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Novas abordagens na pesquisa de alvos terapêuticos frente à infecção por *Trypanosoma cruzi*

FRANCISCA HILDEMAGNA GUEDES DA SILVA

Rio de Janeiro

Maio de 2016

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Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Parasitária.

Orientadora: Dra. Maria de Nazaré Correia Soeiro

RIO DE JANEIRO

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A vida da gente pode ser melhor

Ter o Chagas não é o fim de tudo
Pois saiba que a vida da gente pode ser melhor
A esperança é o motor que move o mundo
Saúde é o mais importante pode acreditar
E existe um lugar
Que alguém pode te ajudar
Vamos juntos sem medo que tem muito chão pra caminhar
O medo não, não pode não
O medo não vai te dar a vida
Seu coração batendo são
Que você merece ser feliz
O medo não, não pode não
O medo não vai te dar a vida
Seu coração batendo são
Que você merece ser feliz.

Compositor: Luna Cohen

Dedico este trabalho...

À minha mamãe Maria Socorro da Silva (*in memoriam*) por todo amor e por me ensinar que o estudo é o caminho da liberdade.

Estarás sempre comigo em pensamento e coração.

Agradecimentos

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"É preciso amor pra poder pulsar.
É preciso paz pra poder sorrir.
É preciso a chuva para florir..."
(Almir Sater e Renato Teixeira)

À Deus e à Virgem Maria pelas oportunidades e por toda luz durante a minha caminhada.

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À minha família, meu pai Heronilson, meus irmãos Hildemárcio e Hildemarques e cunhada Francinara pelo apoio e incentivo.

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Resumo

A doença de Chagas (DC), endêmica em 21 países da América Latina, é a principal responsável por mortes no tocante a cardiopatias infecciosas. A terapia atual é insatisfatória devido à limitada atividade na fase crônica, alta toxicidade, e ocorrência de isolados naturalmente resistentes aos nitroderivados de referência. Nesse sentido, dentre os principais desafios a serem enfrentados destacam-se a padronização de modelos experimentais que possam melhor predizer a ação de novos candidatos terapêuticos, assim como a urgente identificação de novas terapias alternativas mais seletivas e promissoras. Estas temáticas, portanto, representaram o objeto da presente tese. Assim, nossos estudos *in vitro* e *in vivo* foram divididos em dois blocos. O primeiro explorando a interferência de diferentes modelos experimentais sobre a ação tripanocida de novos compostos e do medicamento de referência e no segundo bloco, investigando a eficácia (em esquemas de monoterapia e em combinação) de duas diferentes classes de compostos: (a) compostos heterocíclicos da classe das arilimidamidas (AIA), moléculas aromáticas que interagem com fenda menor do DNA e, (b) inibidores imidazólicos da biossíntese de esteróis (CYP51), como VNI e VFV. Dados *in vitro* revelaram que das AIAs estudadas, a 35DAP073 (derivado *m*-terfenil) foi a mais promissora exibindo atividade (EC_{50}/24 h) na faixa de 40 nM e índice de seletividade (IS) de 480. Esta AIA foi ainda capaz de estimular a gênese de corpúsculos lipídicos embora não tenhamos observado correlação entre este achado e ação tripanocida. 35DAP073 (5-20 mg/kg/dia, via intraperitoneal) reduziu de modo dose-dependente (46-96%) a parasitemia quando administrada por dois dias (uma dose na positivação e a outra no pico da parasitemia). Apesar de suprimir completamente a infecção e conferir 100% sobrevida dos animais, a dose de 10 mg/kg/dia 35DAP073 levou a efeitos neurológicos reversíveis após 10 dias de administração. Os estudos *in vitro* com o imidazol VFV também revelaram forte ação sobre formas intracelulares (EC_{50}/48h 380 nM e IS >263), baixa toxicidade em modelos de toxicidade aguda (NOAEL 200 mg/kg) sendo capaz de suprimir completamente a parasitemia e prover 100% sobrevida em doses de 25 mg/kg (via oral, duas vezes ao dia). Porem ambos compostos (35DAP073 e VFV) não revelaram índices de cura parasitológica quando testados em sistemas de monoterapia. Nossos resultados revelam ainda importantes diferenças relacionadas ao curso da infecção e impacto da terapia experimental a depender do: (a) gênero do animal, (b) início da administração do composto teste e o seu tempo de tratamento, além da (c) natureza do veículo utilizado para diluir o mesmo. De modo geral, fêmeas são menos vulneráveis que machos à infecção pelo *T. cruzi* e mais sensíveis a terapia, a maior solubilidade de um composto resulta na sua melhor biodisponibilidade e potencial sucesso terapêutico, e o tratamento 24 h após infecção (esquema preventivo) resulta em maiores índices de redução de parasitismo, porém é menos preditivo por exibirem uma fraca correlação com as condições reais de infecção humana. Outro importante aspecto refere-se ao uso de protocolos de imunossupressão pós-terapia visando promover a sensibilidade da detecção do parasito por microscopia ótica e/ou PCR. O uso da terapia combinada corroborou dados da literatura sobre aumento da potência de tratamentos quando se atinge distintos alvos pelo uso de associação de distintos agentes, permitindo ainda redução de doses e tempos de exposição, menor toxicidade, além de prevenir ocorrência de resistência a fármacos. Nossos dados revelaram importantes reduções na carga parasitária (Bz + 35DAP073) e mesmo no aumento nos níveis de cura parasitológica (Bz + VFV) utilizando terapia combinada.

Abstract

Chagas disease (CD), endemic in 21 countries of the Latin America, is the main cause of deaths related to infectious cardiomyopathy. Current therapy is unsatisfactory due to the limited activity, during the later chronic phase, high toxicity, and the occurrence of naturally resistant parasite strain to nitroderivatives. In this sense, among the main challenges to be faced we found the standardization of experimental models that can better predict the action of new therapeutic candidates, and the urgent identification of novel more selective and promising alternative therapies. These subjects represent the aim of the present thesis. Thus, our *in vitro* and *in vivo* studies were spliced into two blocks. The first one exploring the interference of different experimental models on the trypanocidal action of new compounds and the second block, through the analysis of the efficacy (monotherapy and combination regimens) of two different classes of compounds: (a) heterocyclic compounds belonging to arylimidamide (AIA) class, which are aromatic molecules that interact with the minor groove of the DNA and (b) imidazole inhibitors of the sterol biosynthesis (towards the CYP51 enzyme) named VNI and VFV. *In vitro* data revealed that among the studied AIAs, the most active was *m*-terphenyl bis-AIA 35DAP073 exhibiting high trypanocidal activity (EC_{50}/24 h in the range of 40 nM) and selectivity index (SI) of 480. This AIA was able to trigger the generation of intracellular lipid bodies, although no correlation between this result and trypanocidal effect was determined. 35DAP073 (5-20 mg/kg/day, i.p.) reduced in a dose-dependent manner (46-96%) the parasitemia peak when administered for only two days (one dose at the parasitemia onset and the second at the parasitemia peak). Although completely suppressing the infection and providing 100% of animal survival, 10mg/kg 35DAP073 exhibit reversible neurological effects after 10 days of administration. *In vitro* findings using the imidazole VFV also showed a strong action upon intracellular forms (EC_{50}/48 h 380 nM and IS> 263), low toxicity on mouse acute toxicity models (NOAEL 200 mg/kg) being able to suppress the parasitemia and reach 100% mice survival at doses of 25 mg/kg (orally, twice a day). However both compounds (35DAP073 and VFV) given as monotherapy did not result in high rates of parasitological cure. Our results also demonstrated important differences related to the course of infection and impact of the experimental therapy according to the (a) the animal's gender, (b) the starting period of drug administration and exposure time, as well as (c) the nature of the vehicle used to dilute the tested molecules. Female mice were less vulnerable than males to *T. cruzi* infection and more sensitive to the therapy, higher solubibity of a compounds leads to higher bioavailability enabling higher therapeutic success. In addition treatment 24 h after infection (preventive scheme) resulted in higher levels of reduction of the parasitism but this protocol is less predictive since there is a weaker correlation with the real conditions of human infection. Another important aspect is related to the use of immunosuppression post-therapy protocols to promote the sensitivity of the parasite detection by light microscopy and/or PCR. Our results about the use of combination therapy corroborate the literature findings regarding this promising approach reaching different targets and allowing the use of reduced doses and shorter periosds of therapy (and thus less toxicity), also avoiding parasite resistance. Our data revealed significant reductions in parasite load (Bz + 35DAP073) and even the increase in parasitological cure rates (Bz + VFV) when this combination approach was used.

Índice

1. Introdução

1.1 *Trypanosoma cruzi*

Há mais de cem anos atrás, Carlos Chagas, cientista e médico sanitarista, publicou um estudo pioneiro na história da parasitologia (Chagas, 1909). Durante uma campanha sanitária contra a malária na cidade de Lassance, Minas Gerais, identificou pela primeira vez a associação de um inseto hematófago com indivíduos doentes que viviam em habitações pobres. No tubo digestório deste inseto, Carlos Chagas encontrou parasitos flagelados, que por fim denominou de *Trypanosoma cruzi,* em homenagem ao seu mentor, Oswaldo Cruz. Em curto período de tempo, o ilustre cientista descreveu: i) o agente etiológico e seu ciclo de vida; ii) hospedeiro invertebrado (vetor) responsável pela transmissão; iii) hospedeiro vertebrado; iv) sinais e sintomas da doença em humanos e ainda v) o provável impacto social da doença (Chagas, 1909; Coura, 2009).

O protozoário hemoflagelado *T. cruzi* pertence ao filo Sarcomastigophora, subfilo Mastigophora, ordem Kinetoplastida e família Trypanosomatidae. Sob condições naturais, este parasito é capaz de infectar mais de 140 espécies de mamíferos, incluindo seres humanos, animais domésticos e silvestres. O vetor conhecido popularmente como barbeiro, pertence ao filo Arthropoda, subfilo Hexapoda, ordem Hemiptera, família Reduviidae e subfamília Triatominae, incluindo: *Triatoma infestans, Panstrongylus megistus, Rhodnius prolixus, Triatoma brasiliensis, Triatoma dimidiata, Triatoma pseudomaculata, Triatoma sordida, Triatoma maculata, Panstrongylus geniculatus, Rhodnius ecuatoriensis* e *Rhodnius pallescens*, distribuídos em distintas áreas da América Latina (Noireau et al., 2009; Coura, 2015). Além da transmissão primária pelo vetor, outros mecanismos de incluem outras vias como a transfusional e de transplante de órgãos, oral e congênita (Altclas et al., 2008; Shikanai-Yasuda & Carvalho, 2012; Carlier et al., 2011).

Sendo o *T. cruzi* um parasito intracelular obrigatório, o ciclo de vida (Figura 1) envolve o repasto sanguíneo de um triatomíneo num indivíduo infectado, através do qual o inseto ingere as formas tripomastigotas sanguíneas. Uma vez no intestino médio, tripomastigotas se diferenciam para as formas epimastigotas, que se multiplicam e, na extremidade posterior do trato digestório, se diferenciam

em tripomastigotas metacíclicos, num processo denominado metaciclogênese, sendo estas formas eliminadas nas fezes e na urina do inseto. Ao se alimentar novamente, o triatomíneo libera as formas infectantes, que são introduzidas mecanicamente pelo ato de coçar o local da picada ou diretamente em áreas de mucosa. No hospedeiro vertebrado, o parasito, inicialmente, infecta células localizadas no sítio de invasão. Intracelularmente, as formas tripomastigotas passam por mudanças morfológicas e bioquímicas, resultando na diferenciação em amastigotas, que se replicam por divisão binária. A seguir, diferenciam-se novamente em tripomastigotas que são as principais formas liberadas durante a ruptura da membrana plasmática da célula hospedeira. Os parasitos podem então infectar células vizinhas e mais distantes (difundidos através dos sistemas sanguíneos e linfáticos), ou ainda, serem novamente ingeridos pelo inseto vetor através do repasto sanguíneo, completando o ciclo (Lima et al., 2010).

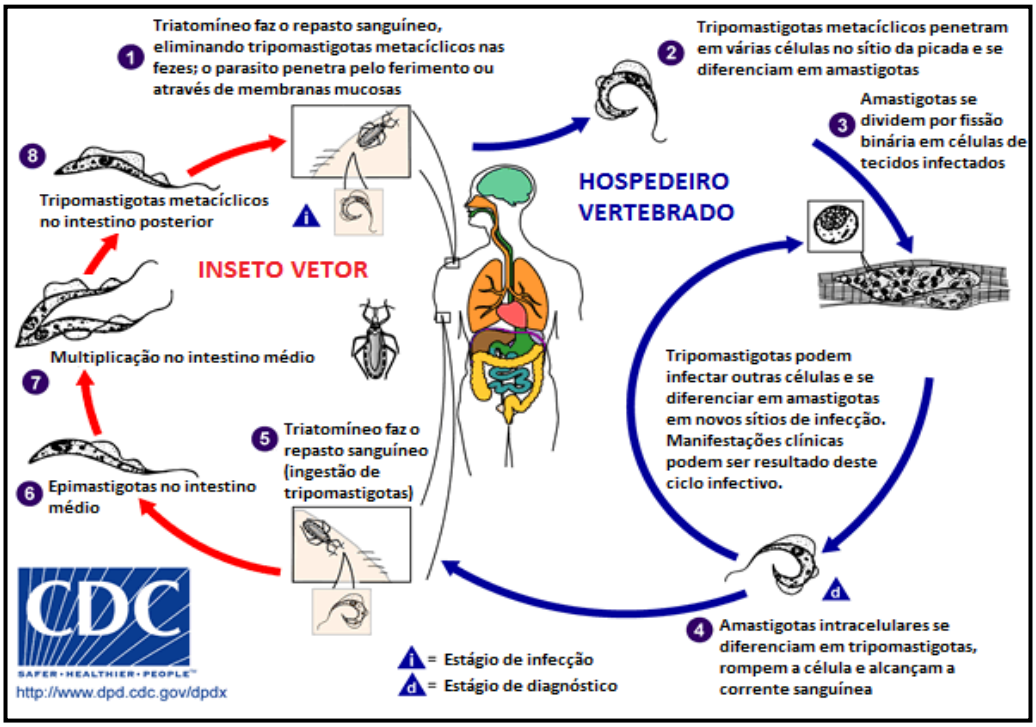


Figura 1. Esquema ilustrando o ciclo de vida do *T. cruzi*. (Fonte: adaptado de *CDC – Centers for Disease Control and Prevention*; disponível em http://www.cdc. gov/parasites/chagas/biology.html).

1.2 A doença de Chagas

A Tripanosomíase Americana ou doença de Chagas (DC) representa um sério problema de saúde pública, destacando-se entre as principais causas de cardiopatia infecciosa em todo o mundo, em especial nos países da América Latina (endêmica em 21 países), mas também relevante em outras regiões não endêmicas (como Europa, América do Norte e Ásia) decorrente da migração de pessoas infectadas para estas áreas. Somente na América Latina, estima-se que 5,7 - 9,4 milhões de indivíduos estejam infectados e 65 milhões estejam sob risco de infecção, com cerca de 10 mil mortes associadas a esta doença todos os anos (WHO, 2015; Pérez-Molina et al., 2015; Pecoul et al., 2016).

Nos indivíduos infectados pelo *T. cruzi*, a DC se inicia com uma fase aguda (duração de cerca de dois meses após a infecção), caracterizada pela presença de parasitos em diferentes tecidos e no sangue (Figura 2). As lesões na fase aguda são principalmente associadas à ruptura direta das células hospedeiras pelo parasito, somada a uma intensa reação inflamatória local (ninhos de parasito nos tecidos).

Na fase aguda a maioria dos pacientes apresenta sintomas leves e inespecíficos (WHO, 2002). Além de febre, os sintomas podem incluir linfoadenopatia, esplenomegalia e quando a inoculação do parasito for próxima à conjuntiva, o indivíduo pode apresentar um inchaço unilateral, indolor e bipalpebral, conhecido como sinal de Romaña e que pode persistir por algumas semanas (Prata, 2001).

No curso da fase aguda, os portadores imunocompetentes desenvolvem uma resposta imune adquirida, resultando no controle da parasitemia e do parasitismo tecidual, e evolução para a fase crônica indeterminada, sendo assintomática e com escasso parasitismo sanguíneo e tecidual (Figura 2). (Junqueira et al., 2010; Rassi et al., 2010; Machado et al., 2012; Dutra et al., 2014). Entretanto, após anos ou décadas, aproximadamente 30-40% dos portadores desenvolvem a forma crônica sintomática: até 30% com distúrbios cardíacos, até 10% exibem alterações digestivas (tipicamente alargamento do esôfago ou do cólon), sendo ainda frequentes as alterações neurológicas e mistas (cardíacas e digestivas). A progressão da patologia resulta na perda da funcionalidade dos órgãos acometidos (coração e sistema digestório) e morte,

no caso da forma crônica cardíaca associada a morte súbita ou insuficiência cardíaca (WHO, 2015).

A patogênese da DC crônica é considerada multifatorial, com interações complexas entre patógeno-hospedeiro, persistência sub-patente do parasito, distúrbios e lesões microvasculares neurogênicas que produzem disautonomia, sendo estes somente alguns dos fatores descritos no processo patogênico (Marin-Neto et al., 2007). Na fase crônica apesar do escasso parasitismo tissular e sanguíneo, ocorrem inflamação e resposta imunológica desbalanceadas e agressivas que contribuem para evolução progressiva dos distúrbios estruturais, e que associadas a outras alterações (ex. de microcirculação), resultam na perda da funcionalidade cardíaca (Tarleton, 2001; Machado et al., 2012; Dutra et al., 2014).

Ainda muitas questões em relação à patogênese da DC carecem de melhor compreensão. Assim, dentre os principais desafios a serem enfrentados destacam-se: i) avanço no conhecimento dos fatores e mecanismos envolvidos na invasão de células hospedeiras pelo *T. cruzi* e sua persistência nos tecidos afetados, sendo o coração um dos principais órgãos acometido nas fases aguda e crônica, ii) identificação dos processos associados à imunopatogênese, remodelamento cardíaco e progressão da cardiomiopatia chagásica crônica, iii) tratamento eficaz e seguro para ambas as fases desta doença, acessível à todos os portadores chagásicos (Junqueira et al., 2010; Urbina 2010; Dutra et al., 2014) e iv) padronização de modelos experimentais que melhor possam predizer ação de novos candidatos a fármacos (Chatelain & Konar, 2015).

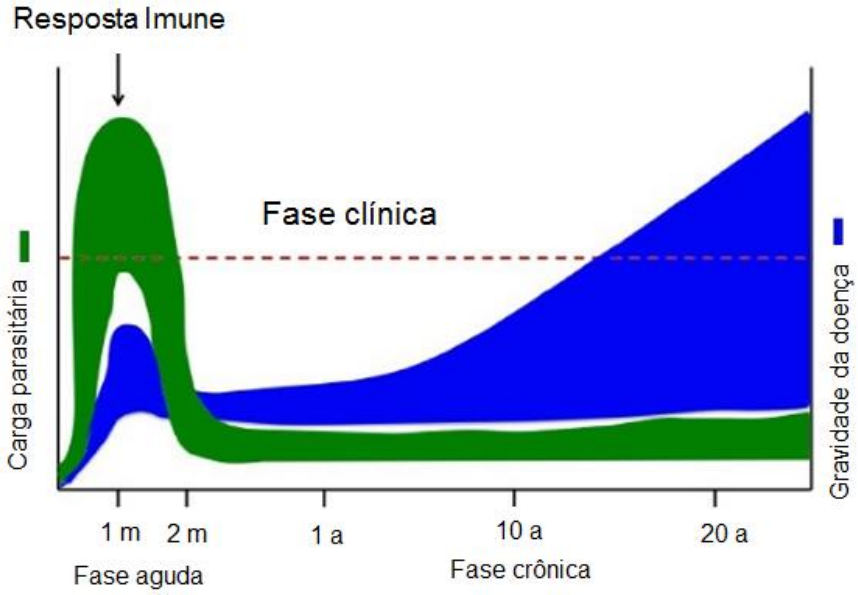


Figura 2. Progressão da doença de Chagas. (Adaptado de Chatelain and Konar, 2015)

1.3 Tratamento da doença de Chagas

O tratamento da DC objetiva a erradicação da infecção, a prevenção do aparecimento de lesões e do o agravamento e progressão de lesões já presentes. A terapia atual, baseia-se em dois nitroderivados, Nifurtimox (Nf) (3-metil-4-(5'-nitrofurfurilidenoamina tetrahidro-4H-1,4-tiazina-1,1-dióxido) e Benznidazol (Bz) (N-benzil-2-nitroimidazol acetamida) (Figura 3), ambos introduzidos na clínica médica há mais de 40 anos (Urbina, 2009).

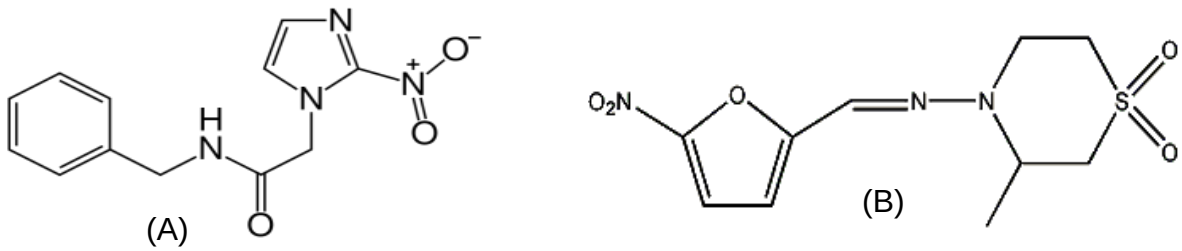


Figura 3. Benzonidazol (A) e Nifurtimox (B), fármacos disponíveis para o tratamento etiológico da doença de Chagas.

O Nf apresenta efeito tripanocida associado ao estresse oxidativo gerado pela transformação de um radical nitroaniônico (via nitrorredutase) por geração de espécies reativas de oxigênio e por peroxidação lipídica (Do Campo & Stoppani, 1979), sendo o *T.cruzi* parcialmente deficiente na detoxificação destes radicais (DoCampo & Moreno, 1979; Fairlamb et al., 1985; Krauth-Siegel et al., 2007). Entretanto, Boiani e colaboradores (2010) observaram que o ciclo redox é apenas detectado em concentrações elevadas de Nf (> 400 µM), enquanto a concentração tripanocida é de 5 µM, sugerindo que aumento do estresse oxidativo não é o principal mecanismo de ação deste fármaco. Ademais, estudos têm descrito outro possível mecanismo envolvendo a ativação redutora de uma nitro-redutase do tipo I em tripanosomatídeos. Em *Trypanosoma brucei* a superexpressão desta enzima induz aumento de susceptibilidade ao Nf (Hall et al., 2011; Cerecetto & González, 2011).

O Bz apresenta como provável mecanismo de ação a inibição da síntese protéica e da cadeia respiratória. Alguns autores sugerem que o efeito tripanocida do Bz está relacionado à geração de metabólitos intermediários (derivados de nitroredução) capazes de interagir com componentes celulares, como DNA, proteínas e lipídeos (Diaz de Toranzo et al., 1988). Igualmente ao Nf, foi caracterizada uma nitrorredutase tipo I insensível a oxigênio responsável pela ativação de Bz no parasito, utilizando NADH como cofator, revelando que a atividade desta enzima conduz à formação de metabólitos altamente reativos. (Wilkinson & Kelly, 2009; Hall & Wilkinson, 2012). Além disto, estudo recente sugere que Bz oxida preferencialmente nucleotídeos e a extensa incorporação destes durante a replicação de DNA conduz potencialmente a fragmentações letais na dupla fita de DNA do *T. cruzi* (Rajão et al., 2014). Ademais, Trochine e colaboradores (2014) observaram que ocorre uma redução nos níveis de vários tióis, incluindo cisteína e tripanotiona após o tratamento do *T. cruzi* com Bz, e essa interferência no metabolismo tiol contribui para a ação tripanocida.

O tratamento com esses fármacos tem sido considerado insatisfatório, principalmente devido à limitada atividade em especial, na fase crônica tardia, alta toxicidade (incluindo hipersensibilidade cutânea, dermatites, intolerância digestiva, depressão da medula óssea, polineuropatia periférica, hepatotoxicidade, anorexia, perda de peso, sonolência ou excitabilidade) além da ocorrência de isolados de parasitos resistentes a ambos nitroderivados (Urbina, 2009; Viotti et al., 2009; Olmo et al., 2015). Esta resistência está relacionada pelo menos em parte, a expressão diferencial de transportadores de ATP, sendo presente em maiores níveis em cepas do parasito, naturalmente resistentes a Bz (Zingales, et al. 2015).

O paradigma científico que relacionava a patogênese da DC crônica somente a geração de uma patologia autoimune foi mudado frente à detecção da presença do parasito (em necropsias de portadores cardíacos crônicos) através de ferramentas moleculares mais sensíveis resultando em recomendações quanto a terapia etiológica para todos portadores agudos e crônicos indeterminados ou ainda com manifestações cardíacas de média a moderada (Viotti et al., 2014). No ensaio clinico com posaconazol analisando em paralelo os portadores tratados com Bz observou-se que Bz foi mais eficaz (Molina et al., 2014). Recentemente, foi também finalizado um estudo multicêntrico, randomizado e prospectivo em portadores chagásicos crônicos

(BENEFIT), com o objetivo de avaliar os efeitos do Bz sobre a progressão da cardiomiopatia chagásica. Neste estudo, os portadores foram tratados por até 80 dias, e acompanhados por uma média de 5,4 anos. Como acima relatado, os achados revelaram que a terapia com Bz reduziu a carga parasitária, mas não inibiu a frequência e evolução dos danos cardíacos (Morillo et al. 2015).

Assim, se faz urgente o desenvolvimento de agentes tripanocidas mais eficazes e seguros, fundamental para o tratamento do portador e o manejo clínico da DC. Como consenso entre instituições de pesquisa e agências não governamentais envolvidas na busca de novos medicamentos para DC (como DNDi), o fármaco ideal deve apresentar várias características incluídas no "Target Product Profile" (TPP, Chatelein, 2014), incluindo: i) indução de cura parasitológica nas fases aguda e crônica e sobre as diferentes cepas do parasito; ii) eficácia em uma ou poucas doses (≤ 60 dias); iii) apresentar baixo custo; iv) não ter efeitos colaterais graves/irreversíveis ou teratogênicos; v) não induzir resistência, e vi) aplicável para uso adulto e pediátrico, entre outras (DNDi, 2015). Buckner & Navabi (2010) ressaltam que, além de ativo e seguro, é essencial a baixa interação do composto-candidato com enzimas do complexo citocromo P450, evitando, desta forma, sinergismos ou antagonismos com outros fármacos, como antiarrítmicos e anticoagulantes.

Nenhum novo medicamento para DC foi licenciado desde a introdução dos nitroderivados de referência nos anos 1960-70. Infelizmente, apesar dos dados promissores *in vitro* e *in vivo* com inibidores de biossíntese de lipídeos (ex. posaconazol e ravuconazol) (Urbina, 2009), estudos clínicos recentemente conduzidos revelaram níveis superiores de falha terapêutica destes inibidores em relação ao Bz (Molina et al., 2015), o que denota a relevância de uso de ensaios pré-clínicos mais reprodutíveis e de aplicação de modelos experimentais que possam melhor traduzir o translado entre resultados de dados pré-clínicos e clínicos (Chatelein & Konar, 2015).

Apesar do forte avanço das ferramentas metodológicas para a descoberta de fármacos, há ainda, uma carência de recursos para identificação de novos medicamentospara o tratamento do grupo das 17 patologias negligenciadas do qual a DC faz parte. Estima-se que do total de portadores chagásicos diagnosticados com DC, apenas 1% têm acesso ao tratamento, evidenciando a necessidade urgente de ampliar o acesso e acelerar o desenvolvimento de medicamentos realmente inovadores além de prover melhor acesso e

disponibilidade dos produtos já disponíveis como o Bz (DNDi, 2015; Pecoul et al., 2016). Assim, estratégias alternativas para o desenvolvimento terapêutico precisam ser consideradas e desenvolvidas urgentemente, assim como análise e padronização de metodologias e modelos experimentais *in vitro* e *in vivo* que melhor reproduzam a condição de infecção humana (Chatelain & Konar, 2015).

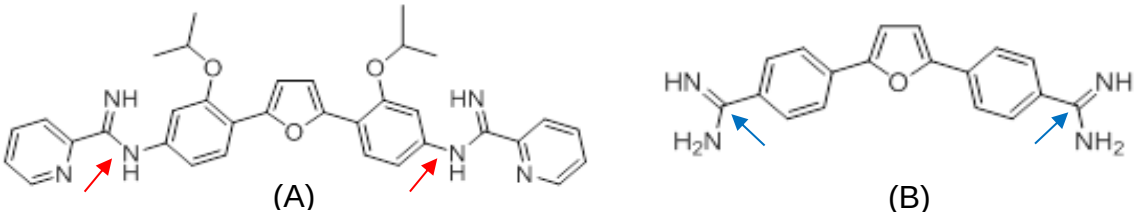
1.4 Quimioterapia na infecção experimental pelo *Trypanosoma cruzi*

Diferentes estratégias conduzidas a partir de diferentes abordagens têm sido amplamente utilizadas visando alternativas terapêuticas para DC, incluindo i) aplicação de novos regimes e formulações dos fármacos atuais (Davanço et al., 2016), ii) ensaios *in silico* e fenotípicos (colorimétricos, flurescentes e luminescentes), em média e larga escala através de de *high-throughput screening* (HTS) e *high content screening* (HCS) (neste caso para com sistemas de imagem *in vitro* e *in vivo*) a partir de bibliotecas de entidades públicas e privadas (Buckner et al., 1996; Allonso-Padilla & Rodriguez, 2014), iii) reposicionamento de fármacos já licenciados para outras patologias e que apresentem alvos em comum (Kaiser et al., 2015; Alberca et al., 2016), iv) síntese e triagem de inibidores de alvos ou via metabólicas seletivas ao parasito (Tagoe et al., 2015; Keenan & Chaplin, 2015) e v) terapia combinada, tem sido também aplicado em várias patologias, possibilitando utilização de pelo menos dois compostos que podem agir sobre diferentes alvos reduzindo concentrações dos fármacos e número de doses contribuindo para amenizar efeitos tóxicos, e minimizando o risco de desenvolvimento de resistência a drogas (Coura, 2009; Urbina, 2009; Bahia et al., 2014; Urbina, 2014).

Entre a variedade de classes de compostos com ação antiparasitária e e fármacos já utilizados na clínica médica contra outras patologias que compartilhem alvos em comum ao *T. cruzi*, destacam-se os inibidores de biossíntese do ergosterol (Urbina, 2009) e moléculas heterocíclicas amidínicas derivadas da pentamidina (Soeiro et al., 2013). Assim, essas duas classes foram estudadas na presente tese, ambas com base no desenvolvimento racional de novas drogas para a DC.

1.4.1 Amidinas aromáticas

Pentamidina (King et al., 1937), sintetizada em 1937 como análogo de insulina, e a furamidina (DB75) são potentes agentes sobre diferentes patógenos incluindo tripanosomatideos, como o *Trypanosoma brucei* e *Leishmania* (Soeiro et al., 2013). Estas moléculas da classe das diamidinas aromáticas (DAs), são compostos dicatiônicos que apresentam forte ligação não covalente e não intercalante à fenda menor do DNA (e ao kDNA em cinetoplastídeos) em regiões ricas em AT (Wilson et al., 2008; Soeiro et al., 2009; Soeiro & De Castro, 2011, Soeiro et al., 2013). A natureza dicatiônica das DAs permite o acúmulo na mitocôndria dos cinetoplastídeos podendo desencadear um colapso do potencial da membrana interna mitocondrial, resultando assim na permeabilização desta organela e deflagrando a morte do parasito (Soeiro et al., 2008). Estudos anteriores mostraram que dentre os análogos das DAs testados, as arilimidamidas (AIAs), anteriormente denominadas de “amidinas reversas”, são as mais ativas sobre parasitos intracelulares como o *T. cruzi e Leishmania* (Wang et al., 2010, Batista et al., 2010). As AIAs diferem estruturalmente das DAs (Figura 4) por apresentarem o grupo imino terminal ligado ao anel arila através de um átomo de nitrogênio e não diretamente pelo átomo de carbono (Rosypal et al., 2008).



(A) (B)

Figura 4. Estruturas da arilimidamida DB766 (A) e da diamidina aromática DB75 (B). Enquanto um átomo de nitrogênio (setas vermelhas) conecta a amidina à estrutura aromática central em (A), esta função é cumprida por um átomo de carbono (setas azuis) em (B) (Fonte: adaptado de De Souza et al., 2004 e Batista et al., 2010a).

Com relação aos alvos celulares desta classe de compostos, estudos bioquímicos, moleculares e funcionais têm sido realizados em diferentes

patógenos e os dados apontam para a ação sobre DNA, mitocôndria, topoimerases, citoesqueleto e síntese de poliaminas, biossíntese de lipídeos, entre outros (Pandharkar et al., 2014; Wilson et al., 2014). Nosso grupo avaliou a interação e afinidade de várias amidinas e análogos através de ensaios termodinâmicos e revelou a falta de correlação entre atividade destas moléculas e sua direta associação ao kDNA do parasito, sugerindo que a forte afinidade não é suficiente para gerar e desencadear a atividade tripanocida (Daliry et al., 2011).

Estudos têm demonstrado que AIAs como a DB766 são extremamente efetivas sobre formas sanguíneas e amastigotas intracelulares do *T. cruzi*, alcançando sobre a cepa Y em ensaios a 37°C, valores de EC_{50} de 60 e 25 nM após incubação por 24 e 72 h, respectivamente com a DB766 (Batista et al., 2010). O efeito tripanocida de algumas AIAs como a DB745 ocorre mesmo após curtos períodos de tratamento, alcançando elevados índices de lise dos tripomastigotas após 5 minutos de incubação (Silva et al., 2011).

Estudos *in vivo*, revelaram que a administração da BD766 reduziu a parasitemia e parasitismo cardíaco, apresentando atividade sobre as cepas Y e Colombiana maior ou igual ao Bz. Entretanto, apesar dos excelentes resultados não foi observada significativa cura parasitológica (avaliada por hemocultivo e PCR) com o tratamento por até 20 dias consecutivos (Batista et al., 2011a).

A combinação de DB766 com o Bz demonstrou reduções superiores a 99,5% na parasitemia e no parasitismo cardíaco dos animais sendo não tóxica (marcadores bioquímicos de lesões teciduais (hepáticas e cardíacas) (Batista et al., 2011b). Outros dados do nosso grupo têm confirmado a terapia combinada como abordagem promissora . DB1831 e seu análogo mesilato (DB1965) exibem altos índices de seletividade contra as formas tripomastigotas e amastigotas, alcançando valores de EC_{50} na ordem de 5 nM (Da Silva et al., 2012). DB1965 apresentou elevada atividade sobre a infecção aguda e a sua combinação com Bz, resultou na supressão total da parasitemia e sobrevivência de todos os animais (Da Silva et al., 2012).

Outro estudo *in vitro* do nosso grupo, analisando a influência da presença de um (mono) ou dois grupamentos (bis) catiônicos terminais com novas AIAs demonstraram que todas as bis-AIAs foram mais efetivas em relação as mono-AIAs. Dentre estes os compostos mais ativos, DB1967 e DB1989 foram

avaliadas *in vivo*, e ambas reduziram a parasitemia e protegeram contra morte induzida pela infecção (De Araújo et al., 2014).

Estudos conduzidos com outras AIAs revelaram que as moléculas 18SAB075 e16DAP002, exibiram ação *in vitro* sobre diferentes cepas do parasito (Y e Tulahuen) e suas formas relevantes para infecção de hospedeiros mamíferos (Timm et al., 2014). Uma delas, a 18SAB075 foi avaliada em modelos experimentais de infecção aguda pelo *T. cruzi,* porém se revelou moderadamente ativa alcançando somente 50% de redução da parasitemia (Timm et al., 2014).

Além dos resultados encontrados com os estudos experimentais sobre o *T. cruzi*, têm-se observado que as DAs também apresentam atividade sobre outros patógenos. Wenzler et al. (2014) demonstraram que a diamidina 28DAP010 foi efetiva sobre modelos experimentais de infecção pelo *Trypanosoma brucei*, agente da doença do sono. Esta molécula apresentou eficácia semelhante a DB829 em estudos de dose-resposta em modelo murino de primeiro e segundo estágio desta doença (Wenzler et al. 2014). As bis-AIAs apresentam também excelente atividade leishmanicida *in vitro*, e a DB766 apresenta significativa ação sobre modelos de leishmaniose visceral, quando administrado por via oral (Pandharkar et al., 2014; Zhu et al., 2016).

Estes dados estimulam a continuidade de estudos com esta classe de compostos, sobre distintas cepas representantes das diferentes linhagens do parasito (incluindo aquelas naturalmente resistentes a nitroderivados) objetivando a identificação de candidatos promissores para o tratamento da DC.

1.4.2 Inibidores de ergosterol

Desde a década de 80 do século passado, inibidores da biossíntese de ergosterol têm sido extensivamente estudados sobre modelos experimentais (murino e canino) de infecção (aguda e crônica) pelo *T. cruzi* (Diniz et al., 2010; Diniz et al., 2013; Guedes et al., 2014; Assíria et al., 2015). Assim como os fungos, estes protozoários requerem ergosterol endógeno, a inibição das etapas da via biossintética deste lipídeo é letal ao parasito e tem sido utilizada como um alvo terapêutico (Urbina, 2009). Vários inibidores de ergosterol atuam bloqueando, a enzima 14α-desmetilase (CYP51), uma citocromo que catalisa a remoção oxidativa do grupo 14α-metilo a partir de precursores de esterol

ciclizado, sendo sua inibição resultando além da falta de produção de esteróis, no acúmulo de esteróis citotóxicos metilados (Urbina, 2009; Lepesheva et al., 2011). Estes inibidores interferem na sobrevivência e proliferação de fungos (uso agrícola e clínico) e de protozoários, uma vez que este esterol é componente biológico essencial destes parasitos, apresentando papel chave no controle da fluidez e permeabilidade da membrana celular, na funcionalidade de canais de íons, bem como, nos ciclos de divisão celular (Adler et al., 1977; Lepesheva et al., 2007; Urbina, 2009).

Os antifúngicos triazólicos representam alguns dos principais agentes usados na clínica médica e muitos atuam sobre a CYP51. Em 1981, Do Campo e colaboradores demonstraram que miconazol e econazol, dois derivados imidazólicos inibiram o crescimento da cepa Tulahuen do *T. cruzi* na faixa de 20 µM, revelando uma redução do teor de 5,7-dieno esterol em epimastigotas, além de alterações celulares como o condensamento da cromatina nuclear, vacuolização e perda de eletrodensidade citoplasmática. *O*utro estudo demonstrou em macrófagos infectados com *T. cruzi* e tratados com cetoconazol inibição da multiplicação intracelular de amastigotas, e *in vivo* redução damortalidade de camundongos infectados e tratados (McCabe et al., 1983).

Como acima mencionado, os antifúngicos posaconazol e ravuconazol revelaram eficácia nas fases aguda e crônica na infecção experimental pelo *T. cruzi* (Urbina, 2009), porém revelaram falha terapêutica superior ao Bz em ensaios clínicos prospectivos e randomizados (fase II) conduzidos para verificar a eficácia e segurança de destes azóis. O estudo revelou que, em comparação com portadores chagásicos crônicos (cerca de 75% na forma assintomática) tratados com Bz, um número significativamente maior de pacientes tratados com posaconazol apresentou falha terapêutica durante o período de acompanhamento (Molina et al., 2014). As taxas de falha terapêutica nos “endpoint” primário e secundário foram de cerca de 90 e 80% dos portadores que receberam a menor 200 mg/kg (mpk) e a maior dose (800 mpk) de posaconazol, respectivamente, em comparação com 38% dos que receberam Bz (300 mpk) (Molina et al., 2014). A falta de correlação entre os dados pré-clínicos e clínicos levantou várias hipóteses quanto ao (a) uso de modelos experimentais de baixa estringência, (b) baixa efetividade destes triazóis sobre formas tripomastigotas (em comparação a sua excelente ação na faixa nanomolar sobre amastigotas intracelulares) (Molina et al., 2015), (c) exposição

sub-óptima e/ou curta duração do tratamento (Urbina, 2014). Desta forma, denotando a necessidade de padronização mais adequada de modelos experimentais (Chatelain & Konar, 2015).

Novos inibidores da CYP51 têm sido identificados a partir de bibliotecas de compostos (ex. Novartis) sendo alguns deles potentes e seletivos sobre a CYP51 de *T. cruzi* e de outros tripanosomatídeos. Um destes agentes sintetizados a partir de um novo "scaffold", foi o imidazol VNI [(R) N-(1-(2,4-diclorofenil)-2-(1H-imidazol-1-il)etilo)-4-(5-fenil-1,3,4-oxadiazol 2-il) benzamida] que exibiu potente seletividade sobre a enzima e em ensaios fenotípicos em tripanossomatídeos (Lepesheva et al. 2007; Lepesheva & Waterman, 2011).

Estudos do nosso grupo revelaram que o VNI apresentou baixa toxicidade *in vitro* sobre células de mamíferos (Lepesheva et al., 2007) e *in vivo*, exibindo NOAEL de 400 mg/kg em camundongos (Hargrove et al., 2012). Diferentemente de outros inibidores mais seletivos para fungos, tais como o fluconazol e posaconazol, estes novos imidazóis (VNI e análogos) não induzem aumento da expressão do gene CYP51 em *T. cruzi* (Lepesheva et al,. 2010), sugerindo uma baixa propensão a geração de resistência. Além disto, VNI foi efetivo na curar das fases aguda e crônica da infecção murina com a cepa Tulahuen (Villalta et al., 2013). Porém, Soeiro et al. (2013) explorando o potencial do VNI sobre cepas do *T. cruzi* resistentes aos nitro-derivados (Y e Colombiana), em protocolos de infecção aguda com início de tratamento quando do estabelecimento da infecção, demonstrou que apesar da eficácia de VNI e de seu derivado VNF (reduções > 80% na parasitemia e 100% sobrevida), não houve cura parasitológica para todos os grupos testados (incluindo Bz) após 26 dias de tratamento. Ademais, estudos de microscopia eletrônica de transmissão e testes de mutação reversa, foram realizados com VNI. Os dados demonstraram ausência de potencial mutagênico do VNI observado pelo teste de Ames e que o dano mais frequente e intenso induzido no *T. cruzi* foi relacionado a alterações no Golgi e desorganização do retículo endoplasmático, com presença de *blebs* na membrana, com características de morte celular por autofagia (Soeiro et al., 2013). Portanto, estes resultados preliminares com VNI justificam a continuidade de estudos com esta classe de compostos, explorando tempos de tratamento mais longos, objetivando a identificação de novos agentes mais seletivos e potentes que possam ser utilizados no futuro para a terapia dos milhões de portadores chagásicos.

1.4.3 Modelos experimentais e monitoramento do tratamento

Apesar de ter mais de um século desde sua descrição, poucos ensaios pré-clínicos seguiram para as etapas clínicas de infecção chagásica. Os recentes resultados dos primeiros ensaios de fase II nos últimos 40 anos, avaliando posaconazol e E1224 (pró-droga do ravuconazol) foram, infelizmente, sem sucesso (Torrico, 2013; Molina et al., 2014). Para suprir a crítica carência de novos medicamentos para tratar a DC, é fundamental uma melhor compreensão da progressão da infecção pelo parasito e da patologia e de sua reprodutibilidade em modelos animais para a triagem *in vivo*. Tais medidas possivelmente melhorariam a comparação dos dados gerados e a previsibilidade de testar hipóteses e modelos, necessários para uma melhor translação para os ensaios clínicos (Chatelain & Konar, 2015).

Os modelos animais existentes abordam tanto a patologia da doença quanto a eficácia do tratamento, entretanto, os modelos disponíveis têm ainda, valor preditivo limitado para a avaliação pré-clínica de novas terapias. A falta de metodologias padronizadas e marcadores disponíveis para avaliação do perfil de cura e ainda para diagnóstico precoce da a progressão da patologia têm dificultado o desenvolvimento novas drogas (Chatelain & Konar, 2015). O que temos hoje disponível e que foi utilizado para estudos clínicos do Bz (BENEFIT) e do posaconazol e E1224 são os ensaios moleculares de PCR e qPCR (quantitative Polymerase Chain Reaction) que indicam falha terapêutica, mas não podem precisar a cura, sobretudo em portadores crônicos, tendo em vista a infecção sub-patente e intermitente nesta fase mais tardia da infecção. Além disso, os ensaios sorológicos somente resultam em negativação (caso haja cura) após longos períodos pós-tratamento dos portadores crônicos, podendo necessitar anos ou mesmo décadas de acompanhamento (Rassi et al., 2010). A eficácia do tratamento na DC pode ser avaliada pela análise de títulos de anticorpos específicos anti-*T. cruzi*, entretanto, porém uma redução destes títulos pode levar vários anos, tornando a avaliação do tratamento insensível e demorada.

Por outro lado, não há entre os grupos de pesquisa, um consenso a respeito da escolha dos ensaios *in vitro* e *in vivo*. Os protocolos em diferentes estudos são variáveis no que diz respeito aos regimes terapêuticos, cepas do

parasito e os critérios de avaliação de cura, dificultando uma análise comparativa da eficácia e potência dos compostos estudados, bem como a falta de biomarcadores para avaliar a cura e a eficácia do tratamento e a previsão de pacientes com risco de progressão da doença (Pinazo et al., 2014; Chatelain & Konar, 2015). Entretanto em 2010, Romana e colaboradores desenvolveram pela primeira vez uma nota técnica com descrição de protocolos padronizados e de etapas mínimas necessárias para os ensaios pré-clínicos de novos agentes tripanocidas visando contribuir para a harmonização neste campo.

De fato, a falha terapêutica do posaconazol e do E1224 (em comparação com Bz) conduziu a uma reavaliação das etapas e modelos experimentais durante o processo de descoberta de novos fármacos, e características desejáveis para progressão de ensaios *in vitro* para *in vivo*, bem como a procura de novas tecnologias e métodos mais sensíveis, como por exemplo, o uso de sistemas de bioimagem (Chatelain, 2015).

Assim, a falta de padronização metodológica para diagnostico de cura é igualmente uma situação crítica no campo do desenvolvimento de uma terapia eficaz para a DC, sendo necessária a identificação de marcadores mais precisos e reprodutíveis.

2. Justificativa

Estima-se que mais de um bilhão de pessoas, aproximadamente um sétimo da população mundial, sofra de alguma doença tropical negligenciada. Dentre estas, a DC é responsável por mais mortes em todo mundo do que qualquer outra doença parasitária no tocante à cardiopatia infecciosa, situando-se entre as dezessete patologias negligenciadas agrupadas pela Organização Mundial da Saúde. Estas patologias apresentam em comum as características, de carência de investimentos financeiros para pesquisa e desenvolvimento de novas drogas por parte das indústrias farmacêuticas e ainda de negligência por parte do poder público em relação a medidas de controle e acesso a diagnóstico e terapias disponíveis. Estima-se que das milhões de pessoas infectadas na América Latina, apenas 1% têm acesso ao tratamento, evidenciando a igual urgência em ampliar o acesso e acelerar o desenvolvimento de medicamentos realmente inovadores. A falta de tratamento precoce resulta um impacto social e econômico custando à saúde pública brasileira um custo de US$130 milhões por ano e, em todo mundo, a DC representa perdas que atingem cerca de US$7 bilhões de dólares anualmente. E ainda no Brasil, o custo estimado da hospitalização por portador com cardiomiopatia chagásica é cerca de US$ 467 por dia, sendo mais elevado que paciente com insuficiência cardíaca não chagásica. Diante do exposto, o presente trabalho busca contribuir na validação de modelos experimentais murino para o estudo de novos fármacos na infecção experimental pelo *T. cruzi* e na investigação de propostas terapêuticas alternativas.

3. Objetivos

3.1 Objetivo Geral

Contribuir para padronização de modelos experimentais que possam predizer a ação de novos candidatos terapêuticos para doença de Chagas, assim como identificar terapias alternativas mais seletivas e promissoras, avaliando ação *in vitro* e *in vivo* de novas AIAs e de inibidores da síntese de lipídeos no curso da infecção pelo *Trypanosoma cruzi*.

3.2 Objetivos específicos

i) Avaliar *in vitro* a atividade de novos compostos (AIAs e inibidores da CYP51) sobre formas sanguíneas e intracelulares de diferentes cepas do *T. cruzi,* e seu perfil de toxicidade sobre células de mamíferos.

ii) Determinar o índice de seletividade dos compostos testados *in vitro* visando seleção dos mais promissores (IS >50) para condução dos estudos *in vivo*.

iii) Determinar sobre modelo murino experimental a toxicidade aguda (NOAEL) dos compostos selecionados para exclusão de moléculas e doses tóxicas *in vivo*.

iv) Avaliar *in vivo* a eficácia do tratamento dos compostos no da infecção aguda murina experimental pelo *T. cruzi* utilizando cepas com distintos perfis de susceptibilidade (cepa Y e Colombiana) através de diferentes esquemas terapêuticos (monoterapia e terapia combinada) sobre parâmetros parasitológicos (mortalidade e parasitemia) e carga parasitária (qPCR após imunossupressão).

v) Explorar a interferência de diferentes modelos experimentais sobre a atividade anti-*T.cruzi* de novos compostos e do medicamento de referência (Bz), avaliando impacto da terapia experimental a depender do gênero do animal, do início da administração do composto e seu tempo de exposição, além da natureza do veículo utilizado para diluir as moléculas teste.

4. Resultados

Artigo I:

Guedes-da-Silva FH, Batista DG, Da Silva CF, Meuser MB, Simões-Silva MR, Araújo JS, Ferreira CG, Moreira OC, Britto C, Lepesheva GI, Soeiro MN. Different Therapeutic Outcomes of Benznidazole and VNI Treatments in Different Genders in Mouse Experimental Models of *Trypanosoma cruzi* Infection. **Antimicrob Agents Chemother. 2015;59(12):7564-70.**

A falha dos estudos clínicos de fase II (Ps e E1224) sobre portadores chagásicos crônicos revelou a falta de tradução entre ensaios pré-clínicos e clínicos, indicando a necessidade de protocolos e modelos experimentais mais padronizados e com maior grau de estringência. Neste estudo, avaliamos os efeitos do tratamento com Bz e com o imidazol VNI sobre a infecção murina experimental com cepas do *T. cruzi* moderadamente ou altamente resistentes a nitroderivados, usando distintos gêneros (macho e fêmea) e administrando os compostos em diferentes esquemas terapêuticos.

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Different Therapeutic Outcomes of Benznidazole and VNI Treatments in Different Genders in Mouse Experimental Models of *Trypanosoma cruzi* Infection

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The lack of translation between preclinical assays and clinical trials for novel therapies for Chagas disease (CD) indicates a need for more feasible and standardized protocols and experimental models. Here, we investigated the effects of treatment with benznidazole (Bz) and with the potent experimental *T. cruzi* CYP51 inhibitor VNI in mouse models of Chagas disease by using different animal genders and parasite strains and employing distinct types of therapeutic schemes. Our findings confirm that female mice are less vulnerable to the infection than males, show that male models are less susceptible to treatment with both Bz and VNI, and thus suggest that male models are much more suitable for selection of the most promising antichagasic agents. Additionally, we have found that preventive protocols (compound given at 1 dpi) result in higher treatment success rates, which also should be avoided during advanced steps of *in vivo* trials of novel anti-*T. cruzi* drug candidates. Another consideration is the relevance of immunosuppression methods in order to verify the therapeutic profile of novel compounds, besides the usefulness of molecular diagnostic tools (quantitative PCR) to ascertain compound efficacy in experimental animals. Our study aims to contribute to the development of more reliable methods and decision gates for *in vivo* assays of novel antiparasitic compounds in order to move them from preclinical to clinical trials for CD.

Chagas disease (CD), discovered by Carlos Chagas (1), is a neglected pathology caused by the obligately intracellular protozoan parasite *Trypanosoma cruzi*. The disease is endemic in 21 countries of Central and South America, where about 8 million people are infected and more than 12,000 die annually (http://www.dndial.org). The treatment for CD is limited to the use of two nitroderivatives (benznidazole [Bz] and nifurtimox [Nf]), which are largely unsatisfactory, indicating a need for novel, safer, and more efficient therapies (2). Although a large number of *in vitro* and *in vivo* studies have been performed on experimental chemotherapy of novel drug candidates for CD, besides Bz and Nf, very few compounds have moved to clinical trials (3).

Recently, two antifungal drugs, posaconazole and E1224 (the prodrug of ravuconazole), which are inhibitors of fungal sterol 14a-demethylase (CYP51), were evaluated as potential antichagasic drugs on chronic patients, but unfortunately both displayed rather high (70 to 80%) rates of therapeutic failure (4, 5). It has been suggested that at least part of this unexpected failure could be due to the lack of translation from *in vitro* and *in vivo* models to the clinic and that a redesign of the current screening strategy during the drug discovery process should be considered (5). On the other hand, recent data demonstrated the potency and selectivity of a novel experimental inhibitor of *T. cruzi* CYP51, the VNI molecule, which yielded promising *in vivo* findings even with highly resistant *T. cruzi* strains (6). In this vein, we evaluated the effects and outcomes of the treatment with benznidazole and VNI using mouse models of *T. cruzi* infection assaying different animal genders and parasite strains and employing distinct types of drug administration schemes (preventive, i.e., starting therapy at 1 day postinfection [dpi], and therapeutic, i.e., starting at parasitemia onset, 5 to 6 dpi).

Our results confirm that female mice are less vulnerable to *T. cruzi* infection than males (7) and show that male mice are also less susceptible to the antiparasitic effects of Bz and VNI. We found that the use of preventive models may be less sensitive in detecting therapeutic efficiency and therefore should be avoided at the advanced stages of preclinical trials. Also, immunosuppression methods are highly recommended to assess the therapeutic success of novel compounds, since these protocols allow the parasite reactivation and easier detection through the analysis of parasitological parameters such as parasitemia relapse and blood and tissue parasite DNA detection by PCR.

MATERIALS AND METHODS

Drugs. Benznidazole (Bz) was purchased from Laboratório Farmacêutico do Estado de Pernambuco, Brazil. Bz was dissolved in distilled and sterile water supplemented with 3% Tween 80, which does not have any detectable effect on mice (8). VNI was synthesized as reported elsewhere (9), and the compound was suspended in 20% Trappsol (CTD, Inc., USA). Cyclophosphamide (Cy) (Genuxal) was purchased from Baxter Oncology (Frankfurt, Germany) and prepared in distilled and sterile water.

***In vivo* infection.** Male and female Swiss mice (18 to 20 g) were obtained from the animal facilities of the Oswaldo Cruz Foundation (CE-

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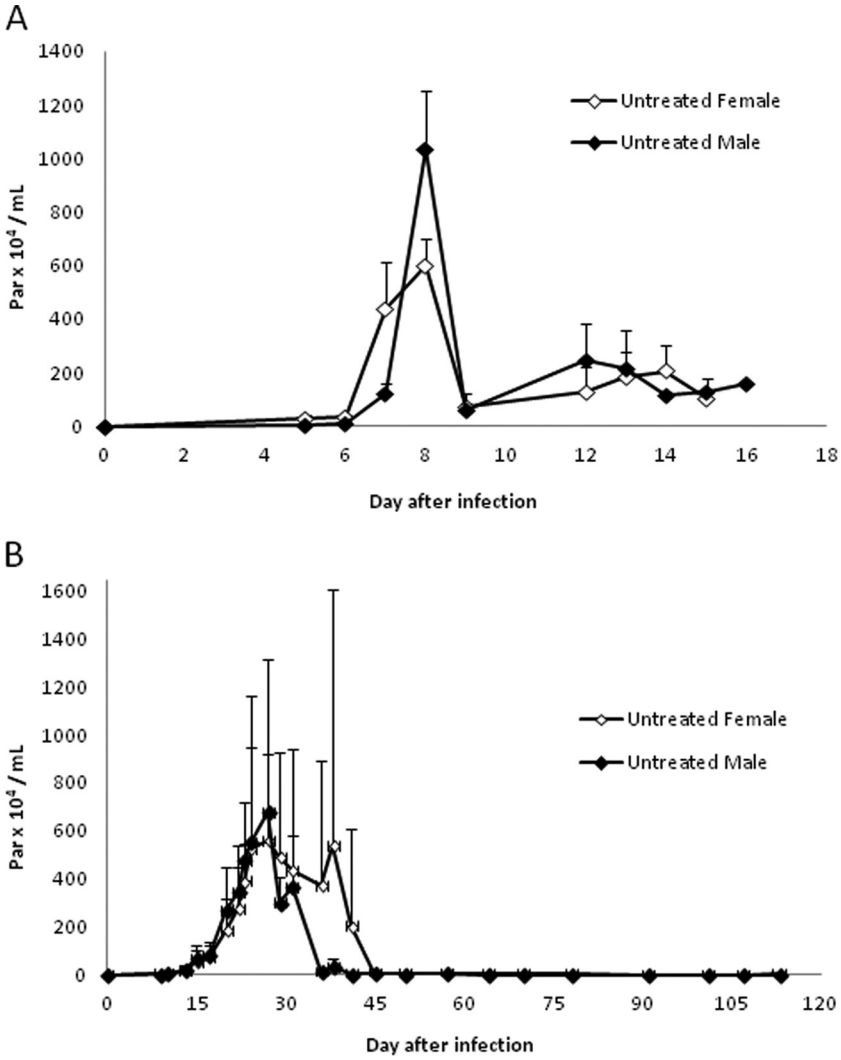

FIG 1 Parasitemia levels in experimental mouse models of *T. cruzi* infection from female and male groups infected with strains Y (A) and Colombiana (B). The high standard deviation in the female group with the Colombiana infection is due to one animal that exhibited high parasitemia.

CAL, Rio de Janeiro, Brazil). Mice were housed at a maximum of five per cage and were kept in a conventional room at 20 to 24°C under a 12-h/12-h light/dark cycle. The animals were provided with sterilized water and chow *ad libitum*. Infection was performed by intraperitoneal (i.p.) injection of 10^4 and 5×10^3 bloodstream trypomastigotes (Y and Colombiana strains, respectively). The animals were divided into the following groups: uninfected (noninfected and nontreated), untreated (infected with *T. cruzi* but treated only with vehicle), and treated orally (p.o.) with 100 mg/kg benznidazole once a day or 25 mg/kg VNI twice a day.

Treatment schemes. Bz therapy was started at 1 day postinfection (dpi) or at the onset of the parasitemia, which corresponds to 5 to 6 dpi for strain Y and 10 dpi for strain Colombiana. Bz was administered using daily consecutive doses (30 and 60 days for Y and Colombiana infections, respectively). VNI was administered for 30 days, with the treatment starting at 5 dpi. In all assays, only mice with positive parasitemia were used in the infected groups.

Parasitemia and mortality rates. The level of parasitemia was checked by the Pizzi-Brener method. Mice were individually checked by direct microscopic counting of parasites in 5 μl of blood. The mortality rates were checked daily and expressed as cumulative mortality (CM) (10).

Cure assessment. Mice that exhibited consistent negative parasitemia up to 30 days after treatment with Bz and VNI were subjected to three cycles of cyclophosphamide exposure (50 mg/kg/day), each with four consecutive days of administration (i.p.) and with 3 days between cycles (11). As reported previously (8), cure criteria were based on parasitemia negativation observed (i) by light microscopy and (ii) by quantitative real-time PCR (qPCR). Animals with negative results for all tests were considered cured. For qPCR, 500 μl blood was diluted 1:2 in a guanidine solution (6 M guanidine-HCl–0.2 M EDTA) and heated for 90 s in boiling water in order to promote the denaturation of the parasite kinetoplast DNA network-associated minicircles (12). Guanidine-EDTA blood samples (GEB) were processed using the QIAamp DNA minikit (Qiagen), as described by in reference 13. qPCR multiplex assays were performed targeting the *T. cruzi* satellite nuclear DNA and the internal amplification control (IAC) (plasmid pZErO-2 containing an insert from the *Arabidopsis thaliana* aquaporin gene), as described by Duffy et al. (14). The standard curves for the absolute quantification were constructed with serial 1/10 dilutions of total DNA obtained from a negative GEB sample spiked with 10^5 parasite equivalents per milliliter of blood.

Ethics. All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14).

TABLE 1 Analysis of biological parameters of male and female Swiss mice infected with *Trypanosoma cruzi* and treated with benznidazole or left untreated

Parasite strain	Experimental group	Highest parasitemia level (10^4 parasites/ml)[a]	Day of highest parasitemia
Y (TcII)	Untreated females	602 ± 99	8
	Bz-treated females	66 ± 146	7
	Untreated males	1,035 ± 215	8
	Bz-treated males	564 ± 241	8
Colombiana (TcI)	Untreated females	560 ± 357	27
	Bz-treated females	1.6 ± 1.3	10
	Untreated males	675 ± 642	27
	Bz-treated males	2 ± 1	10

[a] Values are means ± standard deviations.

TABLE 2 Cure assessment of benznidazole in a murine model of acute *T. cruzi* Y infection[a]

Gender	Treatment initiation (dpi)	Treatment	No. of animals with negative parasitemia/no. of surviving animals[b]	No. of qPCR-negative blood samples/no. of mice[b]
Female	1	Vehicle	0/4	ND
		Bz	4/5	3/5
	6	Bz	5/5	2/5
Male	1	Vehicle	0/5	ND
		Bz	4/4	1/4
	6	Bz	5/5	1/5

[a] Swiss male and female mice were inoculated with 10^4 bloodstream trypomastigotes (strain Y). Treatment was initiated at 1 and 6 dpi (parasitemia onset) and followed by 30 consecutive daily oral doses.
[b] Analysis was performed 30 days posttreatment after cyclophosphamide administration. ND, not determined (0% animal survival).

RESULTS

The analysis of parasitological parameters (parasitemia and mortality rates) showed that Swiss male mice are more vulnerable to strain Y (discrete typing unit [DTU] II) *T. cruzi* infection than age-matched female animals inoculated with the same parasite load. The light microscopy analysis indicated that although both genders exhibited similar prepatent periods with a parasitemia peak at 8 dpi, males displayed higher blood parasite loads (1.7-fold) than females (Fig. 1A; Table 1). Regarding cumulative mortality (CM), although both groups reached 0% survival, only 25% of the females died at 15 dpi, while in the male group, CM at the 15 dpi was 80% (data not shown). When animals were infected using the same inoculum with a well-known highly resistant strain of the parasite (Colombiana [DTU I]), again the males displayed higher parasitemia levels at the peak (at 29 dpi), as levels were about 20% lower for the female group than for the male group (Fig. 1B; Table 1), in addition to the higher mortality rates: 20 and 40% for females and males, respectively. The molecular analysis of the surviving animals by qPCR showed much lower (about 19-fold) blood parasite load in females than in males infected by strain Colombiana (27 ± 43 and 504 ± 693 parasite equivalents/ml of blood, respectively), confirming gender-related differences in the parasite infection vulnerability.

Next, potential gender-related differences during Bz therapy were evaluated using Y and Colombiana experimental models of *T. cruzi* acute infection. In these assays, the treatment was started at the onset of parasitemia. In the strain Y infection, the treatment began on day 6 and lasted for 30 consecutive days. Although no difference in survival rates (100% survival) was found between the sexes (data not shown), there was a huge suppression of the parasitemia in the female group (>90%), while the male group displayed only about a 50% decrease in parasitemia (Table 1). According to cure parameters, females also had higher cure rates (2 out of 5 mice) than the male group (1 out of 5 mice) (Table 2). Also, qPCR findings demonstrated lower parasite load in the blood of females than males (Fig. 2A). In the infection with Colombiana, the treatment was started on day 10 and lasted for 60 days. Although similar levels of parasitemia reduction (>99%) were observed in both treated animal groups, qPCR showed lower blood parasitism (1.9-fold for the mean values) in females (117 ± 60 parasite equivalents/ml) than in males (217 ± 289 parasite equivalents/ml) (Fig. 2B). When the treatment was finished, all Bz-treated mice from both genders relapsed, as assayed by light microscopy analysis (Table 3). However, one animal from the male Bz-treated group, although displaying positive relapse (only one parasite counted into 50 fields by light microscopy), had no detectable parasite DNA in the corresponding qPCR (Fig. 2B), possibly because the DNA amount was under the limit of detection of the qPCR assay (13). It is worth mentioning that despite the higher strain Y inoculum (10^4 parasites/mouse versus 5×10^3 parasites/mouse in the case of Colombiana) and shorter period of treatment (30 versus 60 days), animals treated with Bz (both male and female) exhibited lower parasite loads, as determined by qPCR, when strain Y was used, confirming the naturally resistant profile of the Colombiana strain (Fig. 2). Once more, qPCR confirmed that the drug efficacy is higher in females (0.066 and 117 parasite equivalents/ml for Y and Colombiana, respectively) than in males (0.278 and 217 parasite equivalents/ml for Y and Colombiana, respectively) (Fig. 2).

Next, in order to evaluate the impact of earlier drug administration on the parasite load, mice of both sexes were infected with *T. cruzi* strain Y and then treated with Bz starting at 1 dpi. In this preventive scheme, in both genders, Bz completely suppressed parasitemia (Fig. 3) and resulted in 100% animal survival (data not shown). The qPCR analysis also revealed the superior outcome when Bz was given earlier (at 1 dpi): both male and female groups displayed much lower parasite loads than with the treatment at 6 dpi, which corresponded to parasitemia onset (Table 4). As expected, when Bz therapy was started at 1 dpi, females still showed lower (3-fold) parasitemia (qPCR analysis) than males (Table 4), confirming that male mice are more vulnerable to *T. cruzi* infection and at the same time less sensitive to Bz therapy.

Finally, in order to ascertain if lower sensitivity to etiological treatment of male mice is also observed upon treatment with other trypanocidal compounds, we evaluated gender-related effects of the *T. cruzi* CYP51 inhibitor VNI. The activity of VNI was assayed in male and female mice infected with strain Y, and the treatment was started at 5 dpi (Fig. 3). The data show that at the peak of parasitemia, VNI induced an 86% reduction in the number of circulating bloodstream trypomastigotes in the male mice, while in the female group, the suppression of parasitemia reached 99.8%. Moreover, when qPCR analysis was performed at the endpoint (30 days after the end of therapy with VNI and followed by

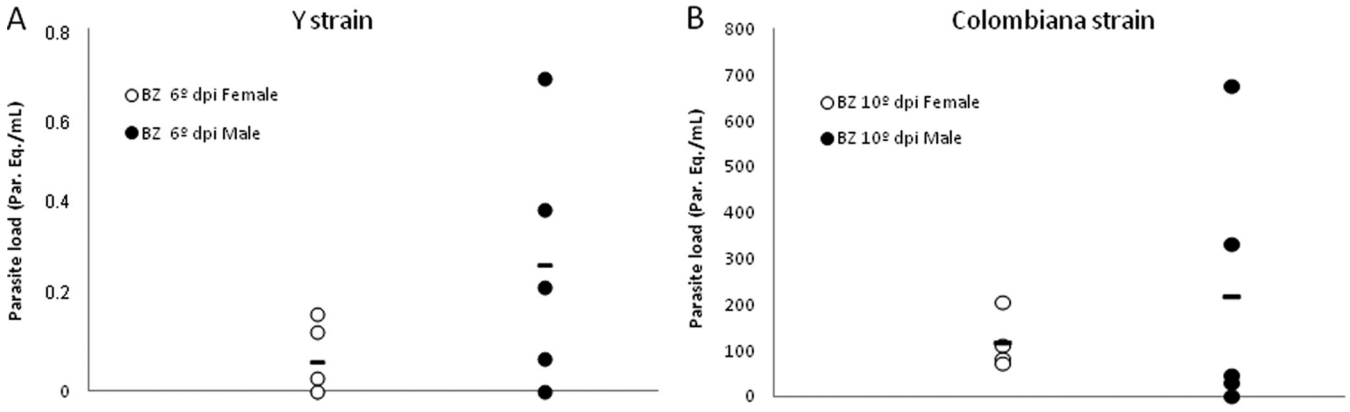

FIG 2 Parasite burden (parasite equivalents [Par. Eq.]/ml) in the blood samples of male and female mice infected with strains Y (A) and Colombiana (B) of *T. cruzi* and either left untreated or subjected to benznidazole therapy, starting at parasitemia onset (6 and 10 dpi, respectively) and lasting for 30 days and 60 days for Y and Colombiana infections, respectively.

three cycles of immunosuppression with cyclophosphamide), the findings were 151 ± 18 and 0.35 ± 0.5 parasite equivalents/ml for blood samples from male and female mice (Table 4), respectively, corroborating the data regarding gender drug susceptibility obtained during Bz treatment.

DISCUSSION

The therapy for Chagas disease (CD) remains unsatisfactory, with only two available options based on nitroderivative compounds (benznidazole [Bz] and nifurtimox) introduced into clinical use more than 4 decades ago. Limited efficiency and significant toxicity of these two drugs justifies the search for novel anti-*T. cruzi* candidates. However, despite the huge number of *in vitro* and *in vivo* assays performed on experimental chemotherapy for CD, very few compounds moved to clinical trials (15). Recent data demonstrated that although the antifungal azoles posaconazole and E1224 (the prodrug of ravuconazole) displayed very promising preclinical results, both displayed high rates of therapeutic clinical failure (4). The lack of translation from preclinical to clinical trials raised different points, including the need to use more stringent and standardized screening protocols and experimental models (5). In our opinion, the extremely high variability of *T. cruzi* populations (>70 genetically diverse strains are known) (2) represents another serious concern. Thus, a 30-day treatment was sufficient for VNI to cure, with 100% efficiency, the acute and chronic mouse models of CD caused by the Tulahuen strain of *T. cruzi* (BALB/c female mice) (16), but it failed to do so in the strain Y infection in both the male and female mouse models. One possible reason for this failure could be the lower susceptibility of strain Y CYP51 to inhibition, which may also be the case in other strains of the parasite due to high genetic variability of CYP51 across the *T. cruzi* population (17). Other possibilities would be strain-related differences in tissue distribution and the presence of possible dormant nonmultiplying forms of the parasite. Altogether, these observations call for thorough testing of new potential antichagasic drug candidates against various *T. cruzi* strains by using the most stringent animal model protocols before proceeding to clinical trials.

In this context, we evaluated the effects and outcomes of the treatment with Bz and VNI in mouse models of *T. cruzi* infection using different parasite strains and animal genders and employing different drug administration schemes (preventive and therapeutic).

Regarding animal gender, a previous study demonstrated that *T. cruzi*-infected BALB/c, C3H, and C57BL/6 female mice have a longer survival time than males (18). Those authors reported that as alterations in the humoral *T. cruzi*-specific response were ruled out, hormones like estradiol could play a relevant role on the higher vulnerability of male than female mice (19). Also, because *T. cruzi* infection in mammalian hosts leads to diverse clinical manifestations, and because this may be partly due to the occurrence of different parasite strains, experiments evaluating vaccination or chemotherapy should take into consideration the use of distinct DTUs relevant for human infection (20). Thus, in our study, we compared the outcome of parasite infection in female and male mice using two strains, the reticulotropic strain Y (DTU II) and the myotropic strain Colombiana (DTU I) (21). The present findings, obtained by using an outbred mouse lineage (Swiss), confirmed that female mice are less vulnerable to *T. cruzi* infection than males regardless of the parasite strain (Y or Colombiana). We found that the parasitemia levels (determined by light microscopy and qPCR measurements) were higher in males than females, and mortality was more prominent and/or earlier in the former group. In both males and females, qPCR analysis confirmed the natural Bz-resistant profile *in vivo* of Colombiana compared to Y (22, 23). Lower numbers of DNA parasite equivalents per milliliter were

TABLE 3 Cure assessment of benznidazole in murine model of acute *T. cruzi* Colombiana infection[a]

Gender	Treatment	No. of animals with negative parasitemia/no. of surviving animals[b]	No. of qPCR-negative blood samples/no. of mice[b]
Female	Vehicle	2/4	0/4
	Bz	0/5	0/4
Male	Vehicle	0/3	0/3
	Bz	0/5	1/5

[a] Swiss male and female mice were inoculated with 5×10^3 bloodstream trypomastigotes (strain Colombiana). Treatment was initiated at 10 dpi (parasitemia onset) and followed by 60 consecutive daily oral doses.
[b] Analysis was performed 30 days posttreatment after cyclophosphamide administration.

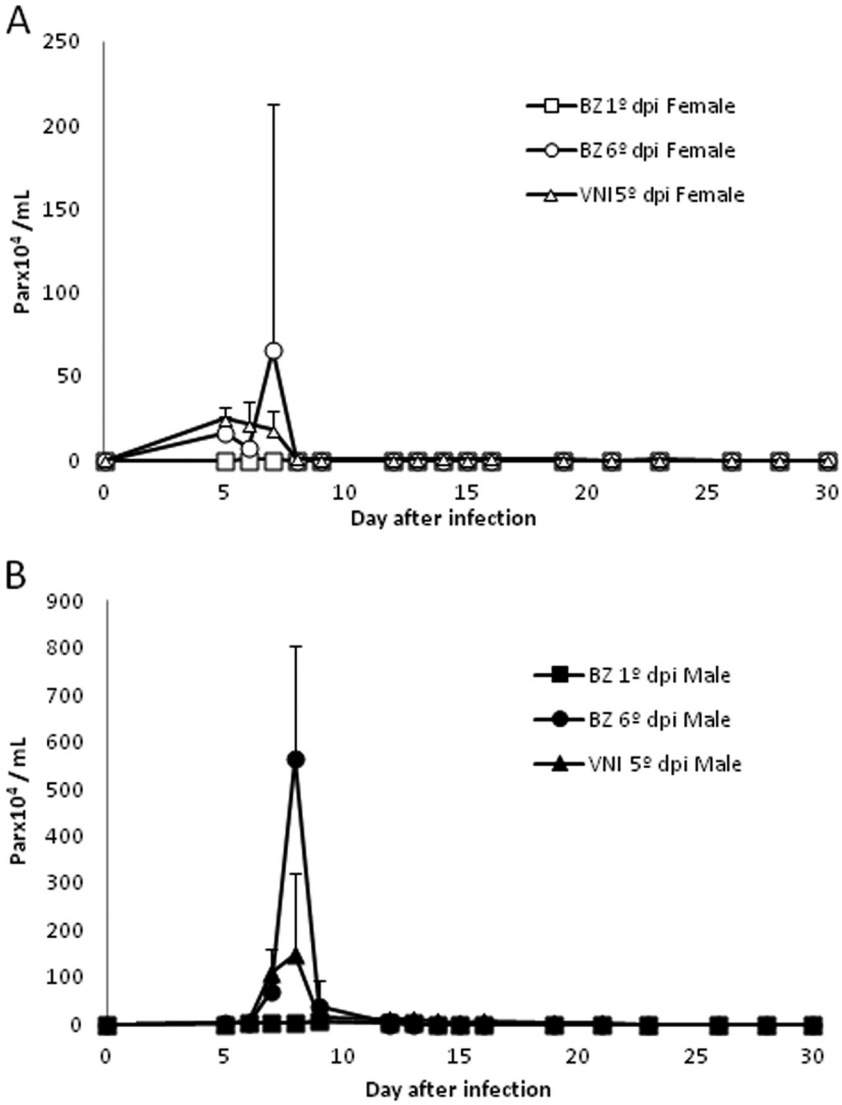

FIG 3 Parasitemia levels in experimental female (A) and male (B) mouse models of Y strain *T. cruzi* infection upon treatment with benznidazole or VNI for 30 days. The corresponding levels of parasitemia in untreated animals are shown in Fig. 1.

found when Bz was given to animals infected with Y than to animals infected with Colombiana, although twice the number of parasites were used to infect the mice (inocula of 10^4 and 5×10^3 bloodstream trypomastigotes per mouse, respectively) and longer periods of treatment (30 and 60 days, respectively) were employed.

TABLE 4 Parasite burden DNA from blood samples of *T. cruzi* (Y strain)-infected male and female mice subjected to benznidazole and VNI therapy for 30 consecutive days, starting at 1 dpi and at parasitemia onset

Compound	Gender	Burden (parasite equivalents/ml) when treatment started at[a]:	
		1 dpi	Parasitemia onset
BZ	Female	0.027 ± 0.05	0.066 ± 0.08
	Male	0.08 ± 0.08	0.278 ± 0.28
VNI	Female	ND	0.35 ± 0.5
	Male	ND	151 ± 18

[a] ND, not determined.

The animal gender must be considered when novel pharmacological entities are being assayed. For instance, data in the literature show that although both sexes most often respond similarly in acute toxicity studies, females are usually more sensitive when differences do exist (24, 25). This highlights the need to compare sexes in experimental chemotherapy studies to ascertain potential drug differences in sensitivity between the genders. In this vein, we tested the outcome of benznidazole and VNI therapy in mouse *T. cruzi* infection by performing a head-to-head comparison with female and male animals. In all assays, we found that males were less susceptible to the effect of Bz than females, with greater differences (parasitemia decreases of about 50 and >90% for males and females, respectively, with Bz and about 86 and 99.8% for males and females, respectively, with VNI) being observed when the Y strain was used and treatment was given for 30 consecutive days. The molecular analysis by qPCR confirmed that the blood

parasite load at the endpoint (30 days posttreatment) was significantly higher in males than females (about 4- and 431-fold for Bz and VNI, respectively).

Another interesting point is the choice of the therapeutic regimen, especially concerning the time when the treatment begins, i.e., at parasitemia onset or immediately after the parasite inoculation (starting at 1 dpi). Our data showed that in both male and females, Bz treatment that was started at 1 dpi resulted in undetectable parasitemia and lower parasite burden detected by qPCR than drug administration started at parasitemia onset, when the infection is already established. This, of course, supports the general knowledge that the earlier treatment begins, the better the outcomes that may be expected. However, in order to identify the most potent drug candidates, drug administration starting at parasitemia onset, when the pathogen has already invaded different tissues and organs, appears to be more sensitive and therefore preferable. The use of immunosuppression protocols (such as cyclophosphamide administration) is another relevant approach that is very helpful in enhancing the assay sensitivity (after treatment parasite detection), expanding the parasitism to detectable levels when adaptive immunity is compromised (8, 26, 27). As reported previously, the parameters for "cure" analysis *in vivo* may be influenced, depending on whether immunosuppression protocols are or are not included (25). As determined by parasitemia analysis, qPCR of blood from surviving male animals at 30 days posttreatment also showed differences in parasitism when animals were treated with Bz starting at 1 and at 6 days after infection with strain Y. Also, qPCR and parasitemia relapses after cyclophosphamide administration (at 30 days after the end of Bz therapy) revealed that, although 0% cure was found for both male and female groups infected with Colombiana, higher cure rates were found in females than males when strain Y was assayed, especially when a preventive scheme was employed (3 out of 5 and 1 out of 4 cured, respectively).

In summary, our findings confirmed that female mice are less vulnerable to *T. cruzi* infection than male animals and present additional evidence that male models are less susceptible to trypanocidal agents and therefore are highly preferable for the selection of more potent compounds. Additionally, we report that the use of preventive protocols in the *in vivo* assays is less sensitive and therefore should be avoided. Another useful consideration is to apply immunosuppression protocols to verify the therapeutic profile of novel compounds besides the use of molecular diagnostic tools (such as qPCR) to investigate compound efficacy in experimental animals. With these findings, we aim to contribute to the design of more reliable methods and decision gates for *in vivo* assays of novel antiparasitic compounds in order to move them from preclinical to clinical trials in CD.

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Artigo II:

Guedes-da-Silva FH, Batista DG, Meuser MB, Demarque KC, Fulco TO, Araújo JS, Da Silva PB, Da Silva CF, Patrick DA, Bakunova SM, Bakunov SA, Tidwell RR, Oliveira GM, Britto C, Moreira OC, Soeiro MN. *In Vitro* and *In Vivo* Trypanosomicidal Action of Novel Arylimidamides against *Trypanosoma cruzi*. **Antimicrob Agents Chemother. 2016;60(4):2425-34**.

DC é a principal causa de mortes no tocante à cardiopatia infecciosa, situando-se entre as dezessete patologias negligenciadas agrupadas pela Organização Mundial da Saúde. Estas patologias apresentam em comum característica, à carência de investimentos financeiros para pesquisa e desenvolvimento de novos fármacos por parte das indústrias farmacêuticas. Amidinas aromáticas como a pentamidina têm sido utilizadas como agentes antiparasitários na clínica médica e veterinária, porém apresentam limitações importantes, tais como a necessidade de administração parenteral e efeitos adversos indesejáveis. Visando superar estas limitações, novas moléculas amidínicas têm sido sintetizadas e avaliadas em ensaios fenotípicos *in vitro e in vivo*. Entre as mais potentes, destacam-se as AIAs que exibem considerável atividade biológica contra vários patógenos intracelulares, incluindo *T. cruzi* Assim objetivando identificar novas alternativas para o tratamento da doença de Chagas, neste estudo, avaliamos *in vitro* e *in vivo* sobre infecção por *T.cruzi* a atividade tripanocida de 14 novas AIAs agrupadas em bis- e mono-AIAs.

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In Vitro and *In Vivo* Trypanosomicidal Action of Novel Arylimidamides against *Trypanosoma cruzi*

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Arylimidamides (AIAs) have been shown to have considerable biological activity against intracellular pathogens, including *Trypanosoma cruzi*, which causes Chagas disease. In the present study, the activities of 12 novel bis-AIAs and 2 mono-AIAs against different strains of *T. cruzi in vitro* and *in vivo* were analyzed. The most active was *m*-terphenyl bis-AIA (35DAP073), which had a 50% effective concentration (EC_{50}) of 0.5 μM for trypomastigotes (Y strain), which made it 26-fold more effective than benznidazole (Bz; 13 μM). It was also active against the Colombiana strain (EC_{50} = 3.8 μM). Analysis of the activity against intracellular forms of the Tulahuen strain showed that this bis-AIA (EC_{50} = 0.04 μM) was about 100-fold more active than Bz (2 μM). The trypanocidal effect was dissociated from the ability to trigger intracellular lipid bodies within host cells, detected by oil red labeling. Both an active compound (35DAP073) and an inactive compound (26SMB060) displayed similar activation profiles. Due to their high selectivity indexes, two AIAs (35DAP073 and 35DAP081) were moved to *in vivo* studies, but because of the results of acute toxicity assays, 35DAP081 was excluded from the subsequent tests. The findings obtained with 35DAP073 treatment of infections caused by the Y strain revealed that 2 days of therapy induced a dose-dependent action, leading to 96 to 46% reductions in the level of parasitemia. However, the administration of 10 daily doses in animals infected with the Colombiana strain resulted in toxicity, preventing longer periods of treatment. The activity of the combination of 0.5 mg/kg of body weight/day 35DAP073 with 100 mg/kg/day Bz for 10 consecutive days was then assayed. Treatment with the combination resulted in the suppression of parasitemia, the elimination of neurological toxic effects, and survival of 100% of the animals. Quantitative PCR showed a considerable reduction in the parasite load (60%) compared to that achieved with Bz or the amidine alone. Our results support further investigations of this class with the aim of developing novel alternatives for the treatment of Chagas disease.

Chagas disease (CD) is caused by the obligate intracellular protozoan *Trypanosoma cruzi*. More than a hundred years after its discovery (1), CD is still an important public health problem, and about 6 million to 7 million people, mostly in Latin America, are estimated to be infected worldwide (2). Of these infected individuals, 30 to 40% develop cardiomyopathy and/or digestive syndromes (3). For over 4 decades, nifurtimox [3-methyl-4-(5′-nitrofurfurylideneamine tetrahydro-4H-1,4-tiazine-1,1-dioxide)] and benznidazole (Bz; *N*-benzyl-2-nitroimidazole acetamide), which have been used as empirical therapy for CD, have remained the sole treatment options (4). Both have several limitations, including a variety of adverse effects and limited efficacy in the later chronic phase of infection (5, 6), characteristics which strengthen the need for new trypanocidal compounds that overcome these limitations.

Classic aromatic diamidines (ADs) are DNA minor groove binders with recognized broad-spectrum antimicrobial and antitumor activities (7). ADs, such as pentamidine, diminazene, and propamidine, have been used as therapies in human and veterinary medicine for decades, but despite their excellent antiparasitic effects, they have relevant drawbacks that include low bioavailability and side effects. To overcome these limitations, new analogues have been synthesized and tested *in vivo* and *in vitro* (7). Several ADs and related compounds have been screened for their activities against *T. cruzi* and have shown promising results, at least in part due to their capacity to alter the kinetoplast DNA molecule (8, 9). Arylimidamides (AIAs; formerly called "reversed" amidines, because of the reversed position of the nitrogen and carbon atoms compared to their orientation in classic aromatic amidines) are the most effective amidine analogues tested against *T. cruzi in vitro* and *in vivo* (9–12).

Lipid bodies (LBs) consist of a nucleus of cholesteryl esters and triglycerides surrounded by a single monolayer of phospholipids. LBs are considered not only lipid storage compartments but also intracellular sites for many biological events, like cellular signaling and activation, regulation of lipid metabolism, membrane trafficking, and regulation of inflammatory mediators (13, 14). Recent studies proposed a dynamic role of LBs in the control of pathogen infections due to their localization inside the parasitophorous vacuoles in intracellular parasites like *T. cruzi* (15).

Our present study focused on an analysis of the activities of 12 novel bis-AIAs and 2 mono-AIAs against bloodstream trypomastigotes (BTs) and intracellular forms of *T. cruzi* (the Tulahuen, Y, and Colombiana strains) *in vitro*. The most potent and most se-

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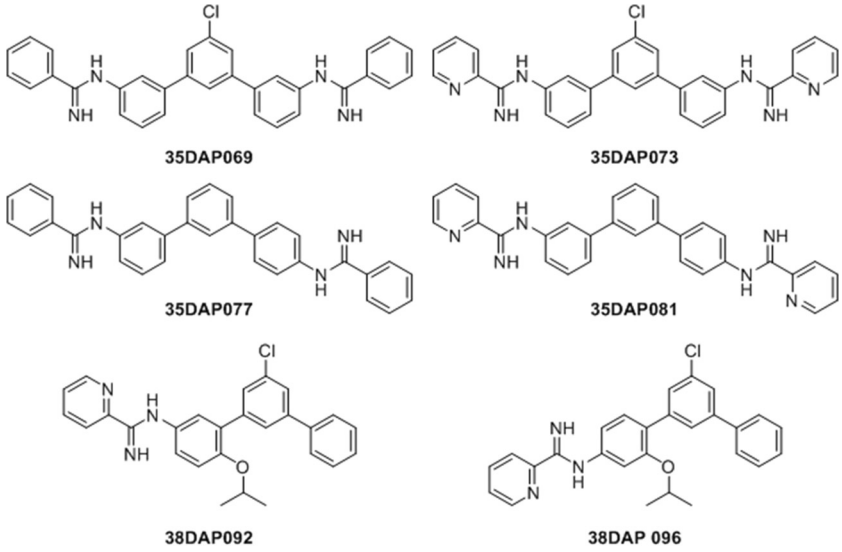


FIG 1 Structures of mono- and bisarylimidamide derivatives of *m*-terphenyl.

lective compound tested, 35DAP073, underwent further screening in experimental mouse models of acute parasite infection (with strains Y and Colombiana) alone or combined with the reference drug (Bz) with the aim of contributing to the search for novel alternative protocols for the treatment of CD.

MATERIALS AND METHODS

Synthesis of the arylimidamides. The synthesis of the four *m*-terphenyl bisarylimidamides 35DAP069, 35DAP073, 35DAP077, and 35DAP081 and the two monoarylimidamides 35DAP092 and 38DAP096 (Fig. 1) has been described previously (16), wherein the final step was the reaction of the appropriate terphenyl amine or diamine with benzonitrile or 2-cyanopyridine using sodium bis(trimethylsilyl)amide in tetrahydrofuran. Similar reactions involving 1,4-diphenylenediamine and 2,5- or 2,6-diaminopyridine were employed for the synthesis of 19SAB003, 19SAB005, 19SAB007, and 28SMB008 (17) (Fig. 2). Compounds 23SMB046, 23SMB050, 26SMB060, and 27SMB005 (Fig. 3) were prepared by a previously described methodology from the methyl imidate derivatives of 2- or 4-cyanopyridine and the appropriate α,ω-diaminoalkane (18). All 14 compounds were isolated as their hydrochloride salts. Experimental details and physical data for the compounds shown in Fig. 2 and 3 are given in Text S1 in the supplemental material.

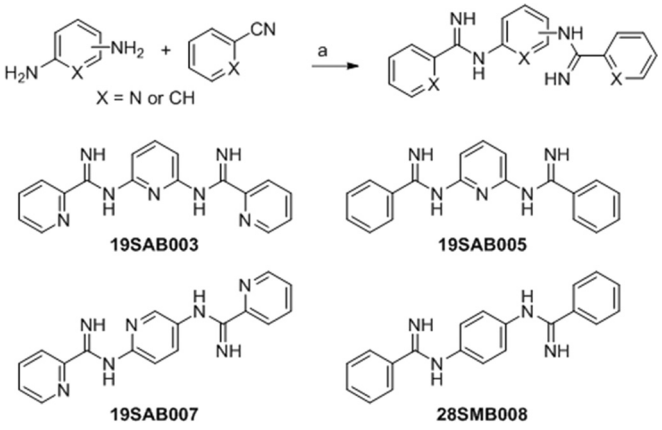


FIG 2 Synthesis of bisarylimidamide derivatives of benzene and pyridine. Reagents and conditions were as follows: a, sodium bis(trimethylsilyl)amide; tetrahydrofuran; and 25°C.

Stock solutions of the tested compounds. The studied molecules were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich), with the final concentration of the solvent (obtained by dilution using RPMI 1640 medium) never exceeding 0.6% and 10% for the *in vitro* and *in vivo* analyses, respectively. The solvent did not exert toxicity on parasites or mammalian host cells (data not shown). Bz was purchased from the Laboratório Farmacêutico do Estado de Pernambuco (LAFEPE), Brazil. Bz was dissolved in distilled and sterile water supplemented with 3% Tween 80 and does not have any detectable effect on mice (19).

Mammalian cell cultures. Primary cultures of embryonic cardiac cells (CCs) were obtained from Swiss Webster mice as previously reported (20). After purification, the CCs were seeded into 24- and 96-well microplates containing gelatin-coated coverslips at densities of 0.2×10^6 and 0.05×10^6 cells/well, respectively, as reported previously (18). The cardiac cell cultures were then sustained at 37°C in Dulbecco's modified Eagle medium (DMEM; without phenol red; Sigma-Aldrich) supplemented with 10% horse serum, 5% fetal bovine serum, 2.5 mM $CaCl_2$, 1 mM L-glutamine, and 2% chicken embryo extract. Additionally, mouse L929 fibroblasts were cultivated (4×10^3 cells/well in 96-well microplates) at 37°C in RPMI 1640 medium (pH 7.2 to 7.4) without phenol red (Gibco BRL) supplemented with 10% fetal bovine serum and 2 mM glutamine (RPMI), as reported previously (21).

***In vitro* cytotoxicity tests.** CCs and L929 cell cultures were incubated at 37°C for different periods of time (24 to 96 h) with increasing concentrations of each compound (up to 96 μM) diluted in DMEM (without phenol red). Next, mammalian cell morphology and spontaneous contractibility (of CCs) were evaluated by light microscopy, and cell viability was determined by a colorimetric assay using 10 μl alamarBlue (Invitrogen), which was added to each well. After incubation for 24 h, the absorbance at 570 and 600 nm was determined and the results were determined following the manufacturer's instructions. Then, the concentration that reduced cell viability by 50% (LC_{50}) was calculated as reported previously (21).

Parasites. Bloodstream trypomastigote (BT) forms of the Y and Colombiana strains of *T. cruzi* were obtained from the blood of infected male Swiss Webster mice at the peak of parasitemia (12). Immediately after the purification step, the parasites were resuspended in RPMI 1640 medium (pH 7.2 to 7.4) without phenol red (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine, as reported previously

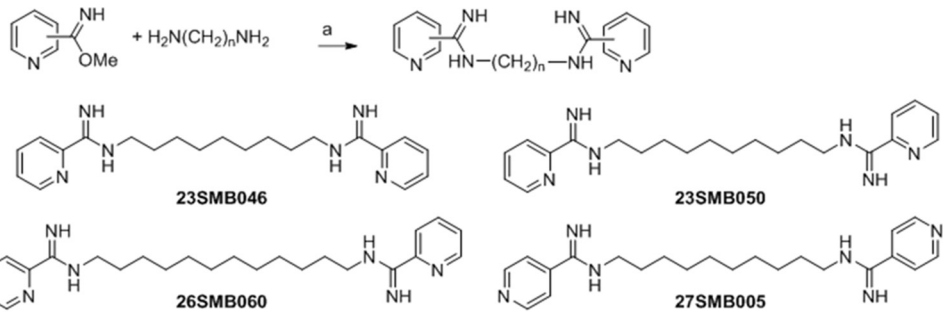


FIG 3 Synthesis of bisarylamidamide derivatives of *n*-alkanes. Reagents and conditions were as follows: a, 4 M HCl in dioxane; methanol; and 25°C.

(18). The effect against the intracellular forms was investigated through the use of L929 cell lineages infected with tissue culture-derived trypomastigotes (the Tulahuen strain expressing the *Escherichia coli* β-galactosidase gene), using a 10:1 parasite/host cell ratio. The incubation with the tested compounds was performed for 96 h, and previously established protocols were followed (21). Alternatively, CCs were infected (ratio, 10:1) with bloodstream trypomastigotes (Y strain). After 24 h of interaction, the infected cultures were rinsed and then exposed for 48 h to nontoxic concentrations of the amidines, which had previously been screened on mammalian host cells.

Trypanocidal analysis. The BT forms of the Y and Colombiana strains (5×10^6 per ml) were incubated for up to 24 h at 37°C in RPMI in the presence of serial dilutions of the compounds (0 to 32 μM), and parasites incubated with culture medium alone were used as controls. After incubation with the compound, 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 compound concentration that reduced the number of parasites by 50% (the 50% effective concentration [EC_{50}]) was calculated. Also, the compound concentration that reduced the number of parasites by 90% (EC_{90}) was further calculated in intracellular assays using the Y strain (18). For the assay with intracellular forms, cardiac cell cultures and cells of the L929 cell line were used as hosts for infection with the Y and Tulahuen strains, respectively. Briefly, Tulahuen-infected L929 cell cultures were exposed to 10 μM (corresponding to the EC_{90} value of Bz, as reported elsewhere [18]) each compound diluted in RPMI, and the compounds that resulted in a ≥50% reduction of the parasite infection index were further screened at increased concentrations to determine the EC_{50}s (18). After 96 h of incubation with the compound at 37°C, chlorophenol red glycoside (500 μM; Sigma-Aldrich) in 0.5% Nonidet P-40 was added to each well, and the plate was incubated for 18 h at 37°C. Next, the absorbance at 570 nm was measured. Uninfected and *T. cruzi*-infected cultures exposed to the vehicle and Bz were run in parallel. The results are expressed as the percentage of *T. cruzi* growth inhibition in compound-tested host cells compared to that in the infected but untreated cells (21). Triplicate samples were run in the same plate, and at least two assays were performed in each analysis. For the analysis of the effects of the test compounds against intracellular amastigotes of the Y strain, after 24 h of parasite-host cell interaction, the infected CCs were washed to remove free parasites and then incubated for another 48 h with increasing concentrations of the test compounds. CCs were maintained at 37°C in an atmosphere of 5% CO_2 and air, and the medium was replaced every 24 h. Then, the samples were fixed and stained with Giemsa solution (Sigma-Aldrich), and the number of infected host cells and the number of parasites per infected host cell were determined by light microscopy analysis (18). Only characteristic *T. cruzi* cells with nuclei and kinetoplasts were counted as surviving parasites, since irregular structures could indicate parasites undergoing death. Compound activity was estimated by calculating the percentage of inhibition upon the infection index (the percentage of infected host cells multiplied by the average number of intracellular amastigotes per infected host cell) (12). At least two assays were performed in duplicate in each analysis.

LB labeling. To determine the biogenesis of lipid bodies (LBs) in cardiac cell cultures exposed to amidines, untreated CCs were incubated for 2 to 48 h with the studied compounds at the corresponding EC_{50}s (against intracellular forms of the Y strain determined earlier) and then fixed for 10 min with 3.7% formaldehyde in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (pH 7.4). Next, the samples were rinsed twice using distilled water, incubated for 5 min in absolute propylene glycol, and stained for 10 min in 0.5% oil red (Sigma-Aldrich). The cultures were incubated for 3 min with 85% propylene glycol and, finally, rinsed twice using distilled water. The samples were incubated or not with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for DNA staining (host cells nuclei), rinsed with saline buffer, dried, and mounted using aqueous mounting medium [2.5% 1.4-diazabicyclo-(2.2.2)octane (DABCO)], and the fluorescence was analyzed with a ×63 oil objective in a Zeiss photomicroscope (AxioCam) equipped with epifluorescence (Zeiss Inc., Thornwood, NY) and by using a filter set for UV-excited probes. Images were captured using the software AnalySIS OPTI. As positive control, 2 μM oleic acid (Sigma-Aldrich) was used to trigger the biogenesis of the lipid bodies (22).

***In vivo* acute toxicity.** In order to determine the no-observed-adverse-effect level (NOAEL), increasing doses of the tested compounds (up to 200 mg/kg of body weight) were injected by the intraperitoneal (i.p.) route individually in female Swiss Webster mice (weight, 20 to 23 g; $n = 2$ mice per assay for two assays). Treated animals were inspected for toxic and subtoxic symptoms according to the Organization for Economic Cooperation and Development (OECD) guidelines. At 48 h after compound injection, the NOAEL values were determined as reported previously (23).

Biochemical analysis. At 48 h after compound administration, mouse blood was collected and immediately submitted to biochemical analysis for determination of the levels of plasma markers, including urea (blood urea nitrogen [BUN]), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine kinase (CK), which was performed at the animal facilities (CECAL/Fiocruz platform) of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) using an Ortho Clinical Vitros 250 chemistry system (Johnson & Johnson), as reported previously (23).

***In vivo* infection.** Male Swiss Webster mice (weight, 18 to 20 g) obtained from the animal facilities of CECAL were housed at a maximum of 6 per cage, kept in a specific-pathogen-free (SPF) room at 20 to 24°C under a 12- light and 12-h dark cycle, and provided sterilized water and chow *ad libitum*. The animals were allowed to acclimate for 7 days before starting the experiments. Infection was performed by i.p. injection of 10^4 and 5×10^3 bloodstream trypomastigotes (the Y and Colombiana strains, respectively). Age-matched noninfected mice were maintained under identical conditions (12).

Treatment schemes. AIAs were first dissolved in DMSO and then freshly diluted with sterile distilled water. The stock solution of benznidazole (*N*-benzyl-2-nitroimidazole acetamide) was prepared in sterile distilled water with 3% Tween 80 (Sigma-Aldrich). The animals were divided into the following groups (5 animals per group): uninfected (noninfected and nontreated), untreated (infected but treated only with vehicle), and treated (infected and treated with the compounds) groups. The therapy (once a day) was performed by the use of different schemes (see Text S2 in

TABLE 1 *In vitro* activities (EC_{50}s) and SIs of the tested compounds against BT forms of *Trypanosoma cruzi* Y

Compound	EC_{50} (μM)		SI at 24 h
	2 h	24 h	
35DAP096	>32	>32	>1
35DAP073	0.7 ± 0.14	0.5 ± 0.2[a]	64
35DAP077	28 ± 0.3	10 ± 5.5	2
35DAP081	2 ± 0.8	0.6 ± 0.2[a]	53
38DAP092	16 ± 11	7 ± 1.2[a]	5
38DAP096	>32	24 ± 1	1.3
19SAB003	>32	>32	>1
19SAB005	>32	>32	>1
19SAB007	>32	>32	>1
28SMB008	>32	>32	>1
23SMB046	>32	>32	>1
23SMB050	>32	>32	>1
26SMB060	>32	>32	>1
27SMB005	>32	>32	>1
Benznidazole	>100	13 ± 2	77[b]

[a] $P < 0.05$ by ANOVA.
[b] The datum is from Timm et al. (18).

the supplemental material). In the first set of assays, *T. cruzi* (Y strain)-infected mice were treated for only 2 days (at 5 days postinfection [dpi] and at 8 dpi, which correspond to the time of parasitemia onset and the time of peak parasitemia in this experimental model, respectively), using 5 to 20 mg/kg/day AIA (administered i.p.) and 100 mg/kg/day Bz (administered orally). In the second set of experiments, mice were infected with the Colombiana strain and the therapy started at 10 dpi (the time of parasitemia onset in this animal model) were treated for 10 consecutive days with 35DAP073 alone (5 mg/kg/day) or in combination with Bz (0.5 mg/kg/day AIA plus 100 mg/kg/day of Bz). In all assays, only mice with positive parasitemia were used in the infected groups.

Parasitemia and mortality rates. Parasitemia was individually checked by direct microscopic counting of the number of parasites in 5 μl of blood, and the mice were checked for mortality daily until 30 days posttreatment. Mortality is expressed as the percent cumulative mortality (CM) as described before (18).

Determination of blood parasite load by qPCR. For quantitative real-time PCR (qPCR), 500 μl blood was diluted in a 1:2 volume of guanidine solution (6 M guanidine-HCl, 0.2 M EDTA; Sigma-Aldrich) and heated for 90 s in boiling water. Blood samples in guanidine-EDTA were processed using a QIAamp DNA minikit (Qiagen) (24). Quantitative real-time multiplex PCR assays using TaqMan probes targeting *T. cruzi* satellite nuclear DNA and the internal amplification control (IAC; plasmid pZErO-2 containing an insert from the *Arabidopsis thaliana* aquaporin gene) were performed as described previously (25). The standard curves for absolute quantification were constructed with serial dilutions of total DNA ranging from 10^5 parasite equivalents to 0.5 parasite equivalents per ml of blood obtained with a negative blood sample in guanidine-EDTA spiked with 10^5 parasite equivalents per milliliter of blood.

Ethics. All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14).

Statistical analysis. The data represent the means ± standard deviations (SDs) from 2 experiments run in duplicate, and statistical analysis was performed by analysis of variance (ANOVA), with significance being set at a P value of ≤0.05 (23).

RESULTS

The phenotypic analysis of BT forms (Y strain, discrete typing unit [DTU] II) showed that all studied compounds were active against the parasite. The response to compounds 35DAP073, 35DAP081, 35DAP077, and 35DAP092 was time dependent, with the compounds exhibiting EC_{50}s ranging from 0.7 to 28 μM and 0.5 to 10 μM after 2 and 24 h of incubation, respectively, while the EC_{50} of Bz was 13 μM after 24 h (Table 1). Among these four compounds, the most active were 35DAP073 and 35DAP081, and both also displayed the highest selectivity indexes (SIs; 64 and 53, respectively). Statistical analysis revealed that 35DAP073, 35DAP081, and 35DAP092 presented efficacy superior to that of Bz ($P < 0.05$). When BT forms of the Colombiana strain (DTU I) were assayed, both amidines showed a trypanocidal effect, with the EC_{50}s of 35DAP073 and 35DAP081 being 3.8 ± 2.8 and 1.9 ± 0.4 μM, respectively. 35DAP073 and 35DAP081 were very active against intracellular forms of both the Tulahuen (Table 2) and Y (Table 3) strains of *T. cruzi*, which are representatives of DTUs VI and II, respectively, and were more potent than Bz ($P \leq 0.05$). The *m*-terphenyl bis-AIA 35DAP073 showed higher activity, indicated by the lower EC_{90} values (87 nM) after 48 h of incubation, and was about 126-fold more effective than Bz, and it also exhibited the highest SI (SI, 2,000) (Table 3).

TABLE 2 *In vitro* activities (EC_{50}s) and SIs of the tested compounds against intracellular forms of *Trypanosoma cruzi*[a]

Compound	EC_{50} (μM)	SI
35DAP073	0.04 ± 0.005[b]	480
35DAP081	0.79 ± 0.06[b]	18
Benznidazole	2 ± 0.8	51

[a] The *Trypanosoma cruzi* Tulahuen strain expressing the *Escherichia coli* β-galactosidase gene was used to infect L929 cells, which were treated for 96 h at 37°C.
[b] $P < 0.05$ by ANOVA.

As 35DAP073 was more effective against intracellular forms than BT forms (Y strain; Tables 1 and 3) and the biogenesis of LBs may be induced by different stimuli (26), the induction of LB biogenesis during incubation of uninfected CCs with this amidine was compared with that during incubation of uninfected CCs with another molecule not active against *T. cruzi in vitro* (26SMB060). After incubation for different lengths of time (2 to 48 h), our data revealed that both amidines were able to induce LB biogenesis at similar levels very quickly, since strong LB formation compared to that in the untreated group was seen as soon as only 2 h posttreatment, and the profile was similar to that obtained with the positive control (oleic acid) (Fig. 4). A large number of LBs was also observed after 24 h of amidine exposure, and the levels were maintained up to 48 h, although oleic acid caused the progressive and continuous formation of LB (increases in the number and volume) (Fig. 4).

Next, due to the high activity and selectivity of 35DAP073 and 35DAP081, Swiss Webster mice were used to determine the NOAEL values of the two compounds. 35DAP073 proved to be

TABLE 3 *In vitro* activities (EC_{50}s) and SIs of the tested compounds against intracellular forms of *Trypanosoma cruzi* Y in CCs at 48 h after incubation at 37°C

Compound	EC_{50} (μM)	EC_{90} (μM)	SI[a]
35DAP073	0.016 ± 0.007[b]	0.087 ± 0.009[b]	2,000
35DAP081	0.23 ± 0.06[b]	0.9 ± 0.05[b]	139
Benznidazole	3.6 ± 1.7	11 ± 2.7	277

[a] The SI data are related to the EC_{50} values.
[b] $P < 0.05$ by ANOVA.

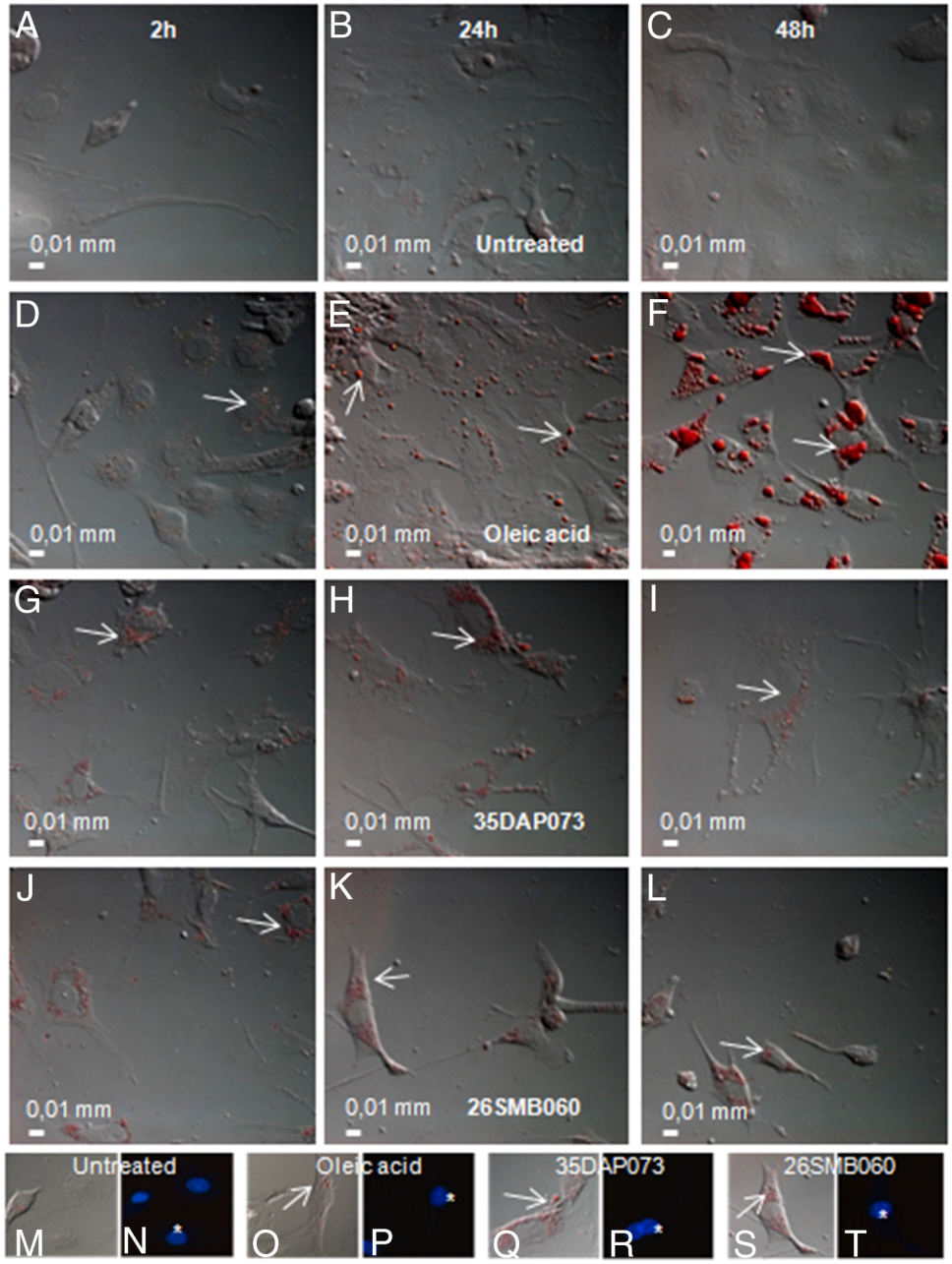


FIG 4 Differential interference contrast (DIC) microscopy analysis of oil red labeling of cardiac cells untreated (A to C) and exposed to oleic acid (D to F), 35DAP073 (G to I), and 26SMB060 (J to L) for 2 h (A, D, G, J), 24 h (B, E, H, K), and 48 h (C, F, I, L) at 37°C. (M to T) Detailed oil red labeling of cardiac cells by differential interference contrast (M, O, Q, and S) and fluorescence microscopy using DAPI staining of mammalian cell nuclei (N, P, R, and T) after 24 h of incubation with 35DAP073 (Q and R) and 35DAP060 (S and T), showing the accumulation of lipid bodies randomly distributed throughout the cytoplasm.

less toxic at all doses tested, presenting an NOAEL of 25 mg/kg, while 35DAP081 showed an NOAEL of <12.5 mg/kg (Table 4). Plasma biochemical analysis ($P \leq 0.1$) after 48 h of compound administration did not show any statistically significant differences in the levels of ALT, AST, CK, and BUN among all tested groups (Table 5). Due to the findings of these preliminary acute toxicity studies, only 35DAP073 was evaluated in *in vivo* models of acute *T. cruzi* infection using nontoxic doses (up to 25 mg/kg/day). In parallel, a control group was orally treated with Bz (100 mg/kg/day) (Fig. 5).

Our data demonstrated a dose-dependent effect of treatment with 35DAP073 at concentrations of up to 20 mg/kg/day, which led to 96 to 46% reductions in the levels of parasitemia (Fig. 5). The animal survival rates achieved 100% when the mice were treated with 5 and 10 mg/kg/day of 35DAP073, which was similar to the results obtained with 100 mg/kg/day Bz (Fig. 5). However,

TABLE 4 Results of acute toxicity analysis[a]

Compound	Result after treatment at the following dose (mg/kg): 0	12.5	25	50	100	200	NOAEL (mg/kg)
35DAP073	NDE	NDE	NDE	Loss of mouse body wt	Tremors, ataxia	Death	25
35DAP081	NDE	Ataxia, tremors, excitation and vocalization[b]	Ataxia, tremors, excitation and vocalization[b]	Ataxia, tremors, excitation and vocalization[b]	Ataxia, tremors, excitation and vocalization[b]	Ataxia, tremors, excitation and vocalization[b]	<12. 5

[a] Escalating doses starting at 12.5 mg/kg and increasing to 200 mg/kg were administered i.p. in a 0.1-ml final volume to female Swiss Webster mice, using a single mouse per dose. NOAEL, no-observed-adverse-effect level (for noninvasive parameters); NDE, no detectable effect.
[b] These effects were observed after 2 h of compound administration.

as the tested compound did not result in the complete suppression of the blood parasite load, a longer period of therapy (10 days using 5 mg/kg/day) was next investigated with mice infected with 5×10^3 bloodstream trypomastigote forms of the Colombiana strain. Also, 35DAP073 combined with Bz (treatment for 10 days using 0.5 mg/kg/day 35DAP073 and 100 mg/kg/day Bz) was assayed in parallel using the same experimental model described above, with the therapy being started at the time of parasitemia onset. Our findings showed that 5 mg/kg/day of 35DAP073 completely suppressed the parasitemia and gave 100% animal survival, as did Bz (Fig. 6). However, at the end of the therapy period (the last day of 35DAP073 administration), the mice presented some undesirable neurological toxic disorders (tremors, shaking, ataxia) that were no longer observed after 48 h posttreatment (data not shown). The combination of 0.5 mg/kg/day of this amidine with Bz resulted in parasitemia suppression, 100% animal survival, and no toxic events (Fig. 6). Analysis by qPCR showed that although no statistically significant difference in blood parasite loads was found among the tested groups ($P > 0.05$), monotherapy with Bz and 35DAP073 resulted in mean values of 204 and 265 parasite equivalents (Par. Eq.)/ml, respectively, while the association of Bz with the AIA induced a higher reduction (60%) of the blood parasite load (82 Par. Eq./ml) (Fig. 7).

DISCUSSION

The current therapies for Chagas disease remain unsatisfactory due to their various side effects and limited efficacies (27). Amidines have been used as antiparasitic agents for decades in human and veterinary medicine but show important limitations, such as the need for parenteral administration and undesirable side effects (7). In order to overcome these limitations, novel amidines have been synthesized and phenotypic analysis of novel amidines has been conducted *in vivo* and *in vitro* (12, 28). Among these, arylimidamides (AIAs) showed considerable biological activity against several intracellular pathogens, including *T. cruzi* (29), with the bis-AIAs being the most effective of the group (19).

A previous study reported the synthesis of dicationic *m*-terphenyl derivatives and their biological effect against *Trypanosoma brucei rhodesiense*, *Plasmodium falciparum*, *Leishmania amazonensis*, and *T. cruzi* (16). Among these, bispyridylimidamides were the most potent against the intracellular amastigote form of the *T. cruzi* (Tulahuen strain, DTU VI). In the present study, we further explored the phenotypic activity of 14 bis- and monoarylimidamides against BTs and intracellular forms of *T. cruzi in vitro* and *in vivo* and extended the analysis to other parasite strains (the Y and Colombiana strains, which belong to DTUs II and I, respectively).

Structural variations of the test compounds used in this study included those in AIA moieties as well as the central cores of the molecules. Maximum potencies were observed with 35DAP073 and 35DAP081, each of which has two 2-pyridylimidamide groups attached to an *m*-terphenyl nucleus and in which at least one of the two AIA groups is *meta* to the central ring. These two compounds were more potent than the correspond-

TABLE 5 Plasma biochemical analysis of female mice after 48 h of compound administration

Biochemical marker	Compound	Result after treatment with the following dose (mg/kg)[a]: 0[b]	12.5	25	50	100	200
ALT	35DAP073	57 ± 8	58 ± 0	51 ± 0	88 ± 51	77 ± 41	ND
	35DAP081		56 ± 0.7	67 ± 13	82 ± 8	102 ± 23	116 ± 11
CK	35DAP073	935 ± 226	635 ± 80	368 ± 0	701 ± 260	870 ± 646	ND
	35DAP081		503 ± 145	509 ± 145	737 ± 0	1,681 ± 310	1,162 ± 452
AST	35DAP073	183 ± 16	109 ± 0	113 ± 0	152 ± 37	242 ± 105	ND
	35DAP081		105 ± 16	143 ± 21	285 ± 55	422 ± 132	376 ± 229
BUN	35DAP073	46 ± 11	51 ± 16	34 ± 0	43 ± 2	55 ± 12	ND
	35DAP081		37 ± 7	29 ± 7	31 ± 10	29 ± 0	60 ± 29

[a] Data are in milligrams per deciliter for blood urea nitrogen and units per liter for all other biochemical markers and are means ± SDs from two independent assays. Reference values (CECAL/Fiocruz) are as follows: blood urea nitrogen (BUN), up to 29 mg/dl; alanine aminotransferase (ALT), up to 132 U/liter; aspartate transaminase (AST), up to 247 U/liter; creatine kinase (CK), up to 1,070 U/liter. *P* was <0.05 by ANOVA for all samples. ND, not determined due to animal death at the higher dose.
[b] Results are from one representative assay.

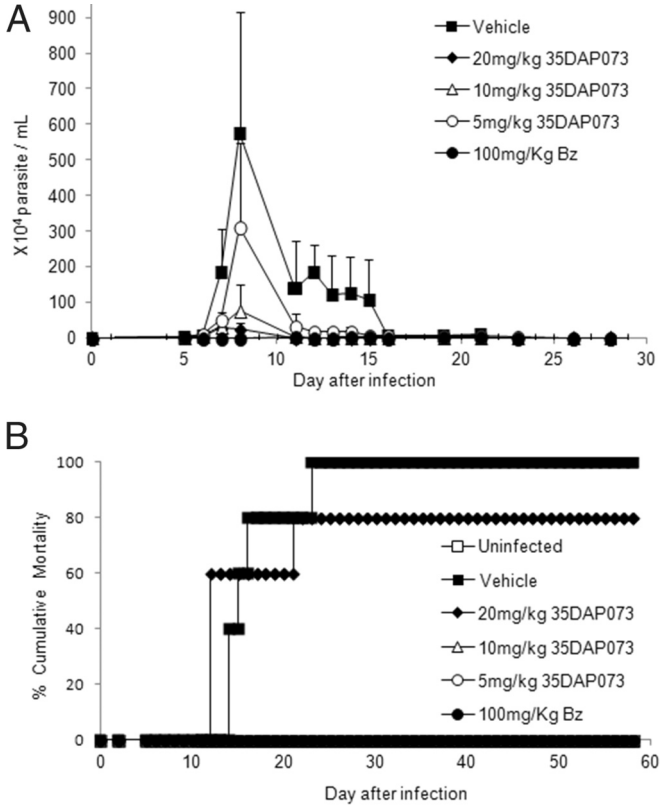


FIG 5 Effect of 35DAP073 (20 to 5 mg/kg/day) and of benznidazole (100 mg/kg/day) administration at 5 and 8 dpi in a mouse model of experimental *T. cruzi* infection (Y strain). (A) Parasitemia levels; (B) percent cumulative mortality.

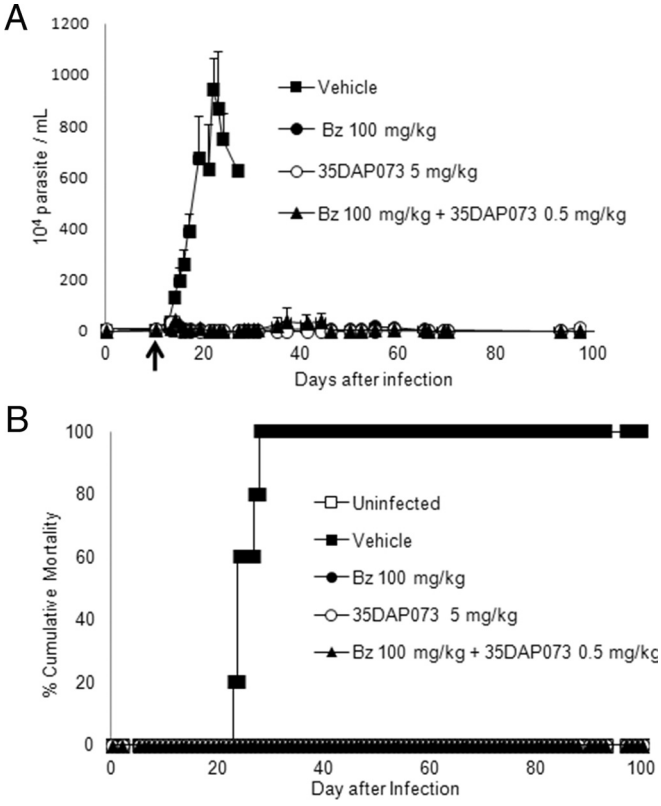
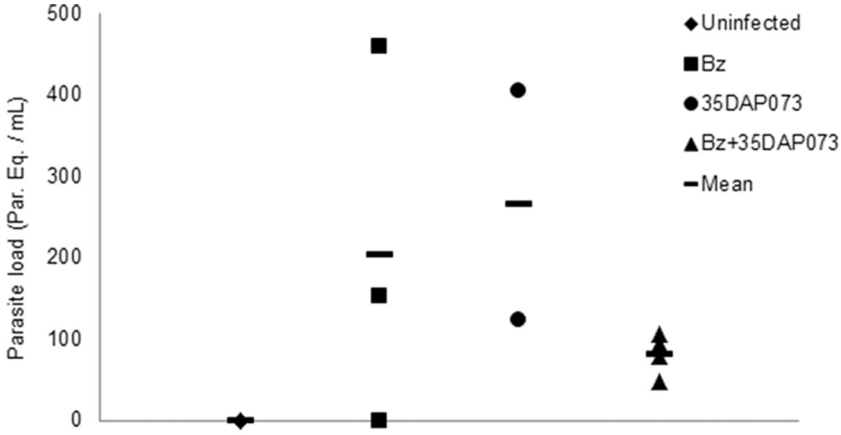


FIG 6 Effect of daily administration of 35DAP073 (5 mg/kg/day), benznidazole (100 mg/kg/day), and 35DAP073 plus Bz (0.5 mg/kg/day and 100 mg/kg/day, respectively) for 10 days in mouse models of experimental *T. cruzi* infection (Colombiana strain) when compounds were administered starting at 10 dpi. (A) Parasitemia levels; (B) percent cumulative mortality.

ing bisphenylimidamides, 35DAP069 and 35DAP077. The difference in potencies between 35DAP073 and 35DAP069 was greater than 60-fold but was less pronounced than that between 35DAP081 and 35DAP077. The bispyridylimidamides 35DAP073 and 35DAP081 were also more potent than the monopyridylimidamides 35DAP092 and 38DAP096, thus demonstrating the importance of two AIA moieties. Compound 38DAP092, in which the AIA group is *meta* to the central ring, was more potent than its regioisomer, 38DAP096, which bears a *para*-AIA moiety. None of the bis-AIAs with lone aromatic rings or aliphatic chains between the two AIA functionalities (Fig. 2 and 3) showed detectable activity.

The most active molecule, the *m*-terphenyl bis-AIA 35DAP073, showed an EC_{50} of 0.5 μM against trypomastigotes (Y strain) after 24 h and was more effective than the reference drug, Bz (13 μM). This amidine was also active against a highly naturally resistant strain (Colombiana), exhibiting an EC_{50} of 3.8 μM. Analysis of its activity against intracellular forms (Tulahuen strain) showed that this bis-AIA (EC_{50} = 0.04 μM) was about 50-fold more active than Bz (2 μM). The greater effect against intracellular forms than BT forms was dissociated from the ability of the amidines to induce LB accumulation within mammalian host cells. The biogenesis of lipid bodies is a central event in several processes of cellular homeostasis, as well as during intracellular pathogen infection (30, 31). LBs are recognized to be dynamic organelles composed of a triglyceride and cholesteryl ester core with a surrounding monolayer of phospholipid, cholesterol, and a variety of associated proteins with diverse functions in cell metabolism, signaling, and inflammation (14, 32). Under inflammatory and infectious conditions, prostaglandins and other lipid mediators are mainly produced by LBs (33). Also, recent findings showed that antimicrobial compounds may induce an accumulation of lipid droplets in the cytoplasm of *Candida* spp. (34) and protozoans (35). In *Mycobacterium leprae*-infected cells, LBs showed a strong ability to fuse, forming giant lipid droplets (36). Since amidines can modulate the functional activities of mammalian host cells linked to the control of parasite proliferation and/or survival (37, 38) and LBs act on cellular metabolism, inflammation, and infectious conditions (14, 32), in the present study we investigated whether the studied AIAs could interfere with LB biogenesis and parasitism control *in vitro*. Our data also demonstrated that 35DAP073 and 28SMB060 were able to induce LB biogenesis in cardiac cells at all times tested (2 to 48 h), with cells treated with the two compounds exhibiting similar morphological profiles. No correlation between the trypanocidal effect of amidines and LB accumulation in CCs was found, but further studies are desirable to better understand the consequences of the higher LB levels induced by the amidines in the parasite as well the consequences on the mammalian cell physiology, since this modulation may influence the growth of intracellular parasites (36).

Next, due to the excellent selective indexes of 35DAP073 and



Mean and standard deviation (±) values of the parasite equivalents per milliliter of blood (Par.Eq/mL) by qPCR analysis

Mice Groups	Par.Eq/mL
Uninfected	0 ± 0
35DAP073	265 ± 199
Benznidazole	204 ± 233
Benznidazole + 35DAP073	82 ± 24

FIG 7 qPCR analysis of the parasite load (in numbers of parasite equivalents per milliliter [Par. Eq./ml]) in the blood of uninfected mice and mice infected with the Colombiana strain submitted to each different treatment regimen: monotherapies (benznidazole at 100 mg/kg/day and 35DAP073 at 5 mg/kg/day) and combined therapy (benznidazole at 100 mg/kg/day plus 35DAP073 at 0.5 mg/kg/day). Each symbol represents an individual value, and the bars represent the respective mean values.

35DAP081, both were tested for acute toxicity. The results showed NOAEL values of 25 and 12.5 mg/kg for 35DAP073 and 35DAP081, respectively. Then, *in vivo* efficacy studies were conducted with 35DAP073 at concentrations of <25 mg/kg. Our data demonstrated that 35DAP073 treatment of mice infected with the Y and Colombiana strains resulted in a dose-dependent action of 35DAP073, leading to 96 to 46% reductions of parasitemia and 100% animal survival, with Bz showing a similar effect. Unfortunately, as the 10 daily doses (at 5 mg/kg i.p.) were toxic for the animals, combined therapy with 35DAP073 and Bz was tested with the aim of reducing the toxic effects of the amidine. Monotherapy with 35DAP073 at only 0.5 mg/kg/day was not attempted, as previous data with related amidines demonstrated that doses of ≤5 mg/kg/day had only a mild effect on parasitemia control (23). As was also found by our group (28) using the combination of Bz with the amidine DB289, the association of 0.5 mg/kg 35DAP073 with Bz in the present study improved the control of parasite proliferation, providing a 9-fold enhancement of activity compared to that of Bz alone. qPCR analysis showed that the combination therapy was the best regimen, exhibiting parasitemia suppression with no detectable toxicity to the animals. In this sense, our results support further experimental investigations of this compound class in association with licensed antiparasitic drugs with the aim of contributing to the identification of novel alternatives for the treatment of neglected parasitic diseases, such as Chagas disease.

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Artigo III:

Guedes-da-Silva FH, Batista DGJ, Da Silva CF, Meuser MB, Pavão BP, Moreira OC, Souza LRQ, Britto C, Lepesheva GI; Soeiro MMC. Combined therapy of sterol 14 α-demethylase (CYP51) inhibitor VFV associated to benznidazole on mouse experimental models using drug-resistant strain of *Trypanosoma cruzi* **Current Topics in Medicinal Chemistry, submetido para publicação**.

A alta variabilidade genética das populações do *T. cruzi* representa importante desafio a ser considerado nas ações relacionadas a descoberta de novos candidatos a fármacos para doença de Chagas. O tratamento de várias doenças incluindo de origem infecciosa como tuberculose, lepra, doença do sono e SIDA atinge a melhor eficácia com combinações de fármacos com diferentes mecanismos de ação. O tratamento com a combinação de medicamentos pode não somente aumentar a ação dos diferentes compostos terapêuticos usados isoladamente, mas também contribuir para prevenção no desenvolvimento da resistência do parasito aos quimioterápicos por atingir diferentes alvos dos parasitos, reduzir tempo de administração e doses empregadas. Visando explorar o aspecto promissor do esquema combinatório de novos compostos para ampliar o arsenal de novas terapias para DC, neste estudo, nosso objetivo foi avaliar a potente eficácia de VNI and VFV (inibidores da CYP51) sobre modelos experimentais de infecção pelo *T. cruzi* usando uma cepa naturalmente resistente a nitroderivados, a Colombiana, sob regime de monoterapia ou associado com Bz.

Combined Therapy of Sterol 14 α-Demethylase (CYP51) Inhibitor VFV Associated to Benznidazole on Mouse Experimental Models Using Drug-Resistant Strain of *Trypanosoma cruzi*

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Summary

Up to now no vaccines are available and the current therapy is largely unsatisfactory for Chagas disease. Novel imidazoles scaffolds of sterol 14α-demethylase (CYP51) inhibitors have demonstrated potent anti-parasitic activity. Presently our aim was investigate the potential effectiveness of the novel CYP51 inhibitor VFV against mouse models of *Trypanosoma cruzi* infection using naturally resistant Colombiana strain under monotherapy and on association with the reference drug, benznidazole (Bz). VFV was as potent as VNI resulting in complete parasitemia suppression and giving 100% animal survival when administered orally at 25 mg/kg for 60 days. As VFV did not reach parasitological cure and combined therapy may result in higher efficacy due to the different targeting of parasite metabolism, the association of VFV with Bz was assayed under two different protocols: giving simultaneously (for 60 consecutive days) or sequentially (Bz for 30 days followed by VFV for another 30 days) by oral route. The findings showed that all tested mice groups resulted in >99.9% of parasitemia decrease and 100% survival. However, qPCR analysis performed in cyclophosphamide immunosupressed mice revealed that although presenting lack of cure, VFV given as monotherapy was 14-fold more active than Bz, reducing largely the blood parasite load. Also, the combination of VFV plus Bz (given sequentially or simultaneously) resulted in 225 and 106-fold lower blood parasitism respectively, as compared to the monotherapy of Bz. Another interesting data was the parasitological cure in 40% of those animals treated sequentially with Bz and VFV confirming the promising aspect of drug combinatory scheme to improve the efficacy of therapeutic arsenal against *T. cruzi*.

Introduction

Chagas disease affects more than 6 million people mostly in poorest areas of Latin America. The available therapy is based on two nitroderivatives, nifurtimox (N) and benznidazole (Bz) introduced more than 4 decades ago and that are quite unsatisfactory since both display limited activity (especially in the later chronic stage), high toxicity besides the occurrence *Trypanosoma cruzi* strains that are naturally resistant to nitroderivatives (Zingales et al., 2014) which represents a special concern for the identification of novel trypanocidal candidates.

CYP51 (sterol 14α-demethylase) has proven a relevant target for fungi and protozoan infection (Lockhart et al., 2016, Lepesheva et al., 2011). Previous studies showed the high antiparasitic efficacy of the CYP51 inhibitor VNI against *T. cruzi in vitro* and *in vivo* infection, using 25 mg/kg dose twice a day (b.i.d.) (Soeiro et al., 2013; Villalta et al., 2013). However, although being able to cure mice infected with the susceptible Tulahuen strain (DTU VI) and treated for 30 days (Villalta et al., 2013), VNI was not able to reach high parasitilogical cure rates when *T.cruzi* resistant strains were employed under similar period of therapy and same dose (Soeiro et al., 2013).

Combined therapy has been successfully used to treat different pathologies including those triggered by parasitic infections. This scheme has also been largely recommended as promising alternative therapy for CD (Coura, 2009) aiming to improve drug efficacy allowing (i) to target different cellular elements and metabolic pathways (ii) the reduction of doses and exposure periods of drug administration, thus contributing to the lowering of toxic effects, and (iii) minimizing the risk of drug resistance (McKerrow et al. 2009; Bahia el al., 2014). In this sense, presently we investigate the anti-parasitic effect of a novel CYP51 inhibitor molecule (VFV) in mouse model of *T.cruzi* infection, using schemes of monotherapy and in association with Bz (concomitant or sequential therapeutic protocols). Our findings demonstrated the benefits of the association of Bz plus VFV leading to high suppression of *in vivo* infection reaching considerable cure rates (40%) when a high stringent model was employed, the highly resistant *T. cruzi* Colombiana strain.

Materials and methods

Compounds

Synthesis of VNI and VFV was performed as reported (Hargrove et al. 2012). In this study the CYP51 inhibitors were diluted using 10% of dimethyl sulfoxide (DMSO) plus 5% Gum Arabic (Soeiro et al., 2013). Benznidazole (Bz) was purchased from Laboratório Farmacêutico do Estado de Pernambuco, LAFEPE, Brazil and dissolved in distilled and sterile water supplemented with 3% Tween 80, which does not cause any detectable effect on mice (Da Silva et al., 2011). Cyclophosphamide (Cy) (Genuxal) was purchased from Baxter Oncology (Frankfurt) and prepared in distilled and sterile water (Guedes-da-Silva et al., 2015).

Parasites

Bloodstream trypomastigote (BT) forms of Colombiana (discrete typing unit – DTU I) strain of *T. cruzi* was obtained from the blood of infected male Swiss mice at the peak of parasitemia (Batista et al, 2010). Immediately after the purification step, the parasites were ressuspended in RPMI-1640 medium (pH 7.2-7.4) without phenol red (Gibco BRL) supplemented with 10% fetal bovine serum and 2 mM glutamine, as reported previously (Timm et al, 2014).

In vivo infection

Male Swiss Webster mice were obtained from the Fundação Oswaldo Cruz (FIOCRUZ) animal facilities (CECAL/FIOCRUZ, Rio de Janeiro, Brazil). Mice were housed at a maximum of 05 per cage and kept in a conventional room at 20 to 24°C under a 12 h/12 h light/dark cycle. The animals were provided with sterilized water and chow ad libitum. Mice (18 to 20 g) were infected by intraperitoneal (i.p.) route with $5x10^3$ bloodstream trypomastigotes from the Colombiana strain. Age-matched non-infected mice were maintained under identical conditions (Soeiro et al, 2013).

Treatment schemes

The animals were divided into the following groups (5 animals per group): uninfected (non-infected and non-treated); untreated (infected but treated only

with vehicle - 10% of dimethyl sulfoxide (DMSO) plus 5% Gum Arabic); and treated (infected and treated with the compounds). The therapy was performed by 60 consecutive days starting at 10 dpi (corresponding to the parasitemia onset in this animal model) given per oral (po), comparing the effectiveness of the monotherapy of each compound (100 mg/kg/day of Bz and 25mg/kg/day bid of VNI and VFV) with the simultaneous (60 daily doses given by po, using Bz and after 15 min giving VFV, only once a day) or sequential (30 daily doses of Bz (once a day) and then 30 daily doses of VFV bid) administration of the drugs using same doses above described. Only mice with positive parasitemia were used in the infected groups.

Parasitemia and mortality rates

Parasitemia was individually checked by direct microscopic counting of parasites in 5 µL of blood, and mortality rates checked daily until 30 days post treatment and expressed as percentage of cumulative mortality (% CM) as described before (Guedes-da-Silva et al, 2015).

Cure assessment

Mice that presented consistent negative parasitemia up to 30 days post treatment were submitted to three cycles of cyclophosphamide exposure (50 mg/kg/day) each, with four consecutive days of administration (ip) and with three days of intervals between each cycle (Guedes-da-Silva et al., 2015). As reported, cure criteria was based on the following parasitological methods: blood parasitemia negativation observed (i) by light microscopy, and (ii) by quantitative real time polymerase chain reaction (qPCR). Animals presenting negative results for all tests were considered “cured”. For qPCR 500 µL blood was diluted in 1:2 volume of guanidine solution (guanidine-HCl 6M/EDTA 0.2M), and heated for 90 sec. in boiling water (Britto et al., 1993). Guanidine-EDTA Blood Samples (GEB) were processed using the QIAamp DNA mini kit (QIAGEN), as described by (Moreira et al., 2013). Quantitative Real Time PCR Multiplex assays were performed targeting the *T. cruzi* satellite nuclear DNA and the internal amplification control - IAC (pZErO-2 plasmid containing an insert from the *A. thaliana* aquaporin gene), as described by Duffy et al (2013).

The standard curves for absolute quantification were constructed with 1/10 serial dilutions of total DNA obtained from a negative GEB sample spiked with 10^5 parasite equivalents per milliliter of blood (par. eq./mL).

Ethics

All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14).

Results

Previous studies performed with the small molecule VNI administered orally at 25 mg/kg for 30 days demonstrated a very promising trypanocidal activity even against *in vivo* infection using the highly resistant Colombiana strain (Soeiro et al., 2013). Thus presently the effect of a novel imidazolic derivative named VFV was investigated and due to its similar pharmacological profile as VNI, the same dosage was used as in the previous study (25 mg/kg b.i.d.) but now for longer periods of treatment - 60 consecutive days (Soeiro et al. 2013). In parallel, the use of Bz (at its optimal dose of 100mg/kg/day) and VNI was performed to establish a head-to-head comparison in potency aspects. Our data showed that VFV given as monotherapy scheme was able to suppress completely the parasitemia on infected animals similarly as VNI (Fig. 1A, Table 1) and Bz (Table 1). According to mice mortality induced by the parasitic infection, all treated groups reached 100% of mice survival during the whole period of analysis (Figure 1B, Table 1) while those animals that only received the vehicle reached > 40 % of mortality rates (Fig. 1 B). After the end of the monotherapy drug administration, a natural parasitemia relapse was found in all animals exposed to Bz and VFV but in only 3 out of five mice in the VNI group (Table 1). In order to confirm the therapeutic failure levels, the animals were further immunosuppressed by cyclophosphamide (Cy) cycles and then monitored for another 4 weeks to search for blood relapse. We found that 20% of the VNI group displayed a sustained negative parasitemia (through blood counting at light microscopy) which was also consistently negative for parasite DNA amplification through qPCR blood analysis (data not shown). Next, to further explore the effectiveness of the novel inhibitor VFV that presented about

14-fold reduction on blood parasite load measured by qPCR as compared to Bz (Table 2), combinatory assays were performed using Bz. The simultaneous administration of VFV plus Bz once a day revealed that besides reaching >99% parasitemia decline at the peak and 100% animal survival (Figure 2), two of five mice remained negative after the end of the combined therapy (Table 1). After Cy administration, although none remained negative during light microscopy analysis, the qPCR demonstrated a huge reduction in the blood parasite load reaching more than 100-fold decrease as compared to the monotherapy of Bz (Table 2). Next, another protocol was employed based on a methodology previously reported regarding the use of sequentially combined therapy, with Bz followed by the administration of another CYP51 inhibitor, the posaconazole (Diniz et al., 2013). In order to improve the potency of VFV, it was given twice a day (50 mg/kg/day) while Bz was kept once a day (100 mg/kg). Our findings showed that Bz plus VFV suppressed parasitemia (100%), avoided mortality (100% survival) leading to 2 out of 5 mice without exhibiting relapses (Tables 1 and 2) even after Cy injection (data not shown). Importantly, the qPCR analysis confirmed that 40% of the VFV-treated mice did not present therapeutic failure (Table 2) and the remaining positive mice from this group displayed more than 200-fold decrease in the blood parasitism detected by qPCR as compared to Bz monotherapy (Table 2).

Discussion

There is an urgent need for more safe and efficacious drugs for Chagas disease, a neglected silent life-threatening illness that causes more than 7,000 annual deaths, with about 25 million people at risk of infection in endemic areas of Latin America, affecting mainly rural populations in areas of low resources, where human contact with vectors is frequent (WHO, 2016). Despite vector control and blood transmission regional initiatives have successful achieved substantial reduction in the number of new acute cases (Pinto Dias, 2009), other concerns still remains as challengers such as the disease globalization and the existence of alternative routes such as mother-to-child and oral transmission through contaminated food (Gascón et al., 2010, WHO, 2015). Thus, as the available chemotherapy for CD have substantial side-effects and

variable efficacy, diverse pre-clinical approaches have been used in an attempt to identify new therapeutic alternatives, including the screening of natural and chemical libraries (Soeiro & De Castro, 2011), the repositioning of drugs already licensed to other diseases (Kaiser et al., 2015), the synthesis and validation of specific inhibitors targeting parasite molecules (Tagoe et al., 2015), as well as, the use of combination of licensed drugs with promising novel candidates (Coura, 2009; Bahia et al., 2014). Among the targeted-directed anti-parasitic approach, inhibitors of the sterol biosynthesis pathway, in particular, those related to the CYP51 enzyme, have been widely studied on experimental infection with *T. cruzi* (Hoekstra et al., 2015, Bahia et al. 2014) and two of them, posaconazole and the prodrug of the ravuconazole (E1224) were recently moved to clinical trials but unfortunately failed to display a sustained parasite suppression in chagasic chronic patients (Molina et al., 2015).

Through the screening of a Novartis library, our group has identified a new imidazole scaffold, the VNI that is a CYP51 inhibitor. VNI kills efficiently both bloodstream and intracellular forms of *T. cruzi* i*n vitro* in micromolar range concentration (Soeiro et al., 2013). The oral administration of VNI (up to 400 mg / kg) did not induce animal mortality neither triggered detectable side effects in acute toxicity mouse models (Hargrove, et al. 2012). VNI administrated up to 26-30 daily consecutive under optimum doses of 25mg/kg twice a day, gives more than 99% parasitological clearance and 100% survival in both acute and chronic mouse models of infections using susceptible (Tulahuen) and resistant *T. cruzi* strains (Y and Colombiana) (Villalta et al., 2013, Soeiro et al., 2013). However, VNI only provide parasitogical cure when mice were infected with the susceptible strain (Villalta et al., 2013). However, there is a consensus that a novel candidate for CD must act towards the diverse parasite strains belonging to the distinct *T.cruzi* DTUs, at least for those relevant for human infection, including those naturally resistant strains (DNDi, 206, Don & Loset, 2013, Zingales et al., 2013). Therapeutic failures are well documented in Chagas disease under the use of Bz and nifurtimox which seems to depend on the interplay of the genetic background of the *T. cruzi* strains and their mammalian hosts, the drug access and accumulation in different environments, and the host immune response (Romanha et al., 2010; Coura & De Castro 2002). In this context, to further explore the potential effectiveness of VNI and of the VFV,

both were investigate upon the high intrinsic resistant Colombiana strain. Presently the studies were conducted employing a longer period of treatment (60 days) using Bz (as reference drug) and the imidazole molecules under monotherapy regimen also addressing the scheme of drug combination (simultaneously – once a day and sequentially – Bz for 30 days followed by VFV for another 30 days by oral route, the later twice a day to improve pharmacological status). All tested mice groups treated using monotherapy approach resulted in >99.9% of parasitemia suppression and 100% mice survival. However, although not reaching sterile cure, VFV was more effective than Bz in reducing blood parasitism measured by qPCR analysis of treated mice submitted to Cy injection. VFV resulted in more than 14-fold reduction of parasite DNA that correlates to lower and subpatent blood parasitism. Another important finding was the improved efficacy of VFV when administrated in combination with Bz (given sequentially or simultaneously) resulting in >100-200-fold less blood parasitism measured by qPCR than Bz alone. Interestingly, the group sequentially exposed to Bz and then VFV (bid) induced 40% of parasitological cure confirming the promising aspect of drug combinatory scheme to improve the efficacy of therapeutic arsenal against *T. cruzi*. Other studies have revealed the successful use of drug combination using amidine compounds and Bz (Guedes-da-Silva et al., 2015) and also other compound classes including CYP51 inhibitors like posaconazole plus Bz in animals experimental models of *T. cruzi* infection (Diniz et al., 2013, Batista et al., 2011, Bustamente et al., 2014, Guedes-da-Silva et al., 2015).

Our study gives support for the use of combinatory schemes of anti-parasitic drugs as future potential clinical evaluation of Chagas disease. The curative action of Bz/VFV combinations was explored in a well-established acute infection model with the Colombiana strain using optimal doses of both compounds that did not exert any signs of animal toxicity until the endpoint (>120 days of following up), while no considerable cure rates were observed using the single drugs. Our findings accumulate evidences that it is possible to achieve a better therapeutic outcome using Bz in combination of other drug candidates that target other metabolic pathways of the parasite, including the ergosterol biosynthesis, also corroborating previous literature data (Diniz et al., 2013). In fact, several other pathogens induced diseases such as tuberculosis,

leprosy, HAT and HIV infection reached better efficacy with combinations of drugs with different mechanisms of action (Harries et al., 2015; Kumar et al., 2013; *Guia de Vigilância Epidemiológica da Tuberculose,* 2009). The combined drug treatment can not only boost the action of the different therapeutic compounds, but may also aid in avoiding the development of parasite chemotherapeutic resistance (Coura, 2009). Indeed, more than one new drug is needed for each so that combination therapy can be employed to avoid drug resistance and to provide back-up drugs when resistance emerges (WHO, 2015). In summary, our results support further experimental approaches towards this line of investigation aiming to achieve therapeutic and affordable drugs, orally administrated, as alternatives for those millions of chagasic patients.

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Legends:

Figure 1: Effect of VFV and VNI (25 mg/kg/day) administered as monotherapy for 60 days on mouse experimental model of *Trypanosoma cruzi* infection using Colombiana strain. (A) Parasitemia levels and (B) Percentage of cumulative mortality.

Figure 2: Effect of VFV (25 mg/kg/day - once a day) administered simultaneously with Benznidazole (Bz) - 100mg/kg/day – once a day) for 60

days on mouse experimental model of *Trypanosoma cruzi* infection using Colombiana strain.(A) Parasitemia levels and (B) Percentage of cumulative mortality.

Artigo IV:

Guedes-da-Silva FH, Batista DGJ, Da Silva CF, Araújo JS, Pavão BP, Simões-Silva MR, Meuser MB, Demarque KC, Moreira OC, Britto C, Lepesheva GI, Soeiro MNC. Anti-trypanosomal activity of sterol 14α-demethylase (CYP51) inhibitors VNI and VFV in experimental murine infection. **Antimicrobial Agents and Chemotherapy, submetido à publicação.**

A falta de correlação de resultados pré-clínicos e clínicos recentemente observados com posaconazol e a prodroga do ravuconazol, o perfil tóxico do fexinidazol a falta de predição de modelos experimentais mais reprodutíveis sobre a real eficácia e segurança de novos candidatos anti-*T.cruzi* demandam uma melhor compreensão e a padronização de modelos projetados para a descoberta de novas drogas. Neste estudo, exploramos o perfil de eficácia de VNI e VFV através de diferentes regimes de tratamento em modelos murino de infecção pelo *T. cruzi*, comparando gêneros dos animais (fêmea e macho), utilizando regime preventivo e terapêutico, avaliando a sua biodisponibilidade através de diferentes veículos para melhorar a solubilidade além da administração por longos períodos de tempo (até 120 dias) buscando caracterizar os eventos de sucesso/falha terapêutica com o tipo de abordagem experimental utilizada nos estudos de atividade *in vivo*.

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EXPERIMENTAL THERAPEUTICS

Antitrypanosomal Activity of Sterol 14α-Demethylase (CYP51) Inhibitors VNI and VFV in the Swiss Mouse Models of Chagas Disease Induced by the *Trypanosoma cruzi* Y Strain

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AQ: aff Laboratório de Biologia Celular[a] and Laboratório de Biologia Molecular e Doenças Endêmicas, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil[b]; Department of Biochemistry, School of Medicine, Institute for Global Health, Vanderbilt University, Nashville, Tennessee, USA[c]

AQ:A **ABSTRACT** Chagas disease is a life-threatening infection caused by a variety of genetically diverse strains of the protozoan parasite *Trypanosoma cruzi*. The current treatment (benznidazole and nifurtimox) is unsatisfactory, and potential alternatives include inhibitors of sterol 14α-demethylase (CYP51), the cytochrome P450 enzyme essential for the biosynthesis of sterols in eukaryotes and the major target of clinical and agricultural antifungals. Here we performed a comparative investigation of two protozoon-specific CYP51 inhibitors, VNI and its CYP51 structure-based derivative VFV, in the murine models of infection caused by the Y strain of *T. cruzi*. The effects of different treatment regimens and drug delivery vehicles were evaluated in animals of both genders, with benznidazole serving as the reference drug. Regardless of the treatment scheme or delivery vehicle, VFV was more potent in both genders, causing a >99.7% peak parasitemia reduction, while the VNI values varied from 91 to 100%. Treatments with VNI and VFV resulted in 100% animal survival and 0% natural relapse after the end of therapy, though, except for the 120-day treatment schemes with VFV, relapses after three cycles of immunosuppression were observed in each animal group, and quantitative PCR analysis revealed a very light parasite load in the blood samples (sometimes below or near the detection limit, which was 1.5 parasite equivalents/ml). Our studies support further investigations of this class of compounds, including their testing against other *T. cruzi* strains and in combination with other drugs.

KEYWORDS Chagas disease, chemotherapy, sterol 14α-demethylase, inhibitors, VNI, VFV, *Trypanosoma cruzi*

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AQ: B Chagas disease (CD), or American trypanosomiasis, is a zoonosis caused by multiple strains of the protozoan parasite *Trypanosoma cruzi*, which are transmitted to more than 150 mammalian species by the triatomine insect vector (kissing bugs). *T. cruzi* has been infecting humans in South America for at least 9,000 years (1) and was discovered 107 years ago by Carlos Chagas (2). Although, according to the WHO, the number of infected patients has dropped significantly within the past decades, most likely because of successful vector control programs (3), CD still represents an important public health issue, remaining endemic in 21 Latin American countries (more than 6 million patients, with the largest estimated numbers in Argentina, Brazil, and Mexico [4]) and spreading outside the area where CD is endemic as a result of human migration (5–7). The broadening of the area of the insect vector habitat is particularly alarming in the United

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States (8), where kissing bug bites were reported in 43 states; studies in Louisiana (2007) revealed that 30% of armadillos and 38% of opossums were infected, and although no epidemiological study of humans has been performed, some estimates suggest that the number of chagasic patients in the United States is more than 1 million, most of them remaining undetected (6, 9–12).

The current therapeutic options for CD are limited to two nitroderivatives, benznidazole (Bz) and nifurtimox. These compounds, however, have several limitations, including serious adverse side effects, which lead to treatment discontinuation in 15 to 30% of patients (10, 13), lack of efficacy in the later chronic phase of the disease, and the occurrence of several naturally resistant strains of *T. cruzi* (14–16). In both nitroderivatives (as well as the nitroimidazole fexinidazole, which was under recent clinical trial for CD), the nitro group is expected to undergo reductive metabolism catalyzed by parasite nitroreductases, leading to the formation of biologically active species exerting their trypanocidal activity (15). Because of their toxicity, neither Bz nor nifurtimox is approved by the FDA and therefore they are not sold or prescribed in the United States. Although the possibility of obtaining these drugs from the Centers for Disease Control (parasites@cdc.gov; www.cdc.gov/parasites/chagas) has been reported (10), most physicians appear to be unaware of this option.

Within the past decades, after the parasitological nature of the chronic stage of CD has been confirmed (17), different alternative therapeutic strategies for the disease have been considered (18), though only a few new candidates entered clinical trials and the results so far have been quite disappointing. For example, a clinical trial of another nitroderivative, fexinidazole, that was very active in preclinical assays (15) has been stopped and the compound was discarded as a drug candidate because of its severe toxic profile in chagasic patients (19). The results of clinical trials of two antifungal agents, posaconazole and ravuconazole, have not met expectations. Thus, although treatment with posaconazole revealed a clear dose-dependent effect in the randomized Chagazol trials, only ~20% of the patients (versus ~60% in the Bz group) were found to be negative by follow-up reverse transcription-PCR assays, though *T. cruzi* DNA was undetectable in the blood of all of the posaconazole-treated patients during the treatment period (16). Currently, largely because of its much better safety profile and clear antiparasitic effect in the Chagasol clinical trials, posaconazole is considered for potential use in combination or in sequential therapies (20). Posaconazole inhibits the fungal sterol 14α-demethylase (CYP51), the cytochrome P450 enzyme that is required for the production of sterols, which in turn serve as essential components of eukaryotic membranes and regulators of the cell cycle and development (21, 22). Posaconazole is used as a systemic clinical drug to treat invasive fungal infections, and its repurposing for CD could be highly advantageous (23). However, the procedure of its synthesis is long and low in yield (24), making posaconazole very costly.

Recently, we have shown that a potent inhibitor of Tulahuen *T. cruzi* CYP51 (25), VNI {lsqb]*N*-[(1*R*)-1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethyl]-4-(5-phenyl-1,3,4-oxadiazol-2-yl)benzamide}, given orally at 25 mg/kg for 30 days, cures Tulahuen *T. cruzi* infection with 100% efficiency in (BALB/c) mouse models of both acute and chronic CD (26). VNI is easy to synthesize and nontoxic (25, 27), has favorable pharmacokinetics (26), and does not upregulate *CYP51* gene expression (28). However, VNI was found to be less potent against infection with the Y strain of *T. cruzi* (short-term treatment) (29). Sequencing of the genomic DNA of the Y strain of *T. cruzi* revealed the presence of two *CYP51* genes, gene A (NCBI accession no. JQ434483) and gene B (accession no. JQ434484). While CYP51B was identical to CYP51B in the CL-Brener strain of *T. cruzi* (NCBI accession number XP_821219.1), CYP51A was found to carry one amino acid sequence variation (P355S) that involves the surface of interaction between the enzyme and its inhibitors. Gene A was expressed in *Escherichia coli*, and the CYP51A protein was purified and characterized, confirming lower susceptibility to inhibition by VNI, posaconazole, and all of the other compounds tested, except for VFV (30). VFV [(*R*)-*N*-(1-(3,4′-difluorobiphenyl-4-yl)-2-(1H-imidazol-1-yl)ethyl)-4-(5-phenyl-1,3,4-oxadiazol-2-yl)benzamide] is the CYP51 structure-based VNI derivative designed to fill the deepest

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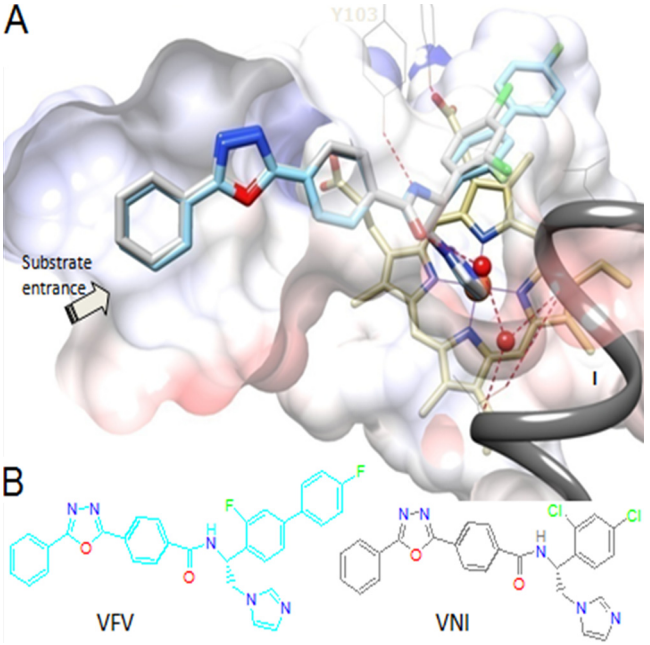

FIG 1 (A) VNI (gray) and VFV (cyan) bound in the CYP51 active site (Protein Data Bank codes 3GW9 and 4G7G, respectively). Shown is a slice through the semitransparent protein surface, distal cytochrome P450 view. The heme is depicted in yellow; the catalytic iron atom is presented as an orange sphere. The H-bond network connecting the carboxamide fragment of the inhibitors with the CYP51 B′ helix (Y103) and helix I (proton delivery route) is displayed as dashed red lines, and water molecules are shown as red spheres. (B) Structural formulas. The color code for the atoms is the same as in panel A.

F1 portion of the CYP51 substrate binding cavity (Fig. 1) to broaden its spectrum of activity. Similar to VNI, VFV was proven to be 100% efficient in curing Tulahuen *T. cruzi* infection and displayed higher potency than VNI in a mouse model of visceral leishmaniasis (31). In this study, we compared the effects of VNI and VFV in mouse models of infection with the Y strain of *T. cruzi* by using both genders (32), different drug delivery vehicles, and different treatment schemes.

RESULTS

The rapid acute toxicity assay *in vivo* (48 h after administration *per os*) demonstrated a low toxicity profile of VFV (no-observed-adverse-effect level [NOAEL] of 200 mg/kg), with no detectable side effects assayed by clinical observation (animal behavior and
T1 body weight analysis) and biochemical plasma measurements (Table 1). The comparative testing of VNI and VFV in the mouse models of infection with the Y strain of *T. cruzi* was performed by using both genders, different drug delivery vehicles, and different
AQ:D/T2 treatment schemes (Table 2). Both genders were included in the experiments because male mice infected with the Y strain of *T. cruzi* display about 2-fold greater parasite loads at the peak of parasitemia (which corresponds to day 8 after infection with the Y strain of *T. cruzi* in this experimental mouse model) and appeared to be more suitable for screening of antiparasitic compounds (32). Indeed, VNI, particularly if the treatment

TABLE 1 Plasma biochemical analysis of female mice after 48 h of VFV administration

Enzyme	Mean level ± SD[a] after treatment with VFV at (mg/kg):					
	0[b]	12.5	25	50	100	200
ALT	106 ± 39	72 ± 21	73 ± 13	62 ± 0	83 ± 38	161 ± 118
CK	396 ± 32	1,000 ± 30	446 ± 102	540 ± 255	369 ± 228	407 ± 336
AST	123 ± 6.4	168 ± 14	115 ± 16	92 ± 0	104 ± 40	70 ± 0

[a]Values of two independent assays are shown. Reference values (CECAL/Fiocruz): ALT, up to 132 U/liter; AST, up to 247 U/liter; CK, up to 1,070 U/liter.
[b]Results of one representative assay are shown.

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TABLE 2 Effects of Bz, VNI, and VFV on male and female Swiss mice infected with the Y strain of *T. cruzi* and subjected to different treatment schemes with different drug delivery vehicles

Gender and treatment scheme	Drug	% Suppression at peak parasitemia	% Animal survival	No. of mice with natural relapse at end of therapy/surviving animals	No. of mice with relapse after Cy immunosuppression/surviving animals
Males					
6 dpi, 30 days	Bz[a]	54	100	0/5	0/5
5 dpi, 30 days	VNI[b]				
	DGAT	93.4	100	0/4	1/4
	HPCD	94	100	0/3	2/3
	DGA	91	100	0/4	0/4
1 dpi, 30 days	DGAT	99.8	100	0/4	2/4
5 dpi, 120 days	HPCD	98	100	0/5	4/5
5 dpi, 30 days	VFV[b]				
	DGAT	99.9	100	0/5	4/5
	HPCD	99.9	100	0/5	4/5
	DGA	99.9	100	0/4	3/4
1 dpi, 30 days	DGAT	100	100	0/4	2/4
5 dpi, 120 days	HPCD	99.8	100	0/5	(0/4)[c]
	Bz[a]	99.6	100	0/5	0/5
Females					
6 dpi, 30 days	Bz[a]	>90	100	0/5	0/5
5 dpi, 30 days	VNI[b]				
	DGAT	99.7	100	0/4	1/4
	HPCD	99.8	100	0/5	0/5
	DGA	NT[d]			
1 dpi, 30 days	DGAT	100	80	0/4	4/4
5 dpi, 120 days	HPCD	99.8	100	0/5	1/5
5 dpi, 30 days	VFV[b]				
	DGAT	100	100	0/5	4/5
	HPCD	99.9	100	0/5	3/5
	DGA	NT			
1 dpi, 30 days	DGAT	100	80	0/4	0/4
5 dpi, 120 days	HPCD	100	100	0/5	0/5
	Bz[a]	100	100	0/5	0/5

[a]Administered at 100 mg/kg.
[b]Administered at 25 mg/kg/bid.
[c]One mouse was euthanized after it lost an eye because of mouse aggression.
[d]NT, not tested.

was started 5 days postinfection (dpi) (3 days before peak parasitemia), caused a >91% peak parasitemia reduction in all male mouse groups versus a >99% peak parasitemia reduction in the female mouse groups (Table 2). Interestingly, although it is well known that CYP51 inhibitors have a relatively slower mode of action (since several cycles of pathogen multiplication are necessary to exhaust the preexisting resource of cellular sterols), the suppressive effect of VFV was >99.8% in both genders even when the treatment was started 5 dpi and reached 100% if the treatment was started 24 h after infection (Table 2).

No drastic alterations in the course of parasitemia or mortality rates were observed during the use of different drug delivery vehicles in these experiments, but arabinosylated gum arabic (DGA) resulted in greater parasite loads measured by real-time quantitative PCR (qPCR) assay of the blood of VNI-treated male animals (Fig. 2 and 3A). In this male mouse model, all three formulations of VFV produced a 99.9% peak parasitemia decline, though for VNI, DGA appeared to be slightly weaker (91% peak parasitemia suppression versus 94% and 93.4% for hydroxypropyl-β-cyclodextrin (HPCD) and 5% DGA plus 0.5% Tween 80 (DGAT), respectively (Table 2 and Fig. 2A), all three being more efficient than when VNI was added from 10% DMSO alone (see Fig. 4 in reference 29). Because of the weaker effect of VNI in DGA, only DGAT and HPCD formulations were tested in the female mouse model.

All of the treatment schemes used protected against animal death, and there was no natural relapse of parasitemia after completion of the 30-day therapies. However, after

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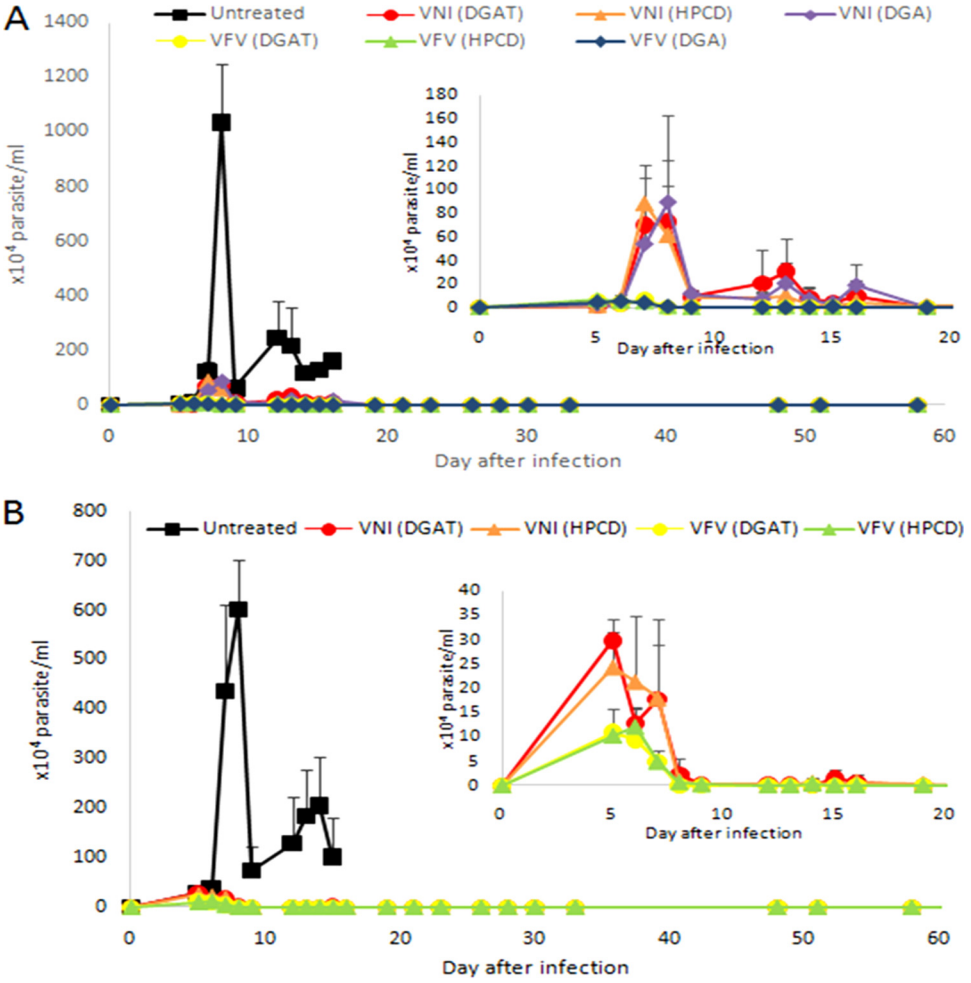

FIG 2 Parasitemia levels in experimental male (A) and female (B) mouse models of Y strain *T. cruzi* infection treated with VNI and VFV (25 mg/kg/day) with different vehicles for 30 days starting at 5 dpi.

a 1-month waiting period, when the animals were immunosuppressed with 12 injections of cyclophosphamide (Cy), most of them relapsed and the presence of parasite DNA was confirmed by qPCR analysis of blood samples (Fig. 3A). Although it is impossible to draw any solid conclusions because of the high variability of the qPCR data (quite expected, as outbred animal models were used), but the qPCR results suggest that in the case of the treatments with VNI (opposite to its effects on peak parasitemia suppression), there was no major decrease in the parasite burden when the treatment was started 1 versus 5 dpi, while in the case of VFV, the early treatment scheme appears to be more beneficial (at least in the female model) (Fig. 3A). In the hope that longer drug exposure would produce better outcomes, the treatment period was extended to 120 days (HPCD vehicle, starting at 5 dpi, both genders). All of the animals survived the treatment and showed no natural relapse after the completion of therapy, and in both VFV-treated groups (males and females), no relapses after immunosuppression were detected by light microscopy, while in the VNI-treated groups, four out of five males and one out of five females still relapsed (Table 2 and Fig. 3B). qPCR analysis, however, still displayed residual traces of parasite DNA in blood samples, 2.2 $\pm$ 3.6 parasite equivalents/ml for VFV-treated males and 0.94 $\pm$ 0.49 parasite equivalents/ml for VFV-treated females (Fig. 3B). Quite peculiarly, in the blood samples of VNI-treated animals (where the presence of the parasite was detected by light microscopy [see above]), qPCR assays revealed lighter loads of *T. cruzi* DNA (0.001 $\pm$ 0 parasite equivalents/ml in males and 0.095 $\pm$ 0.14 parasite equivalents/ml in females). Overall,

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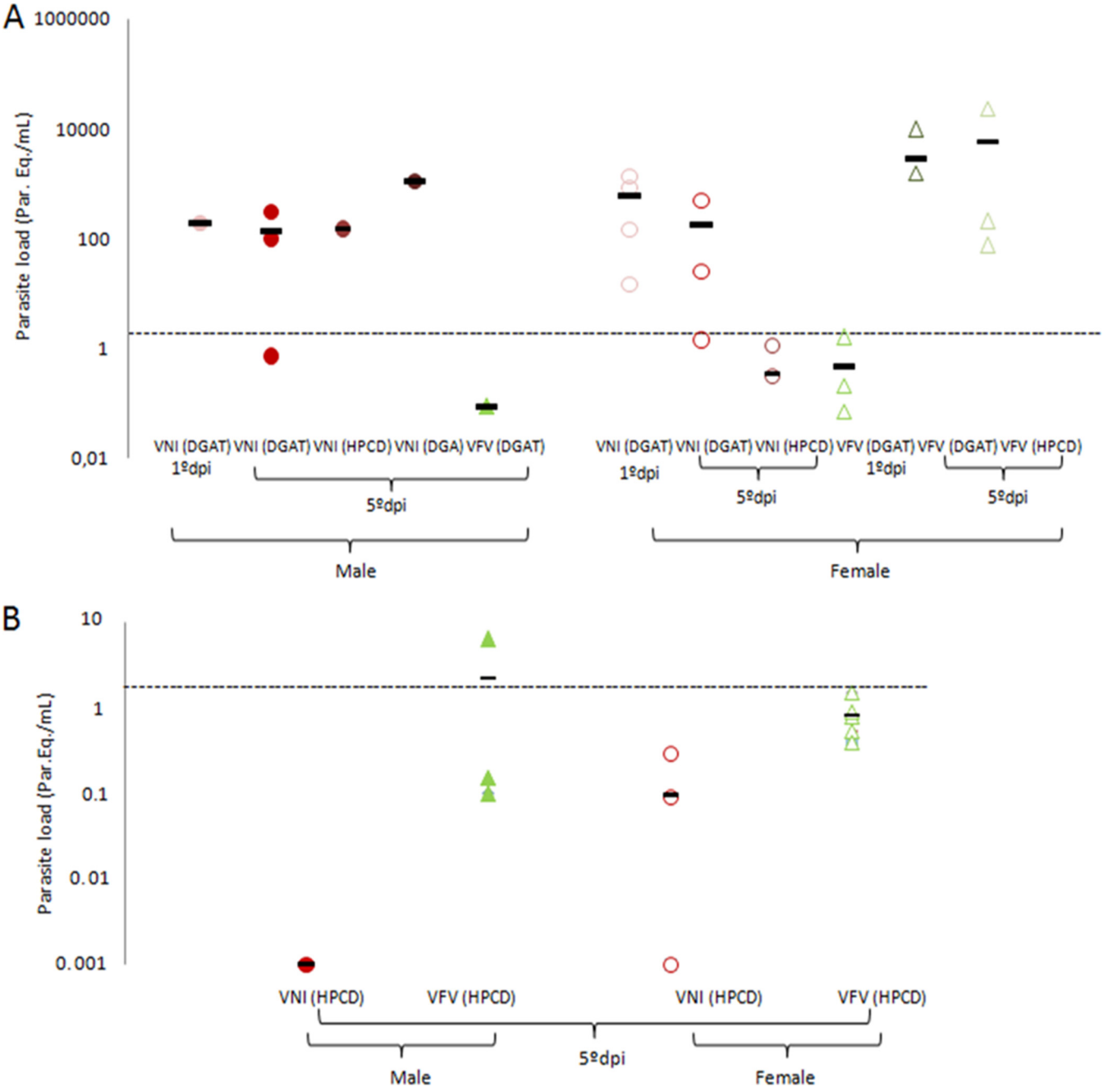

FIG 3 Cure assessment by qPCR analysis of the load parasite burdens (parasite equivalents per milliliter) of male and female mice infected with the Y strain of *T. cruzi* and submitted or not to VNI and VFV therapies started at the onset of parasitemia (5 dpi) or 24 h after parasite infection (1 dpi) and continued for 30 days (A, log) or 120 days (B). *, statistically significant at $P = 0.02$. Dashed lines: limit of qPCR detection (1.5 parasite equivalents/ml). Treatment of female and male mice with Bz did not result in qPCR positivity or parasite load detection.

all of the values determined by qPCR after the 120-day therapies were below or near the limit of parasite detection (1.5 parasite equivalents/ml). The statistical analysis only demonstrated a significant difference in the amount of parasite DNA when females were treated for 120 days with VFV compared to VNI ($P = 0.02$). Treatment of mice (both genders) with Bz for 120 days resulted in no qPCR amplification in any of the mice tested.

DISCUSSION

The fact that Bz will now have a much broader use in the treatment of CD is, overall, highly positive. However, we tend to agree with the notion (13, 20, 33) that searches for

new drugs should not be discouraged and less toxic alternatives for chagasic patients must still be considered, especially regarding the combination approach of two drugs targeting different mechanisms. CYP51 inhibitors remain one of them, because unlike Bz (or any nitroderivatives), whose very mode of action is based mainly on the generation of toxic nitroreduction intermediates, CYP51 inhibitors are potentially much less harmful, as they act selectively. Thus, although negative follow-up PCR results after the Chagazol clinical trials were achieved in only 20% of the posaconazole-treated patients, none of the patients had to discontinue therapy because of adverse side effects, which was not the case for the Bz-treated group (16). Longer treatment periods and greater doses of posaconazole were recommended (16), and variability of the *T. cruzi* population was suggested as a possible reason for the poor treatment outcomes (13, 20).

It is well known that there are more than 70 different strains of *T. cruzi* (see http://www.dbbm.fiocruz.br/TcruziDB/strain.html). Depending on the strain, the infection varies in its progression (the times of onset and peak parasitemia), the severity of the acute stage, tissue tropism, and chronic symptoms (cardiac versus gastrointestinal), etc. Some of the *T. cruzi* strains, including Y, CL, and Colombiana, similar to filamentous fungi (34), have been found to carry two *CYP51* genes (29, 30), and the high CYP51 sequence variability across the strains suggests that they should really be regarded as different species (30).

The results of the present study confirm that infection with the Y strain of *T. cruzi* is less susceptible to treatment with CYP51 inhibitors than is infection with the Tulahuen strain (26) and show that although VFV is more potent than VNI in killing the parasite at the peak of infection (>99% versus >91% peak parasitemia reduction), a much longer treatment period is required to prevent a relapse of parasitemia after immunosuppression. One more possible reason for the various susceptibilities of *T. cruzi* strains to CYP51 inhibitors may be connected to the differences in the propensity of parasites to form metabolically quiescent amastigotes (20). Hidden within different organs and tissues to evade the host immune system, these dormant forms cannot be targeted efficiently by CYP51 inhibitors because, as is well known in mycology, CYP51 inhibitors act largely on metabolically active cells that require a permanent supply of newly synthesized sterols to build their membranes and regulate intracellular processes. In a similar sense, if these supposedly latent intracellular forms could more likely be trypomastigotes and as CYP51 inhibitors have less of an effect on these nonproliferative forms, this could also result in different outcomes with different parasite strains.

Studies including infections with a broader variety of *T. cruzi* strains, testing greater dosages of VNI and VFV (e.g., 50 mg/kg), and perhaps the use of mild immunosuppression during the treatment period (as was done in the case of successful treatment of immunosuppressed chronic human CD with posaconazole [35]) should provide useful information on the prospective treatment of CD. In the meantime, combination therapy appears to be the most obvious step to proceed because, since it is by now quite clear that a decrease in the parasite burden leads to a significant reduction in the development of the symptomatic chronic form of CD (13). CYP51 inhibitors would definitely fulfill this function and besides, given in combination with Bz, they should allow lowering of the Bz dosage and shortening of the therapy period, which, in turn, may decrease the severity of side effects.

MATERIALS AND METHODS

Compounds. VNI and VFV were synthesized at Vanderbilt University as described previously (27). For *in vivo* analysis, the compounds were diluted with three different vehicles, (i) 20% HPCD (CTD, Inc., USA) (36), (ii) 10% DMSO and 5% DGA (as reported in reference 29 for Colombiana *T. cruzi* infection), and (iii) 5% DMSO and DGAT (26). Cy (Genuxal) was purchased from Baxter Oncology (Frankfurt, Germany) and prepared in sterile distilled water (32). Bz was purchased from Laboratório Farmacêutico do Estado de Pernambuco, LAFEPE, Brazil, and dissolved in sterile distilled water supplemented with 3% Tween 80 as described previously (32).

Parasites. Bloodstream trypomastigotes (BTs) of the Y strain (discrete typing unit II) of *T. cruzi* were obtained from the blood of infected male Swiss mice at the peak of parasitemia (37). Immediately after the purification step, the parasites were resuspended in RPMI 1640 medium (pH 7.2 to 7.4) without

phenol red (Gibco BRL) and supplemented with 10% fetal bovine serum and 2 mM glutamine as reported previously (38).

***In vivo* acute toxicity.** To determine the NOAEL, increasing doses of the test compounds (up to 200 mg/kg of body weight) were injected by the intraperitoneal (i.p.) route individually into female Swiss Webster mice (20 to 23 g, two per assay, two assays). Treated animals were inspected for toxic and subtoxic symptoms according to the Organization for Economic Cooperation and Development guidelines. Forty-eight hours after compound injection, the NOAELs were determined as reported previously (27).

Biochemical analysis. Forty-eight hours after compound administration, mouse blood was collected and immediately submitted to biochemical analysis for determination of plasma tissue markers, including, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine kinase (CK), which was performed at the animal facilities of the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil, CECAL/Fiocruz platform) with Vitros 250 (Ortho Clinical-Johnson & Johnson) as reported previously (27).

***In vivo* infection.** Female and male Swiss Webster mice (18 to 20 g) were obtained from the Fundação Oswaldo Cruz (FIOCRUZ) animal facilities (CECAL/FIOCRUZ, Rio de Janeiro, Brazil). Mice were housed at a maximum of five per cage and kept in a conventional room at 20 to 24°C under a 12-h light–12-h dark cycle. The animals were provided with sterilized water and chow *ad libitum*. Infection was performed by i.p. injection of 10^4 BTs of the Y strain. Age-matched uninfected mice were maintained under identical conditions (29).

Treatment schemes. The animals (female and male) were divided into the following groups (five animals per group): uninfected (uninfected and nontreated), untreated (infected but treated only with each respective vehicle), and treated (infected and treated with the test compounds). Therapy, given orally (VNI and VFV at 25 mg/kg/day twice a day [bid] and Bz at 100 mg/kg/day once a day), was performed starting at 1 or 5 dpi. For the 5-dpi treatment schemes, only mice with positive parasitemia were used in the infected groups.

Parasitemia and mortality rates. Parasitemia was individually checked by direct microscopic counting of parasites in 5 μl of blood, and mortality rates were checked daily until 30 days posttreatment and expressed as percent cumulative mortality as described before (32).

Immunosuppression. Mice that presented consistent negative parasitemia up to 30 days posttreatment were submitted to three cycles of immunosuppression with Cy (50 mg/kg/day), with each cycle including 4 consecutive days of Cy administration (i.p.) and a 3-day interval (32).

Cure assessment. Cure assessment criteria were based on negative blood parasitemia observed by light microscopy, and blood samples from consistently negative mice were subjected to real-time qPCR. For qPCR, 500 μl of blood was diluted 1:2 in a volume of guanidine solution (6 M guanidine-HCl, 0.2 M EDTA) and heated for 90 s in boiling water. Guanidine-EDTA blood (GEB) samples were processed with the QIAamp DNA minikit (Qiagen) as previously described (39). Multiplex real-time qPCR assays targeting the *T. cruzi* satellite nuclear DNA and the exogenous internal amplification control (plasmid pZErO-2 containing an insert from the *Arabidopsis thaliana* aquaporin gene, 40 amplification cycles) were performed as previously described (40). The standard curves for absolute quantification were constructed with 1/10 serial dilutions of total DNA obtained from a negative GEB sample spiked with 10^5 parasite (Y strain) equivalents/ml of blood.

Ethics. All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14).

Statistical analysis. The data represent means ± standard deviations from two experiments run in duplicate, and statistical analysis was performed by analysis of variance with the level of significance set at $P \leq 0.05$ (40).

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AQA—Au/Carefully read the entire text to verify that the editorial changes made have not altered the intended meaning.

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AQB—Au/Cite refs. 41 & 42 (were 36 & 37) in text. Please check renumbered references throughout. If any of the uncited references should be eliminated, please sequentially renumber the end of the reference list (if necessary) and cite the renumbered reference(s) in the text. Please check throughout the manuscript. If any references should be deleted from the beginning or middle of the list, please mark "Reference deleted" in the margin next to that entry; do not renumber subsequent references.

AQC—Au/?Should "efficiency" be "efficacy"?

AQD—Au/?Definition of "NT" added to Table 2 OK? If not, supply correct one.

AQE—Au/?Change to "arabinosylated gum arabic (DGA)" OK? If not, supply correct definition.

AQF—Au/Supply URL for ref. 3.

5. Discussão

Apesar do sucesso das iniciativas regionais na América Latina de controle do vetor e da transmissão em bancos de sangue alcançando redução substancial do número de novos casos agudos (Pinto Dias, 2009), outras preocupações ainda permanecem desafiadoras, como a globalização da doença e a existência de vias alternativas de transmissão, tais como a materno-fetal além da transmissão oral através de alimentos contaminados (Gascon et al., 2010, WHO, 2015).

Adicionalmente, o estudo BENEFIT revelou que apesar da diminuição da carga parasitária com o tratamento com Bz, os resultados do estudo clínico de fase II em portadores chagásicos crônicos não demonstrou proteção contra a progressão das alterações cardíacas, o que reforça a busca por novas terapias para a DC (Torrico, 2013; Morillo et al., 2015; Molina et al., 2016), bem como uso de terapia combinada com o fármaco de referência associado a novos promissores candidatos anti-*T.cruzi* (Coura, 2009; Diniz et al., 2014). Outro ensaio clínico usando fexinidazol, um composto nitroheterocíclico, muito ativo em ensaios pré-clínicos foi interrompido devido ao perfil tóxico grave em portadores chagásicos (Molina et al., 2016) e, portanto, descartado como novo candidato para terapia.

Assim, após cento e sete anos da descoberta da DC, a atual terapia baseada nestes dois nitroderivados (Bz e Nf) introduzidos na clínica médica há mais de quatro décadas atrás, continua sendo insatisfatória. A limitada eficácia e a elevada toxicidade destes dois fármacos justificam a busca de novos candidatos anti-*T. cruzi*. No entanto, apesar do significativo número de ensaios *in vitro e in vivo* realizados na quimioterapia experimental para CD, poucos compostos moveram para os ensaios clínicos (Soeiro et al., 2013; Chatelain et al., 2015). Dados recentes demonstraram que a despeito dos promissores resultados pré-clínicos dos azóis antifúngicos (posaconazol e E1224 - pró-fármaco do ravuconazol), ambos apresentaram altas taxas de falha terapêutica nos ensaios clínicos (Torrico, 2013; Molina et al., 2014). A falta de tradução dos ensaios pré-clínicos para clínicos levanta alguns pontos, incluindo a necessidade de utilizar modelos experimentais mais rigorosos e padronizados (Chatelain, 2015). Além disto, a alta variabilidade genética das populações do *T. cruzi*

representa outro desafio a ser considerado nas ações relacionadas a descoberta de novas drogas (Zingales et al., 2012).

Nos estudos *in vivo* realizados na presente tese somente utilizamos modelos murino de infecção aguda pelo *T. cruzi*, uma vez que estes são os mais utilizados nos ensaios de desenvolvimento de novos fármacos, sendo mais apropriados que modelos crônicos para análises nas etapas iniciais de seleção e optimização de novos compostos tripanocidas (Gulin et al., 2015; Chatelain & Konar, 2015).

Iniciamos nossos estudos pela análise de algumas variáveis experimentais no tratamento de modelos murinos de infecção aguda pelo *T.cruzi* utilizando duas moléculas já conhecidamente efetivas sobre este parasito: Bz, medicamento de referência da DC, e VNI, inibidor da CYP51 previamente testado por nós (Soeiro et al., 2013) e por outros grupos (Villalta et al., 2013). Uma das tentativas para identificar novas alternativas terapêuticas para DC inclui o rastreio de bibliotecas de compostos naturais e químicos (Soeiro & De Castro, 2011), o reposicionamento de fármacos já licenciado para outras patologias (Kaiser et al., 2015), a síntese e validação de inibidores específicos de moléculas do parasito (Tagoe et al., 2015), além do uso da combinação de fármacos (Coura, 2009; Bahia et al, 2014; Salomão et al., 2016). Entre as abordagens antiparasitárias direcionadas a alvos dos parasitos, inibidores da biossíntese dos esteróis, em particular, da enzima CYP51, têm sido amplamente estudados na infecção experimental pelo *T. cruzi* (Bahia et al. 2014; Hoekstra et al., 2015). Como acima relatamos os dois inibidores, o posaconazol e a prodroga do ravuconazol avaliados em ensaios clínicos, infelizmente, não exibiram supressão definitiva da carga parasitária em portadores chagásicos crônicos (Torrico, 2013; Molina et al., 2014).

O VNI foi identificado e sintetizado pelo grupo da Dra. Galina Lepesheva ia partir da análise *in silico* de uma biblioteca da Novartis, sendo um novo "scaffold" imidazólico inibidor da CYP51. Esta molécula é ativa *in vitro* sobre as formas sanguínea e intracelulares de *T. cruzi* em concentrações micromolares (Soeiro et al., 2013) e sua administração via oral até dose de 400 mg/kg não induziu mortalidade dos animais ou desencadeou efeitos colaterais em modelos murino de toxicidade aguda (Hargrove et al., 2012).

Dados prévios *in vivo* revelaram que o tratamento por 30 dias consecutivos com VNI foi suficiente para obter 100% de cura em camundongos

BALB/c fêmeas frente a infecção aguda e crônica pela cepa Tulahuen do *T. cruzi* (Villalta et al., 2013). Entretanto, este resultado não foi reproduzidol quando nosso laboratório avaliou este inibidor em outro modelo murino: Swiss machos infectados com cepa Y e Colombiana do *T. cruzi* e tratados por 26 dias (Soeiro et al., 2013). Algumas hipóteses podem ser sugeridas de modo a entender a diferença quanto ao perfil de cura dos animais testados. A a primeira hipótese seria a variabilidade genética presente nas diferentes populações do *T. cruzi* testadas (cepas Y (DTU II), Colombiana (DTU I) e Tulahuen (DTU VI)) quanto à susceptibilidade da inibição da CYP51. A segunda hipótese seria relativa as diferenças dos modelos experimentais empregados nos dois estudos, com VNI incluindo distintas linhagens e gêneros de camundongos, tipos de veículos usados para solubilizar os compostos, tempo de exposição, entre outras variáveis.

De fato, a infecção pelo *T. cruzi* conduz a diversas manifestações clínicas que têm sido relacionadas, pelo menos em parte, à variabilidade genética das diferentes cepas do parasito somada à genética populacional dos hospedeiros. Neste sentido, sugere-se que estudos de novas vacinas e quimioterápicos devam levar em consideração o uso de distintas DTUs (I, II, V e VI) relevantes para a infecção humana (Zingales et al., 2009; Romanha et al., 2010). Dados já publicados revelaram diferenças no sequenciamento da CYP51 entre cepas de *T. cruzi*, sendo não somente na natureza de aminoácidos, mas no número de cópias gênicas (Soeiro et al., 2013). Estas diferenças na expressão de CYP51 podem ter contribuído para os níveis de cura discrepantes induzidos por VNI em animais infectados com cepa Y, Colombiana e Tulahuen.

Visando aprofundar nossos estudos quanto a segunda hipótese, seguimos nossas análises explorando o impacto de diferentes modelos experimentais sobre a ação antiparasitária de Bz e de VNI. Avaliamos simultaneamente sobre modelos murino de infecção pelo *T. cruzi* (cepas Y e Colombiana) emfêmeas e machos submetidos a dois esquemas de administração dos compostos: (i) preventivo, como usado por Villalta e colaboradores (2013) com administração a partir do primeiro dia pós-infecção (dpi) e (ii) terapêutico, com inicio a partir da positivação da parasitemia como reportado em Soeiro et al., (2013).

Em relação ao gênero do animal, estudos anteriores demonstraram que camundongos fêmeas de diferentes linhagens (BALB/c, C3H, C57BL/6)

infectados com *T. cruzi* apresentam uma maior sobrevida que os seus pares machos utilizando o mesmo inóculo e cepa do parasito (De Souza et al., 2001). Através de ensaios pela administração de estradiol em machos antes da infecção, os autores sugeriram que diferenças hormonais poderiam influenciar os distintos perfis de susceptibilidade, resultando na menor vulnerabilidade das fêmeas a infecção por este patógeno em modelos experimentais (De Souza et al., 2001).

Nossos resultados (Artigo I, Guedes-da-Silva et al., 2015) corroboraram os dados publicados (De Souza et al., 2001) que revelaram que camundongos fêmeas são menos vulneráveis à infecção pelo *T. cruzi* que machos, independentemente da cepa utilizada (Y ou Colombiana). Observamos que os níveis parasitêmicos (microscopia óptica e qPCR) são maiores nos machos que em fêmeas, sendo a mortalidade mais proeminente e/ou precoce no primeiro grupo. Observamos também menor carga parasitária (qPCR) em animais tratados com Bz que foram infectados com a cepa Y em relação aqueles infectados com Colombiana. A menor carga parasitária no caso da cepa Y foi observada mesmo tendo sido utilizado inóculo maior e menor tempo de tratamento(10^4 parasitos/animal; 30 dias) do que no caso da cepa Colombiana ($5x10^3$ parasitos/animal; 60 dias). Assim, confirmamos as diferenças quanto ao perfil de susceptibilidade a nitroderivados das duas cepas, nos dois grupos testados (machos e fêmeas), sendo a Colombiana e Y classificadas como altamente e moderadamente resistentes ao Bz, respectivamente (Brener & Chiari et al., 1967).

Nosso estudo confirma que o gênero do animal deve ser considerado quando novos compostos forem avaliados em estudos pré-clínicos. Os dados da literatura mostram que, embora ambos os gêneros respondam com alta sensibilidade aos estudos de toxicidade aguda de novos candidatos á fármacos, as fêmeas são geralmente mais susceptíveis aos efeitos colaterais (Lipnick et al., 1995; Lewis et al., 2015). Isto destaca a necessidade de se comparar gêneros nos estudos de quimioterapia experimental. Nos ensaios realizados frente a infecção com a cepa Y e tratamento por 30 dias consecutivos observamos que os machos foram menos susceptíveis ao tratamento que as fêmeas: administração de Bz a partir da positivação da parasitemia resultou em redução no pico da parasitemia de cerca de 50 para machos e > 90% para fêmeas. Já com o VNI obtivemos supressão de parasitemia na faixa de 86 e 99,8% para

machos e fêmeas, respectivamente. A análise molecular por qPCR confirmou que a carga parasitária no sangue dos animais 30 dias após o final do tratamento foi significativamente maior em machos do que fêmeas (cerca de 4 vezes para Bz e 431 vezes para VNI).

Outro ponto relevante é a escolha do regime terapêutico, especialmente quanto ao momento em que se inicia o tratamento: logo após o inóculo de parasitos (1 dpi) ou após positivação da parasitemia (5-6 dpi para cepa Y e 10-12 dpi para cepa Colombiana, com os inóculos anteriormente citados). Nossos dados revelaram que em ambos os gêneros (machos e fêmeas), o tratamento com Bz administrado a partir do 1 dpi resultou em completa supressão de parasitemia (avaliada ao microscópio óptico) e inferiores cargas parasitárias mensuradas por qPCR em comparação a administração do fármaco quando a infecção está estabelecida. Este dado é compatível com o consenso de que com o tratamento precoce, melhores são os prognósticos de cura. No entanto, a fim de identificar os potenciais candidatos a fármacos para a DC, a administração precoce pode mascarar resultados de eficácia sendo portanto, um esquema menos rigoroso de análise além do fato de poder fazer uso de animais negativos para a infecção devido a alguma falha no inóculo. A utilização de protocolos de imunossupressão (tais como administração de ciclos ciclofosfamida) é também uma relevante abordagem visando garantir a sensibilidade do ensaio haja vista a amplificação do parasitismo (Batista et al., 2010; da Silva et al., 2012; Caldas et al., 2012).

Outra consideração visando investigar a eficácia de novos compostos em modelos experimentais é a análise de carga parasitária por ferramentas moleculares de diagnóstico tais como a qPCR que permite a identificação não somente falha terapêutica mas também a comparação da potência de distintos fármacos através da quantificação da carga residual nos animais tratados.

Com relação ao objetivo de identificar alternativas para terapia da DC, investigamos a ação *in vitro* e *in vivo* de novas AIAs (Artigo II, Guedes-da-Silva et al., 2016). Amidinas aromáticas como a pentamidina têm sido utilizadas por décadas como agentes antiparasitários na clínica médica e veterinária, porém apresentam algumas limitações importantes, tais como a necessidade de administração parenteral e efeitos adversos indesejáveis (Soeiro et al., 2013). A fim de superar estas limitações, novas moléculas amidínicas têm sido sintetizadas e avaliadas em ensaios fenotípicos *in vitro* e *in vivo* (Batista et al.,

2010; da Silva et al., 2008). Entre as mais potentes, destacam-se AIAS que exibem considerável atividade biológica contra vários patógenos intracelulares, incluindo *T. cruzi* (Batista et al., 2011; da Silva et al., 2011). Na presente tese, avaliamos por ensaios *in vitro* e *in vivo* a atividade fenotípica de 14 novas bis e mono AIAs contra formas tripomastigotas sanguíneas e amastigotas intracelulares de diferentes cepas (Y e Colombiana). Destas as mais ativas foram as moléculas 35DAP073 e 35DAP081 que apresentam como principal característica estrutural a presença de dois grupos 2-piridilimidamida ligados ao núcleo *m*-terfenil. 35DAP073 exibiu EC_{50} de 0,5µM contra tripomastigotas (cepa Y) após 24 h de incubação sendo mais ativa que Bz (13 µM). Esta AIA também foi efetiva *in vitro* sobre a cepa Colombiana com EC_{50} de 3,8 µM. Análise da sua atividade contra as formas intracelulares (cepa Tulahuen DTU VI) mostrou que essa bis-AIA (EC_{50} 0,04 µM) foi cerca de 50 vezes mais ativa que o Bz (2 µM). Dados anteriores do nosso grupo revelaram que amidinas podem modular a fisiologia de células hospedeiras contribuindo para controle da proliferação de parasitos (De Souza et al., 2004; Leepin et al., 2008). Assim, a excelente atividade, sobretudo contra formas intracelulares nos levou a estudar o potencial modulador desta AIA sobre alguns aspectos da fisiologia das células hospedeiras (como por exemplo capacidade de induzir formação de corpúsculos lipídicos – CL) e sua possível relação com a habilidade de controlar a infecção. A biogênese de CLs é um evento central em diversos processos celulares incluindo metabolismo celular, sinalização, e inflamação, bem como durante a infecção intracelular de patógenos (Listenberger et al., 2003; Bozza et al., 2007). CLs são organelas dinâmicas compostas por núcleo de colesterol e triglicerídeos com uma monocamada circundante de fosfolipídeos, colesterol, e uma variedade de proteínas (Bozza et al., 2009; Farese et al., 2009). Sob algumas condições inflamatórias e infecciosas, ocorre a produção de prostaglandinas e outros mediadores lipídicos nos CLs (Bozza et al.,1996). Compostos antimicrobianos podem induzir um acúmulo de gotas lipídicas no citoplasma de fungos como *Candida spp*. (Ishida et al., 2011) e protozoários (De Machado-Silva et al., 2013). Em células infectadas por *Mycobacterium leprae*, ocorre a fusão ativa de CLs (Elamin et al., 2012). Assim, como CLs agem sobre o metabolismo celular, inflamação, e podem ser modulados frente ao curso de algumas patologias infecciosas (Bozza et al., 2009; Farese et al., 2009), no presente estudo investigamos se as AIAs estudadas poderiam interferir na biogênese de CLs e

no controle do parasitismo *in vitro.* Nossos dados demonstraram que o superior impacto de AIAs como a 35DPA073 sobre as formas intracelulares de *T. cruzi* foi dissociado de sua capacidade em induzir biogênese de CLs em células cardíacas haja vista que outra AIA inativa (28SMB060) foi igualmente capaz de induzir a formação destas estruturas intracelulares.

Em seguida, devido aos excelentes índices seletivos de 35DAP073 e 35DAP081, ambas foram selecionadas para estudos *in vivo*, sendo inicialmente testadas quanto ao perfil de toxicidade aguda. Os resultados mostraram NOAEL valores de 25 mg/kg para 35DAP073 e 12,5 mg/kg para e 35DAP081, e portanto maior potencial de toxicidade da última AIA que foi então excluída das futuras análises. A seguir, realizamos estudos de eficácia *in vivo* com 35DAP073 em concentrações de 25 mg/kg. Os nossos dados, demonstraram que camundongos machos infectados e tratados com 35DAP073 por 2 dias (5 e 8 dpi no caso da cepa Y) apresentaram redução dose-dependente no pico da parasitemia, exibindo inibições de 46-96% com doses de 5-20 mg/kg/dia e alcançando nas menores doses testadas 100% de sobrevida, efeito semelhante aos animais infectados e tratados com Bz. Visando obter cura parasitológica, seguimos com tratamento por 10 dias consecutivos com a dose de 5 mg/kg (i.p.) utilizando a infecção por Colombiana. Porém apesar de suprimir completamente a parasitemia e alcançar 100% de sobrevida, os animais tratados com 35DAP073 apresentaram alterações neurológicas reversíveis ao final da terapia. Assim de modo a reduzir a dose e consequentemente os efeitos colaterais desta molécula, realizamos estudos de terapia combinada da 35DAP073 com Bz. Como também observado pelo nosso grupo utilizando a combinação deste fármaco com outras amidinas (Batista et al., 2010, da Silva et al., 2008), a associação de 35DAP073 com Bz proporcionou aumento na potência antiparasitária alcançando reduções na carga parasitária sanguínea mensurada por qPCR (após imunossupressão com ciclofosfamida) em cerca de 60% em relação ao uso de Bz em esquema de monoterapia, sem que houvesse manifestação de toxicidade nos animais. Nossos resultados justificam a continuidade de estudos com esta classe de compostos em associação com medicamentos antiparasitários já licenciados (como o Bz) com o objetivo de contribuir para a identificação de novas alternativas para o tratamento de doenças parasitárias negligenciadas, como a DC.

Como discutimos acima, há um consenso de que um novo candidato para DC deve ser efetivo sobre as diferentes cepas pertencentes as diferentes DTUs do *T.cruzi*, pelo menos para aqueles relevantes para a infecção humana, incluindo ainda as cepas naturalmente resistentes a nitroderivados (Don & Loset, 2013; Zingales et al., 2013; DNDi, 2016). Falhas terapêuticas estão bem documentadas na DC sob o uso de Bz e Nf e tem sido relacionadas à genética das cepas do *T. cruzi* e de seus hospedeiros, além da distribuição e acúmulo dos fármacos em diferentes tecidos e órgãos (Romanha et al., 2010; Coura & De Castro 2002). Neste contexto, para aprofundar a análise pré-clínica do VNI e de um novo derivado, o VFV, realizamos estudos compilados no terceiro e quarto manuscritos. Nestes estudos estes inibidores foram avaliados sobre cepas Colombiana e Y, utilizando diferentes protocolos, modelos experimentais além da terapia combinada. Os estudos compilados no terceiro manuscrito (Artigo III, Guedes-da-Silva et al., 2016) foram conduzidos empregando períodos mais longos de tratamento (60 dias), utilizando Bz e os dois imidazóis sob esquema de monoterapia e terapia de combinação. Os ensaios de combinação também seguiram diferentes protocolos: (a) terapia simultânea de 100 mg/kg/dia Bz e 25 mg/kg/dia VFV uma vez por dia, via oral e (b) terapia sequencial, com administração de 100 mg/kg Bz durante 30 dias (1 vez ao dia, via oral) seguindo pela terapia por 30 dias com 25 mg/kg/dia VFV (duas vezes ao dia, via oral). Nossos dados mostram que todos os grupos tratados com abordagem de monoterapia resultaram em > 99,9% de supressão de parasitemia e 100% de sobrevida dos camundongos. No entanto, apesar de não alcançar cura estéril, VFV foi mais eficaz que Bz na redução do parasitismo sanguíneo avaliado por qPCR após imunossupressão com ciclofosfamida. VFV foi 14 vezes mais eficaz em reduzir carga parasitária (medida pela qPCR) em relação ao Bz. Outro achado importante está relacionado com a superior eficácia de VFV administrado em combinação com Bz (sequencial- ou simultaneamente), resultando em >1000-2000 vezes menor parasitismo sanguíneo analisado por qPCR quando comparado com a monoterapia de Bz. Curiosamente, o grupo tratado sequencialmente com Bz e, em seguida, VFV (duas vezes ao dia) induziu 40% da cura parasitológica nos animais tratados, confirmando o aspecto promissor do regime combinatório de compostos. Nosso estudo corrobora resultados de combinação entre compostos amidínicos e Bz (Artigo I) e também de outras

classes de compostos, incluindo inibidores da CYP51 como posaconazol e Bz (Batista et al., 2011; Diniz et al., 2013; Bustamante et al., 2014; Artigo I).

Nossos estudos reforçam o conceito do uso de esquemas combinatórios como futuro potencial para o tratamento clínico da DC. A ação curativa da combinação Bz/VFV explorada em um modelo de infecção aguda com uma cepa naturalmente resistente (Colombiana) revela sua superior eficácia em relação aos esquemas de monoterapia corroborando dados anteriores da literatura (Diniz et al., 2013). De fato, o tratamento de várias outras doenças infecciosas causadas por patógenos tais como tuberculose, lepra, doença do sono e SIDA atingem a melhor eficácia com combinações de fármacos com diferentes mecanismos de ação (Guia de Vigilância Epidemiológica da Tuberculose, 2009; Kumar et al., 2013; Harries et al., 2015). O tratamento com a combinação de medicamentos pode não somente aumentar a ação dos diferentes compostos terapêuticos usados isoladamente, mas também contribuir para prevenção no desenvolvimento da resistência do parasito aos quimioterápicos por atingir diferentes alvos dos parasitos, reduzir tempo de administração e doses empregadas (Coura, 2009; OMS, 2015).

Por último, a falta de correlação dos resultados pré-clínicos e clínicos acima referida pode ser, pelo menos, em parte devido à falta de predição mais reprodutível sobre a real eficácia de novos candidatos anti-*T.cruzi* que requer uma melhor compreensão da progressão da infecção causada pelo parasito, além da padronização de modelos animais projetados para a descoberta de novos compostos para o tratamento da DC (Chatelain & Konar, 2015). Uma mais precisa predição permitiria melhor correlação entre os dados pré-clínicos e os achados clínicos, traduzindo melhor os eventos relacionados a doença humana (Romanha et al., 2010; Chatelain & Konar, 2015).

Como discutimos no Artigo I, estudos anteriores (Villalta et al., 2013, Soeiro et al., 2013) demonstraram diferentes taxas de cura de VNI ao usar diferentes modelos murino de infecção pelo *T. cruzi*, e assim, o objetivo deste último manuscrito (Artigo IV) foi explorar, através de diferentes abordagens metodológicas, a eficácia de candidatos promissores para DC (VNI e seu derivado VFV) empregando diferentes parâmetros e comparando com Bz, utilizando períodos mais curtos (30 dias) e longos (120 dias) de exposição aos compostos e utilizando diferentes veículos de diluição.

O derivado VFV foi altamente efetivo contra formas intracelulares do *T. cruzi* em concentração na faixa de nanomolar, sendo mais ativo e seletivo que o VNI. A administração oral de VFV corroborou a previsão *in vitro* de baixa toxicidade atingindo um NOAEL 200 mg/kg, apresentando o perfil de segurança semelhante ao VNI (Hargrove et al., 2012). VFV também foi mais ativo que VNI *in vivo* quando ambos os gêneros macho e fêmea foram tratados por períodos curtos de exposição (30 dias consecutivos).

A diferença na eficácia *in vivo* de ambos os candidatos observado após 30 dias de terapia (VFV mais ativo do que VNI) não pode ser detectada após longos períodos de tratamento como 120 dias. É possível que longos períodos de terapia resultem na prolongada e contínua exposição (no plasma e tecidos dos hospedeiros) aos compostos leve à exaustão da viabilidade das diferentes populações nas cepas de *T. cruzi* reduzindo, assim, a nossa capacidade de detectar diferenças quanto a potência entre os compostos estudados o que pode ser mais facilmente detectado quando curtos períodos de terapia foram oferecido.

Com relação ao gênero, novamente confirmamos diferentes taxas de eficácia de novos candidatos a fármacos em modelo murino de infecção aguda pelo *T. cruzi* (De Souza et al., 2000). Os camundongos machos e fêmeas utilizados confirmam a influência do gênero nos níveis de infecção do parasito: embora, as fêmeas não tratadas tenham uma taxa de mortalidade de 100%, os machos morreram mais precocemente. No 18 dpi a percentagem de mortalidade cumulativa foi de 25% para fêmeas e 100% para machos. Além disto, modelo de fêmea foi mais sensível para descriminar a eficácia dos veículos utilizados em relação aos machos: VNI diluído com DGAT e Trappsol revelou diferenças na carga parasitária medida por qPCR em fêmeas tratadas enquanto que não detectamos diferenças nos machos frente a exposição de VNI com ambos veículos, especialmente quando foram avaliados no menor período de tratamento com os compostos (30 dias consecutivos).

A semelhança do observado com Bz (Artigo I), nossos dados com VNI e VFV mostraram diferentes impactos no efeito tripanocida *in vivo* a depender do tipo de regime terapêutico, sendo o preventivo (ou profilático) menos rigoroso como sugerido na literatura (Chatelain & Konar, 2015), exibindo níveis mais elevados de redução do no pico de parasitemia. O início do tratamento logo após um curto período de tempo da inoculação do parasito pode ser menos preditivo

do que a utilização de modelo murino com a colonização já difusa do parasito no animal (Artigo I). Neste sentido, o regime preventivo não exibe as condições reais da infecção humana, e deve ser evitado no desenvolvimento de fármacos para DC. Como também discutimos neste artigo, outro ponto importante está relacionado à imunossupressão após o fim de tratamento, permitindo aamplificação e proliferação de parasitos e a detecção de recidivas através da quantificação ao microscópio óptico da parasitemia e por qPCR. Conforme relatado, a diversidade de modelos animais associada à heterogeneidade dos métodos para tratar e mesmo avaliar a resposta às moléculas tripanocidas (ex: parasitemia ao microscópio óptico, PCR e qPCR no sangue e nos tecidos dos animais tratados) podem conduzir a resultados variáveis e dificultar a identificação de novos candidatos a fármacos de fato promissores para DC (Chatelain & Konar, 2015). Além disso, a diferença entre os achados de ensaios pré-clínicos e clínicos pode ser explicado, em parte, pela escassa informação contida na maioria dos experimentos que utilizam animais de laboratório (informações cruciais relacionadas com as espécies, linhagem, genética, estado microbiológico, condições de criação e procedimentos) e que não são adequadamente descritas nos artigos publicados nesta área (Gulin et al., 2015). A etapa de imunossupressão pela administração de ciclofosfamida após o final da terapia dos compostos permite detectar diferenças entre a eficácia de novos fármacos (ex. VNI *versus* VFV) mesmo em estudos utilizando associação de diferentes compostos tripanocidas *in vivo* (Artigo #3). Neste contexto, a terapia de combinação parece ser uma estratégia terapêutica muito promissora, e uma grande expectativa são as futuras conclusões do ensaio clínico STOP CHAGAS (ClinicalTrials.gov Identificador: NCT01377480) que está avaliando a progressão da cardiopatia em portadores chagásicos assintomática tratados com Ps associado ao Bz (Molina et al., 2016). Apesar de VNI e VFV serem muito potentes *in vitro* e apresentarem alta potência *in vivo* como presentemente observado corroborando os estudos anteriores usando cepa resistente do *T. cruzi* (Soeiro et al., 2013), atingindo 100% de cura parasitológica sobre infecção murina aguda e crônica com a cepa Tulahuen (Villalta et al. 2013), esquemas de terapia combinada usando estes inibidores com Bz ou com outros candidatos pode representar uma esperança para encontrar uma terapia alternativa para o tratamento desta doença negligenciada.

Em resumo, os nossos resultados suportam mais investigações experimentais destes compostos, visando a identificação de novas alternativas para o tratamento da doença de Chagas e apontam para a importância do uso de modelos mais rigorosos e padronizados, a fim de alcançar modelos mais viáveis e preditivos para uma melhor tradução dos ensaios pré-clínicos para clínicos.

6. Conclusões

a) O gênero do model murino deve ser considerado nos estudos pré-clínicos de novos compostos *anti-T.cruzi*. Camundongos fêmeas são mais resistentes à infecção pelo *T. cruzi* e mais vulneráveis a ação de fármacos do que machos;

b) A escolha do regime terapêutico deve ser cuidadosamente considerada, sendo preferível a administração do composto a partir do início da parasitemia quando se tem a infecção já estabelecida nos modelos experimentais, com invasão dos diferentes tecidos e órgãos pelo parasito;

c) O uso de diferentes veículos na preparação de compostos antiparasitários como presentemente investigado com uso de VNI e VFV (DGAT, DGA, Trappsol) revelou diferenças na carga parasitária final, revelando a importância de se definir qual melhor veículo permite melhor solubilidade e disponibilidade dos compostos, permitindo ainda discriminar a potência entre diferentes compostos tripanocidas;

d) O longo período terapêutico (120 dias) de Bz possibilitou a detecção da cura parasitológica de camundongos machos e fêmeas infectados com a cepa Y do *T. cruzi* o que não foi observado frente tratamento com VNI e VFV. Porém, com ambos imidazóis, os animais somente exibiram baixas concentrações residuais dos parasitos abaixo ou próximo ao limite de detecção pela qPCR revelando ação bastante promissora destes inibidores de CYP51.

e) A terapia combinada do Bz associado a outros compostos (ex. VFV ou AIA) é uma importante abordagem terapêutica para alcançar maior eficácia antiparasitária resultando em modelos de infecção com cepas naturalmente resistentes do *T. cruzi* índices de cura em torno de 40% enquanto esquemas de monoterapia alcançaram 0% após 60 dias de exposição,

f) A utilização de protocolos de imunossupressão (administração de ciclofosfamida) representa uma metodologia relevante para aumentar a sensibilidade do ensaio após o tratamento visando detecção de processo de reagudização da infecção, amplificando o parasitismo em níveis detectáveis ao microscópio óptico ou por metodologias moleculares (ex. qPCR);

g) As bis-AIAs *m*-terfenil foram mais potentes que as correspondentes moléculas com um único grupamento catiônico terminal contra formas sanguínea e intracelular do *T. cruzi,* sendo algumas delas mais ativas que o Bz;

h) Compostos ativos e inativos sobre formas intracelulares induziram de modo semelhante a gênese de corpúsculos lipídicos em culturas de células cardíacas, dissociando assim, o efeito tripanocida da ativação da fisiologia do metabolismo lipídico em células hospedeira;

i) 35DAP073 induziu a supressão da parasitemia e protegeu contra a mortalidade nas infecções com as cepas Y e Colombiana porém exibiu toxicidade reversível após a décima dose administrada. Além disto, a terapia combinada (35DAP073 + Bz) foi efetiva em reduzir a carga parasitária no sangue avaliado pela qPCR sendo amis efetiva em relação ao uso de Bz em monoterapia;

j) Novos estudos devem ser realizados com estas duas classes de compostos em associação com o fármaco de referência, na tentativa de buscar alternativas promissoras tratamento para a DC;

k) Em resumo, nossos resultados suportam futuras investigações experimentais nesta linha de estudo com o objetivo de contribuir para identificação de novos compostos terapêuticos para os milhões de pacientes chagásicos.

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Anexo

Durante a realização desta tese, participei das seguintes publicações:

Artigo 1

Timm BL, da Silva PB, Batista MM, **da Silva FHG**, da Silva CF, Tidwell RR, Patrick DA, Jones SK, Bakunov SA, Bakunova SM, Soeiro MdeN. *In vitro* and *in vivo* biological effects of novel arylimidamide derivatives against *Trypanosoma cruzi***. Antimicrob Agents Chemother. 2014**; (7):3720-6.

Artigo 2

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In Vitro and *In Vivo* Biological Effects of Novel Arylimidamide Derivatives against *Trypanosoma cruzi*

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Chagas disease (CD), a neglected tropical disease caused by *Trypanosoma cruzi*, remains a serious public health problem in several Latin American countries. The available chemotherapies for CD have limited efficacy and exhibit undesirable side effects. Aromatic diamidines and arylimidamides (AIAs) have shown broad-spectrum activity against intracellular parasites, including *T. cruzi*. Therefore, our aim was to evaluate the biological activity of eight novel AIAs (16DAP002, 16SAB079, 18SAB075, 23SMB022, 23SMB026, 23SMB054, 26SMB070, and 27SMB009) against experimental models of *T. cruzi* infection *in vitro* and *in vivo*. Our data show that none of the compounds induced a loss of cellular viability up to 32 μM. Two AIAs, 18SAB075 and 16DAP002, exhibited good *in vitro* activity against different parasite strains (Y and Tulahuen) and against the two relevant forms of the parasite for mammalian hosts. Due to the excellent selective indexes of 18SAB075, this AIA was moved to *in vivo* tests for acute toxicity and parasite efficacy; nontoxic doses (no-observed-adverse-effect level [NOAEL], 50 mg/kg) were employed in the tests for parasite efficacy. In experimental models of acute *T. cruzi* infection, 18SAB075 reduced parasitemia levels only up to 50% and led to 40% protection against mortality (at 5 mg/kg of body weight), being less effective than the reference drug, benznidazole.

Chagas disease (CD) is a neglected tropical disease caused by the intracellular flagellate protozoan *Trypanosoma cruzi*, which presents a complex life cycle with distinct morphological stages in its obligatory passage through vertebrate and invertebrate hosts (1). Currently, there are approximately 10 million infected individuals in areas of Latin America where CD is endemic, and many reports also point to the occurrence of CD in geographical areas where it is not endemic, such as the United States and Europe, mainly attributed to migration of infected people (2–6). CD can be transmitted by *Triatominae* insect feces, blood transfusion, organ transplantation, and laboratory accidents and through oral and congenital routes (7, 8). This pathology has two successive phases, a short, acute phase characterized by a patent parasitemia, followed by a chronic phase in which most of the infected individuals remain asymptomatic (indeterminate form), but about one-third may later manifest cardiac and/or digestive complications, developed progressively for years or decades after infection (9, 10). Benznidazole (Bz) and nifurtimox, introduced into clinical therapy about 40 years ago, are the only available drugs. Both have several shortcomings related to their required long periods of treatment, high toxicity, variable results, and low efficacy during the chronic phase, justifying the identification of novel therapies (11–13). Aromatic diamidines and analogues exhibit broad-spectrum activity against pathogenic microorganisms, including *T. cruzi* (14). Among the different tested derivatives of amidine compounds, the most effective against *T. cruzi* have been the bis-arylimidamides (AIAs) like DB766 (15) and DB1831 (16). Thus, in this study, we assayed the biological activities of eight novel AIAs (16DAP002, 16SAB079, 18SAB075, 23SMB022, 23SMB026, 23SMB054, 26SMB070, and 27SMB009) against experimental models of *T. cruzi* infection *in vitro* and *in vivo*, using different parasite strains, and also explored their toxicities in cardiac cell cultures and mouse models of acute toxicity.

MATERIALS AND METHODS

Synthesis of the arylimidamides. The eight arylimidamides, all isolated as their hydrochloride salts, were prepared by three general methods (see the supplemental material). Briefly, phenylimidamides 16DAP022 and 16SAB079 (Fig. 1) were prepared by the reaction of 2,7-diaminocarbazole (prepared by reduction of 2,7-dinitroocarbazole) (17) or *m*-xylylenediamine with (2-naphthyl)methyl benzothioimidate hydrobromide (18). The 2-pyridylimidamide 18SAB075 (Fig. 2) was prepared by the reaction of 4,4′-diaminodiphenylacetylene (19, 20) with 2-cyanopyridine by the method of Lange et al. (21) but using lithium (rather than sodium) bis (trimethylsilyl)amide. The reaction of the methyl imidate derivatives of 2- or 4-cyanopyridine with the appropriate α,ω-diamines or spermidine gave 2-pyridylimidamides 23SMB022, 23SMB026, and 23SMB054 and 4-pyridylimidamides 26SBM070 and 27SMB009 (Fig. 3).

Mammalian cell cultures. For the *in vitro* analysis of compound toxicity and effects against intracellular parasites (Y strain), primary cultures of embryonic cardiomyocytes (CM) were obtained from Swiss mice as previously reported (22). After purification, the CM were seeded at a density of 0.2×10^6 and 0.05×10^6 cells/well, respectively, in 24- and 96-well microplates containing gelatin-coated coverslips. The cultures were then maintained at 37°C in Dulbecco's modified medium supplemented with 10% horse serum, 5% fetal bovine serum, 2.5 mM $CaCl_2$, 1 mM L-glutamine, and 2% chicken embryo extract (DMEM). For the further analysis of the effect on intracellular parasites of the Tulahuen strain

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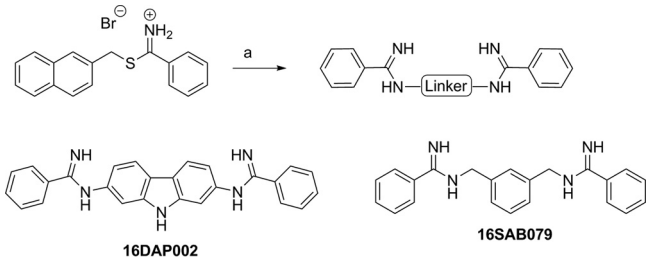

FIG 1 Synthesis of 16DAP002 and 16SAB079. Reagents and conditions: (a) appropriate diamine, ethanol (plus acetonitrile for 16DAP022), 0 to 25°C, overnight (66 to 71%).

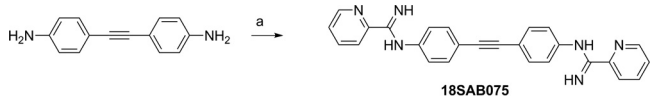

FIG 2 Synthesis of 18SAB075. Reagents and conditions: (a) 2-cyanopyridine, lithium bis(trimethylsilyl)amide, tetrahydrofuran, 25°C, 2 days, (16%).

(parasites expressing the *Escherichia coli* β-galactosidase gene), monolayers of mouse L929 fibroblasts were cultivated (4×10^3 cells/well in 96-well microplates) at 37°C in RPMI 1640 medium (pH 7.2 to 7.4) without phenol red (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine (RPMIS), as reported previously (23).

Parasites. Bloodstream trypomastigote (BT) forms of the Y strain were obtained from the blood samples of infected albino Swiss mice at the peak of parasitemia. The purified parasites were resuspended in Eagle's medium modified by Dulbecco's medium (DME) supplemented with 10% FBS (DMES) as reported previously (22). The effect on the intracellular forms was investigated by both the infection of L929 cell lineages with tissue culture-derived trypomastigotes (Tulahuen strain expressing the *E. coli* β-galactosidase gene) and the infection of CM with bloodstream trypomastigotes (Y strain) using a 10:1 ratio, following previously established protocols (15, 23). Stock solutions were prepared in dimethyl sulfoxide (DMSO) with the final concentrations of the solvent never exceeding 0.6% and 10% for *in vitro* and *in vivo* analysis, respectively, which did not exert any toxicity (data not shown). Benznidazole (Bz) (2-nitroimidazole; Laboratório Farmacêutico do Estado de Pernambuco [LAFEPE], Brazil) was used as a reference drug.

Cytotoxicity *in vitro* tests. CM were incubated for 24 h at 37°C with different concentrations of each compound (up to 96 μM) diluted in DMEM (without phenol red), their morphology and spontaneous contractibility were evaluated by light microscopy, and then their cellular viability was determined by the alamarBlue assay. For this colorimetric bioassay, 10 μl alamarBlue (Invitrogen) was added to each well, and the plate was further incubated for 24 h, after which the absorbances at 570 and 600 nm were measured. As negative controls, alamarBlue assays were also performed in the absence of cells, using only DMEM and DMEM containing each tested compound (at 96 μM). The results are expressed as the percent differences in the decreases between compound-treated and vehicle-treated cells by following the manufacturer's instructions and the EC_{50} value corresponds to the concentration that reduces the cellular viability by 50% (23).

Trypanocidal analysis. Bloodstream trypomastigotes of the Y strain (5×10^6/ml) were incubated for 24 h at 37°C in RPMI medium 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 direct quantification of the numbers of live parasites using a Neubauer chamber, and the EC_{50} (compound concentration that reduces 50% of the number of parasites) was then calculated.

For the assays on intracellular forms, different protocols were performed with the Y and Tulahuen strains. For Tulahuen-infected L929 cells, the cultures were exposed to 1.0 μg/ml diluted in RPMIS, and those compounds that presented ≥50% reductions in the parasite infection index were further screened using increasing concentrations with the aim of determining the EC_{50}s (23). After 96 h of compound incubation at 37°C, chlorophenol red glycoside (500 μM) in 0.5% Nonidet P40 was added to each well, and the plate was incubated for 18 h at 37°C. Next, the absorbance was measured at 570 nm. Uninfected and infected cultures submitted to vehicle and Bz exposure were tested in parallel. The results are expressed as the percentage of *T. cruzi* growth inhibition in compound-tested cells compared to that in the infected cells and untreated cells (23).

For the analysis of the effect against intracellular amastigotes from the Y strain, after 24 h of parasite-host cell interaction, the infected cultures were washed to remove free parasites and then incubated for another 48 h with increasing concentrations of the test compounds. CM were maintained at 37°C in an atmosphere of 5% CO_2 and air, and the medium was replaced every 24 h. Then, untreated and treated infected CM were fixed and stained with Giemsa solution, and the mean numbers of infected host cells and of parasites per infected cell were scored as reported previously (16). Only characteristic *T. cruzi* nuclei and kinetoplasts were counted as surviving parasites since irregular structures might mean parasites undergoing death. The compound activity was estimated by calculating the infection index (II) (the percentage of infected cells times the average number of intracellular amastigotes per infected host cell) (24). Triplicate assays were run on the same plate, and at least two assays were performed in each analysis.

Mouse acute toxicity. In order to determine the no-observed-adverse-effect level (NOAEL), each dose of the tested compounds (12.5 to 200 mg/kg of body weight) was injected by the intraperitoneal (i.p.) route individually in Swiss Webster female mice (20 to 23 g). On days 2 and 3, mice were inspected for toxic and subtoxic symptoms according to the Organization for Economic Co-operation and Development (OECD) guidelines. Forty-eight hours after compound injection, the NOAEL values were determined, and plasma biochemical analysis was performed for alanine aminotransferase (ALT), urea, and creatine kinase (CK) as reported previously (16).

Mouse infection and treatment schemes. Male Swiss mice were obtained from the Fundação Oswaldo Cruz (FIOCRUZ) animal facilities (Rio de Janeiro, Brazil). Mice were housed at a maximum of 6 per cage and kept in a conventional room at 20 to 24°C under a 12 h/12 h light/dark cycle. The animals were provided with sterilized water and chow *ad libitum*. Infection was performed by i.p. injection of 10^4 bloodstream trypomastigotes (Y strain). The animals (18 to 21 g) were divided into the following groups: uninfected (noninfected and untreated), untreated (infected with *T. cruzi* but treated only with vehicle), and treated (infected and treated i.p. with 0.5 to 20 mg/kg/day test compound [up to 0.2 ml] or with 100 mg/kg/day Bz orally [p.o.]). The mouse treatment started at 5 days postinfection (dpi) followed by (i) a 1-s dose at 8 dpi or (ii) five consecutive daily doses. For Bz treatment, infected mice received a 0.2-ml oral dose (gavage) following the same therapeutic schemes as described above (15).

Parasitemia, mortality rates, and ponderal curve analysis. Parasitemia was individually checked by direct microscopic counting of parasites in 5 μl of blood, as described before (25). Body weight was evaluated weekly, and mortality was checked daily until 30 days posttreatment and expressed as a percentage of cumulative mortality (%CM) (15).

Ethics. All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW-16/2013).

RESULTS

The screening of the eight novel arylimidamides (Fig. 1 to 3) against bloodstream trypomastigotes (Y strain) revealed that after 2 h of incubation at 37°C, 18SAB075 and 16DAP002 showed considerable activity, displaying EC_{50}s of 11 and 14 μM (Table 1).

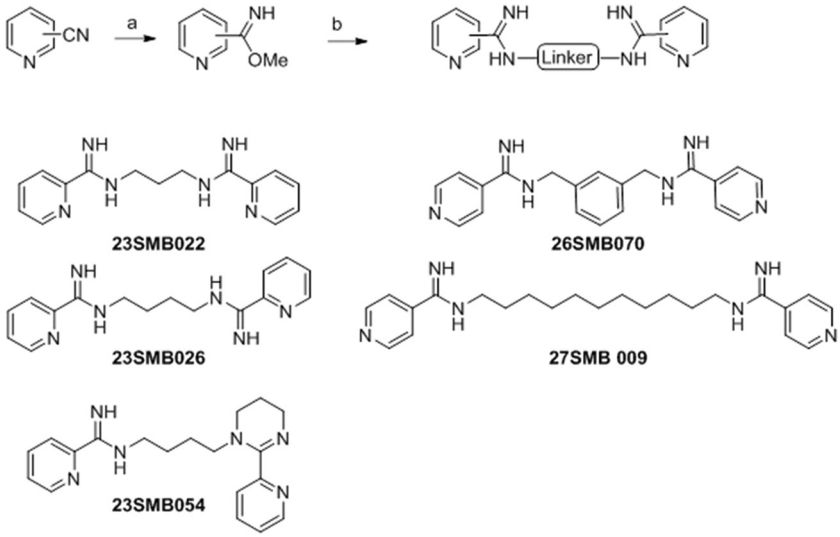

FIG 3 Synthesis of 23SMB022, 23SMB026, 23SMB054, 26SMB070, and 27SMB009. Reagents and conditions: (a) sodium methoxide, methanol, 25°C, 1 week; (b) appropriate α,ω-diamine (or spermidine for 23SMB054), 4 M HCl in dioxane, methanol, 25°C (5 to 92%).

After 24 h of incubation, both were more effective than Bz, displaying EC_{50}s of 0.3 and 3 μM, respectively, with the former molecule exhibiting about 43-fold higher trypanocidal effect than the reference drug Bz (Table 1). *In vitro* analysis of cytotoxicity on cardiac cell cultures performed by the alamarBlue assay showed that up to 32 μM, none of the studied compounds caused loss of cellular viability (Table 1). An alamarBlue assay performed in the absence of cells, with DMEM containing or not containing each tested compound (at 96 μM), showed no alterations in the absorbance measurements (data not shown). When tested on *T. cruzi*-infected host cells (the Tulahuen strain transfected with a β-galactosidase gene) under a standardized screening protocol using a fixed concentration of 1 μg/ml (23), again both 16DAP002 and 18SAB075 were effective (Table 2). Next, the two AIAs were screened against Tulahuen-infected host cells in order to determine the EC_{50}s. The findings showed that 18SAB075 was the most active with values of 1.5 μM after 96 h of incubation, comparable to those for Bz and 16DAP002 (Table 3). Further analysis of the effect of 18SAB075 against *T. cruzi*-infected cardiac cells using another strain (Y strain) confirmed the promising activity (EC_{50} = 0.9 μM) of this AIA, which was especially notable in the data for the mean number of infected cardiac cells when the infected cultures were incubated with concentrations up to 32 μM without causing loss of host cell viability (Fig. 4A and B). Although no loss of viability was noted up to ≤32 μM as seen by the alamarBlue method, light microscopy analysis demonstrated that 16SAB002 at concentrations >3.5 μM induced cellular vacuolization and impaired the contractibility of the cardiac cells *in vitro*. Also, no effect on the levels of *T. cruzi* infection was observed when nontoxic doses (≤3.5 μM) of this AIA were tested (data not shown).

Next, due to the higher selectivity indexes of 18SAB075 on bloodstream trypomastigotes (>106; Table 1) and on intracellular forms (40; Fig. 4), this compound was moved to *in vivo* analysis.

Our first *in vivo* step was to evaluate aspects of the acute toxicity of 18SAB075, aiming to determine its NOAEL value. The administration of this AIA via the i.p. route to Swiss female mice (12.5 to 200 mg/kg) followed for 48 h showed that none of the doses caused

TABLE 1 Trypanocidal activity (EC_{50}) of the studied arylimidamides against bloodstream trypomastigotes of *T. cruzi* (Y strain) and their selectivity index related to cardiomyocytes (EC_{50})

Compound	EC_{50} (mean ±SD) (μM) in:			
	Trypomastigotes		Cardiomyocytes	
	2 h	24 h	(24 h)	SI (24 h)[a]
18SAB075	11 ± 1	0.3 ± 0	>32	>106
16DAP002	14 ± 3	3 ± 1	>32	>11
27SMB009	>100	85 ± 21	>32	>0.4
23SMB054	>100	>100	>32	
23SMB022	>100	>100	>32	
23SMB026	>100	>100	>32	
26SMB070	>100	>100	>32	
16SAB079	>100	>100	>32	
Benznidazole	>100	13 ± 2[b]	1,000	77

[a] Treatment for 24 h at 37°C. Selectivity index (SI) = EC_{50} for cardiomyocytes/EC_{50} for parasites.
[b] Value from da Silva et al. (16).

TABLE 2 Activity of arylimidamides (at 1 μg/ml) against intracellular forms of *T. cruzi* (Tulahuen strain transfected with β-galactosidase) after treatment for 96 h at 37°C

Compound	Inhibition of the host cell infection (mean ± SD) (%)
16DAP002	58 ± 31
18SAB075	50 ± 7
26SMB070	15 ± 8
16SAB079	4 ± 5
23SMB054	3 ± 4
23SMB022	1 ± 1
27SMB009	0 ± 0
23SMB026	0 ± 0
Benznidazole	82 ± 3

TABLE 3 *In vitro* effect (EC_{50}s) of arylimidamides against intracellular forms of *T. cruzi* (Tulahuen strain transfected with β-galactosidase) after treatment for 96 h at 37°C

Compound	EC_{50} (mean ± SD) (μM)
16DAP002	4.1 ± 1.8
18SAB075	1.5 ± 0.2
Benznidazole	2.6 ± 0.8

death. Up to 50 mg/kg, no alteration in animal behavior (compared to that of vehicle-treated mice) was found. However, increased doses induced visible neurological disorders like tremors, ataxia, and hyperactivity (data not shown). The gross pathological examination performed at 48 h after drug administration showed that 200 mg/kg induced hemorrhagic hepatic signs (data not shown). This hepatic injury was confirmed by the biochemical plasma analysis of the higher dose as increased levels of aspartate transaminase (AST) and ALT were found compared to those for the animals from the other groups (data not shown). Then, the efficacy of 18SAB075 was tested in Swiss male mice inoculated with 10^4 bloodstream parasites using a therapeutic scheme employing doses administered once a day, at 5 and 8 dpi (Fig. 5) that correspond to the onset and parasitemia peaks, respectively, in this animal model (15). Only parasitemia-positive mice were used, and Bz treatment was run in parallel using standard protocol (100 mg/kg p.o.), following the same therapeutic scheme as described above (Fig. 5). Although 20 and 10 mg/kg did not reduce or only slightly impaired (20%), respectively, the parasitemia levels, the lower dose (5 mg/kg) resulted in a 50% decrease in the parasitism peak decrease, also leading to 40% animal survival while all the other mice (except for the Bz group) died (Fig. 5).

Then, aiming to allow a longer plasma exposure of the compound but taking into consideration the use of maximum nontoxic doses (up to 50 mg/kg), we performed additional studies, providing the AIA for 5 daily consecutive days, starting at 5 dpi (Fig. 6). As expected for this experimental mouse model of *T. cruzi* acute infection, infected and vehicle-treated animals presented high parasitemia and no animal survival (Fig. 5 and 6). When 18SAB075 was administered via the i.p. route, none of the doses reduced the parasitemia or protected against mortality. As expected, Bz completely suppressed the parasitemia and conferred 100% survival (Fig. 6). Also, none of the doses were able to protect the animals against the body weight loss (data not shown) induced by the *T. cruzi* infection in this experimental acute model (26).

DISCUSSION

Structural variations in the presently available AIAs include seven different linkers between the two AIA moieties and three different outer aromatic rings. Those molecules containing multiple or fused aromatic rings in their linkers were active against the bloodstream and intracellular parasites. The most active compound, 18SAB075, contains a diphenylacetylene linker, which offers extended conjugation. The second most active compound, 16DAP002, contains a carbazole system as its linker, which also offers extended conjugation but a more rigid conformation due to the fused rings. Both compounds displayed AIA nitrogen atoms attached directly to aromatic rings. The xylene derivative 26SMB070, which was less active, bears an aromatic ring in its linker, but the nitrogen atoms are attached to aliphatic carbons. Analogues of the two most active compounds containing the same linkers but different outer rings were not included in this study.

18SAB075	EC_{50} (μM)	SI
% Host cell Infection	0.8 ± 0.1	40
Mean number of parasite/per infected host cell	6.0 ± 2.0	5
Infection Index	0.9 ± 0.1	36

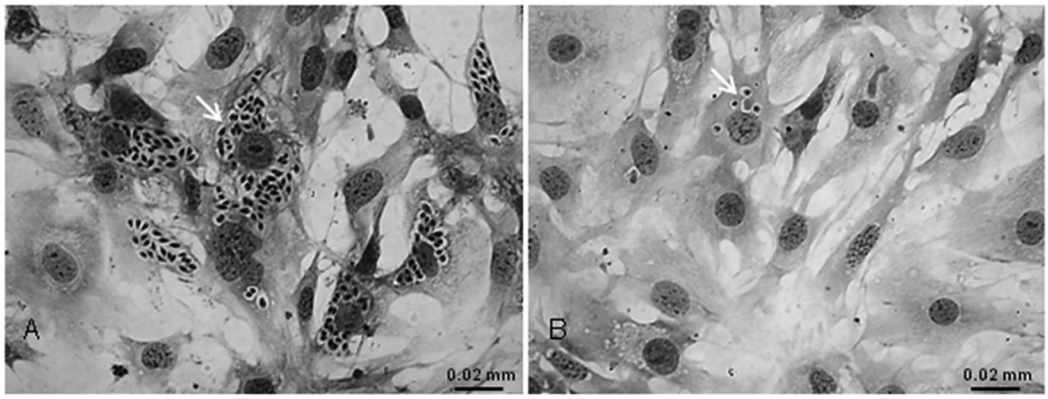

FIG 4 Activity (EC_{50}s) and selective index (SI) of 18SAB075 against intracellular forms of *T. cruzi* (Y strain) after treatment for 48 h at 37°C. (A) *T. cruzi*-infected cardiac cells incubated with vehicle. (B) *T. cruzi*-infected cardiac cells incubated with 32 μM 18SAB075. The arrows point to intracellular parasites. Infection index, host cell infection × number of parasites/infected host cell, as reported in Batista et al. (15).

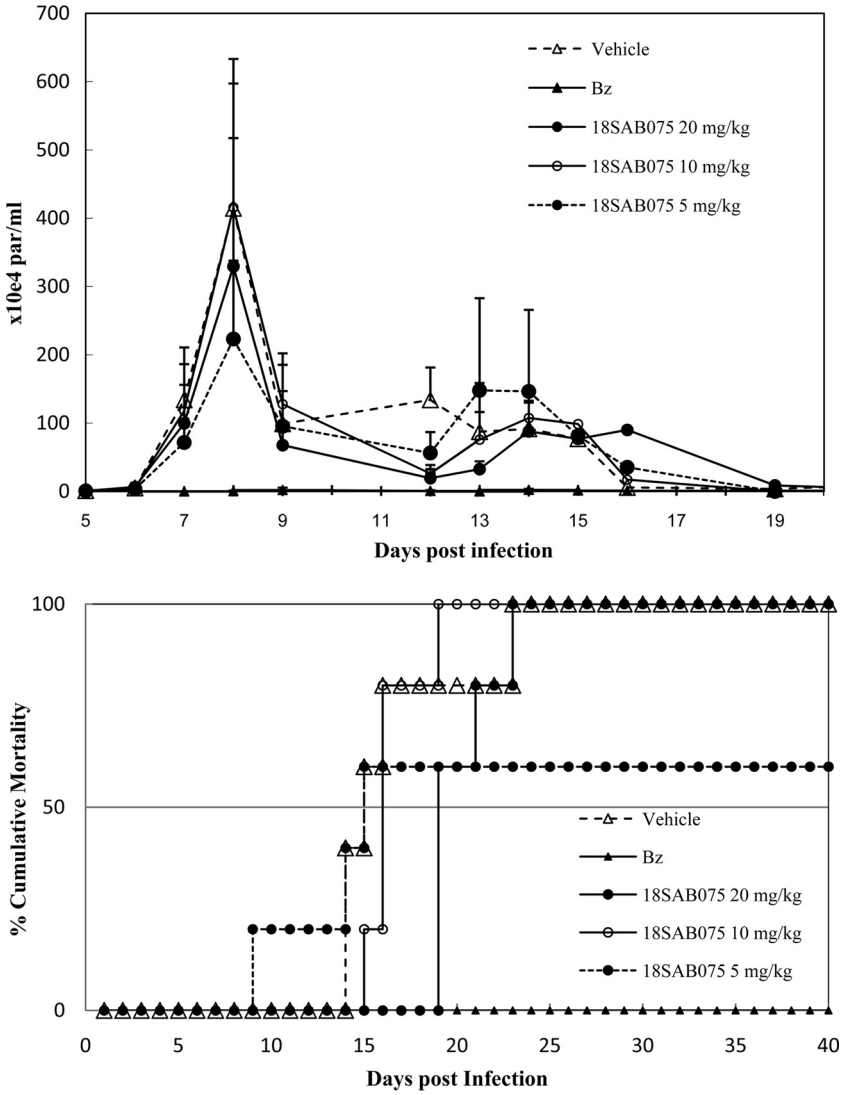

FIG 5 *In vivo* effect of 18SAB075 on *T. cruzi* infection using male Swiss mice inoculated with 10^4 bloodstream trypomastigotes (Y strain). The activities of 5, 10, and 20 mg/kg/day 18SAB075 (i.p.) and of benznidazole (100 mg/kg/day by gavage) given at 5 and 8 dpi was evaluated through parasitemia levels (top) and cumulative mortality (bottom).

Chemotherapeutic options for treating chagasic patients currently depend on only two nitro derivative drugs, namely, benznidazole (2-nitroimidazole; Laboratório Farmacêutico do Estado de Pernambuco [LAFEPE], Brazil) and nifurtimox (5-nitrofuran, Bayer 2502; Bayer, Germany) (27). Both cause severe side effects, resulting in discontinuation of the treatment and low effectiveness, especially in the later chronic phase of the disease, demanding the identification of novel drugs to treat this devastating chronic pathology. However, there is a lack of interest from most pharmaceutical companies for discovery of new anti-*T. cruzi* agents mainly due to the long time and high costs associated with the drug pipeline for this and other neglected parasitic illnesses that afflict millions of the poorest people worldwide (28). In this context, our aim was to explore the possibility of finding new amidine derivatives with considerable activity against *T. cruzi* in *vitro* and *in vivo* which possess the potential to act as clinical candidates against this neglected parasitic disease. In this vein, eight novel AIAs were screened against the bloodstream and intracellular forms of the parasite and two of them, 18SAB075 and 16DAP002, demonstrated higher activity, with EC_{50}s of <4 μM. The two compounds were effective against different strains (Y and Tulahuen), and due to its high selectivity, 18SAB075 was further examined in *in vivo* analyses for preliminary ascertainment of acute toxicities and for efficacy determination using an acute mouse model of *T. cruzi* infection (male Swiss mice infected with Y strain). The follow-up of acute toxicity signs for 48 h after 18SAB075 administration via the i.p. route showed that although at up to 50 mg/kg, no alterations were noticed, considerable toxic side effects like ataxia, hyperactivity, and tremors were found with doses of 100 and 200 mg/kg. The gross pathological examination confirmed that the higher dose also induced hemorrhagic hepatic signs that were corroborated by increased plasma levels of hepatic markers like AST. Thus, efficacy analysis was further explored using only doses ≤20 mg/kg/day and never exceeding cumulative doses of 50 mg/kg. Bz was included as a reference drug, given at its effective dose of 100 mg/kg/day (23). The i.p. administration of

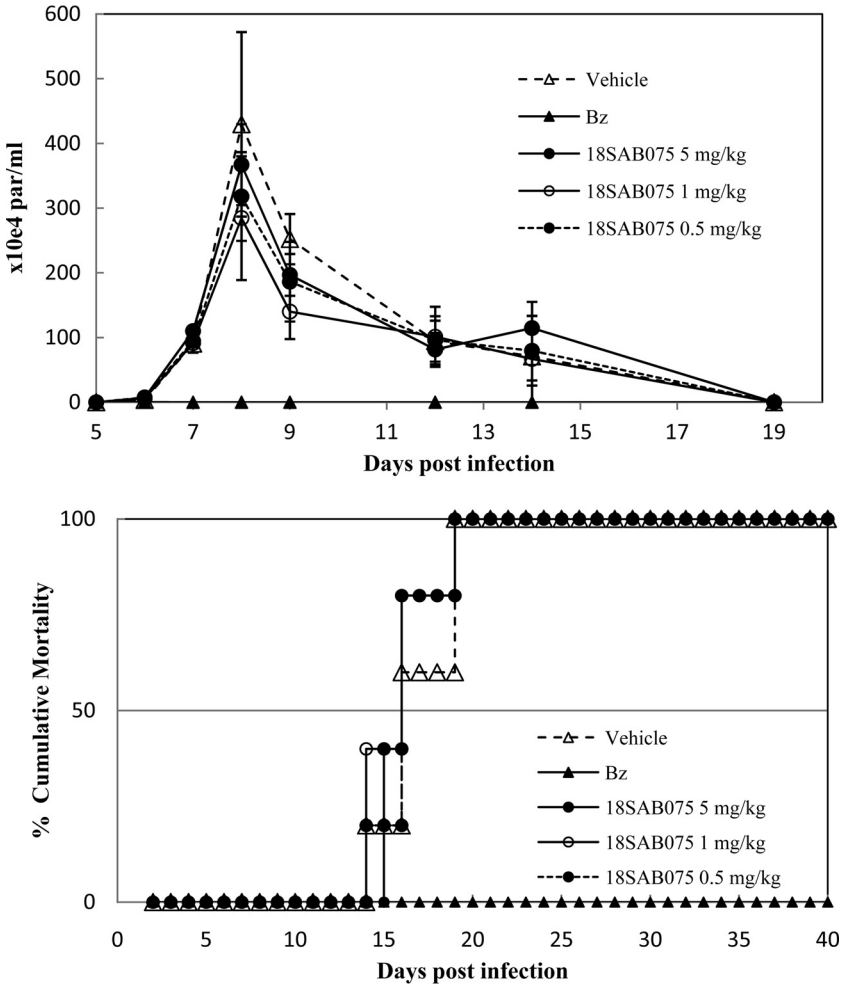

FIG 6 *In vivo* effect of 18SAB075 on *T. cruzi* infection using male Swiss mice inoculated with 10^4 bloodstream trypomastigotes (Y strain). The activities of 0.5, 1, and 5 mg/kg/day 18SAB075 (i.p.) and of benznidazole (100 mg/kg/day by gavage) given for 5 consecutive days (5 to 9 dpi) were evaluated through parasitemia levels (top) and cumulative mortality (bottom).

18SAB075 in all assayed therapeutic schemes (2 and 5 days of treatment) using nontoxic doses failed to demonstrate the activity of this diphenylacetylene AIA *in vivo*, while Bz not only suppressed the parasitemia but also induced 100% protection against mortality due to the experimental infection as described previously (26). Previous data using bis-AIAs like 2,5-bis[2-(2-propoxy)-4-(2-pyridylimino)aminophenyl]furan (DB 766) (15) and a related analog (DB 1831) (16) showed their high activity *in vivo* in the acute experimental mouse models of *T. cruzi*, exhibiting trypanocidal effects similar to that of Bz. In fact, with the same highly stringent experimental *in vivo* model as presently used for 18SAB075, the two previous studies reported that the presence of pyrimidine and pyridine units of bis-AIAs seemed to be advantageous for *T. cruzi* activity (14), supporting the continuity of preclinical studies of novel related molecules with the aim of finding new alternatives for treating Chagas disease.

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The Search For New Drug Candidates To Treat Chagas Disease

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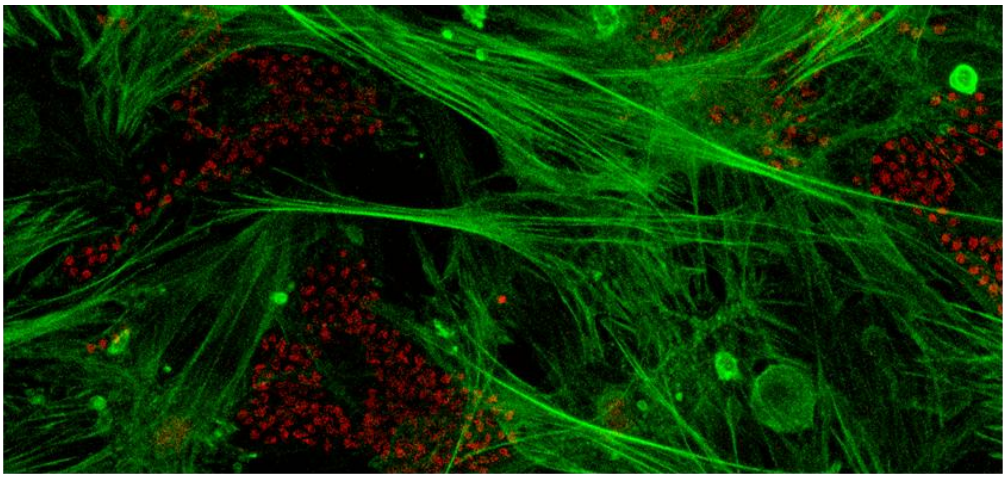
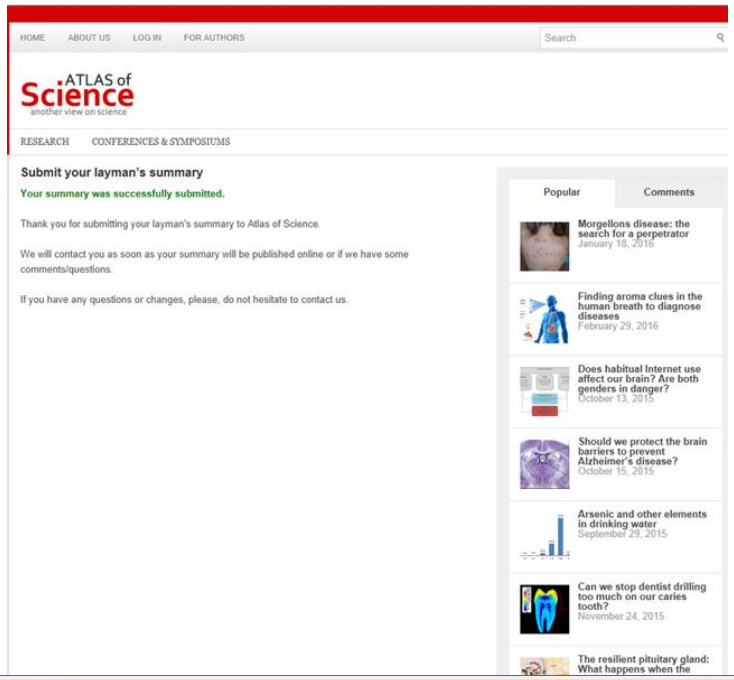
Chagas disease (CD) is a parasite-born pathology caused by a unicellular microorganism called *Trypanosoma cruzi.* This illness causes a significant morbidity and mortality in the developing world, being endemic in 21 countries across Latin America, representing also a relevant public health problem in non-endemic countries (as Australia, Canada, Japan, Spain, and the United States) mainly in consequence of the immigration of infected people to these regions. CD is one of the seventeen neglected diseases and is primarily transmitted by blood-sucking insects through contact with the feces of infected bugs, deposited on the skin after their blood meal. These insects typically hide in crevices of poorly-constructed homes in rural or suburban areas and thus CD is mainly a poverty-associated disease. Blood, organ transplant, and congenital transmission also occur, and cases of oral transmission through ingestion of food infected by bugs/feces are well documented. CD has two clinical phases: the acute phase that starts as soon as the infection occurs and is usually oligosymptomatic, and may display fever, malaise, facial edema, generalized lymphadenopathy, and hepatosplenomegaly. As result of the immune response, there is a control of the parasite proliferation (but not eradication) and often spontaneously resolves in few weeks. However, some people may present a serious cardiopathy leading to about 5% of death (mostly in children). Next, the

infected people move to a second stage called the chronic phase in which the majority keep asymptomatic (indeterminate form). However, years or even decades after the initial infection, untreated individuals may develop a chronic progressive pathology with about 10% to 30% exhibiting severe heart and/or gastrointestinal disorders that results in death. Today more than 6-8 million people are infected with *T.cruzi*, 7,000 die annually and about 70 million people are at risk of infection only at Latin America. Despite this serious problem, until now only two drugs can be used to treat this pathology: two nitroderivatives named nifurtimox and benznidazole (Bz). Both are far from being ideal since are not active upon the last chronic phase, have several toxic effects (cutaneous hypersensitivity, ringworm, digestive intolerance, bone marrow depression, peripheral neuropathy, hepatotoxicity, anorexia, weight loss, drowsiness or excitability), require long periods of therapy and there is several parasite strains that are naturally resistant against both drugs. Another great concern is that today less than 1% of the chagasic patients have access to the treatment, underlining the urgent need to expand access and accelerate the development of truly innovative safer and more potent medicines to treat CD. In this context, our group is involved in scientific studies using *in vitro* (from Latin: “in glass”) and *in vivo* (from Latin: "within the living") models aiming to identify novel drug candidates and presently we will briefly report some of these results obtained with 14 aromatic heterocyclic compounds named arylimidamides (AIAs) assayed against different strains and forms of *T. cruzi*. We found that one of the most promising was the m-terphenyl bis-AIA 35DAP073. *In vitro* analysis showed that it was about 26-100 fold more potent than the reference drug (Bz), being also active against those naturally resistant parasite strains. *In vivo* findings using mouse models of acute *T.cruzi*-infection revealed that 35DAP073 induced a dose-dependent action, leading to 96 to 46% reductions in the level of blood parasitism in mice, but unfortunately longer periods of treatment designed to reach parasitological cure demonstrated reversible animal neurological side effects. The combination of this AIA with Bz aiming to reduce drug toxicity and improve efficacy resulted in suppression of blood parasitism in the animal models also providing elimination of toxic effects, besides leading to 100 % of mice survival. Although this combination was not able to cure the infected mice, it resulted in a great reduction of the total parasitism measured

by sensitive molecular tools like qPCR (quantitative polymerase chain reaction). Our laboratory results support further investigations of this class of compounds with the aim of developing novel alternatives for the treatment of Chagas disease.



Fluorescent miscroscopy image of primary cultures of mouse cardiac cells labeled (in green with phalloidin) infected with *Trypanosoma cruzi* (stained in red) *in vitro.*



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