containing 0.75% (wt/vol) Poloxamer 188 and mixed. All solvents were evaporated under reduced pressure (Laborota 4000; Heidolph Instruments, Germany) to render a colloidal NC suspension (10 ml). NC sterically stabilized with a corona of PEG were prepared using PLA-PEG diblock polymer as reported by Mosqueira et al. (25). Briefly, a 1:1 mixture of PLA-PEG and Resomer 203 (1.2% [wt/vol]) was dissolved in acetone containing Epikuron 170 (0.4% [wt/vol]), Miglyol 810N (2.5% [vol/vol]), and LYC. This organic phase was poured into an external aqueous phase and mixed for 10 min. The solvents were then eliminated under reduced pressure to render the desired concentration of LYC and polymers in the colloidal suspension (2.0 mg/ml). The unloaded NC were prepared by the same methods described above, but without LYC in the formulation. The mean size and the polydispersity index of the NC were determined as previously described (25, 26).

Extraction, purification, and characterization of benznidazole and preparations of BZ solution. BZ was not obtained from suppliers in its pure form, so it was extracted from tablets and subjected to purification according to the procedure described below. Rochagan tablets (20) were pulverized, dissolved in MeOH, and stirred in the dark for 20 min. The resulting suspension was filtered through quantitative paper filter (Whatman filter), and the filtrate was concentrated in a rotary evaporator until dried (Heidolph Instruments, Germany). The material was recrystallized twice in MeOH-H_2O to render acicular crystals. The crystals were filtered and dried under a vacuum in a desiccant containing anhydrous silica. Melting points were determined, and TLC was used for chemical identification and determination of BZ purity. BZ purity was verified by comparison with a standard BZ in TLC of silica gel eluted with AcOEt-MeOH (85:15 [vol/vol]), with a detection system consisting of UV light ($\lambda = 254$ nm), tin(II) chloride, and *N*-(1-naphthyl)-ethylenediamine-dihydrochloride (27).

The BZ solution for i.v. administration was prepared similarly to a method described by Leite et al. (23). The solution contained DMA-PEG 300 mixture at 40:60 proportions was diluted in isotonic 5% (wt/vol) glucose up to 4.0 mg/ml. Subsequently, the solution was filtered in a sterile 0.80-μm-pore-size filter before i.v. injection in mice at a dose of 50 mg/kg of bodyweight.

Parasites. The CL and Y strains of *T. cruzi* weren determined to be susceptible and partially resistant, respectively, to BZ by Filardi and Brener (8) and were therefore used in the present study. The original isolates were maintained as blood trypomastigotes in liquid nitrogen and

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TABLE 1 Treatment schedules for *T. cruzi*-infected mice with lychnopholide and control formulations during the acute phase of infection

Expt no. (treatment scheme) and *T. cruzi* strain	Drug/formulation[a]	Treatment period (days)	Dose (mg/kg/day), i.v.[b]
I[c] (24 h after infection), CL strain (sensitive to BZ)	LYC-PCL NC		
	Free LYC	10	2.0
	BZ	10	50.0
	Unloaded NC	10	2.0*
	DMA-PEG 300 (control i.v. solution)	10	50.0*
	Untreated control		
II (prepatent period, 7th day), CL strain (sensitive to BZ)	LYC-PCL NC	20	2.0
	LYC-PLA-PEG NC	20	2.0
	Free LYC	20	2.0
	BZ	20	50.0
	Unloaded NC	20	2.0*
	DMA-PEG 300 (control i.v. solution)	20	50.0*
	Untreated control		
III (24 h after infection), Y strain (partially resistant to BZ)	LYC-PCL NC	10	2.0
	Free LYC	10	2.0
	BZ	10	50.0
	Unloaded NC	10	2.0*
	DMA-PEG 300 (control i.v. solution)	10	50.0*
	Untreated control		2.0
IV (prepatent period, 4th day), Y strain (partially resistant to BZ)	LYC-PCL NC	20	2.0
	LYC-PLA-PEG NC	20	2.0
	BZ	20	50.0
	Unloaded NC	20	2.0*
	DMA-PEG 300 (control i.v. solution)	20	50.0*
	Untreated control		

[a] NC, nanocapsules.
[b] *, the amount of excipients used in DMA-PEG solution and in unloaded NC was the same as that used in BZ and LYC NC formulations.
[c] Infection was confirmed by hemoculture in all animals.

cultured in liver infusion tryptose (LIT) medium (28). Mice were subsequently inoculated with trypomastigotes from the culture.

Mouse infection. Groups of eight female Swiss mice aged 28 to 30 days and weighing 20 to 25 g were used and maintained according to the guidelines established by the Colégio Brasileiro de Experimentação Animal. Mice were maintained in a specific-pathogen-free room at 20 to 24°C under a 12/12-h light/dark cycle and were provided with sterilized water and chow *ad libitum*. Infection was performed via intraperitoneal injection of 10^4 blood trypomastigotes. Uninfected and age-matched mice were maintained under identical conditions as a control. The experiments were approved by the Ethical Committee on Animal Experimentation of the Universidade Federal de Ouro Preto, Brazil (protocol 2009/13).

Treatment schedules. The dose of LYC used in all experiments was determined after a pilot experiment performed with *T. cruzi*-infected Swiss mice at 1, 2, and 4 mg/kg/day for three consecutive days, evaluating only the parasitemia level. Since the parasitemia reduction rate and extent were not improved above 2 mg/kg/day, this dose of LYC was adopted for the treatment of the animals during 10 and 20 days, as shown in Table 1. All animals were treated during the acute phase of the infection. Four independent experiments were carried out to evaluate the *in vivo* efficacy of free LYC, LYC-PCL NC, LYC-PLA-PEG NC, BZ (as a reference drug), and the following controls: control untreated (infected but not treated), control unloaded NC (UN-NC), and control DMA-PEG 300 (control solution). Four experiments of each treatment were used for each *T. cruzi* strain: in experiments I and III, the infection was carried out with the CL and Y strains, respectively. Treatment started 24 h after infection according to the method of Filardi and Brener (8). In experiments II and IV, mice were infected with the CL and Y strains, and treatment was started in the prepatent period of the *T. cruzi* strains used (CL strain, 7 days; Y strain, 4 days after inoculation) as described by Filardi and Brener (8). All formulations were administered i.v. in the tail veins at the doses described in Table 1.

Parasitemia and survival rates. The level of parasitemia was checked by fresh blood examination using the Brener method (29). Mice were individually examined daily by direct counting of parasites in 5 μl of blood under optical microscopy. Mortality of the animals was checked daily until 6 months postinfection in order to determine the percentage of survival expressed in percentage.

Parasitological cure assessment. The cure criterion was based on parasitological methods (fresh blood examination, hemoculture, and PCR on peripheral blood) and conventional serology. These methodologies are described below. Animals with negative parasitological and serological outcomes were considered cured.

Parasitological methods. (i) Fresh blood examination. To determine the reduction, suppression, and/or reactivation of parasitemia in treated animals, fresh blood examination was performed before the prepatent period, throughout experimental period, and for 5 days after complete negative parasitemia. Parasitemia curves were plotted by using the mean parasitemia values of eight mice recorded daily.

(ii) Hemoculture. Hemoculture was carried out 30 days after the end of treatment as described by Filardi and Brener (8). Blood samples collected from the orbital sinus vein were inoculated into 3 ml of LIT medium and maintained at 28°C. Each tube was examined under microscopy for parasites detection at 30, 60, 90, and 120 days after culture.

(iii) PCR on peripheral blood. Blood samples were collected from the orbital sinus veins 60 and 120 days after treatment. DNA extraction and PCR protocols were adapted and standardized for rodent samples as previously reported (30). Briefly, 200-ml portions of blood were diluted in a 1:2 volume of guanidine solution (guanidine-HCl, 6 M; EDTA, 0.2 M) and heated for 90 s in boiling water to cleave the parasite kDNA (DNA of the kinetoplast). PCR was performed using the primers 5′-AAATAATGT ACGGG[T/G]GAGATGCATGA-3′ and 5′-GGTTCGATTGGGGTTGG TGTAATATA-3′ (Invitrogen, São Paulo, Brazil), which amplify a 330-bp sequence from kDNA as previously described (31). A 2-μl blood DNA sample was added to the reaction mixture, which was then overlaid with 30 ml of mineral oil to avoid evaporation. Following an initial denaturation step of 5 min at 94°C, 35 amplification cycles consisting of 1 min at 95°C for DNA denaturation, 1 min at 65°C for primer annealing, and 1 min at 72°C for primer extension were performed, followed in turn by a final extension performed in a thermal cycler (MJ Research, model PTC-150). The amplified DNA was visualized by electrophoresis on a 6% polyacrylamide gel and revealed by silver staining (32). Positive and negative controls and reagents were included in each test. The presence of inhibitors in negative samples was evaluated by the addition of 100 fg of DNA of *T. cruzi* in 30 samples randomly selected from different samples with negative results followed by a new PCR.

Serological methods. Conventional serology using a modified enzyme-linked immunosorbent assay (ELISA) approach based on that described by Voller et al. (33) was performed. Serum samples were collected 3 and 6 months after treatment and stored at −20°C. Samples were tested at 1:80 dilution in phosphate-buffered saline using the antigen of *T. cruzi* Y strain cultivated in LIT medium and prepared by alkaline extraction in the exponential growth phase. Antibody binding was detected by peroxidase-labeled anti-mouse immunoglobulin G (Sigma Immunochemical Reagents, St. Louis, MO). The absorbance was read in a spectrophotometer with a 490-nm filter (model 3550; Bio-Rad). Positive and negative controls were processed in parallel with each assay. The cutoff value calculated for each plate was the mean absorbance of 10 negative-control serum samples plus two standard deviations.

Statistical analysis. Statistical analyses of data were carried out using Prism software v5.02 (GraphPad Software, San Diego, CA). The data were

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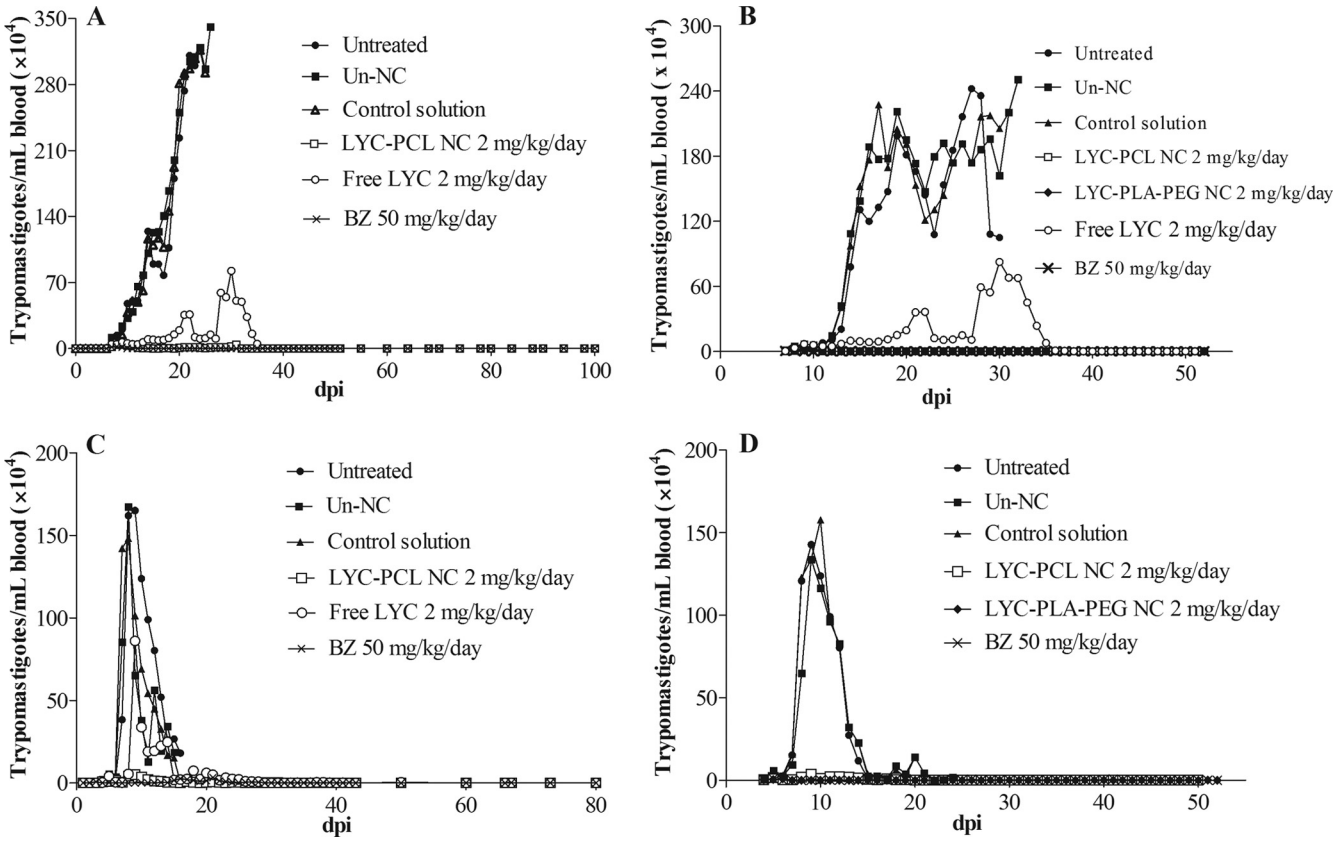

FIG 2 Parasitemia curves of *T. cruzi*-infected mice treated i.v. with lychnopholide at 2.0 mg/kg/day and benznidazole at 50 mg/kg. (A) Mice infected with CL strain treated with 10 doses (started 24 h after infection). (B) Mice treated with 20 doses (started in the prepatent period [day 7 after infection]). (C) Mice infected with Y strain treated with 10 doses (started 24 h after infection). (D) Mice treated with 20 doses (started in the prepatent period [day 4 after infection]). dpi, days postinfection; LYC-PCL NC, lychnopholide loaded in conventional NC; LYC-PLA-PEG NC, lychnopholide loaded in PEG sterically stabilized NC; free LYC, lychnopholide in i.v. solution; BZ, benznidazole i.v. solution; control groups, untreated (infected and not treated) or Un-NC (unloaded NC); control solution, DMA-PEG 300.

initially assessed by one-way analysis of variance (ANOVA). When interactions were significant, a Tukey test was used to determine specific differences between mean values. The Kolmogorov-Smirnov test was used to compare parasitemia between infected groups that were either treated or untreated. One-way ANOVA or Mann-Whitney U tests were used to compare maximum peak values of parasitemia between the different groups. The log-rank (Mantel-Cox) method was used to estimate the media of survival for the different experimental groups. Values were expressed as means ± the standard deviations. Differences in mean values were considered significant at $P \leq 0.05$.

RESULTS

LYC was encapsulated in NC, at a high yield, more than 95% in conventional PCL NC (13) and with 100% loading in PLA-PEG NC. The mean diameter of LYC PCL NC was 182.5 ± 3.2 nm and that of PLA-PEG NC was 105.3 ± 2.3 nm, as determined by quasi-elastic light scattering method. The nanoparticle populations were monodispersed with a polydispersion index lower than 0.3.

Since pure BZ was not provided in sufficiently large amounts to perform *in vivo* experiments, the drug was extracted and purified from Rochagan tablets. This process yielded 99.0% ± 1.2% acicular yellowish crystals. The purity of the BZ crystals was confirmed by its melting point of 188.5 to 190°C (methanol SM Lux Leitz apparatus) determination that was performed in accordance with the literature (27) and also by TLC. TLC showed only one stain correspondent in position (retention factor = 9.48 cm), color, and intensity to that obtained with standard BZ.

Toxicity at the injection site with the parenteral BZ formulation was observed in mice during the first days of treatment. Itch and edema around the site were verified immediately after the injection and disappeared within 15 min. No similar effects were observed in the control formulations of NC excipients or in the parenteral solution, LYC solution, or LYC-NC formulations.

Curve of parasitemia and survival rates. (i) Animals infected with CL strain (sensitive to BZ). Animals treated as described in the experiment I schedule (24 h after infection for 10 days) (Fig. 2A) exhibited a significant parasitemia reduction ($P < 0.05$), which became subpatent during and after treatment with BZ (Table 2). Mice treated with LYC-PCL NC showed higher parasitemia ($P < 0.05$) than animals treated with BZ, but this parasitemia was significantly lower ($P < 0.05$) than that observed in the control groups (untreated control, unloaded-NC, and control solution). Four animals of eight treated with LYC-PCL NC showed subpatent parasitemia during and after treatment (Table 2). Five and one animal(s) of eight treated with LYC-PCL NC and free LYC, respectively, showed subpatent parasitemia during and after treatment (Table 2). Analyses of the parasitemia peak of the treated groups revealed a reduction ($P < 0.05$) of MPP (maximum peak of parasitemia) of 98.6% (BZ), 56.3% (free

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TABLE 2 Parasitemia and maximum peaks of parasitemia in *T. cruzi*-infected mice treated with lychnopholide i.v. using different treatment schemes during acute infection[a]

Treatment (dose [mg/kg])[d]	Strain and treatment scheme[b]							
	CL strain, expt I		CL strain, expt II		Y strain, expt III		Y strain, expt IV	
	Subpatent PAR/total[c]	MPP ± SE (10^4) (% reduction)[e]	Subpatent PAR/total	MPP ± SE (10^4) (% reduction)	Subpatent PAR/total	MPP ± SE (10^4) (% reduction)	Subpatent PAR/total	MPP ± SE (10^4) (% reduction)
Untreated	0/8	223.4 ± 40.8 (ND)	0/8	244.7 ± 24.3 (ND)	0/8	163.9 ± 12.9 (ND)	0/8	123.8 ± 7.8 (ND)
Benznidazole (50)	8/8	2.8 ± 0.9 (98.6)^{A}	8/8	1.2 ± 0.6 (99.2)^{A}	0/8	5.3 ± 1.3 (94.35)^{A}	7/8	2.2 ± 0.5 (97.9)^{A,D}
Free LYC (2)	1/8	88.6 ± 26.8 (56.3)^{A,B,C}	1/8	86.5 ± 27.4 (57.7)^{A,B,C,D}	0/8	90.6 ± 19.4 (37.85)^{A,B,C}	0/8	ND
LYC-PCL NC (2)	5/8	7.1 ± 2.7 (96.3)^{A,C}	8/8	3.3 ± 1.3 (98.3)^{A,C,D}	4/8	4.0 ± 1.7 (96.9)^{A}	6/8	5.3 ± 1.4 (94.9)^{A,D}
LYC-PLA-PEG NC (2)	ND	ND	8/8	1.5 ± 0.2 (99.3)^{A}	ND	ND	8/8	0.6 ± 0.1 (99.7)^{A}

[a] Female Swiss albino mice (20 to 25 g) were infected with 10^4 blood trypomastigotes/ml.
[b] Treatment schemes are shown in more detail in Table 1.
[c] Subpatent PAR/total, number of mice with a negative fresh blood examination during the acute phase of the infection divided by the total number of infected animals; MPP, maximum peak of parasitemia; ND, not determined.
[d] Untreated, in this table the untreated group represents all the control groups, including untreated, unloaded PCL NC, unloaded PLA-PEG, and control solution.
[e] The percent parasitemia reduction was based on untreated controls because there were no significant differences between the control groups (untreated, unloaded NC, and control solution). *P* values of <0.05 indicated significant differences and are denoted by superscript capital letters as follows: A, different from controls; B, different from LYC-PCL NC; C, different from BZ; and D, different from LYC-PLA-PEG).

LYC), and 96.3% (LYC-PCL NC) compared to the untreated control group (Table 2).

The percent animal survival for infected and treated groups is shown in Table 3. The untreated control group showed a mean survival time of 20 days. In groups treated with LYC-PCL NC and BZ, 100% survival was observed until 6 months posttreatment, when mice were necropsied. In animals treated with free LYC, the survival was 75% at the same period, and the other two mice (25%) survived 24 days. No significant difference ($P > 0.05$) in survival was observed between groups treated with the formulations of LYC-PCL NC, free LYC and BZ. Taking into consideration that the results of all control groups (untreated, unloaded NC, and excipients of i.v. solution) were similar ($P > 0.05$), Tables 2 and 3 show only the results of the untreated group.

TABLE 3 Efficacy of lychnopholide in mice experimentally infected with *T. cruzi* and treated with lychnopholide i.v. at 2.0 mg/kg/day using different schemes and formulations during the acute phase of the infection

Expt (strain)[a]	Expt group[b]	Parasitological cure (%)[c]	Survival (%)
I (CL strain)	Controls	0	0
	LYC-PCL NC	50	100
	Free LYC	0	75
	BZ (50 mg/kg, i.v.)	100	100
II (CL strain)	Controls	0	0
	LYC-PCL NC	100	100
	LYC-PLA-PEG NC	100	100
	Free LYC	0	50
	BZ (50 mg/kg, i.v.)	100	100
III (Y strain)	Controls	0	0
	LYC-PCL NC	50	100
	Free LYC	0	50
	BZ (50 mg/kg, i.v.)	0	100
IV (Y strain)	Controls	0	0
	LYC-PCL NC	62.5	100
	LYC-PLA-PEG NC	100	100
	BZ (50 mg/kg, i.v.)	75	100

[a] For more detail about the treatment schemes, see Table 1.
[b] Controls refers to untreated mice, unloaded NC, and control solution.
[c] Defined as a negative HC, PCR, or ELISA result.

The animal treated as described in the experiment II schedule (started at the patent period [the seventh day] and continued for 20 days) (Fig. 2B) with LYC-PCL NC, LYC-PLA-PEG NC, or BZ showed similar parasitemias ($P > 0.05$). Parasitemia decreased in the three groups of mice and became subpatent during and after treatment in 100% of these animals (Table 2). Treatment with free LYC reduced parasitemia, but the parasitemia was higher and significantly different compared to animals treated with LYC loaded in NC (PCL and PLA-PEG) or even compared to the BZ-treated group (Table 2). Control groups always showed higher patent parasitemia compared to all treated groups. Significant reductions in MPP ($P < 0.05$) of 99.2% for BZ, 57.7% for free LYC, 98.2% for LYC-PCL NC, and 99.30% for LYC-PLA-PEG NC were observed compared to the untreated control group (Table 2).

Animal survival is illustrated in Table 3. The untreated control groups showed a mean survival time of 27 days. All animals (100%) treated with the formulations LYC-NC (PCL and PLA-PEG) or BZ survived the acute phase and showed similar survival rates until be necropsied 6 months after treatment. In animals treated with free LYC, 50% (4/8) of the animals survived up to 34 days posttreatment.

(ii) Animals infected with Y strain (partially resistant to BZ). Animals treated, as described in experiment III schedule (started 24 h postinfection with Y strain for 10 days) with the formulation of LYC-PCL NC and BZ presented more intense and significant ($P < 0.05$) reduction of the parasitemia than control groups (Fig. 2C). No significant difference in the MPP (maximum peak of parasitemia) of mice treated with LYC-PCL NC and BZ. The parasitemia of animals treated with free LYC was higher than that of mice treated with LYC-PCL NC and BZ ($P < 0.05$) but was significantly lower than that observed in control groups.

Animal survival is shown in Table 3. The control group showed a mean survival time of 16 days, while 100% of the animals treated with BZ or LYC in NC formulations survived the acute phase up to necropsy at 6 months posttreatment. MPP was reduced in 94.3% by BZ, 37.8% by free LYC, and 96.9% by LYC-PCL NC relative to the untreated control group (Table 2). Animals treated as described in the experiment IV schedule (after patent parasitemia [4

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days for 20 days]) with LYC PCL NC or LYC-PLA-PEG NC exhibited a reduction in parasitemia similar to that observed in animals treated with BZ (Fig. 2D).

All animals (100%) treated with LYC-PLA-PEG NC, 75% of the animals treated with LY-PCL NC, and 87.5% of the animals treated with BZ displayed subpatent parasitemia (Table 2). Compared to the untreated control group, MPP was reduced in 99.55, 95.76, and 98.20% in mice treated with LYC-PCL NC, LYC-PLA-PEG NC, or BZ, respectively (Table 2). Free LYC was not tested in this experiment because it did not improve the survival of mice and was not effective against the Y strain compared to BZ in experiment III. The animal survival in experiment IV is shown in Table 3. The control group showed a mean survival time of 16 days, while 100% of the animals treated with NC or BZ formulations survived the acute phase up to necropsy at 6 months after treatment.

Treatment efficacy. (i) Animals infected with CL strain (susceptible to BZ). In experiment I (Table 3), the parasitological cure assessed by HC, PCR, and ELISA revealed that mice treated with either LYC-PCL NC or BZ formulations displayed cure rates of 50% (4/8) and 100% (8/8), respectively, whereas animals treated with free LYC were not cured. In experiment II (Table 3) mice treated with LYC-PLA-PEG NC, LYC-PCL NC, or BZ displayed 100% cure, whereas no parasitological cure was observed in animals treated with free LYC.

(ii) Animals infected with Y strain (partially resistant to BZ). In experiment III (Table 3), animals treated with LYC-PCL NC showed 50% (4/8) parasitological cure. No cure was observed in mice treated with BZ or free LYC. In experiment IV (Table 3), mice treated with LYC-PLA-PEG NC or LY-PCL NC showed parasitological cures of 100% (8/8) and 62.5% (5/8), respectively. Animals treated with BZ showed a 75% percent cure, and no cure was observed in any control group for all experiments.

DISCUSSION

There is an urgent need for the development of new compounds or new strategies to increase the effectiveness and reduce the toxicity of chemotherapy against Chagas disease (6). Developing such strategies was the main purpose of the present study, in which we investigated the efficacy of a sesquiterpenic lactone named lychnopholide of natural origin isolated from *Lychnophora trichocarpha*.

The tests for evaluating the therapeutic efficacy of LYC in mice infected with strains of *T. cruzi* were motivated by the *in vitro* results reported by Chiari et al. (34) and Oliveira et al. (14). Oliveira et al. conducted an *in vitro* study wherein the lactone lychnopholide, isolated from *L. trichocarpha*, inhibited 50% of the growth of blood trypomastigotes of CL and Y strains at a concentration of 150 μg/ml (0.42 μM) (14). In this first work reporting lychnopholide activity, the MIC of LYC was overestimated, because the 100% inhibitory concentration (IC_{100}) could not be precisely determined due to the LYC low solubility in the test conditions against bloodstream trypomastigotes (14). The IC_{50} was then approximately determined to be 150 μg/ml for the Y and CL strains, using a parasite density of 2×10^6 parasites/ml, even if LYC precipitation in test media had been detected. Considering the parasitemia curves of untreated animals infected with CL and Y strains (Fig. 2), we determined that LYC was able to reduce parasitemia to very low levels just after the first dose, even at LYC concentrations estimated to be approximately 25 μg/ml (2 mg/kg) in the total blood of mice immediately after i.v. administration, assuming the blood volume of mice to be 78 ml/kg (35). Table 2 shows that *in vivo* the maximum peaks of parasitemia (untreated animals) are around 2 million and 1.6 million trypomastigotes/ml of blood for the CL and Y strains, respectively, similar to the findings of the parasite density studies of Oliveira et al. (14). In fact, blood parasite exposure to LYC *in vivo* at these daily concentrations was enough to reduce parasitemia by 56 to 99% and by 37 to 99% in CL and Y strains, respectively, depending on the type of LYC formulation used and dosage schedule. If we take into consideration the complexity of *in vivo* animal model with high efficacy at 25 μg/ml, the *in vitro* MIC found in previous studies was probably overestimated. On the other hand, an increasing in dose could provide better efficacy results in CL and Y strains by increasing LYC blood exposure. Blood and tissue exposures were not identified here, as a limitation of the present work. However, pharmacokinetic and biodistribution experiments are being performed by our group to clarify this point. In the present study, highly stringent *in vivo* protocols were used to perform the LYC efficacy studies of two parasite strains with different sensitivities to BZ and NF according to methods of Filardi and Brener (8).

In the recent years, the use of nanocarriers loaded with substances of vegetal origin has attracted much attention because of their several advantages (36). The association of LYC with the polymeric colloidal carrier NC improved the water dispersion of this poorly soluble substance and allowed its safe i.v. administration. NC provided also a modified LYC release profile (13, 19). LYC NC formulations were prepared by interfacial deposition of preformed polymers, followed by solvent displacement (13, 19), as first reported by Fessi et al. (21). This method is notably simple and provides homogeneous dispersion of colloidal particles containing lipophilic drugs suitable for parenteral administration (37). Compared to the parasitic disease leishmaniasis, only a small number of studies have reported the treatment of *T. cruzi* experimental infection using nanocarriers (38–41).

Under our experimental conditions, all animals survived the treatment without signs of general toxicity related to LYC administration. Transitory toxicity with the BZ i.v. formulation was observed at the injection site in mice, as observed in humans with the antimalarial drug halofantrine i.v. solution in the same excipients (42). In contrast, no signs of toxicity or abnormal behavior were observed when free LYC or LYC-NC (LYC-PCL NC or LYC-PLA-PEG NC) was injected into mice. Furthermore, these NC preparations were stable for 6 months at 4°C.

Surprisingly, BZ in solution at a dose of 50 mg/kg/day cured 75% of the mice, more than when administered by oral route at a dose of 100 mg/kg/day where the index of cure is 50%. This different result could be attributed to the difference in the administration schedule, which could not be compared in different doses and administration routes. The reason for this difference in efficacy, observed with a nitroheterocyclic compound (BZ) in our experiments, was not explained and has not already been determined in other circumstances (7). However, these results may be related to unfavorable pharmacokinetic properties of BZ, such as a relatively short half-life and limited tissue penetration (7, 11, 20) in the conventional dosage form.

Analysis and comparison of the results of treated animals infected with the CL strain (experiment I) showed a reduction in parasitemia and an increase in animal survival among animals treated with free LYC or LYC-PCL NC compared to control

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groups. These effects, however, were not better than those obtained with BZ. A parasitological cure of 50% in animals treated with LYC-PCL NC and of 100% in animals treated with BZ were obtained. The better efficacy of BZ in this experiment may be attributed to the high sensitivity (100%) of the CL strain to this drug (8) and to the short 10-day period of treatment compared to 20 days normally used in experimentally infected mice treated with BZ and NF. In this dosage schedule, blood exposure to LYC was probably not enough to eliminate blood and tissue parasites (pseudocysts), even in a prolonged drug release NC formulation.

On the other hand, in experiment II LYC-PCL NC, LYC-PLA-PEG NC, and BZ showed 100% subpatent parasitemia, full survival, and complete parasitological cure, whereas animals treated with free LYC were not cured, when the treatment started at day 7 after infection (prepatent period) and continued for 20 days. It is important to highlight that in this case better therapeutic effects were observed, as measured by the higher index of parasitological cure and survival compared to the treatment 24 h after inoculation. These results are in agreement with the findings of Urbina et al. (43) and may indicate that the etiological treatment is more effective, particularly at the moment that a higher number of blood trypomastigotes are exposed to drug, and immediately after the rupture of higher number of pseudocysts (in the prepatent period), besides a longer period of treatment (20 days). Under these conditions, drug would be more effective than in the beginning of infection (24 h after inoculation) when the number of pseudocysts is lower and the majority of the parasites are still under amastigote forms within host cells (44), as in the case of experiment I. These same effects were observed in experiments conducted with the Y strain, which is partially resistant to BZ. The efficacy of LYC NC compared to free LYC, particularly when loaded in sterically stabilized PLA-PEG NC, may be attributed to the ability of the nanocarriers to maintain the LYC release for longer times in biological media compared to free LYC, as we recently reported (13, 19). LYC diffusion from polymeric NC to biological media was reduced in rate by 20-fold compared to apparent LYC dissolution in the same media. The nanocapsule property of controlling LYC release *in vitro* could be the main factor responsible for LYC's greater parasitological effects *in* vivo, probably by improving the time of body exposure to the drug. Consequently, the results of the efficacy of LYC on experimental CD are probably related to the LYC association with these nanometrical carriers (13).

In experiment III, mice were infected with the BZ partially resistant Y strain and treated 24 h after infection for 10 days. The number of animals with subpatent parasitemia was higher when treated with LYC-PCL NC (50%) relative to BZ (0%) and free LYC (0%). Parasitological cure was obtained only with LYC-PCL NC (50%). One of the possible reasons for the therapeutic failure of free LYC in experiments I and III may be due to the highly stringent protocols used, in which treatment periods of 10 days instead of 20 days were used (8, 29).

In experiment IV, mice were infected with the Y strain but underwent treatment beginning at the fourth day after infection for 20 days. All animals (100%) treated with LYC-PLA-PEG NC formulation showed subpatent parasitemia and showed the highest reduction in MPP compared to the CL strain (experiment II). Again, an evident improvement of treatment efficacy (100%) and parasitological cure was observed with LYC-PLA-PEG NC compared to BZ (75%) and LYC-PCL NC (62.5%). Free LYC was not used for treatment in this experiment because previous experiments showed that the therapeutic efficacy of NC formulations was always better.

In all experiments, animals treated with free LYC showed higher peaks of parasitemia, a significant number of animals showed patent parasitemia, and 50% did not survive. None of the animals receiving free LYC exhibited parasitological cure compared to animals treated with LYC loaded in NC formulations.

Taken together, these results clearly demonstrated that LYC-NC showed therapeutic effects leading to the reduction of parasitemia, the improvement of animal survival in experimental *T. cruzi* infections, and higher rates of cure in mice infected with a *T. cruzi* strain partially resistant to BZ and NF (8), even when used in very low doses (2.0 mg/kg/day) compared to BZ (50 mg/kg/day) i.v. The mechanism of sesquiterpene lactones action against *T. cruzi*, including LYC, is still unknown. However, the presence of alkylant groups in the LYC molecule could be responsible for these activities (14, 16, 17, 18).

In general, LYC-PLA-PEG NC (experiments II and IV) showed higher efficacy than LYC-PCL NC, except in animals infected with sensitive *T. cruzi* strain as CL. Both types of NC were important to maintain LYC efficacy in sensitive and in partially resistant strains. However, PLA-PEG NC showed greater efficacy. This result confirms the previously reported long-circulating properties of PLA-PEG NC (24). PLA-PEG NC have already improved the antiprotozoal efficacy of halofantrine by increasing the blood circulation times of this drug (37). Halofantrine is also a very lipophilic molecule, and PLA-PEG NC was able to modify its efficacy in experimental model of malaria (37). The effect of LYC was probably improved by the NC long circulating property in blood, resulting in pharmacokinetic profile modification. It may facilitate the contact of the active substance with the target parasite. In LYC-PLA-PEG NC formulations, the NC surface is modified by PEG. PEG chains retard the rapid removal of NC from the bloodstream by macrophages, consequently prolonging the drug-associated plasma half-life (24, 25, 37). Thus, these nanoparticles have the chance to extravasate to tissues infected by *T. cruzi*, particularly at inflammatory sites (26). LYC pharmacokinetics are under investigation by our group.

Our results showed that treatment with LYC-NC was able to produce higher levels of parasitological cure in infected mice. This result was particularly striking in studies with a partially resistant *T. cruzi* strain (Y) during the acute phase of experimental Chagas infection. The results for the efficacy of LYC in the present study are better than the current investigational treatments with several ergosterol biosynthesis inhibitors, particularly the inhibitors of C14-α-demethylase (CYP51), such as posaconazole and ravuconazole, under similar experimental conditions. The ergosterol biosynthesis inhibitor drugs are currently the most promising alternative drugs studied in mice (43–46) and dogs (47, 48). However, they are extremely expensive for the poor population normally affected by CD in Latin American countries (49). It is also important to note that a potent drug may completely lose its efficacy when inappropriately delivered or even when evaluated in an inadequate treatment scheme. Thus, one of the main contributions of the present study is that LYC represents an important alternative for treatment of CD that should be further evaluated in a BZ-resistant strain. At dose of 2.0 mg/kg/day, LYC associated with NC is a potent, fast-acting drug that is useful in short treatment regimens.

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Finally, we demonstrated here for the first time the *in vivo* efficacy of LYC against *T. cruzi* infection, as well as the important contribution of nanotechnology to the improvement of LYC therapeutic effects for treating Chagas disease. Our results suggest that the use of an isolated substance of vegetal origin encapsulated in polymeric NC formulations may offer promising possibilities for the treatment of experimental CD in mice.

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Different therapeutic outcomes of benznidazole and VNI treatment in distinct genders of mouse experimental models of *Trypanosoma cruzi* infection

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Running title: Effects of bz and VNI on T. cruzi infected-mice