INSTITUTO OSWALDO CRUZ

Doutorado em Biologia Parasitária

Estudos *in vitro* e *in vivo* da atividade biológica de fluorquinolonas, tiossemicarbazonas, diamidinas aromáticas e arilimidamidas sobre *Trypanosoma cruzi*

Denise da Gama Jaén Batista

Rio de Janeiro
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Orientadora: Dra. Maria de Nazaré Correia Soeiro

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Rio de Janeiro, 07 de outubro de 2009.

Aos meus pais, às minhas tias, à minha irmã e ao meu namorado, por todo amor, apoio, paciência e compreensão.

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“Tudo vale a pena se a alma não é pequena.”
Fernando Pessoa

“A vida é para quem topa qualquer parada. Não para quem para em qualquer topada.”
Bob Marley

“Nas grandes batalhas da vida, o primeiro passo para a vitória é o desejo de vencer!”
Mahatma Gandhi

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2. **Batista, D.G.J.**, Silva, P.B., Lachterb, D.R., Silva, R.S., Aucélio, RQ., Louro, S. R.W., Beraldo, H., Soeiro, M.N.C., Teixeira, R.T. (2009). Coordination of Manganese(II) with N^4-methyl-4-nitrobenzaldehyde, N^4-methyl-4-nitroacetofenone, and N^4-methyl-4-nitrobenzophenone thiosemicarbazone and their biological *in vitro* Activity upon *Trypanosoma cruzi*. *Polyhedron 29:2232-2238.*

3. **Batista, D.G.J.**, Pacheco, M.G.O., Kumar, A., Branowska, D., Ismail, M.A., Hu, L., Boykin, D.W., Soeiro, M.N.C. (2009). Biological, Ultrastructural Effect and Subcellular Localization of Aromatic Diamidines in *Trypanosoma cruzi. Parasitology 137(2):251-259. doi: 10.1017/S0031182009991223.*

4. **Batista, D.G.J.**, Batista, M.M., Oliveira, G.M., Borges, P., Lannes-Vieira, J., Britto, C.C., Junqueira, A., Lima, M.M., Romanha, A.J., Sales Junior, P.A., Stephens, C.E., Boykin, D.B., Soeiro, M.N.C. (2009). Arylimidamide DB766: A Potential Chemotherapeutic Candidate for Chagas disease treatment. Submetido à PLOs Pathogens. *Antimicrob Agents Chemother. 54(7): 2940-2952. dói: 10.1128/AAC.01617-09.*

5. **Batista, D.G.J.**, Batista, M.M., Oliveira, G.M., Britto, C.C., Rodrigues, A.C.M., Boykin, D.B., Soeiro, M.N.C. (2009). Combined Treatment of Heterocyclic Dications and Benznidazole upon *Trypanosoma cruzi in vivo. Submetido à PLOS One.*

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3. XIII Internacional Congresso of Protistology/XXV Annual Meeting of the Brazilian Society of Protozoology/XXXVI Annual Meeting on Basic Research in Chagas' Disease (Agosto de 2009).

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1. Soeiro, M.N.C., de Castro, S.L., de Souza, E.M., **Batista, D.G.J.**, Silva, C.F., Boykin, D.W. (2008). Diamidine Activity Against Trypanosomes: the State of the Art. Current Molecular Pharmacology 1:151-161.

2. De Souza, E.M., Batista, M.M., **Batista, D.G.J.**, Duarte, B.B., Araújo-Jorge, T.C., Soeiro, M.N.C. (2008). *Trypanosoma cruzi*: Alpha-2-Macroglobulin Regulates Host Cell Apoptosis Induced by the Parasite Infection *in vitro*. *Exp Parasitol* 118(3):331-337.

3. Silva, C.F., Batista, M.M., **Batista, D.G.J.**, de Souza, E.M., Silva, P.B., Oliveira, G.M., Meuser, A.S., Shareef, A.R., Boykin, D.W., Soeiro, M.N.C. (2008). *In vitro* and *in vivo* Studies of the Trypanocidal Activity of a Diarylthiophene Diamidine Against *Trypanosoma cruzi*. *Antimicrob Agents Chemother* 52(9):3307-3314.

4. Soeiro, M.N.C., Daliry, A., Silva, C.F., **Batista, D.G.J.**, Souza, E.M., Oliveira, G.M., Salomão, K., Batista, M.M., Pacheco, M., Silva, P.B., Santa-Rita, R.M., Menna-

Barreto, R.F.S., Boykin, D.W., De Castro, S.L. (2009). Experimental Chemotherapy for Chagas Disease: 15 Years of Research Contributions Through *in vivo* and *in vitro* Studies. *Mem Inst Oswaldo Cruz*, 104 (Suppl. I): 301-310.

5. Coutinho, L., Alves Ferreira, M., Cosson, A., Batista, M.M., **Batista**, **D.G.J**., Minoprio, P., Degrave, W., Berneman, A., Soeiro, M.N.C. (2009). Inhibition of *Trypanosoma cruzi* Proline Racemase Hampers Host-Parasite Interactions. *Submetido* à *Mem Inst Oswaldo Cruz.*

LISTA DE ABREVIATURAS

AIA	Arilimidamidas
BZ	Benznidazol
CCC	Cardiopatia chagásica crônica
CK	Creatinina Quinase
DA	Diamidinas Aromáticas
DC	Doença de Chagas
DND*i*	Drug for Neglected Disease *initiative*
ECG	Eletrocardiograma
Fen	Fenantrolina
FQ	Fluorquinolonas
GPT	Glutamato piruvato transaminase
ip	Intraperitoneal
MS	Ministério da Saúde
NF	Nifurtimox
NOR	Norfloxacina
OMS	Organização Mundial de Saúde
OPAS	Organização Pan-Americana da Saúde
p.o.	*Per* oral
Phen	Phenanthroline
SPAR	Esparfloxacina
TOPO II	Topoisomerase II
TS	Tiossemicarbazonas

RESUMO

No centenário da descoberta da doença de Chagas (DC), esta parasitose negligenciada causada pelo *Trypanosoma cruzi* é importante causa de mortalidade e morbidade na América Latina, sem vacinas ou agentes quimioterápicos satisfatórios. Esta tese objetivou analisar atividade, seletividade e mecanismos de ação sobre o *T.cruzi* de 04 classes de compostos (fluorquinolonas - FQ, tiossemicarbazonas - TS, diamidinas aromáticas - DA e arilimidamidas – AIA – uma nova classe a partir de DA) *in vitro* e *in vivo*. FQ como NOR e SPAR são antibióticos com ampla ação bactericida, atuando sobre o DNA via toposoimerases. Embora SPAR e NOR não tenham sido ativas, seus complexos metálicos de cobre-fenantrolina foram efetivos sobre tripomastigotas e formas intracelulares de *T.cruzi*, (IC_{50} 1,62 a 4,65μM). TS são agentes anti-tumorais e microbicidas que atuam sobre ribonucleotideo redutases e cisteína proteases. Todas TS apresentaram baixa toxicidade (>200μM) sobre células de mamíferos. Embora N^4-metil-4-nitrobenzaldeído-tiossemicarbazona, N^4-metill-4-nitroacetofenona-tiossemicarbazona e seus complexos metálicos de manganês (Mn) não tenham sido ativos, o complexo de Mn da N^4-metil-4-nitrobenzofenona-tiossemicarbazona revelou-se moderadamente ativo sobre formas sanguíneas (IC_{50} 19μM). A terceira classe foi DA, ligantes de DNA com excelente atividade sobre diversos patógenos. Embora todas tenham baixa toxicidade sobre cardiomiócitos, somente três (DB1645, DB1582 e DB1651) foram ativas contra formas sanguíneas e intracelulares (IC_{50} entre 0,15 a 13,3μM), com atividade relacionada a curvatura das moléculas (sendo as moléculas lineares as não efetivas). Todas DA localizam-se no núcleo e kDNA dos parasitos, com maior acúmulo na última estrutura. DB1582 e DB1651 também se acumulam em organelas desprovidas de DNA, possivelmente acidocalcisomas. Alvos ultra-estruturais incluíram mitocôndria, kDNA e microtúbulos. Análise *in vitro* da AIA DB766 sobre *T.cruzi* revelou: (i) excelente atividade sobre formas sanguíneas (60nM) e intracelulares (25nM), mantendo ótima ação mesmo quando o parasito é incubado com 96% de sangue de camundongo, sugerindo assim, um potencial uso profilático, (ii) ação sobre diferentes cepas (peridomiciliares/silvestres), incluindo as naturalmente resistentes ao benznidazole (BZ), com superior eficácia que BZ, (iii) localização em compartimentos ricos em DNA, induzindo danos ultra-estruturais na mitocôndria. Com base no excelente índice de seletividade (>533 e 714), ensaios foram conduzidos durante a infecção aguda experimental por *T.cruzi* (cepas Y e Colombiana), em doses diárias ou alternadas ≤100mg/kg/dia da DB766 sozinha ou associada ao BZ, administradas via ip ou p.o. por até 20 dias, com primeira dose no início da parasitemia. DB766 (25 e 50mg/kg/dia ip e 100mg/kg/dia p.o.) reduziu (>90%) a carga parasitária (sangue e tecido cardíaco) e protegeu 90-100% contra mortalidade, com semelhante eficácia ao BZ. AIA reverteu inflamação e alterações elétricas cardíacas e protegeu contra lesões hepáticas e musculares induzidas pela infecção. Doses sub-ótimas de BZ associado com a DB289 (DA - pró-droga da DB75 – via p.o.) ou com a DB766 (ip) resultaram em ≥99% redução de parasitemia e 100% de proteção sobre mortalidade. BZ (p.o)+DB766 (ip) resultou em cura parasitológica (2 em 15 animais) avaliada por hemocultivo e PCR. AIA foi a mais ativa e seletiva sobre *T.cruzi,* estimulando a continuidade de ensaios pré-clínicos com representantes desta classe visando identificação de novos fármacos para a DC.

ABSTRACT

In the centennial of Chagas disease (CD) discovery, this neglected parasitose caused by *Trypanosoma cruzi* is still an important cause of mortality and morbidity in endemic areas of Latina America, claiming for new safer and more effective prophylactic and therapeutic approaches. Our aim was to evaluate the activity, selectivity and mechanisms of action on *T.cruzi* of 04 different classes of synthetic compounds: (fluoroquinolones-FQ, thiosemicarbazones-TS, aromatic diamidines-AD and arylimidamides–AIA a new class of AD analogues) *in vitro* and *in vivo*. FQ such as NOR and SPAR are antibiotics used against different bacteria strains, acting upon DNA via toposoimerases. Although SPAR and NOR were inactive, their metallic complexes of cupper-phenantroline were effective against bloodstream and intracellular forms of *T.cruzi*, (IC_{50} 1,62 to 4,65μM). TS are anti-tumor and microbicidal agents acting by ribonucleotide reductases and cystein protease inhibitors. All TS showed low toxicity (>200μM) towards mammalian cells. Although N^4-metil-4-nitrobenzaldehydo-thiossemicarbazone, N^4-methyl-4-nitroacetophenone-thiossemicarbazone and their manganese (Mn) complexes were not effective, Mn-complex of N^4-metil-4-nitrobenzophenone-thiossemicarbazone showed moderate activity on bloodstream forms (IC_{50} 19 μM). AD are DNA binders with striking activity upon different pathogens. Although all AD presented low toxicity towards cardiac cells, only three out of them (DB1645, DB1582 and DB1651) showed effect upon both bloodstream and intracellular forms of *T.cruzi (*IC_{50} 0,15 to 13,3μM), with the trypanocidal activity related to molecule shape curvature (linear molecules inactive). AD localized in nuclei and kDNA of treated parasites, being the later structure the one with higher accumulation. DB1582 and DB1651 also labeled non DNA structures, possibly acidocalcisomes. Ultrastructural targets were mitochondria, kDNA and microtubules. *In vitro* analysis of AIA DB766 upon *T.cruzi* showed: (i) excellent activity upon bloodstream (60nM) and intracellular (25nM) forms, maintaining good action even when the parasite was incubated with 96% of mouse blood, suggesting its potential application in blood banks, (ii) effect on different parasite strains (peridomiciliary/sylvatic), and those naturally resistant to benznidazole (BZ), showing superior efficacy than reference drug, (iii) localization within DNA-enriched compartments leading to important ultrastructural mitochondrial damages. Due to the excellent high selective index (>533-714), *in vivo* assays were conducted upon acute experimental *T.cruzi* infection *(*Y and Colombiana strains), using daily and alternative doses (≤100mg/kg/day) of DB766 alone or combined with BZ, given by ip and p.o. up to 20 days, with the first doses at parasitemia onset. DB766 (25 e 50mg/kg/day ip and 100mg/kg/day p.o.) reduced (>90%) parasite load, (from blood and cardiac tissues) besides protecting (90-100%) against mortality rates, showing similar efficacy as BZ. AIA reversed inflammation and electric cardiac alterations and protected against hepatic and muscular lesions induced by parasite infection. Sub optimum doses of BZ associated with DB289 (prodrug of DB75 p.o.) or with DB766 (ip) reduced ≥99% parasitemia levels and gave 100% protection against mice mortality. BZ (p.o.)+DB766(ip) lead to parasitological cure (2 out of 15 animals) evaluated by hemoculture and PCR assays. Our data showed that AIA was the most effective and selective class of compounds presently assayed against *T.cruzi*, supporting further pre-clinical studies with other AIA aiming to identify new potential therapies for CD.

ÍNDICE

Introdução

1. INTRODUÇÂO

1.1. Considerações gerais sobre a doença de Chagas:

Em 1909, o médico e pesquisador Dr. Carlos Chagas publicou o trabalho onde descreveu a descoberta do vetor, do agente etiológico e dos reservatórios naturais (Figura 1 e 2), determinando ainda o quadro clínico de uma nova enfermidade – a tripanosomíase americana – que foi denominada doença de Chagas (DC) (Chagas, 1909).

No ano em que comemoramos 100 anos desta descoberta, esta doença tropical causada pelo protozoário hemoflagelado *Trypanosoma cruzi* e que apresenta duas fases clínicas (aguda e crônica), é ainda considerada importante causa de mortalidade, absentismo e morbidez em indivíduos em plena idade produtiva em áreas endêmicas (Dias, 2007). Segundo dados da Organização Mundial de Saúde (OMS) aproximadamente 15 milhões de indivíduos estão infectados com este parasito, com cerca de 12 000 mortes por ano e cerca de 28 milhões de pessoas expostas ao risco de contrair a infecção em 15 países endêmicos da América Latina (WHO/TDR, 2006; Moncayo e Silveira, 2009).

Alguns estudos apontam também para a globalização desta parasitose com a ocorrência de pessoas infectadas em áreas não endêmicas, como nos Estados Unidos da América (EUA) e na Europa, atribuída principalmente à migração populacional de infectados, sendo no continente Europeu, estimada em cerca de 30 mil pessoas (Dias, 2007; Gáscon et al., 2007; Rodriguez-Morales et al., 2008, Guerri-Guttenberg et al., 2008). Além disso, algumas regiões dos EUA têm uma alta incidência de animais infectados, sendo ainda sugerido que muitos cardiopatas chagásicos tenham sido erroneamente diagnosticados como portadores de "cardiomiopatia dilatada idiopática primária", apontando assim para ameaça da DC também nestas áreas não endêmicas (Milei et al., 2009).

Segundo Coura (2007), a infecção pelo *T. cruzi* ocorreu há milhões de anos como uma infecção enzoótica de animais selvagens que passou a ser transmitida acidentalmente aos seres humanos como uma antropozoonose quando o homem invadiu ecótopos naturais. A prova da infecção humana data de 9.000 anos atrás, quando foi detectado kDNA de *T. cruzi* em tecidos obtidos de múmias da região norte do Chile e da região Sul do Peru (Aufderheide et al., 2004). Contudo, a infecção por este protozoário se tornou uma zoonose endêmica há 200 a 300 anos

atrás, com a adaptação dos triatomíneos ao ambiente doméstico (Coura e Dias, 2009).

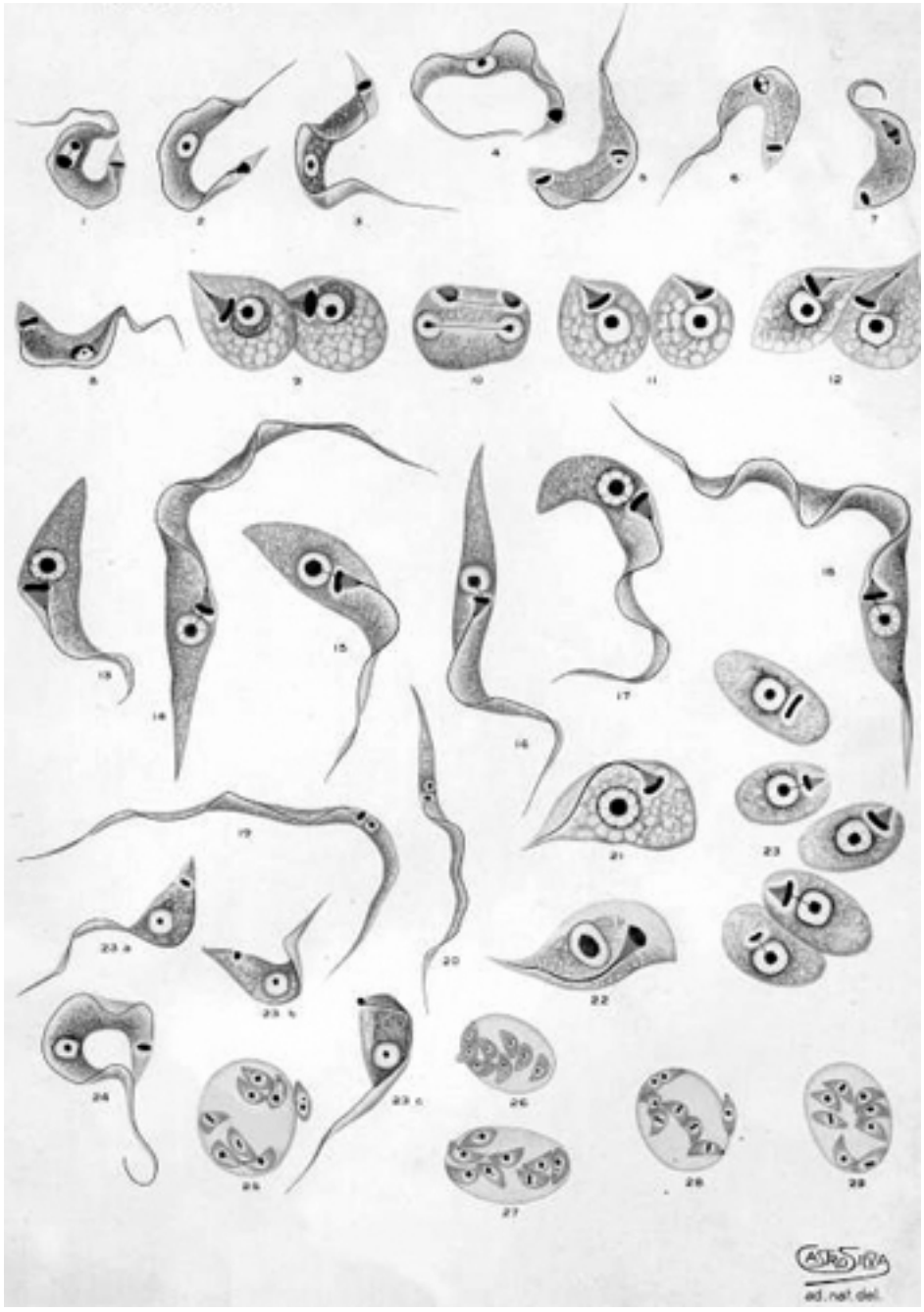

Figura 1: Desenho feito por de Castro Silva dos estágios do *Trypanosoma cruzi*, publicado em: Chagas, Carlos. “Nova tripanossomíase humana. Estudos sobre a morfologia e o ciclo evolutivo do *Schizotrypanum cruzi* n. gen., n. sp., agente etiológico de nova entidade mórbida do homem”, Memórias do Instituto Oswaldo Cruz, 1(2): 159-218.

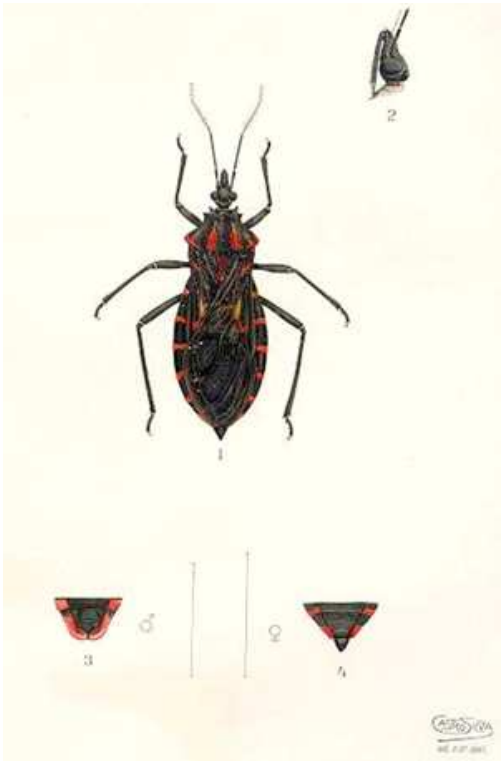

Figura 2: Desenho feito por de Castro Silva do inseto vetor, barbeiro, da doença de Chagas publicado em: Chagas, Carlos. “Nova tripanossomíase humana. Estudos sobre a morfologia e o ciclo evolutivo do *Schizotrypanum cruzi* n. gen., n. sp., agente etiológico de nova entidade mórbida do homem”, Memórias do Instituto Oswaldo Cruz, 1(2): 159-218.

1.2. Taxonomia, ciclo e formas evolutivas

1.2.1. Classificação taxonômica

De acordo com a classificação taxonômica, o *T.cruzi* pertence à ordem Kinetoplastida, família Trypanosomatidae e gênero *Trypanosoma*. O vetor deste parasito é um invertebrado conhecido vulgarmente no Brasil como “barbeiro ou chupança”, que pertence à ordem Hemíptera, família Reduviidae e subfamília Triatominae (WHO, 2002). Atualmente 140 espécies de triatomínios são conhecidas como potenciais vetores do *T. cruzi*, sendo 61 espécies encontradas no Brasil (revisto em Costa e Lorenzo, 2009). A infecção é atribuída principalmente às espécies melhor adaptadas ao ambiente domiciliar que são *Panstrongylus megistus*, *Rhodnius prolixus*, *Triatoma brasiliensis*, *Triatoma dimidiata* e *Triatoma infestans*, que representam os mais importantes vetores de *T. cruzi* em áreas endêmicas. Os

três gêneros são característicos das Américas do Sul e Central, tanto de áreas silvestres quanto de domiciliares (WHO, 2002).

1.2.2. Ciclo evolutivo e formas evolutivas

Além de diferentes hospedeiros invertebrados, o parasito também se desenvolve em distintos hospedeiros vertebrados (100 espécies de mamíferos incluindo o Homem). O ciclo de vida do *T. cruzi* (Figura 3) compreende também diferentes formas evolutivas: duas multiplicativas (amastigotas no hospedeiro vertebrado e epimastigotas no hospedeiro invertebrado) e uma terceira, não multiplicativa, mas altamente infectiva, que é a tripomastigota (Deane et al., 1984; Tyler e Engman, 2001; De Souza, 2002). O hospedeiro invertebrado infecta-se no momento do repasto de sangue a partir de um hospedeiro vertebrado infectado, através da ingestão de formas tripomastigotas sanguíneas (Brack, 1968). No vetor, o parasito se desenvolve na luz do intestino, no qual ocorre a diferenciação de tripomastigotas para epimastigotas que multiplicam por divisão binária. Na porção posterior do intestino ocorre a mudança para tripomastigotas metacíclicos, que são as formas infectivas transmitidas através das fezes e/ou urina do inseto no momento em que se alimenta do sangue de mamíferos. Estas formas invadem células do sistema fagocítico mononuclear e células residentes no local de inoculação, como por exemplo células epiteliais. Depois da ruptura do vacúolo parasitóforo (que abriga temporariamente o parasito), os tripomastigotas diferenciam-se em amastigotas, multiplicando-se ativamente por divisão binária no citoplasma da célula hospedeira. Após ciclos de multiplicações, as formas amastigotas se convertem para tripomastigotas, que são as principais formas evolutivas liberadas com a ruptura da célula no espaço intercelular, sendo capazes de invadir outras células vizinhas, difundir-se pelos tecidos através das correntes sanguínea e linfática e/ou serem ingeridos pelo inseto vetor, assim cumprindo seu ciclo de vida (Brener, 1973; De Souza, 1984; Stuart et al., 2008). Um ciclo alternativo ocorre no hospedeiro vertebrado com a ruptura de células hospedeiras e liberação de formas amastigotas, que podem invadir novas células e manter o ciclo de vida do parasito (Ley et al., 1988; Mortara, 1991; Tyler e Engman, 2001). No entanto, há evidências de que a interação de amastigotas com as células hospedeiras envolva mecanismos e receptores diferentes daqueles utilizados pelas formas tripomastigota (Mortara, 1991; Procópio et al., 1998; Mortara et al., 2005; Alves e Mortara, 2009).

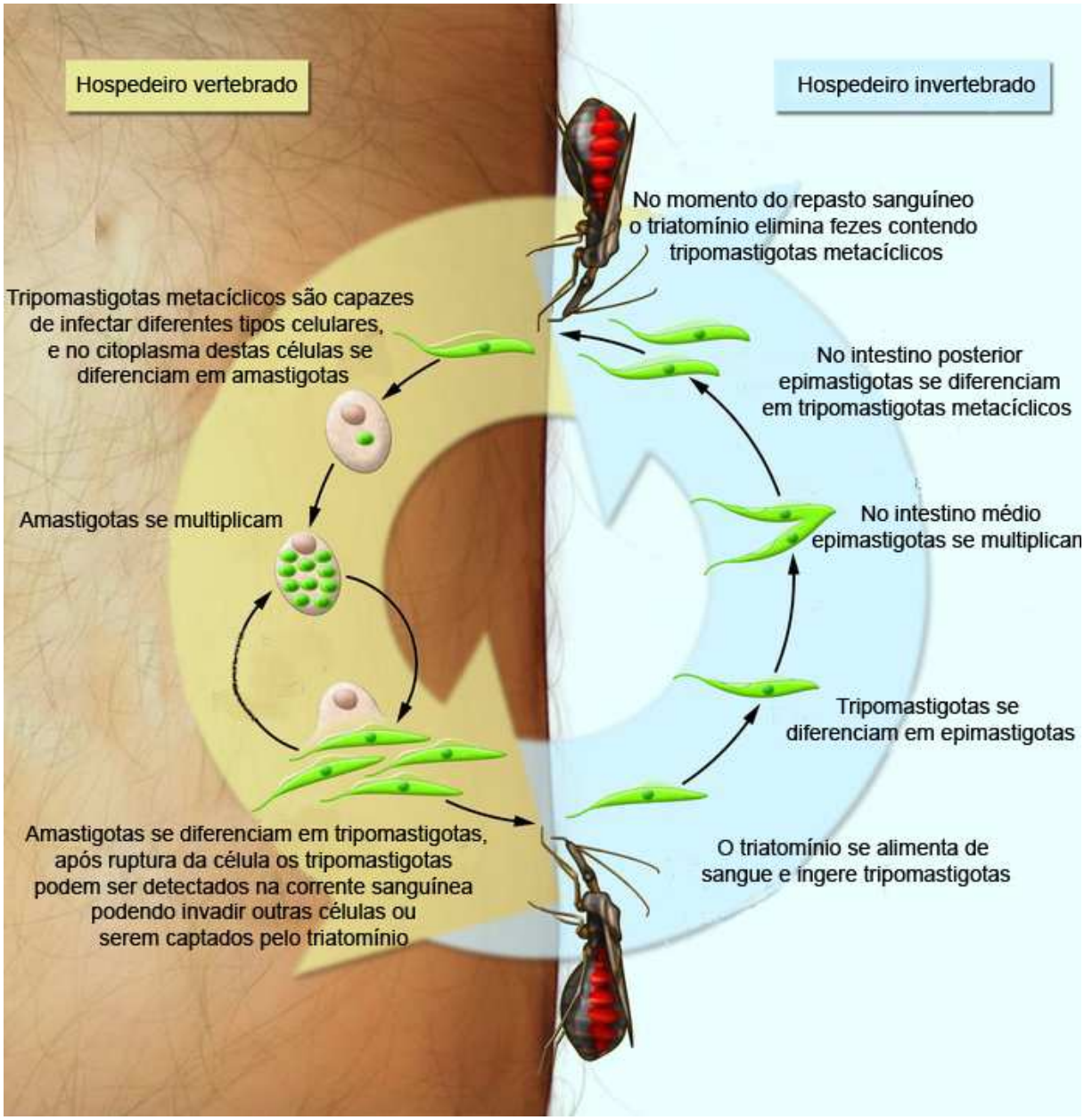

Figura 3: O ciclo evolutivo do *Trypanosoma cruzi:* após se alimentar do sangue de um mamífero, o triatomínio infectado elimina fezes e urina contendo formas infectivas do *Trypanosoma cruzi*, denominadas tripomastigotas metacíclicas. No hospedeiro vertebrado, estas formas são capazes de invadir fagócitos mononucleares e células epiteliais; no citoplasma destas células se transformam em formas multiplicativas: as amastigotas, que, posteriormente, se diferenciam em tripomastigotas. Logo, após a ruptura da célula, os tripomastigotas invadem células vizinhas ou atingem outros tecidos através das correntes sanguínea e linfática ou podem ser ingeridas por outro inseto vetor, assim completando seu ciclo evolutivo. Adaptado de Stuart et al., 2008.

1.3. Transmissão

A via de maior relevância na transmissão da DC é a vetorial que corresponde a 80-90% dos casos de infecção através da invasão de formas metacíclicas em mucosas ou na pele lesionada. Contudo, a infecção pode também ser adquirida por transfusão de sangue, transmissão congênita, transplante de órgãos, infecção acidental em laboratório e, ainda, pela ingestão de comida ou bebida contaminada (Miles et al., 2003; Remme et al., 2006).

1.3.1. Transmissão por vetores

A transmissão vetorial do *T. cruzi* pode se dar por três ciclos: o ciclo doméstico que é responsável pela manutenção da doença em humanos, ocorre principalmente em áreas urbanas e peri-urbanas, tendo como os principais reservatórios do parasito os humanos, os cães e os gatos; o ciclo silvestre, no qual triatomíneos silvestres infectam roedores, marsupiais e outros mamíferos e, o ciclo peridomiciliar, que representa uma conexão entre o ciclo doméstico e o silvestre, onde se observa o fluxo de mamíferos (roedores domésticos, marsupiais, gatos, cachorros, entre outros) entre casas e áreas silvestres, bem como a ocorrência de espécies de triatomíneos silvestres infectados que têm acesso às casas infectando diretamente pessoas, animais e até mesmo alimentos (Remme et al., 2006).

Estudos sugerem que pelo menos dois grupos principais de populações de *T. cruzi* circulem na natureza (Pena et al., 2009). O primeiro grupo está relacionado ao ciclo silvestre sendo aparentemente mais prevalente na América Central e América do Norte; e o segundo, relacionado com o ciclo doméstico. Ainda de acordo com a Organização Mundial de Saúde (OMS), os dados obtidos a partir de trabalhos de campo realizados em áreas endêmicas revelam que o número de vetores infectados está intimamente relacionado ao número de novos casos agudos, principalmente entre os indivíduos jovens, nos quais a morbidade e mortalidade são mais elevadas.

1.3.2. Transmissão por transfusão de sangue e transplante de órgãos

A partir da década de 60, ocorreu na América Latina um grande movimento migratório de indivíduos das áreas rurais para os grandes centros, o que fez mudar a tradicional epidemiologia da transmissão do *T. cruzi* (WHO, 2002). A infecção que era restrita às regiões rurais (causada pelo vetor), passou a ocorrer nas grandes cidades em decorrência da transmissão por transfusão de sangue e derivados e/ou

transplante de órgãos. Neste caso os receptores de órgãos de doadores chagásicos apresentam episódios agudos da doença, podendo o parasito ser identificado no sangue periférico. Assim, como medida de controle adotada por países do Cone Sul (incluindo Brasil, Chile, Argentina, Uruguai, Paraguai e Venezuela), a análise sorológica em bancos de sangue e de doadores tornou-se obrigatória (WHO, 2002). Recentemente, outros países não endêmicos (Canadá e Estados Unidos) adotaram tal medida a fim de prevenir novos casos de infecção por transmissão de sangue/órgãos (Urbina, 2002; Schmunis et al., 2007; Castro, 2009).

1.3.3. Transmissão Congênita

A doença de Chagas congênita ainda representa um sério desafio em vários países da América Latina, em especial na Bolívia, onde a transmissão vertical representa relevante problema epidemiológico (Torrico et al., 2004). Casos de doença de Chagas congênita foram também notificados na Argentina, Brasil, Chile, Colômbia, Guatemala, Honduras, Paraguai, Uruguai e Venezuela. O risco de transmissão congênita está relacionado com diferentes fatores epidemiológicos, tais como a cepa do parasito, fase da doença, existência de lesões na placenta e região geográfica. Dados sobre infecção congênita relatados pela OMS demonstram que no Brasil o risco é de ≤1% enquanto na Bolívia, Chile e Paraguai alcança cerca 7%. A transmissão congênita depende diretamente da prevalência da infecção em mulheres em idade fértil, que geralmente foram infectadas por transmissão vetorial (WHO, 2002).

1.3.4. Transmissão acidental

A transmissão acidental da DC foi relatada em laboratórios e hospitais de países endêmicos e não endêmicos. Dos dados notificados e publicados na literatura ocorridos entre 1938 a 2001, infecções foram documentadas em laboratoristas e médicos, entre outras pessoas que trabalham em serviços de saúde e que manipulam diferentes materiais biológicos infectados, tais como os dejetos de triatomíneos, culturas de parasitos, e sangue de humanos e animais infectados, entre outros (Herwaldt, 2001). Estes dados reforçam a importância de se adotar nestes ambientes laboratoriais e hospitalares, uma séria política de Biossegurança e gestão da qualidade a fim de minimizar a exposição a este agente de risco.

1.3.5. Transmissão oral

A transmissão oral da DC, via ingestão de alimento contaminado com triatomíneos infectados ou seus dejetos, tem sido documentada em vários países tais como no Brasil, Colômbia e México. Esta via de transmissão representa um dos atuais desafios no controle epidemiológico da DC, haja vista que um crescente número de casos tem sido recentemente reportado na região da Amazônia, em especial no Estado do Pará, onde vários surtos com óbitos foram relatados através da contaminação via suco de frutas e cana de açúcar (Pereira et al., 2009; Nóbrega et al., 2009). Estes dados reforçam a importância da manutenção de políticas de controle desta parasitose, incluindo o papel das agências de fiscalização sanitária e de saúde em monitorar as etapas desde a coleta até a comercialização de alimentos/produtos gerados em áreas geográficas endêmicas.

1.4. Controle

A transmissão de doenças tropicais negligenciadas, tal como a DC, se mantém em decorrência de diferentes fatores, incluindo graves contrastes sociais, problemas ambientais e econômicos, e a escassez de recursos orçamentários voltados para o seu controle e tratamento. As doenças infecciosas e as parasíticas representam cerca de 30% do total global de mortalidade no mundo. Segundo Hotez e colaboradores (2004), há a necessidade do controle, da eliminação e da erradicação dessas doenças tropicais. Hotez e colaboradores (2004) definem estes termos como: (a) o controle a redução de incidência, prevalência, morbidade e mortalidade da doença em níveis aceitáveis determinados pelo país ou área em questão; (b) a eliminação a redução a zero da incidência da doença em uma determinada área geográfica, sendo necessárias contínuas ações e políticas de saúde pública de modo a prevenir sua reemergência; (c) erradicação a redução da incidência da doença a zero, logo não havendo mais necessidade de intervenções e, finalmente, a (d) extinção a erradicação do patógeno e destruição de amostras laboratoriais.

Com relação ao controle da transmissão da DC, como acima discutido, vários países do cone Sul tiveram a iniciativa de criar um programa de controle da transmissão vetorial e transfusional, o que resultou em um importante declínio no número de novos casos agudos (Hotez et al., 2004). No Brasil, o programa de controle da doença que teve início em 1975 e término em 1995 custou ao governo aproximadamente 516 milhões de dólares, dos quais 78% foram gastos com controle vetorial e 4% com a melhoria das habitações (Hotez et al., 2004). Este programa resultou em um efeito positivo na prevenção de infecções e de mortes

(Hotez et al., 2004). No Brasil milhões de casas foram inspecionadas, e como consequência houve a redução nas capturas de Triatoma infestans que eram de mais de 80 mil no ano de 1979, para cerca 40 exemplares em 2005 (Moncayo e Ortiz-Yanine, 2006). Assim, no ano de 2006 o Ministério da Saúde do Brasil recebeu a Certificação Internacional de Eliminação da Transmissão da Doença de Chagas conferida pela Organização Pan-Americana da Saúde (OPAS) (Dias, 2006, 2007; OPAS, 2007; Moncayo e Silvera, 2009).

No entanto, mesmo que hoje a transmissão fosse completamente eliminada, o que não é possível devido as diversas características epidemiológicas desta parasitose e seus distintos ciclos biológicos, ainda haveria milhões de pessoas infectadas que dependem de cuidados médicos (Araújo et al., 2000). É também importante lembrar que embora a transmissão pelo *Triatoma infestans*, principal vetor nas áreas domiciliares e peridomiciliares de países da América do Sul, tenha sido controlada no Uruguai, Chile, Brasil e em muitas regiões endêmicas na Argentina, infelizmente outras espécies da subfamília *Triatominae,* tais como *Rhodnius prolixus, Triatoma dimidiata, Triatoma pseudomaculata, Pastrongylus megistus,* entre outras, são também potenciais vetores da doença (Urbina e Do campo, 2003). Assim, apesar da certificação de eliminação vetorial e controle transfusional concedida ao Brasil e à outros países do Cone Sul nas últimas décadas, a doença de Chagas, descrita há 100 anos atrás, continua sendo considerada um sério problema de saúde pública, principalmente em áreas endêmicas das Américas Central e do Sul, logo justificando a busca por uma terapia mais efetiva e de menor toxicidade (Moncayo, 2003; Moncayo e Silveira, 2009)

1.5. A doença: fase aguda e fase crônica

A DC apresenta uma fase aguda e uma crônica, sendo o coração alvo de infecção e inflamação em ambas as fases (Rossi e Bestetti, 1995; Rocha et al., 2007; Rassi et al., 2009). A fase aguda da doença de Chagas tem como principal característica a parasitemia patente, que é evidente após 7 a 15 dias da infecção. Esta fase apresenta baixa mortalidade (<10%), sendo mais observada em crianças; é frequentemente assintomática/oligosintomática (Urbina e Docampo, 2003; Remme et al., 2006) e representa o início da resposta inflamatória aguda, com ativação do sistema fagocítico mononuclear e produção de citocinas inflamatórias (Silva et al.,

1995; Aliberti et al., 1996; Revelli et al., 1998, Cunha-Neto et al., 2009). Em consequência da resposta imune efetiva do hospedeiro, observa-se a redução do parasitismo circulante e tissular, característica do término da fase aguda. Quando a inflamação e a carga parasitária aguda (em média de 2 a 5 meses após infecção) são controladas, o indivíduo infectado ingressa no período indeterminado da fase crônica, onde não há evidências clínico-patológicas, ou seja, sem parasitemia patente e sem sintomatologia evidente, tais como alterações eletrocardiográficas, de raios-X, entre outras, havendo apenas sorologia positiva (Rossi e Bestetti, 1995; Prata, 2001; Dutra et al., 2005). Caso o indivíduo não seja tratado, ele permanecerá infectado, e após anos ou décadas, cerca de 20-30% passam a apresentar sintomatologias crônicas importantes, frequentemente associadas a alterações cardíacas (principal manifestação) e/ou digestivas, estas últimas denominadas de mega-síndromes (Rossi e Bestetti, 1995; Brener e Gazzinelli, 1997; Elizari, 1999; Prata 2001).

Calcula-se que os pacientes que desenvolvem a sintomatologia crônica apresentam redução de cerca de 9 anos na expectativa de vida (Punukollu et al., 2007). Alguns fatores têm sido relacionados à evolução da fase crônica assintomática para a forma sintomática incluindo (i) a genética do hospedeiro vertebrado e (ii) a linhagem/tipo do parasito, haja vista que diferentes isolados do *T. cruzi* possuem diferentes perfis morfológicos, biológicos, patológicos (virulência, mortalidade, tropismo celular, lesões teciduais etc), clínicos (de formas indeterminadas a mega-síndromes), imunológicos, bioquímicos, moleculares e de resistência à drogas (Martinez-Diaz et al., 2001; Devera et al., 2003), além da (iii) exposição do hospedeiro vertebrado a re-infecções que vêm sendo avaliadas em diferentes modelos experimentais (Bustamante et al., 2003).

As análises mais detalhadas demonstraram a presença do parasito e de seus antígenos e DNA em infiltrados inflamatórios cardíacos durante a fase crônica (Higuchi et al., 1993, 1997; Palomino et al., 2000) sugerindo uma estimulação antigênica persistente por toda esta fase. Por outro lado, a discrepância entre a severidade das lesões e a baixa carga parasitária observada na fase crônica sugere que outros fatores além do parasito, possam estar relacionados ao desenvolvimento da patologia chagásica (Dos Santos et al., 2001; Higuchi et al., 2003; Coura, 2007). Neste sentido, vários estudos têm demonstrado que o fenômeno da auto-imunidade seria fator desencadeador da patologia da doença de Chagas. Deste modo, as seguintes hipóteses poderiam ser formuladas para justificar a patogênese da

cardiopatia chagásica crônica (CCC): (i) a persistência do parasito induzindo e mantendo as reatividades inflamatórias crônicas, (ii) a infecção induzindo uma resposta imune inicialmente direcionada somente contra o parasito, mas cuja desregulação torna o próprio tecido do indivíduo alvo de citotoxicidade, levando então ao dano tissular, independente da carga parasitária e/ou (iii) as duas alternativas agindo em paralelo (Urbina, 2001; Urbina e Docampo, 2003; Croft et al., 2005). Em todas as hipóteses, a resposta imune contribui fortemente para as lesões dos tecidos (Zhang e Tarleton, 1999; Tarleton, 2001; Croft et al., 2005; Dos Reis et al., 2005), mas o parasito é o principal fator desencadeador da doença.

Assim, a persistência do parasito (variável a depender de suas características genéticas, incluindo sua virulência) associada a uma resposta imune desbalanceada (dependente das características imunogenéticas do hospedeiro) são fatores críticos que podem determinar a evolução da patologia chagásica crônica (Gomes et al., 2003, 2005; Higuchi et al., 2003; Perez-Fuentes et al., 2003; dos Reis et al., 2005; Marino et al., 2005; Davila-Spinetti et al., 2005; Hyland e Engman, 2006). Estes dados reforçam a necessidade do desenvolvimento de novas estratégias profiláticas e terapêuticas, incluindo a identificação de novas drogas tripanocidas e imunomoduladoras que proporcionem o controle do parasitismo e contribuam para a modulação da inflamação crônica não benéfica. Vale ressaltar que os pacientes se beneficiam com a redução da carga parasitária sendo que, a maioria dos indivíduos infectados que apresenta uma resposta imune apropriada é capaz de controlar a proliferação do parasito e consequentemente a evolução da doença (Engman e Leon, 2002; Tarleton, 2003; Urbina e Docampo, 2003).

1.6. Terapia

A doença de Chagas é ainda considerada uma doença incurável por apresentar uma quimioterapia insatisfatória baseada em compostos nitrofurânicos (nifurtimox, Bayer/WHO) e nitroimidazólicos (benznidazol, LAFEPE) (Figura 4) (Amato Neto, 1999; Coura e De Castro, 2002; Urbina e Do Campo, 2003; Croft et al., 2005; WHO, 2009). Além destes fármacos, outros poucos compostos também têm sido usados, de modo restrito na clínica da DC, incluindo derivados azólicos como o cetoconazol, e o alopurinol, sendo este último utilizado para redução da reativação da parasitemia em pacientes imunossuprimidos, havendo, contudo, controvérsias quanto a sua aplicação por apresentar apenas efeito tripanostático transitório (Stoppani, 1999; de

Alemida et al., 2009). Com relação ao alopurinol, este não apresentou eficácia na fase crônica da doença de Chagas (Rassi et al., 2007).

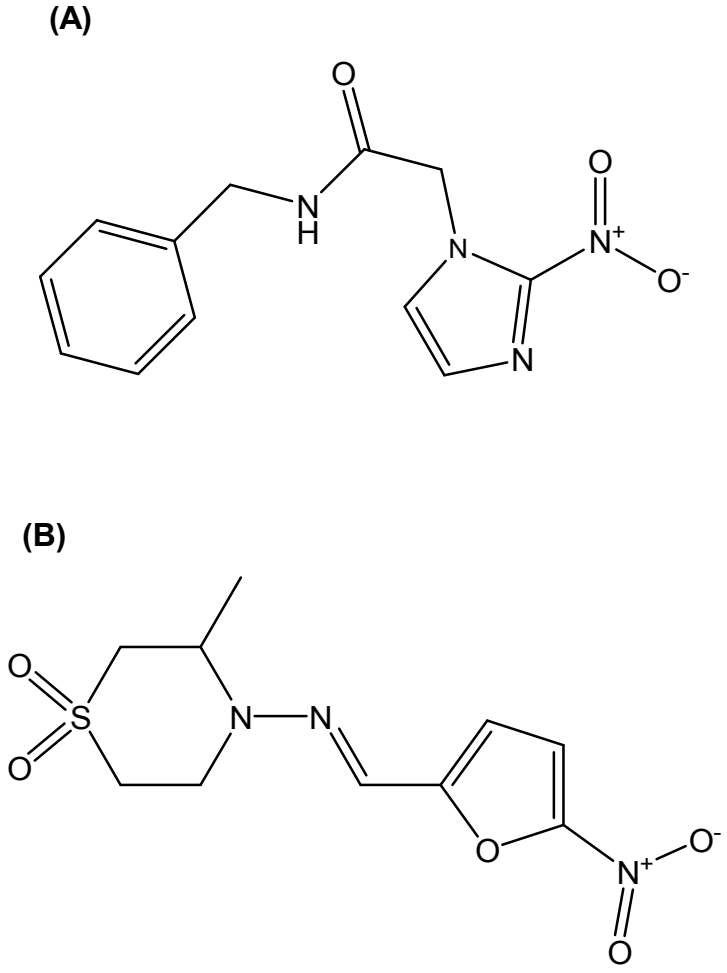

Figura 4: Estrutura química de (A) Benznidazol e (B) Nifortimox.

Nifurtimox (NF) e benznidazol (BZ) foram introduzidos na clínica nas décadas de 60-70 do século passado, sendo que o primeiro teve sua produção descontinuada na década de 80, tendo sido recentemente re-introduzido na clínica e distribuído pela Organização Mundial de Saúde (Urbina e Docampo, 2003; Barret et al., 2003; Steverding e Tyler, 2005; WHO 2009). Nenhum destes compostos é ideal porque (i) não apresentam eficácia na fase crônica da doença e apresentam sérios efeitos colaterais, (ii) requerem administração por longos períodos de tempo (30-60 dias) sob supervisão médica especializada, (iii) diferentes isolados do parasito apresentam grande variação na eficácia, (iv) populações de parasitos resistentes a ambos compostos têm sido relatadas, (v) não há formulações pediátricas, apesar do

fato de que crianças até 12 anos possuírem maiores chances de se beneficiarem com o tratamento por não apresentarem ainda a sintomatologia crônica da doença, e (vi) por apresentarem alto custo (Coura e de Castro, 2002; Stewart et al., 2005; Ribeiro et al., 2009). Violeta de genciana tem sido usada em bancos de sangue, mas sua principal desvantagem é a coloração azulada que confere ao sangue tratado e consequentemente, aos tecidos dos pacientes transfundidos (Soares et al., 2004; Maya et al., 2007).

1.6.1. Nifurtimox

Os primeiros ensaios clínicos com nifurtimox foram realizados em 1965 em pacientes chagásicos agudos oriundos de países endêmicos da América do Sul (Argentina com 657 pacientes, Brasil com 14 pacientes e El Salvador, com 16 pacientes). O tratamento resultou no desaparecimento dos sintomas mais rapidamente em relação ao grupo que recebeu placebo (Wegner e Rohwedder, 1972). Os efeitos colaterais mais frequentes relatados neste estudo e em outros ensaios clínicos incluem anorexia, dor de cabeça, vômitos, perda de peso, insônia, mialgia, manifestações cutâneas, cólicas intestinais, diarreia, entre outros (Coura e De Castro 2002; Castro et al., 2006).

Em geral, a administração da droga (oralmente, três vezes ao dia) segue os seguintes esquemas terapêuticos: (a) crianças de 0-10 anos: 15-20 mg/kg/dia por 60-90 dias; (b) jovens de 11-16 anos: 12,5-15 mg/kg/dia, por 90 dias; e (c) adultos: 8-10 mg/kg/dia, por 60-90 dias (Wegner e Rohwedder, 1972; Amato Neto, 1999).

1.6.2. Benznidazol

Beznidazol é administrado por 60 dias na dose de 5-10 mg/kg/dia podendo ser estendido por até cinco meses no caso de tratamento de pacientes imunocomprometidos. Em casos de indivíduos que foram infectados acidentalmente o tratamento pode ser abreviado, utilizando-se esquema profilático restrito a 10-15 dias (Amato Neto, 1999; Maya et al., 2007). Os principais efeitos colaterais relatados são: anorexia, dor de cabeça, vômitos, insônia, mialgia, manifestações cutâneas incluindo dermatites, edemas generalizados, febre, depressão da medula óssea, trombocitopenia, e polineuropatias periféricas (Castro et al., 2006; Maya et al., 2007, Viotti et al., 2009).

1.6.3. Mecanismo de ação de nifurtimox e benznidazol

Os mecanismos de ação destes compostos estão relacionados à geração de radicais livres e/ou metabólicos eletrofílicos: sabe-se que o grupo nitro de ambos compostos é reduzido a um grupo amino através da ação de nitroredutases do *T. cruzi*, levando à formação de vários radicais e de metabólitos eletrofílicos que se associam aos lipídeos, proteínas e DNA do parasito (Stoppani, 1999; Maya et al., 2007).

Dados *in vitro* sugerem que o principal mecanismo de ação do NF seja através de sua nitroredução, com geração de radical nitroaniônico que em presença de oxigênio leva a produção de íon superóxido ($O_2^{-\cdot}$) e peróxido de hidrogênio (H_2O_2), lesivos para o *T. cruzi* (Maya et al., 2007). O parasito não apresenta o mesmo arsenal de mecanismos de defesa contra o estresse oxidativo que se observa em células de mamífero, sendo deficiente, por exemplo, de catalase e glutationa peroxidase, apresentando baixos níveis da enzima superóxido dismutase (Maya et al., 2007). Assim, o parasito se torna mais suscetível que células de mamíferos aos produtos de redução do oxigênio gerados pelo NF, particularmente o peróxido de hidrogênio e o radical hidroxila.

Já o efeito tripanocida do BZ não parece estar relacionado à geração de radicais livres, sendo mais provável que os metabólitos eletrofílicos gerados a partir deste composto estejam envolvidos na morte do *T. cruzi* através de sua ligação covalente a macromoléculas dos parasitos e/ou associação ao DNA, lipídeos e proteínas (Stoppani, 1999; Maya et al., 2003, 2007; Croft et al., 2005; Murta et al., 2006). Alternativamente, foi também descrita uma ação inibitória direta do BZ sobre a atividade de topoisomerases do *T. cruzi* (Maya et al., 2003; Croft et al., 2005; Murta et al., 2006).

1.6.4. Tratamento clínico

NF e BZ têm sido principalmente utilizados no tratamento de pacientes chagásicos agudos e crônicos recentes (≤ 15 anos), nos quais se observam resultados positivos, principalmente em crianças, calculando-se um percentual médio de cura em torno de 80% (Coura e De Castro, 2002; Dias, 2006; Yun et al., 2009).

Dados revistos por Amato Neto (1999) revelam, com base na sorologia (ensaios de ELISA, fixação de complemento e imunofluorescência, etc) e no xenodiagnóstico, percentuais de cura de 70, 60 e 20% para, respectivamente, o tratamento de casos agudos, crônicos recentes e crônicos tardios.

Além de serem recomendados para quimioterapia dos casos agudos e de crônicos recentes, BZ e NF têm sido também fortemente sugeridos para tratamento de infecções congênitas, transplantes de órgãos de doadores infectados, quadros de re-agudizacão de paciente imunossuprimidos, e como medida de prevenção frente a acidentes (Amato-Neto, 1999; Urbina, 2001, 2002; Coura e De Castro, 2002). E apesar da maioria dos estudos revelar uma baixa eficiência destes fármacos durante a terapia de pacientes crônicos associada à ocorrência de múltiplos efeitos tóxicos que, em muitos casos leva à interrupção do tratamento (Urbina e Docampo, 2003, Viotti et al., 2009), avaliações recentes têm indicado que o tratamento etiológico pode retardar ou mesmo evitar a evolução da doença. Viotti e colaboradores (2006) mostraram que de 566 pacientes crônicos submetidos ou não ao tratamento por BZ (283 em cada grupo) somente um pequeno percentual de pacientes tratados evoluiu para a forma grave da CCC reforçando a ideia de que o tratamento pode regular a evolução para a forma severa da CCC (Dias, 2006). Contudo, há severas críticas relacionadas a generalização desta recomendação para o tratamento de pacientes crônicos, pontuando-se a necessidade de se avaliar caso a caso (Amato Neto, 1999).

As variações observadas quanto à eficiência de cura devida ao tratamento clínico estão, pelo menos em parte, relacionadas às diferenças na suscetibilidade dos diversos estoques de *T. cruzi* às drogas (Amato-Neto, 1999; Coura e De Castro, 2002; Ribeiro et al., 2009). De fato, estudos com diversos isolados do parasito mostram um espectro variável (0-100%) de suscetibilidade *in vitro* e *in vivo* a diferentes fármacos, incluindo o BZ e o NF (Martinez-Diaz et al., 2001; Toledo et al., 2003; Campos et al., 2005; Coronado et al., 2006; Campos et al., 2009). Como o *T. cruzi* apresenta uma população altamente heterogênea, caracterizada por diferenças morfológicas, biológicas, patológicas, clínicas, imunológicas, bioquímicas e moleculares (Devera et al., 2003; Pena et al., 2009), supõem-se que a variável eficácia da quimioterapia entre as diferentes regiões endêmicas possivelmente esteja relacionada à diferenças no perfil de susceptibilidade das diferentes populações do parasito (Urbina, 2001; Ribeiro et al., 2009). De fato, dados recentes referentes ao tratamento de crianças e jovens (até 18 anos de idade) revelaram uma

importante diferença quanto aos níveis de cura observados frente ao tratamento com BZ, alcançando índices de até 87-58% e 5-0% em regiões da América Central (Guatemala e Hounduras) e do Sul (Bolívia), respectivamente (Yun et al., 2009).

Assim, (i) a baixa e variável eficiência destes fármacos em especial durante tratamento de pacientes crônicos, (ii) a ocorrência de múltiplos efeitos tóxicos que em muitos casos leva à interrupção do tratamento, e o (iii) desenvolvimento de clones/isolados de parasitos resistentes a drogas (Coura e De Castro, 2002; Urbina e Docampo, 2003; Ribeiro et al., 2009), são argumentos que justificam e reforçam a busca por compostos mais efetivos e menos tóxicos para o tratamento da doença de Chagas, como por exemplo, de agentes quimioterápicos já utilizados na clínica contra outros patógenos, tais como as fluorquinolonas, tiossemicarbazonas e diamidinas aromáticas e seus análogos (Barret et al., 2003; Beraldo e Gambino 2004; Soeiro et al., 2009; Croft et al., 2005; Wong-Beringer et al., 2009).

1.7. Fluorquinolonas

As quinolonas e seu subconjunto fluoroquinolonas (FQ) são uma importante classe de antibióticos sintéticos, de amplo espectro bacteriano (bactérias gram-positivas e gram-negativas), sendo amplamente utilizada na clínica (Taver et al., 2009). As FQ, sintetizadas a partir da década de 80 do século passado, tem amplo uso na prática clínica humana e veterinária e apresentam como característica química a presença de um átomo de flúor ligado ao sistema do anel central, normalmente na posição 6 ou 7 (Figura 5). O efeito bactericida das quinolonas assim como das FQ é atribuído principalmente à inibição da DNA topoisomerases II (girase de DNA) e IV de procariontes, enzimas envolvidas na replicação do DNA, decatenização, recombinação e reparo (Anquetin et al., 2006; Castora et al., 1983, Alovero et al., 2000; Barnard e Mxwell, 2001).

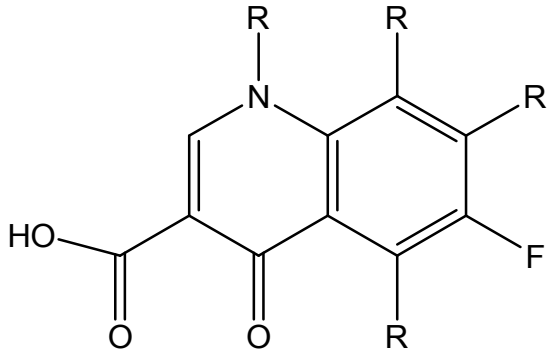

Figura 5: Estrutura química de fluorquinolonas.

R = anel de piperazina.

Os representantes desta classe mais utilizados e relativamente mais seguros tolerados são as norfloxacina (NOR), ofloxacina, ciprofloxacina, levofloxacina, moxifloxacina e gatifloxacina (Anquetin et al., 2006). No entanto, devido às crescentes evidências de cepas bacterianas resistentes a estes antibióticos somadas ao perfil adverso de vários destes agentes (Taver et al., 2009), vários grupos de química medicinal, como o liderado pela Dra. Letícia R Teixeira (Departamento de Química da Universidade Federal de Minas Gerais), têm trabalhado no desenvolvimento de novos derivados que apresentem maior segurança e induza menor toxicidade e que possam ser usados contra um amplo espectro de infecções (Md-Saleh et al., 2009).

Como dados sugerem que o principal mecanismo de ação dessa classe esteja relacionado à capacidade de intervir no DNA, sua atividade pode ser explorada sobre diferentes classes de patógenos, incluindo os tripanosomatídeos. A inibição de topoisomerase II (TOPO II) pelas FQ representa um mecanismo de ação promissor e útil para terapias anti-tumorais e anti-bacterianas (Nernortas et al., 1999; Chollet et al., 2009), abordagem semelhante pode ser aplicada para tripanosomas haja vista que a TOPO II também apresenta importante função nos compartimentos nucleares e mitocondriais dos parasitos.

A membrana externa das bactérias Gram (-) funciona como uma barreira protetora da célula, constituindo a principal barreira no acesso das fluoroquinolonas ao seu local de ação. Apesar da sua extensa utilização terapêutica, permanecem muitas questões no que diz respeito ao mecanismo molecular e cinético da sua entrada na célula bacteriana. Estudos conduzidos sobre várias cepas bacterianas têm demonstrado que a porina OmpF tem papel relevante na captação destes antibióticos na bactéria (Fernandes et al., 2007). Por outro lado, alguns destes antibióticos estão associados a internalização através da bicamada lipídica estando relacionada às características de hidrofobicidade do fármaco (Md-Saleh et al., 2009).

Estudos de síntese de FQ complexadas com diferentes metais de transição têm sido conduzidos visando aumentar a eficácia destes compostos e ampliar seu espectro de ação e os dados biológicos *in vitro* e *in vivo* tem revelado a promissora ação sobre diversos patógenos incluindo *Leishmania sp.* (Cortazar et al., 2007) e *T. cruzi* (Gonzales-Perdomo, 1990).

1.8. Tiossemicarbazona

As tiossemicarbazonas (TS) são análogos de semicarbazonas que contêm a substituição de um átomo de oxigênio por um enxofre. A estrutura química das tiossemicarbazonas e a respectiva numeração de seus átomos podem ser observadas na Figura 6. Estes compostos são de considerável interesse científico, pois apresentam importantes propriedades biológicas. Muitos exemplos dessa classe de pequenas moléculas têm sido avaliados ao longo dos últimos 50 anos, revelando atividade antitumoral (Finch et al., 1999; Feun et al., 2002; Wadler et al., 2004; Odenike et al., 2008; Sen e Chaudhuri, 2009), antibacteriana (Costello et al., 2008; Ramachandran et al., 2009), antifúngica (Ramachandran et al.,. 2009), antiviral (Condit et al., 1991; Teitz et al., 1994; Mishra et al., 2002; Finkielsztein et al., 2008) e anti-protozoário (Greenbaum et al., 2004; Abid et al., 2008; Perez-Rebolledo et al., 2008; Aquino et al., 2008). Além disso, outros estudos demonstraram que TS apresentam significativa atividade *in vitro* contra tripanosomatídeos, tais como *Leishmania sp.* (Dodd et al., 1989), *Trypanosoma brucei* (Greenbaum et al., 2004), e *T. cruzi* (Ceceretto et al., 1998; Otero et al., 2006; Vieites et al., 2008).

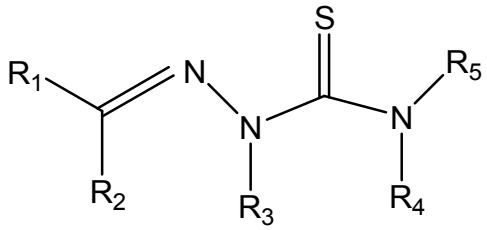

Figura 6: Estrutura química de tiossemicarbazonas

R_1, R_2, R_3, R_4 e R_5 = H, Alquil e aril

Em função de seu amplo espectro de ação, as TS têm sido consideradas como uma classe de compostos bastante promissora. Embora outros alvos também possam estar relacionados ao mecanismo de ação das TS, o mais relevante parece ser devido a sua alta afinidade pela enzima ribonucleotídeo redutase, que é responsável pelo passo crucial na síntese do DNA e, consequentemente, da divisão celular (Beraldo e Gambino, 2004; Greenbaum et al., 2004)

Alguns estudos têm demonstrado a suscetibilidade de diversos protozoários às tiossemicarbazonas associadas a complexos metálicos. Bharti e colaboradores (2002), utilizando como fármaco de referência o metronidazol, realizaram testes com vários derivados de TS e verificaram a boa atividade sobre *Entamoeba histolytica* e *Trichomonas vaginalis*. Neste estudo, o composto 5-nitro-tiofeno-2-carbaldeído-N(4)-hexametilimina tiossemicarbazona (5-NT-HMINTSC) apresentou valores de IC_{50} sobre *Entamoeba histolytica* inferiores aqueles observados com o metronidazol. Outro estudo de atividade antiamoebicida demonstrou que TS complexadas com Pd(II) apresentaram maior atividade do que as TS livres (Bharti et al., 2003).

Também foi relatada atividade de antimalárica de TS: Klayman e colaboradores (1984) observaram a cura de camundongos infectados com *Plasmodium berghei* após o tratamento com a 1-acetilisoquinolina TS.

1.9. Diamidinas aromáticas

Diamidinas aromáticas (DA), como a pentamidina (Figura 7), representam uma importante classe de ligantes de DNA que possuem um amplo espectro de ação (Soeiro et al., 2005). DA foram inicialmente testadas na tripanosomíase Africana (Apted 1980; Bouteille et al., 2003) e desde então sua ação tem sido investigada em diversas parasitoses (Soeiro et al., 2005), incluindo as patologias causadas por *Pneumocystis carinii* (Tidwell et al., 1990), *Saccharomyces cerevisiae* (Lanteri et al., 2004); vírus da diarréia viral bovina (Givens et al., 2003, 2004); *Plasmodiun falciparum, Leishmania amazonensis* (Bell et al., 1990; Macharia et al., 2004), *Toxoplasma gondii* (Lindsay et al., 1991), *Trypanosoma spp* (De Souza et al., 2004, 2006), *Trichomonas vaginalis* (Crowell et al., 2004) e *Giardia lamblia* (Bell et al., 1993).

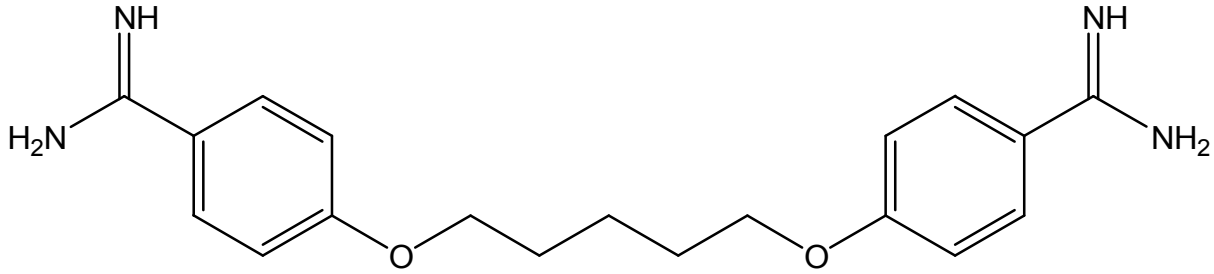

Figura 7: Estrutura química da pentamidina, a primeira diamidina sintetizada.

Os mecanismos de ação das DAs não estão totalmente elucidados. Um dos mecanismos propostos está relacionado à formação de um complexo (não covalente e não intercalante) das DAs à fenda menor do DNA do microrganismo em sequências ricas em bases AT (aquelas regiões de maior eletronegatividade das fendas menores), inibindo assim sua replicação e transcrição através da associação direta ao ácido nucléico e/ou interferindo com enzimas associadas ao DNA como, por exemplo, a TOPO II (Nguyen et al., 2004). No entanto, dados têm demonstrado que diamidinas, como a furamidina (DB75), podem também interagir, menos frequentemente e com menor intensidade, com sequências GC, via intercalação na molécula de DNA (Bailly et al., 2005). Outros mecanismos de ação que têm também sido propostos incluem: inibição de proteases, polimerases, proteína quinase A, inibição de síntese de fosfolipídios e distúrbios no metabolismo de poliaminas (Soeiro et al., 2005; Werbovetz, 2006).

A natureza dicatiônica da pentamidina permite sua acumulação direta na mitocôndria levando ao colapso parcial do potencial da membrana interna, resultando na permeabilização desta organela (Soeiro et al., 2005; Werbovetz, 2006, Tidwell e Boykin 2003; Nguyen et al., 2004; Soeiro et al. 2008). A análise ultra-estrutural da interação entre diversos análogos de pentamidina com parasitos da família Kinetoplastidae, revela a ação destes compostos preferencialmente no núcleo e cinetoplasto dos parasitos (DNA mitocondrial) (Croft e Brazil, 1982; Fusai et al., 1997; De Souza et al., 2004; Mathis et al., 2007; Batista et al., 2009; Daliry et al., 2009), o que sugeriria um mecanismo de ação universal. No entanto, a identificação de algumas DAs em organelas livres de DNA, como acidocalcisomos de *Trypanosoma brucei* pode refletir um novo mecanismo de ação ainda desconhecido destes compostos ou alternativamente significar um sítio de estoque destes compostos (Mathis et al., 2006).

Dados recentes da avaliação da atividade, acúmulo e distribuição de uma série de análogos DB75 e da diamidina DB820 revelaram que independentemente de sua atividade, houve o acúmulo de todos os compostos, em doses millimolares, após 8 horas de tratamento dos tripomastigotas de *Trypanosoma brucei* (Mathis et al., 2007). Porém, alguns compostos menos efetivos apresentaram superiores acúmulos em relação a outros análogos mais ativos *in vitro*. Os autores relataram que apenas os níveis de acúmulo de cada composto não respondem unicamente pela sua atividade tripanocida e que a localização em uma determinada organela (núcleo, mitocôndria ou acidocalcisomas) também não guarda relação com o grau

de sua atividade *in vitro* (Mathis et al., 2007). Outro estudo realizado com DAs em *T. cruzi in vitro* também demonstrou que embora as DAs se localizem preferencialmente no núcleo e kDNA dos parasitos, o maior acúmulo nesta última estrutura não está correlacionado com eficácia sobre o *T. cruzi,* pois alguns dos compostos menos ativos apresentaram superior acúmulo no kDNA em relação a outros compostos mais efetivos (Daliry et al., 2009).

Apesar de sua comprovada atividade microbicida, o tratamento com as diamidinas atualmente utilizadas na clínica, como a pentamidina, gera importantes efeitos colaterais, incluindo cardiotoxicidade, hepatoxicidade e diabetes (Coyle et al., 1996). Assim, uma intensa busca por compostos análogos e menos tóxicos tem sido realizada por alguns grupos de química medicinal, incluindo o do nosso colaborador, o Dr. David W. Boykin (departamento de Química, Laboratório de Química e Ciências Biológicas da Universidade do Estado da Geórgia, Atlanta/USA) onde foram sintetizados os compostos que analisamos nesta tese.

Um destes compostos é a furamidina também conhecida por DB75, que representa o membro da série de compostos de DAs melhor caracterizado em estudos *in vitro* e *in vivo*. Este derivado difenilfurano bis-amidina apresenta atividade antiparasitária contra diversos microorganismos incluindo fungos, bactérias e protozoários (Tidwell e Boykin 2003; De Souza et al., 2004; Soeiro et al., 2005).

Dados do nosso grupo demonstraram que um análogo de furamidina, a DB569 (que apresenta uma substituição da amidina terminal por um grupo fenila), que mantém inalterada a capacidade de ligação ao DNA (Lansiaux et al., 2002), apresenta uma atividade tripanocida potencializada em relação à furamidina *in vitro* (De Souza et al., 2004). Observamos também que frente ao tratamento com furamidina ou com seu análogo DB569, parte das formas tripomastigotas sanguíneas morrem exibindo características de morte celular programada do tipo I ou apoptose (De Souza et al., 2006b) o que é um dado novo com relação à atividade tripanocida de diamidinas em *T. cruzi* e interessante do ponto de vista da regulação da lesão cardíaca chagásica, pois esta via de morte (apoptose) não induz inflamação (De Souza e cols. 2006b). Nossos resultados nos levaram a eleger a DB569 como alvo para investigações em modelos experimentais (camundongos Suíços e cepa Y) cujos resultados revelaram que esta DA é capaz de reduzir parcialmente os níveis de mortalidade e importantemente o parasitismo intracelular cardíaco *in vivo* (este último em níveis semelhantes ao grupo de animais tratados com benznidazol), além de proteger de lesões cardíacas e de alterações

eletrocardiográficas evidenciadas na fase aguda final e crônica da infecção murina (De Souza et al., 2006a, 2007). Esta proteção refletida pelo aumento da sobrevida (40-60%) dos animais tratados com DB569 foi diretamente relacionada à menor expressão de células T $CD8^+$ no coração de animais infectados e tratados sugerindo um possível papel imunomodulador da DA além do seu potencial tripanocida (De Souza et al., 2007). Estes resultados confirmam dados anteriores nos quais observamos que a ação da DB569 contra formas amastigotas presentes em fagócitos profissionais é superior em relação à infecção de cardiomiócitos *in vitro* (De Souza et al., 2004), sugerindo uma potencialização da atividade microbicida dos fagócitos somada a ação tripanocida da diamidina.

Silva e colaboradores (2008) realizaram experimentos que revelaram a atividade *in vitro* e *in vivo* de uma diariltiofeno diamidina (DB1362) sobre formas tripomastigotas de sangue e amastigotas intracelulares de *T. cruzi in vitro* em concentrações que não apresentaram toxicidade para células de mamífero. Com relação ao mecanismo de ação, os dados demonstraram alterações ultra-estruturais no núcleo e na mitocôndria de formas tripomastigotas de sangue. Ensaios de citometria de fluxo utilizando a R123 confirmaram as alterações mitocondriais induzidas por esta DA nos parasitas tratados. Os dados de atividade da DB1362 em estudos de infecção experimental *in vivo* utilizando duas diferentes concentrações do composto e dois diferentes esquemas de tratamento, demonstraram que o melhor resultado foi obtido frente ao tratamento por dois dias (início e pico da parasitemia) utilizando-se a menor dose (25 mg/kg/dia), resultando em 100% de sobrevida. Este tratamento também reduziu o parasitismo cardíaco e inflamação, e protegeu contra alterações eletrocardiográficas (Silva et al., 2008).

1.9.1 Arilimidamidas

Outros exemplos de análogos de diamidinas aromáticas que têm sido estudados sobre o *T. cruzi* são as amidinas reversas, hoje denominadas de arilimidamidas (AIA) (Figura 8) (Pacheco et al., 2009; Silva et al., 2007a,b). AIAs resultam de alterações nos amidinas terminais, que são centros catiônicos das DAs: nas AIAs o grupamento imino (elipse) encontra-se diretamente associado a um nitrogênio do grupo anilino (quadrado), contrastando com a amidina original, na qual o grupo imino está diretamente ligado a um anel arila (quadrado) (Stephens et al., 2001, 2003).

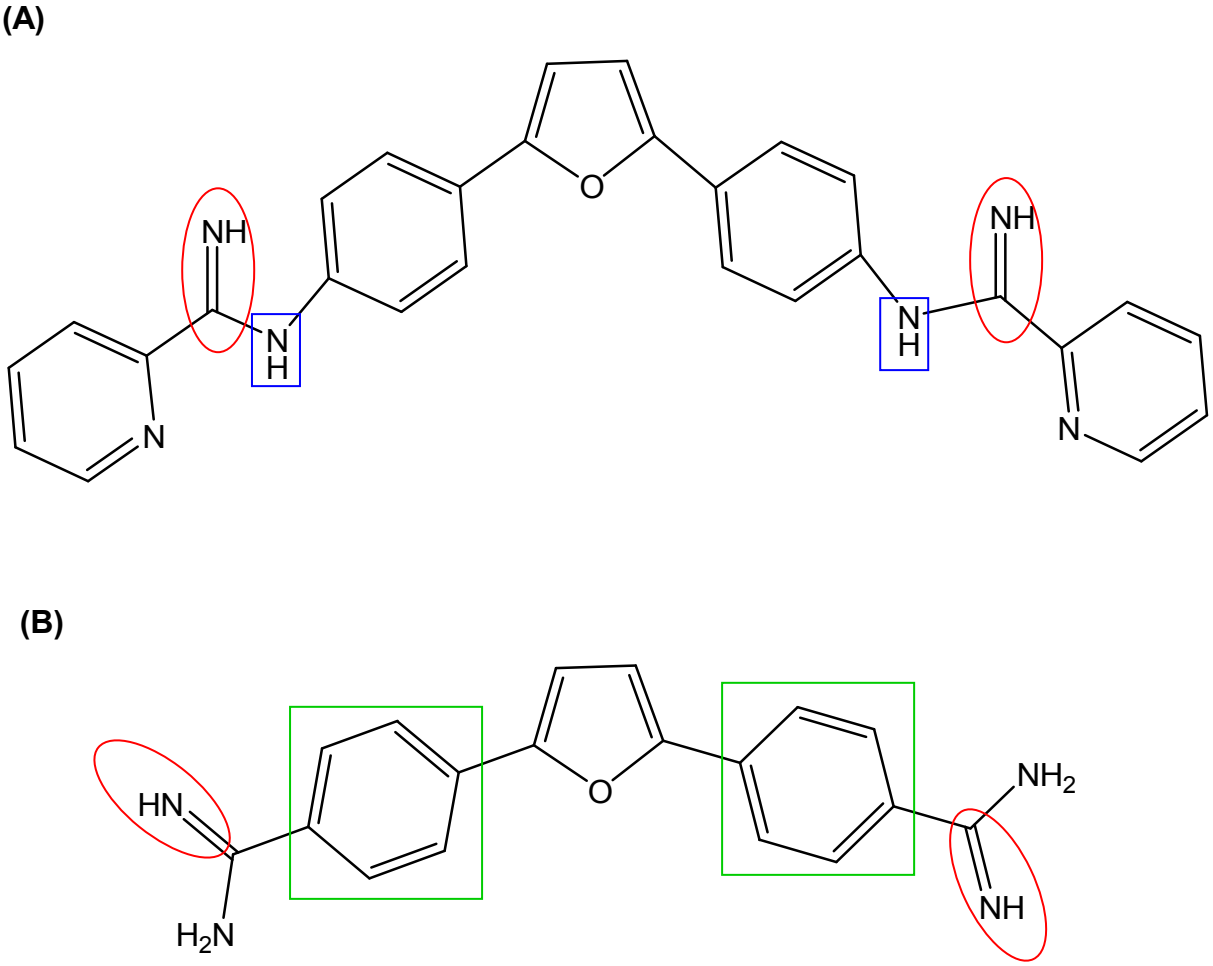

Figura 8: Estruturas químicas de (A) arilimidamidas (AIA) e (B) diamidinas.

Apesar dos poucos estudos feitos, dados da literatura têm revelado que AIAs exibem uma excelente atividade *in vitro*, apresentando superior ação contra formas amastigotas de *Leishmania donovani* e de *T. cruzi*, respectivamente, em relação as drogas de referência (Stephens et al., 2003). As AIAs apresentam também boa atividade contra *Mycobacterium tuberculosis*, *Candida albicans* e *Aspergillus fumigatus* (Setphens et al., 2001). Dados do nosso grupo revelaram que as AIAs exibem importante efeito antiparasitário, na faixa micro e nanomolar, sobre amastigotas e tripomastigotas de *T. cruzi* (estoques Y e Dm28c, representantes dos biodemas *T. cruzi* II e I, respectivamente), em doses que não afetam a viabilidade de células de mamíferos (Silva et al., 2007a). Análise ultraestrutural e por citometria

de fluxo revelou importantes alterações no núcleo e mitocôndria do *T. cruzi* (Silva et al., 2007b).

Objetivos

2. OBJETIVOS

A presente tese teve por objetivo geral analisar a atividade, seletividade e mecanismos de ação sobre o *T. cruzi* de 04 classes de compostos (fluorquinolonas - FQ, tiossemicarbazonas - TS, diamidinas aromáticas - DA e arilimidamidas – AIA – uma nova classe a partir de DA) *in vitro* e *in vivo*. A partir do nosso objetivo geral, destacam-se os seguintes objetivos específicos:

Objetivo específico 1: Determinar o limiar de toxicidade *in vitro* das fluorquinolonas, tiossemicarbazonas, diamidinas aromáticas e arilimidamida contra células de mamíferos (cultivo primário de cardiomíocitos). Para este objetivo analisamos a viabilidade celular: (i) pela análise da morfologia ao microscópio óptico e (ii) pelo teste colorimétrico do MTT para determinação de valores de LC_{50}.

Objetivo específico 2: Avaliar a atividade tripanocida *in vitro* de fluorquinolonas, tiossemicarbazonas, diamidinas aromáticas e arilimidamida sobre formas tripomastigotas sanguíneas, amastigotas intracelulares e epimastigotas de diferentes isolados do *T. cruzi*, comparando-as com a ação das drogas de referências (benznidazol e violeta de genciana). Para este objetivo realizamos a análise quantitativa ao microscópio óptico de parasitos e culturas infectadas incubadas ou não (controles) com os compostos para determinação dos valores de IC_{50}.

Objetivo específico 3: Identificar, por microscopia eletrônica de transmissão e de fluorescência, a localização sub-celular e alvos de ação dos compostos (diamidinas aromáticas e arilimidamida) sobre formas tripomastigotas sanguíneas e intracelulares do *T. cruzi* frente ao tratamento *in vitro*.

Objetivo específico 4: Com base na determinação dos valores de IC_{50} e LC_{50}, estabelecer os índices de seletividade dos compostos visando selecionar quais serão analisados *in vivo* (somente aqueles com IS ≥ 50).

Objetivo específico 5: Investigar *in vivo* a toxicidade aguda e atividade tripanocida da arilimidamida (DB766) sobre diferentes modelos experimentais de infecção aguda causada pelo *T. cruzi*. Para este objetivo utilizamos diferentes doses e esquemas de

tratamento, e analisamos parâmetros parasitológicos (parasitemia e mortalidade), histopatológicos (carga parasitária e inflamação), clínicos (curva ponderal e eletrocardiograma), bioquímicos (dosagem de CK e GPT) e de cura parasitológica (Hemocultivo e PCR).

Objetivo específico 6: Verificar o efeito *in vivo* do co-tratamento da diamidina aromática (DB289) e da arilimidamida (DB766) associadas ao Beznidazol durante a infecção experimental aguda de camundongos pelo *T. cruzi*. Para este objetivo analisamos parâmetros parasitológicos (parasitemia e mortalidade), histopatológicos (carga parasitária e inflamação), clínicos (curva ponderal e eletrocardiograma), bioquímicos (dosagem de CK e GPT) e de cura parasitológica (Hemocultivo e PCR).

RESULTADOS

Artigo#01: Submetido à publicação (Polyhedron)

Título: **"Co(II), Mn(II) and Cu(II) complexes of fluorquinolones: synthesis, spectroscopical studies and biological evaluation against *Trypanosoma cruzi*"**

Estado do conhecimento quando da concepção do trabalho:

- ✓ A doença de Chagas é uma doença tropical negligenciada, mas que apesar de sua importância epidemiológica, ainda não apresenta terapia ideal justificando a busca por novos agentes quimioterápicos.
- ✓ Fluoroquinolonas são representantes de uma importante classe de antibióticos sintéticos de amplo espectro bacteriano sendo amplamente utilizados na clínica, apresentando conhecida atividade (*in vivo* e *in vitro*) anti-tumoral e imunomoduladora atuando ainda sobre diferentes agentes parasitários que causam patologias humanas.

- ➢ **Questões propostas:**

1. Investigar a atividade antiparasitária, toxicidade e a seletividade *in vitro* sobre tripomastigotas de sangue e formas intracelulares de *Trypanosoma cruzi* de fluorquinolonas (esparfloxacina e norfloxacina) e de seus complexos metálicos (Co, Mn e Cu-fenantrolina).
2. Correlacionar atividade tripanocida com a composição química dos compostos.

Co(II), Mn(II) and Cu(II) complexes of fluorquinolones: synthesis, spectroscopical studies and biological evaluation against Trypanosoma cruzi

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Abstract

$[MnCl_2(NOR)(H_2O)_2]$ (**1**), $[MnCl_2(SPAR)(H_2O)_2]$ (**2**), $[CoCl_2(NOR)(H_2O)_2]$ (**3**) $[CoCl_2(SPAR)(H_2O)_2]$ (**4**), $[CuCl_2(phen)(NOR)]$ (**5**) and $[CuCl_2(phen)(SPAR)]$ (**6**) complexes with norfloxacin (NOR) and sparfloxacin (SPAR) were obtained from $MnCl_2.4H_2O$, $CoCl_2.4H_2O$ and $CuCl_2$(phen) (phen = 1,10-phenanthroline). In all cases the fluorquinolones norfloxacin and sparfloxacin coordinate in the neutral zwitterionic form. The electron paramagnetic resonance (EPR) spectra of the Cu(II) complexes (**5**) and (**6**) in aqueous and DMSO solutions indicate mixture of mononuclear and binuclear complex. Complexes (**1**-**6**), together with the corresponding ligands were evaluated for their *in vitro* trypanocidal effect, against both bloodstream trypomastigotes and intracellular forms of *Trypanosoma cruzi*, the etiological agent of Chagas disease. Our present findings showed that although SPAR and NOR were poorly effective upon *T. cruzi*, complexes (**3**) and (**4**) were active against intracellular forms of the parasite lodge within cardiac cells. Importantly, complexes (**5**) and (**6**) displayed a higher activity upon both bloodstream and intracellular forms of *T. cruzi* reaching low IC_{50} values ranging between 1.62 and 4.65 μM. The potency of fluoroquinolones, specially those coordinated to Cu(II)-phen justify further trypanocidal screening assays with this class of compounds *in vitro* as well as upon experimental models of *T. cruzi* infection.

Keywords: *Trypanosoma cruzi*; Chagas disease; chemotherapy, fluoroquinolones, metal complexes

1. Introduction

Quinolones, and their subset fluoroquinolones (FQ) are chemotherapic agents with a fluorine atom attached to the central ring system, which represent an important class of synthetic broad-spectrum antibiotics [1]. These drugs enter cells via porins and have been used in clinical over the past thirty years to treat intracellular pathogens including *Mycobacterium tuberculosis, Clostridium difficile, Legionella pneumophila, Mycoplasma pneumonia* among others [2,3]. Their microbicidal effect is largely attributed to inhibition of DNA topoisomerases II (DNA gyrase) and IV of prokaryotes, enzymes involved in DNA replication, decatenation, recombination and repair [4-8]. The most often used, relatively safe and well-tolerated anti-bacterial agents are the 6-fluoroquinolones, including norfloxacin, ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin, and gatifloxacin [4]. In fact, there are also many restrains mostly associated to adverse safety profile caused by older FQ including nervous, digestive, urinary, cutaneous, muscle-skeletal, cardiovascular, immune system damages also inducing teratogenic effects [1,9]. Then, due to growing evidences of resistant bacterial strains associated to the adverse safety profile of many of these agents [1,9], many groups have been working on the development of new safer derivates that would induce less side effects and could be used against a broader spectrum of infections since DNA topoisomerases represent promising targets for many classes of pathogenic microorganisms, including trypanosomatids. Actually, poisoning these enzymes is the molecular mechanism of action for clinically useful anti-tumor and anti-bacterial agents [10,11]. Similar approach can be applied for trypanosomes since they have abundant topoisomerase II activity in both the nuclear and mitochondrial compartments, and classic topoisomerase inhibitors promote the intracellular formation of protein-DNA complexes, causing cytotoxicity [12,13]. Also, cumulating evidences demonstrated

that FQ, which are derivatives of nalidixic acid, besides acting as large-spectrum antibiotics, also present anticancer [14] and anti-HIV activity [15], and some of them, especially ciprofloxacin, gatifloxacin, and NOR are able to modulate lipopolysaccharide-induced pro-inflammatory cytokine production [16]. The effect of FQ against protozoan parasites was also reported *in vitro* including chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* [4,17,18], *T. cruzi* [19], *Trypanosoma brucei* [10,11,20], *Leishmania spp* [21,22], and *Toxoplasma gondii* [4,23.

Trypanosoma cruzi is an intracellular obligatory parasite that causes Chagas disease, an important public health problem in many countries of Latin American [24]. This anthropozoonosis, discovered a century ago by the Brazilian physician Carlos Chagas, is widespread within the American continent from the southern United States to southern Argentina affecting approximately 12 million cases with 100 million people at risk [24,25]. The only available drugs for the treatment of Chagas' disease are two nitroheterocyclic compounds: nifurtimox and benznidazole. Both were developed empirically over three decades ago and yield variable results which have been attributed to parasite subpopulations and patient susceptibility, besides not being effective against the chronic phase [26]. In addition, although both also exhibit considerable undesirable side effects, very little attention has being dedicated, from the pharmaceutical industries, to the development of new compounds against Chagas disease as well as against other neglected tropical diseases [27,28].

Then, as the identification and screening of new drugs for Chagas disease is urgently needed, the synthesis and the *in vitro* activity of norfloxacin (NOR), sparfloxacin (SPAR) and their Mn(II), Co(II) and Cu(II)-phen (phen = 1,10-phenanthroline) (Fi. 1) complexes were presently evaluated upon *T. cruzi*.

2. Experimental

2.1. Materials and Methods

Partial elemental analyses were performed on a Perkin Elmer CHN 2400 analyzer. Infrared spectra were recorded on a Perkin Elmer FT-IR Spectrum GX spectrometer using KBr pellets; an YSI model 31 conductivity bridge was employed for molar conductivity measurements. Steady state fluorescence measurements were performed on a PTI QM1 fluorescence system and lifetimes were obtained from decay measurements on a Horiba - Jobin Ivon - IBH TCSPC fluorometer. The light source used for excitation of NOR was a 330 nm nanoLED N-16, 1.0 ns nominal pulse duration, 1 MHz repetition rate. Computer programs supplied by the manufacturer were employed to process the time resolved data. X-band electron paramagnetic resonance (EPR) spectra were obtained with a Bruker ESP300E spectrometer with modulation frequency of 100 kHz and modulation amplitude of 1 mT. Frozen aqueous and DMSO solutions of the complexes (1mM) were measured at liquid N_2 temperature (77 K) in Teflon® tubes of 3 mm internal diameter.

2.2 Synthesis

Norfloxacin and sparfloxacin were provided by Sigma–Aldrich. Complexes were obtained by dissolving the desired fluorquinolone (0.025 mmol) in methanol (20 mL) with gentle heating and stirring. After cooling the solution to room temperature $MnCl_2.4H_2O$, $CoCl_2.4H_2O$ or $CuCl_2$(phen) (0.025 mmol) were added. The reaction was stirred at room temperature for 24 h. The solids which precipitate were filtered and washed with diethyl ether and dried *in vacuo.* Stock solutions (50 mM) were prepared in deionized water or DMSO (see Table 1) and fresh dilutions were

prepared immediately before use, with the final concentration of the solvent never exceeding 0.6%, which did not exert any toxicity towards the parasite or mammalian host cells (data not shown).

$[MnCl_2(NOR)(H_2O)_2]$ (**1**). Orange solid. Yeld 64%. Anal.: found, C 39.7, H 4.6, N 8.6. Calc. for $C_{16}H_{22}N_3O_5Cl_2FMn$, C 39.9, H 4.6, N 8.7%. Molar conductivity (1 x 10^{-3} mol L^{-1}, DMF): 31.3 μS.cm^{-1}. IR (cm^{-1}): 3390 s ν(OH); 1566 s ν(C═O); 1630 s $\nu_{ass}(COO^-)$; 1393 m $\nu_s(COO^-)$.

$[MnCl_2(SPAR)(H_2O)_2]$ (**2**). Orange solid. Yeld: 71%. Anal.: found, C 41.0, H 4.6, N 9.8. Calc. for $C_{19}H_{26}N_4O_5Cl_2F_2Mn$, C 41.2, H 4.7, N 10.1%. Molar conductivity (1 x 10^{-3} mol L^{-1}, DMF): 24.3 μS.cm^{-1}. IR (cm^{-1}): 3415 s ν(OH); 1560 s ν(C═O); 1630 s $\nu_{ass}(COO^-)$; 1381 m $\nu_s(COO^-)$.

$[CoCl_2(NOR)(H_2O)_2]$ (**3**). Green solid. Yeld: 76%. Anal.: found, C 39.8, H 4.7, N 8.8. Calc. for $C_{16}H_{22}N_3O_5Cl_2FCo$, C 39.6, H 4.6, N 8.7%. Molar conductivity (1 x 10^{-3} mol L^{-1}, DMF): 39.5 μS.cm^{-1}. IR (cm^{-1}): 3396 s ν(OH); 1573 s ν(C═O); 1630 s $\nu_{ass}(COO^-)$; 1393 m $\nu_s(COO^-)$.

$[CoCl_2(SPAR)(H_2O)_2]$ (**4**). Blue solid. Yeld: 65%. Anal.: found, C 40.8, H 4.7, N 9.9. Calc. for $C_{19}H_{26}N_4O_5Cl_2F_2Co$, C 40.9, H 4.7, N 10.0%. Molar conductivity (1 x 10^{-3} mol L^{-1}, DMF): 41.4 μS.cm^{-1}. IR (cm^{-1}): 3419 s ν(OH); 1568 s ν(C═O); 1633 s $\nu_{ass}(COO^-)$; 1386 m $\nu_s(COO^-)$.

$[CuCl_2(phen)(NOR)].3H_2O$ (**5**). Blue solid. Yeld: 76%. Anal.: found, C 48.5, H 4.9, N 10.1. Calc. for $C_{28}H_{32}N_5O_6Cl_2FCu$, C 48.7, H 5.0, N 10.2%. Molar conductivity (1 x 10^{-3} mol L^{-1}, DMF): 34.4 μS.cm^{-1}. IR (cm^{-1}): 3390 s ν(OH); 1581 s ν(C═O); 1629 s $\nu_{ass}(COO^-)$; 1383 m $\nu_s(COO^-)$.

$[CuCl_2(phen)(SPAR)].3H_2O$ (**6**). Blue solid. Yeld: 72%. Anal.: found, C 49.0, H 4.9, N 10.9. Calc. for $C_{31}H_{36}N_6O_6Cl_2F_2Cu$, C 48.8, H 5.0, N 11.1%. Molar conductivity (1 x

10^{-3} mol L^{-1}, DMF): 40.8 µS.cm^{-1}. IR (cm^{-1}): 3389 s ν(OH); 1574 s ν(C═O); 1632 s $\nu_{ass}(COO^-)$; 1383 m $\nu_s(COO^-)$.

2.3. Parasites

Y strain of *T. cruzi* was used throughout the experiments. Bloodstream forms were harvested by heart puncture from *T. cruzi*-infected Swiss mice at the peak of parasitemia [29].

2.4. Mammalian cell cultures and toxicity assays

For both drug toxicity and infection assays, primary cultures of embryonic cardiomyocytes (CM) were obtained following the previously described method [30]. After purification, the CM were seeded at a density of 0.1 x 10^6 cells/well into 24-well culture plates, or 0.05 x 10^6 cell/well into 96-well microplates, containing gelatin-coated cover slips and sustained 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). All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (License 0099/01). All the cultures were maintained at 37°C in an atmosphere of 5% CO_2, and the assays were run at least three times in duplicate. In order to rule out toxic effects of the compounds on host cells, uninfected CMs were incubated for 24 and 72 h at 37ºC in presence or absence of the compounds (up to 200 µM) diluted in DMEM, then their morphology evaluated by light microscopy and the cell viability measured by the MTT colorimetric assay [31]. The absorbance was measured at 490 nm wavelength with a spectrophotometer (VERSAmax tunable, Molecular Devices, USA) allowing the determination of LC_{50} values (drug

concentration that reduces 50% of cellular viability) and the respective selective indexes (SI = LC_{50}/IC_{50}).

2.5. Trypanocidal analysis

For the analysis *in vitro* on trypomastigotes, the parasites were incubated at 37°C in the presence of increasing doses (0-200 μM) of each compound diluted in Dulbecco's modified medium supplemented with 5% fetal bovine serum and 1 mM *L*-glutamine (DMES) [32]. After 24 h, death rates were determined by light microscopy through the direct quantification of live parasites using a Neubauer chamber, and IC_{50} values (drug concentration that reduces 50% of the number of the parasites) were then calculated. For the analysis of the effect on intracellular parasites, after the 24 h of host cell-parasite interaction of bloodstream trypomastigotes with CM, (using a cell: parasite ratio of 1:10) the cultures were washed to remove free parasites and treated with the compounds (0.2 mL of the drugs in non toxic doses previously determined by MTT assays on uninfected CM). Infected and untreated cultures were used as controls. The culture medium, with or without the drugs, was replaced daily. After 72 h of treatment, supernatants of untreated and treated cultures were recovered and the number of released parasites was determined by light microscopy through the direct quantification using a Neubauer chamber as previously reported [33], and IC_{50} values calculated.

3. Results and Discussion

3.1. Microanalyses and molar conductivity studies

Microanalyses suggest the formation of $[MnCl_2(NOR)(H_2O)_2]$ (**1**), $[MnCl_2(SPAR)(H_2O)_2]$ (**2**), $[CoCl_2(NOR)(H_2O)_2]$ (**3**) $[CoCl_2(SPAR)(H_2O)_2]$ (**4**), $[CuCl_2(phen)(NOR)].3H_2O$ (**5**) and $[CuCl_2(phen)(SPAR)].\ 3H_2O$ (**6**), and the molar

conductivity data indicate that all complexes are non electrolytes in accordance with the proposed formulations. The literature reports the synthesis and characterization of Mn(II), Co(II) and Cu(II)-phen complexes of norfloxacin and sparfloxacin. However, Mn(II) and Co(II) complexes related in the literature are of the type $[M(L)_2]$ (M = Mn(II) or Co(II) and L = NOR or SPAR) [34] while Cu(II)-phen complexes are $[Cu(HNOR)(phen)(NO_3)]NO_3.3H_2O$ and [Cu(SPAR)(phen)Cl] [35,36], and then the complexes related in the present study are quite different.

3.2. Infrared spectral studies

In the IR spectra of norfloxacin and sparfloxacin the valence vibration of the carboxylic stretch $\nu(C=O)_{carb}$ was found at 1732 and 1716 cm^{-1} respectively and the pyridone stretch $\nu(C=O)_p$ at 1638 and 1640 cm^{-1} respectively [34,37]. The characterization of quinolones metal complexes can be achieved by studying the most typical vibrations that are characteristic of the coordination type of quinolones. In the IR spectra of the complexes (**1**-**6**) the absorption of the $\nu(C=O)_{carb}$ vibration has disappeared. Two very strong characteristic bands are present in the 1629-1633 cm^{-1} and 1381-1393 cm^{-1} range assigned to ν(O-C-O) asymmetric and symmetric stretching vibrations, respectively, whereas $\nu(C=O)_p$ is shifted from 1638 and 1640 to 1560-1581 cm^{-1} range upon coordination.

The difference $\Delta = \nu(O\text{-}C\text{-}O)_{asym} - \nu(O\text{-}C\text{-}O)_{sym}$ is a useful characteristic for determining the coordination mode of the quinolone ligands. The Δ values for complexes (**1**-**6**) fall in the 237-249 cm^{-1} range indicating a monodentate coordination mode of the carboxylato group [38] of the norfloxacin and sparfloxacin ligands. Moreover, the infrared spectra of complexes (**1**-**6**) present broad absorptions around 3390 cm^{-1} and a group of weak and medium intensity bands around 2850 and 2492 cm^{-1} assigned to the vibration of the quaternized nitrogen of the piperazinyl group

which indicates that the zwitterionic form of norfloxacin and sparfloxacin are involved in the coordination to the metal ions. The overall changes of the IR spectra suggest that the norfloxacin and sparfloxacin ligands are coordinated to Mn(II), Co(II) and Cu(II)-phen via the pyridone and one carboxylate oxygen in the neutral zwitterionic form (see Fig. 1).

3.3. EPR spectral properties of the Cu(II) complexes **5** and **6**

Room temperature X-band EPR spectra of powdered samples of $CuCl_2$(phen), and complexes **5** and **6** were obtained (Fig. 2). The $CuCl_2$(phen) spectrum is characteristic of axial symmetry, with $g_{\parallel}$ = 2.257 and $g_{\perp}$ = 2.069, and lacks the hyperfine splitting as frequently observed in concentrated solid samples of Cu(II) complexes where exchange interactions are present [39]. The EPR spectra of **5** and **6**, however, show the hyperfine splitting features at the parallel region indicating absence of exchange. Complex **5** presents a multi-component broad spectrum indicating multiple microenvironments. Complex **6** presents a two component spectrum suggesting two different Cu(II) environments.

The EPR spectra of $CuCl_2$(phen) and complexes **5** and **6** in aqueous and DMSO solutions were recorded at 77 K (Fig. 3, A and B). The spectra of Cu (phen) shows that the hyperfine splitting is absent in aqueous solution, suggesting aggregation leading to exchange, but it appears clearly in DMSO, indicating a single mononuclear copper environment with $g_{\parallel}$ = 2.292, $A_{\parallel}$ = 155 gauss, and $g_{\perp}$ = 2.074.

Complex **5** presents signals characteristic of a mixture of binuclear and mononuclear copper complexes [40]. More specifically, the spectrum of the binuclear coupled system consists of two lines indicated by arrows in Fig. 3 corresponding to the $\Delta m_s = \pm 1$ transitions [41]. The much weaker signals from the forbidden $\Delta m_s = \pm 2$ transitions that should appear at ~1600 G were not observed because of the poor

signal to noise ratio. The central derivative line at g~2.08 is attributed to the $g_{\perp}$ component from Cu(II) monomers. The $g_{\parallel}$ component of such species falls in the g ≈ 2.2 region and is expected to be split in four hyperfine lines. Because of the superimposed $\Delta m_s = \pm 1$ signals from the binuclear system this feature is not evident in the spectra of **5**. It is also observed (Fig. 3) that the ratio of mononuclear to binuclear complex is greater in DMSO than in water. Complex **6** in DMSO presents an EPR spectrum similar to that of **5**, but in water the axially symmetric spectrum lacking the hyperfine lines indicates a larger degree of aggregation leading to dipolar and exchange interaction.

The distance between the two Cu(II) ions can be estimated from the zero field splitting parameter *D.* The average distance *r* between the two coupled unpaired electrons can be calculated by using the equation $D = 3/2\ g\beta/r^3 = 1.39 \times 10^4\ (g/r^3)$, where *D* is in gauss and *r* is in Angstroms [42]. The estimated distance *r* for the binuclear complex of **5** is 3.9 Å in water and 4.1 Å in DMSO; the distance for **6** is 4.0 Å in DMSO. These distances are similar to those obtained for Cu(II) dinuclear complexes as previously reported [43,44].

3.4. Association of Cu(phen) and norfloxacina determined by fluorescence quenching

The synthesized metal complexes can dissociate in aqueous solutions so that it is important to evaluate the metal-ligand binding constants. In general, modification of spectroscopic properties of ligands due to interaction with metals can be used to obtain binding parameters. Several fluoroquinolones are fluorescent including norfloxacina (while others such as sparfloxacin are not). Since paramagnetic ions including Cu(II) are well known fluorescence quenchers, it is possible to use fluorescence quenching to obtain information on the interaction between NOR and

the complex Cu(phen). In this section we study the association of NOR with Cu(phen) in aqueous solution at the physiological pH 7.4 using steady-state and time-resolved fluorescence.

Figure 4 shows the fluorescence spectrum of NOR and the effect of titration with Cu (phen). Increasing Cu (phen) concentrations decrease the fluorescence intensity without modifying the peak position and spectral shape. This behavior is characteristic fluorescence quenching, but the Stern-Volmer plot presented in the inset of Fig. 4 is clearly non-linear.

The measurement of fluorescence lifetimes is the most definitive method to distinguish static and dynamic quenching [45]. Dynamic quenching provides an additional decay route leading to a decrease in the fluorescence lifetime, while static quenching removes a fraction of fluorophores from observation, by forming a non-fluorescent complex, and leaves the lifetime unperturbed. Lifetime measurements were performed and the fluorescence decay curves (Fig. 5) were fitted with a single-exponential expression. The lifetime τ_0 in the absence of the quencher was equal to 1.2 ns and did not change with increasing concentration of the quencher. This indicates a static quenching process, due to formation of a non fluorescent complex.

It is worth noting that the Stern-Volmer plot (inset of Fig. 4) should be linear for static quenching, with the Stern-Volmer constant equal to the binding constant ([45,46]. In fact, it would be linear if ($F_0/F \tilde{}$ 1) was plotted against the free concentration of Cu (phen). The free concentration is usually taken as the total added concentration in binding experiments or static quenching experiments, but this is valid only if $K_b\, c_L << 1$, which seems not to be the present situation. In this case, the binding constant K_b can be obtained from the variations of the fluorescence (F_0-F) using the exact expression for the one-site binding model [47]:

$$F_0 - F = \frac{F_0 - F_\infty}{2}\left\{\left(1 + \frac{c_M}{c_L} + \frac{1}{K_b c_L}\right) - \left[\left(1 + \frac{c_M}{c_L} + \frac{1}{K_b c_L}\right)^2 - 4\frac{c_M}{c_L}\right]^{1/2}\right\} \quad (1)$$

where c_L and c_M are the total ligand and metal concentrations, respectively; F_0 , F and F_∞ are the fluorescence intensity of the ligand in the absence of metal, at a given metal concentration, and at $c_M >> c_L$, respectively. Figure 6 shows the plot of $(F_0 - F) \times c_M$, and the nonlinear least squares fit using Eq. 1, which gave $K_b = 1.3 \times 10^6$ mol^{-1} L. It can be noted that the exact expression fits very well the experimental points, showing the adequacy of the model.

3.5. Biological assays

Sparfloxacin and norfloxacin exerted a low trypanocidal effect against bloodstream trypomastigotes exhibiting IC_{50} values for 24 h of 114.1 ± 20.4 and 126.8 ± 30.2 μM, respectively (Fig. 7A, Table 2). The Mn(II) and Co(II) complexes were not able to improve FQ activity displaying similar or higher IC_{50} values to SPAR and NOR, with values between 114.7 and >200 μM (Fig. 7A, Table 2). However, when Cu(II)-phen complexes were assayed we found a striking increase in efficacy against the parasites, with IC_{50} values of 4.65 ±0.12 and 4.36±1.44 μM for [$CuCl_2$(phen)(NOR)] (**5**) and [$CuCl_2$(phen)(SPAR)] (**6**), respectively (Fig. 7B, Table 2). Importantly, the trypanocidal activity of both Cu(II)-phen complexes was about 2.5-fold higher as compared to Benznidazole (Table 2).

The relative toxicity of the free bases and their metal complexes was evaluated in uninfected CM. After 24 h of treatment, except for the Cu(II)-phen complexes (**5** and **6**) that presented toxicity in doses >12.5 μM, the quinolones and their Mn(II) and Co(II) complexes did not induce loss of cellular viability even at the concentration of 200 μM. Additionally, aiming to further evaluated the activity of FQ against intracellular parasites, we next assayed drug toxicity after 72 h hours of

treatment. Our data showed that after longer incubation at 37ºC, complexes (**5**) and (**6**) were again the most toxic to mammalian cells, resulting in LC_{50} values of 5.8 μM and 4.3 μM, respectively, while the other were not toxic even when incubated with 200 μM (data not shown). Then, the assessment of anti-parasitic activity against intracellular parasites was performed only with selected non-toxic doses of each compound.

Next, *T. cruzi*-infected CM were treated with the compounds and the number of viable parasites released into the supernatant quantified after 96 h of infection (corresponding to 72 h of drug exposure). Similarly as noticed with bloodstream trypomastigotes, SPAR and NOR did not show considerable activity against intracellular parasites, with IC_{50} values of 129.6 ±42.95 and 172.5±17.36, respectively (Fig. 7C, Table 2). However, trypanocidal effect was noted when the infected cultures were exposed to $[MnCl_2(SPAR)(H_2O)_2]$ (**2**) and $[CoCl_2(SPAR)(H_2O)_2]$ (**4**) complexes, leading to a considerable reduction in the number of released parasites, showing IC_{50} values of 32.9 ± 22.49 and 24.61 ± 6.98 μM, respectively (Table 2, Fig. 7C). On the other hand, although $[MnCl_2(NOR)(H_2O)_2]$ (**1**) did not improve the anti-parasitic effect of the free norfloxacin, the $[CoCl_2(NOR)(H_2O)_2]$ (**3**) complex displayed a higher activity (4-fold higher than norfloxacin ligand) against intracellular parasite, with IC_{50} of 41.89 ±2.36 □M (Table 2, Fig. 7C). As also found during the incubation of bloodstream trypomastigotes, both Cu(II)-phen complexes showed the highest activity upon intracellular parasites, showing IC_{50} values of 1.62 ±0.16 and 2.24 ±1 □M, being respectively 80 and 77% more active when compared to the free drugs (Table 2, Fig. 7D).

Our data showed that as compared to NOR and SPAR, the Co(II), Mn(II) and Cu(II)-phen complexes exhibited superior biological activity against the two forms of

T. cruzi, which are relevant for mammalian infection: bloodstream trypomastigotes and intracellular amastigotes. This higher activity was specially noticed with the Cu(II)-phen complexes that exhibited striking activity at low micromolar doses. Their higher activity (about 80-fold) as compared to free drugs (NOR and SPAR) may be explained by increased compound ability to penetrate into the parasites and/or different cellular targets (e.g. mitochondrial – kDNA or nuclear DNA), leading to different drug susceptibilities or by the presence of the metal ion. Complexes (**5**) and (**6**) also exerted moderated toxicity against mammalian cells, although toxicity was as much as 2.9 to 2.6-fold greater for trypanosomes than for cardiac cells (Table 2). In fact, although modest, this selective index is encouraging and may be improved in next generation of FQ compounds derived from both complexes since chemical modifications may considerably increase the potency and spectrum of microbicidal activity of FQ [4,10].

Our present findings corroborate previous results that demonstrated FQ activity against trypanosomatids. Recent studies showed that second generation of fluoroquinolones (enoxacin and ciprofloxacin) was active against intracellular amastigotes of *Leishmania (Viannia) panamensis* [21]. In another study, the activity of FQ upon promastigotes of *L. panamensis* was evaluated and the authors suggested that the leishmanicidal effect could be mediated partly through nuclear TOPII inhibition [22].

Fluoroquinolones were also tested for *in vitro* activity against bloodstream-forms of *T. brucei* [11]. The data showed that the tetracyclic analogs were the most potent agents, targeting type II topoisomerase, promoting the formation of protein-DNA covalent complexes, and showing a correlation between complex formation and anti-parasitic activity [11]. Later, FK with pyrrolidinyl substitutions were also assayed

against *T. brucei* and mammalian cells and the findings demonstrated that the bulky substituents at C-7 or a 1-2-bridging thiazolidine ring increased anti-trypanosomal activity and selectivity [20]. They found that these compounds trapped protein-DNA complexes and inhibited nucleic acid biosynthesis, which is characteristics of TOPO II inhibition in these trypanosomes [20]. Interestingly, the activity and index of selectivity of NOR and SPAR found in these bloodstream parasites [11,20] were quite similar to those presently found against *T. cruzi* (Table 2).

Recent data reported the addition of melamine-based P2-targeting motifs to FQ molecules aiming to improve activity through increased selective uptake of these compounds via the P2 transporter of *T. brucei* [10]. Although NOR and SPAR induced only a moderate activity, some analogues, such as melamine-norfloxacin conjugates, were eight times more effective than NOR [10]. Interestingly, fluorescence microscopy showed that treated trypanosomes with melamine-fluoroquinolone derivative but not with unconjugated NOR, became multi-kinetoplastic, suggesting that the effect was not related to inhibition of DNA replication but to some other cellular event that uncoupled DNA replication and kinetoplast-kinesis [10]. The authors suggest that DNA replication proceeds in treated cells, which supports the idea that FQ may be not inhibiting topoisomerases in trypanosomes, at least in the case of the melamine-coupled derivatives [10].

Our study also support previous *in vitro* data that reported trypanocidal effect induced by ofloxacin as well as other bacterial TOPOII inhibitors (nalidixic acid and novobiocin) upon *T.cruzi* [19]. Transmission electron microscopy of treated parasites showed that the main alterations were related to the kinetoplast of both bloodstream and intracellular amastigotes, suggestive that this structure may represent an important target in these trypanosomes [19]. On the other hand, *in vivo* studies

performed with ofloxacin combined or not to benznidazole showed that this FQ neither was able to reduce parasitemiae nor induce parasitological cure in *T. cruzi*-infected mice [48].

Up to now, although different compounds have been assayed against *T. cruzi* both *in vitro* and *in vivo*, since the introduction of nifurtimox and benznidazole, only few (allopurinol, itraconazole and fluconazole) have entered clinical trials [27]. Therefore, despite clearly not of immediate clinical use, our results as well as from others provide evidences that FQ may represent a suitable class of compounds for the future development of much-needed new anti-trypanosomal drugs. The findings also indicate that further structure-activity investigation of new fluoroquinolones, is warranted with the goal of identifying a compound that could replace the only two unsatisfactory drugs that are available for Chagas disease, an illness that mostly affect about 12 million people in poor areas of Latin America.

Acknowledgments

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Figure legends

Figure 1: Synthetic route for complexes (**1**)-(**6**).

Figure 2: Solid state powder EPR spectra (X-band) of $CuCl_2$(phen) and complexes **5** and **6** at room temperature.

Figure 3: EPR spectra of (A) aqueous and (B) DMSO solutions of $CuCl_2$(phen) and complexes (**5**) and (**6**) at 77 K. Concentration 1 mmol L^{-1}.

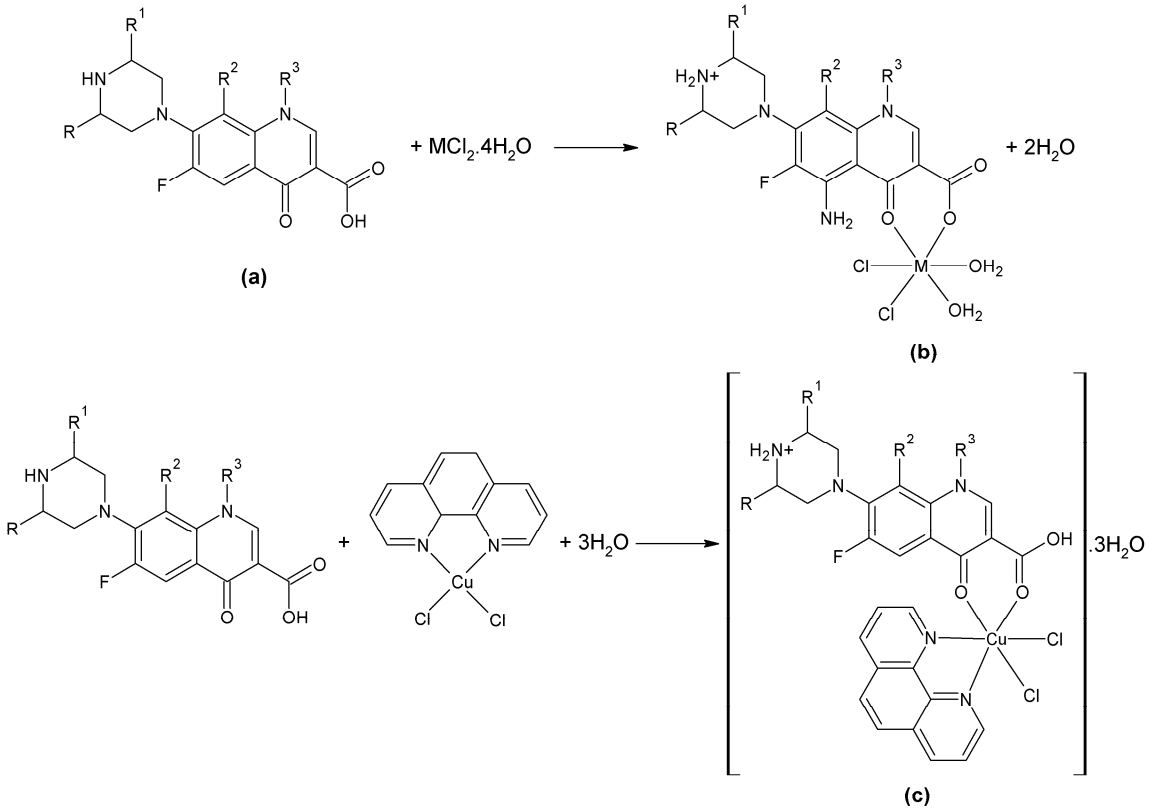
Figure 4: Fluorescence spectrum of NOR in aqueous solution (phosphate buffer pH 7.4) and the effect of titration with $CuCl_2$(phen). Concentration of NOR: 10 μmol L^{-1}; Concentrations of $CuCl_2$(phen) = n × 3.3 μmol L^{-1} , with n= 0, 1, …7. Excitation at 330 nm. The inset is the Stern-Volmer quenching plot.

Figure 5: Fluorescence decay curves of NOR in aqueous solution (phosphate buffer pH 7.4) pure and with added $CuCl_2$(phen). Concentration of NOR: 10 μmol L^{-1}; concentrations of Cu(phen): 0, 10 and 23 μmol L^{-1}. Excitation at 330 nm. Figure 6: Fluorescence of NOR at 407 nm as a function of the total added $CuCl_2$(phen). Total concentration of NOR, c_{NOR} = 10 μmol L^{-1}.

Figure 7: Activity of Sparfloxacin, Norfloxacin, [Mn(SPAR)Cl_2], [Co(SPAR)Cl_2], [Mn(NOR)Cl_2], [Co(NOR)Cl_2] (A and C) and of [Cu(phen)(SPAR)Cl]Cl and [Cu(phen)(NOR)Cl]Cl derivated (B and D) upon bloodstream (A-B) and intracellular (C-D) forms of *T. cruzi in vitro*. The effect upon parasites was evaluated during the treatment at 37°C with the drugs diluted in culture medium. The percentage of dead

bloodstream parasites (A and B) was measured after 24 h of treatment. The effect upon intracellular forms was evaluated by the quantification of parasite release in supernatant of untreated and drug-treated infected cultures (B and D). Data are expressed as mean ± SD of three independent experiments.

Figure 1:



		R,R[1]	R[2]	R[3]	M
(a)	norfloxacin (NOR)	H	H	CH_2CH_3	
	sparfloxacin (SPAR)	CH_3	F		
(b)	$[MnCl_2(NOR)(H_2O)_2]$ (**1**)	H	H	CH_2CH_3	Mn(II)
	$[MnCl_2(SPAR)(H_2O)_2]$ (**2**)	CH_3	F		Mn(II)
	$[CoCl_2(NOR)(H_2O)_2]$ (**3**)	H	H	CH_2CH_3	Co(II)
	$[CoCl_2(SPAR)(H_2O)_2]$ (**4**)	CH_3	F		Co(II)
(c)	$[CuCl_2(phen)(NOR)].3H_2O$ (**5**)	H	H	CH_2CH_3	
	$[CuCl_2(phen)(SPAR)].3H_2O$ (**6**)	CH_3	F		

Figure 2:

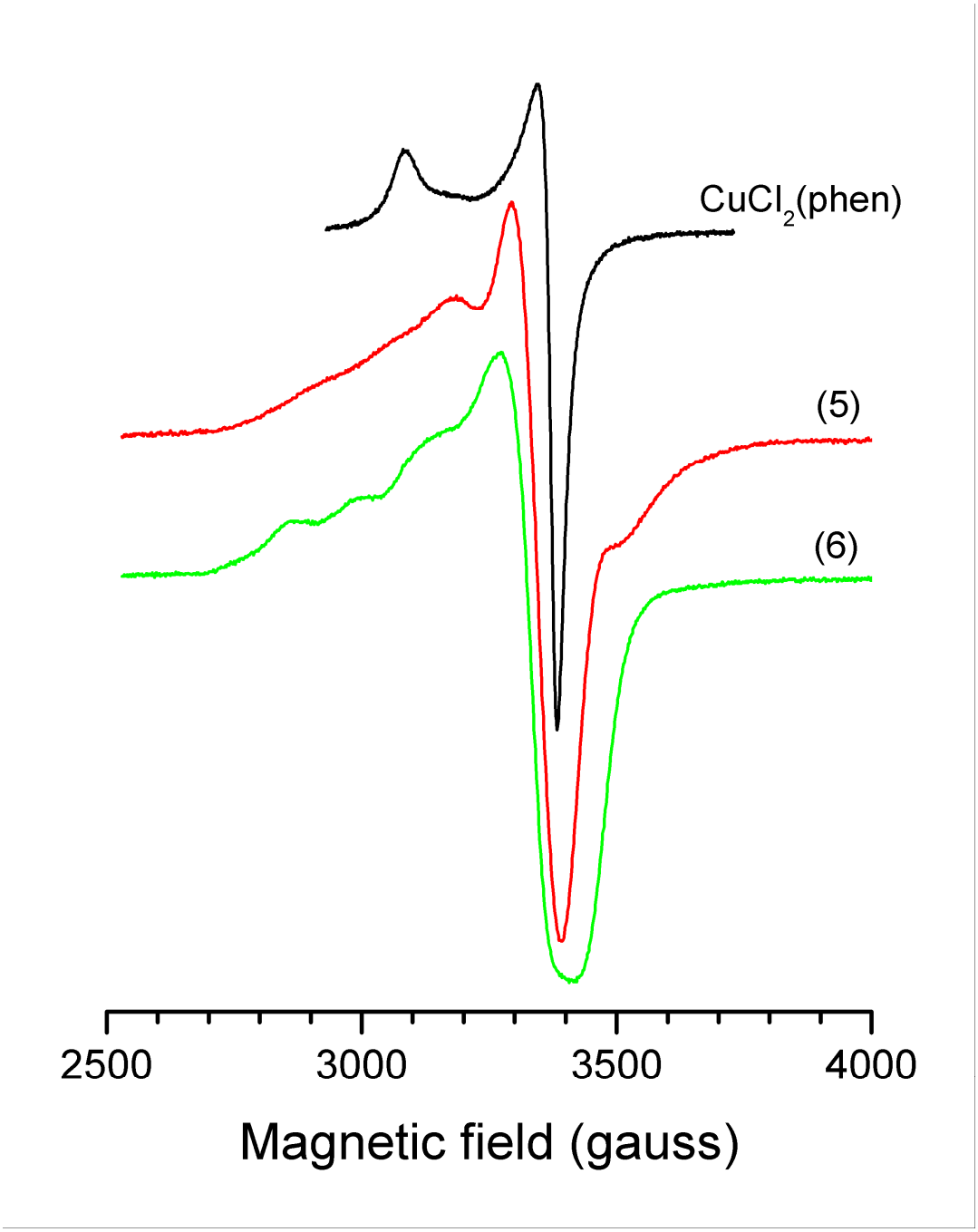


Figure 3:

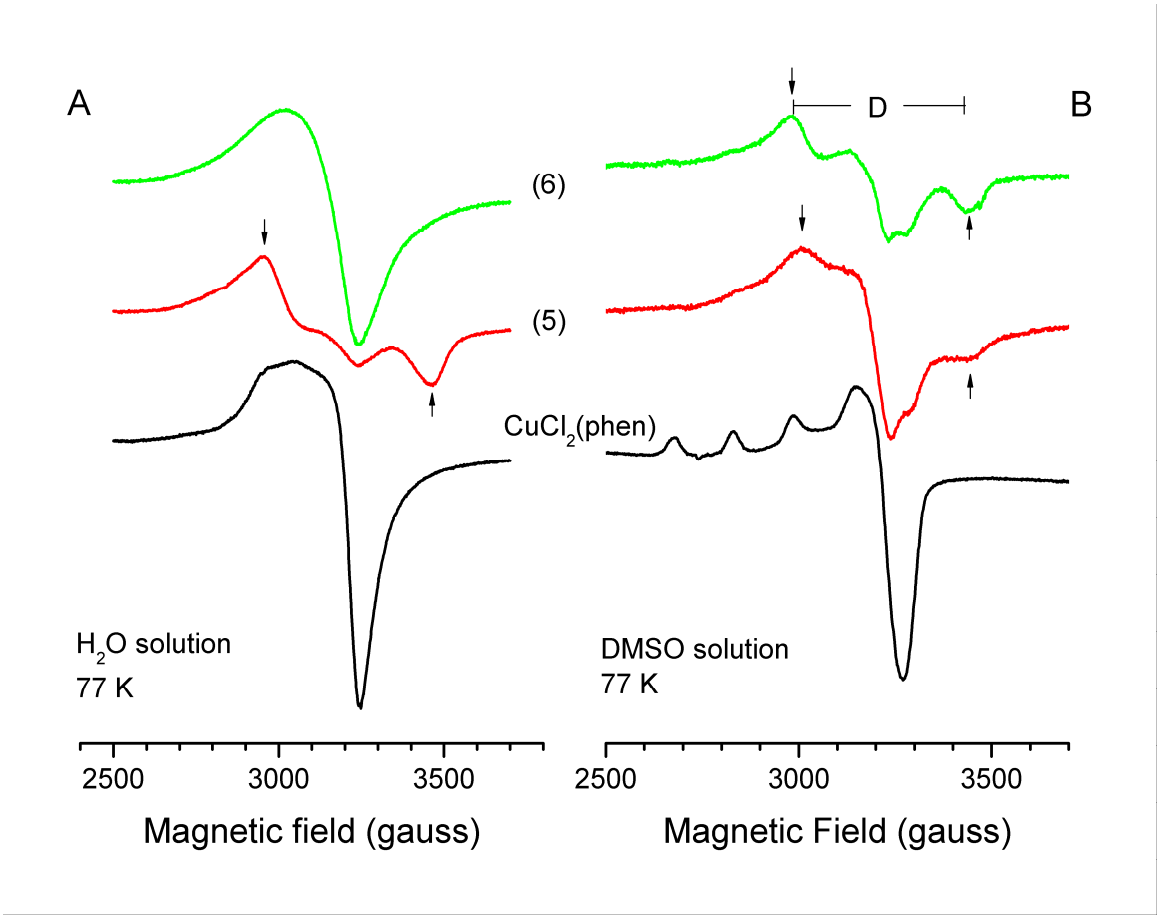

A
B
D
(6)
(5)
CuCl2(phen)
H2O solution
77 K
DMSO solution
77 K
2500
3000
3500
Magnetic field (gauss)
2500
3000
3500
Magnetic Field (gauss)

Figure 4:

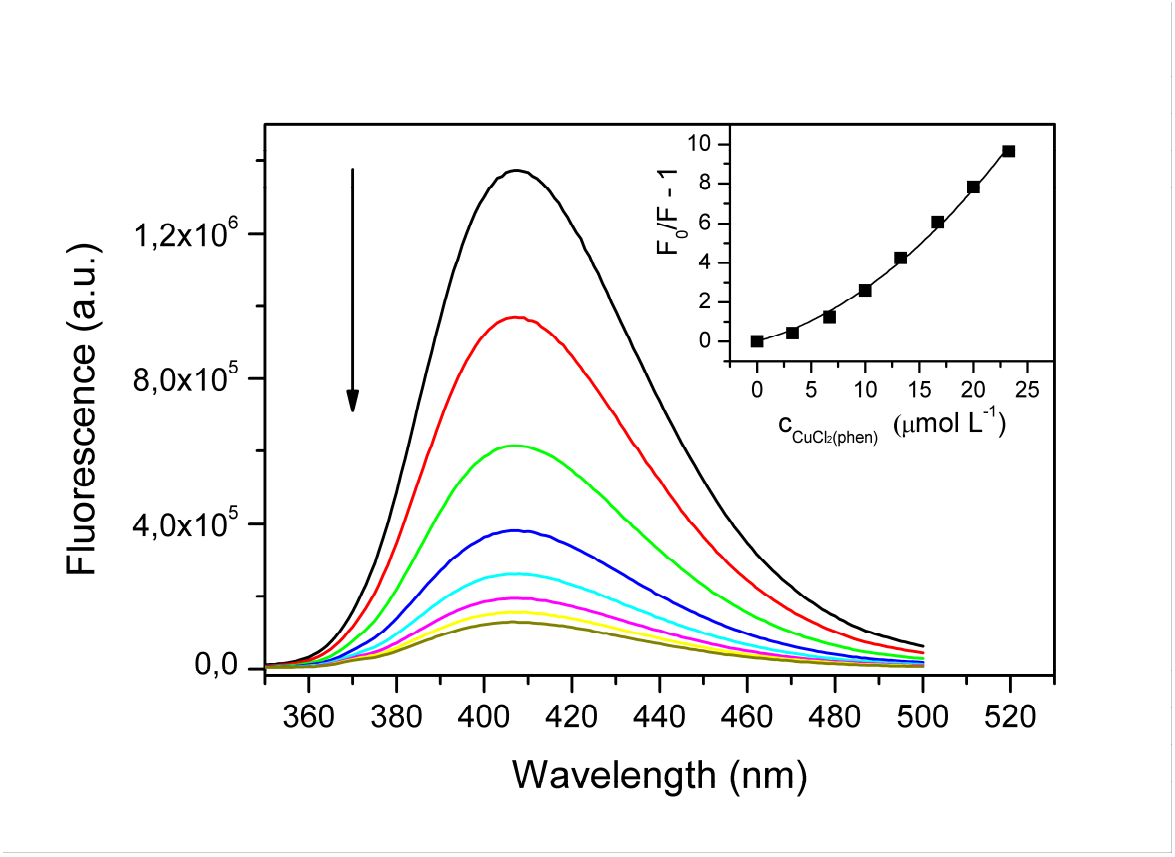

Fluorescence (a.u.)
1,2x10^6
8,0x10^5
4,0x10^5
0,0
360
380
400
420
440
460
480
500
520
Wavelength (nm)
F0/F - 1
0
2
4
6
8
10
0
5
10
15
20
25
cCuCl2(phen) (μmol L-1)

Figure 5:

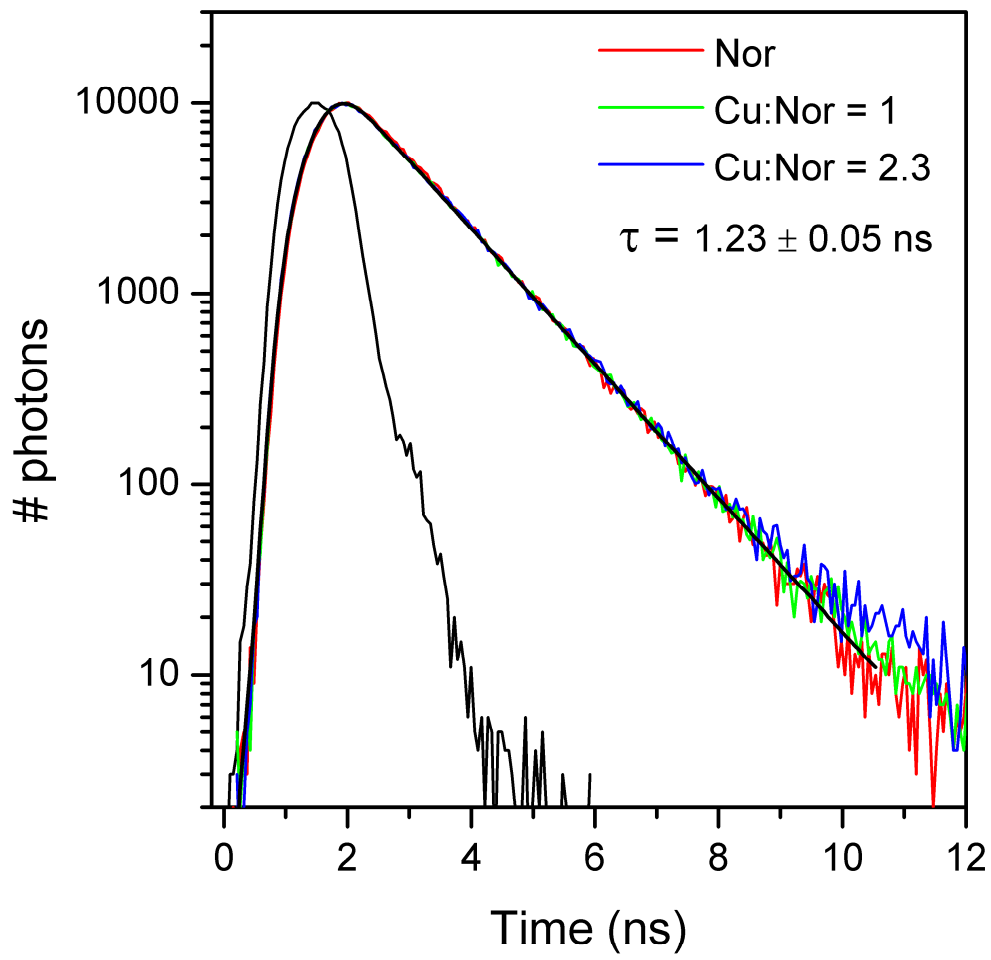

Nor
Cu:Nor = 1
Cu:Nor = 2.3
τ = 1.23 ± 0.05 ns
10000
1000
100
10
photons
0
2
4
6
8
10
12
Time (ns)

Figure 6:

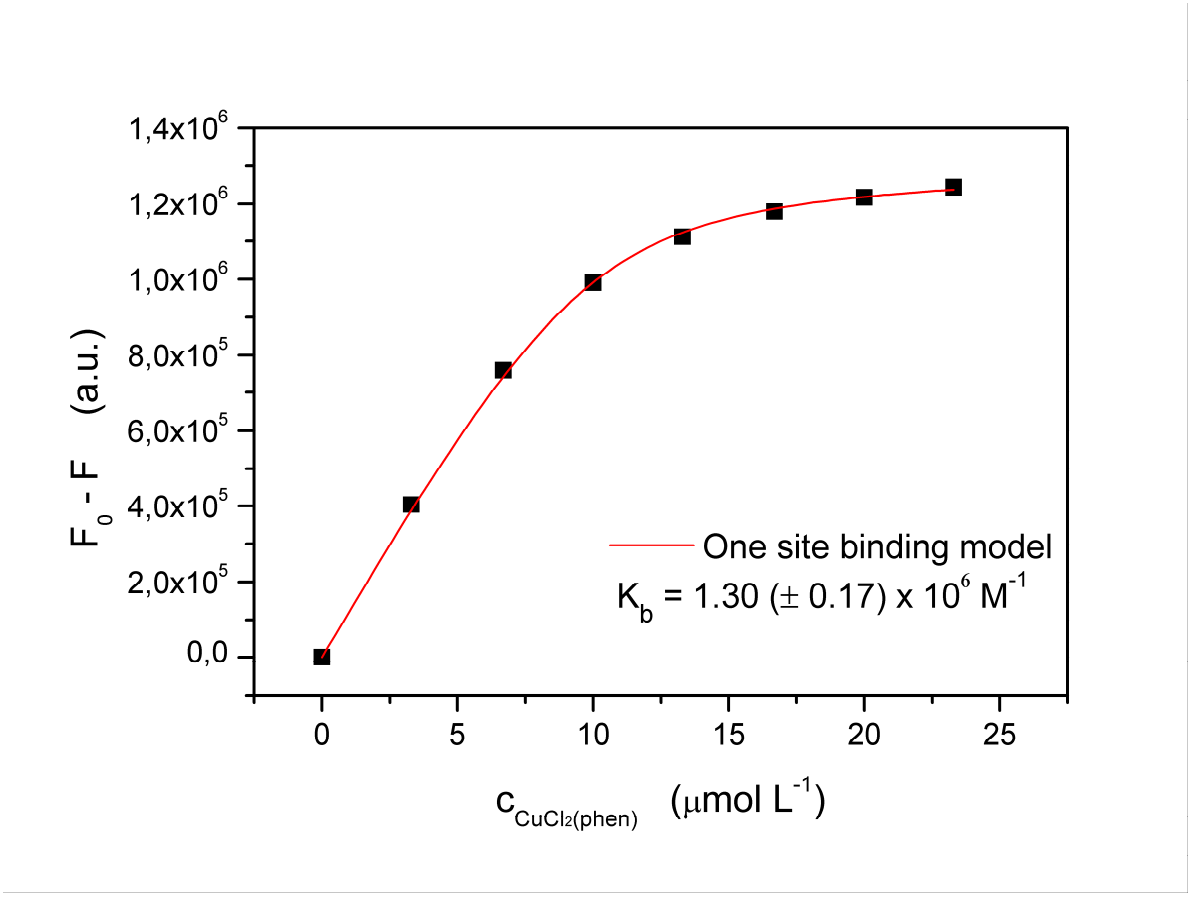

1,4x10^6
1,2x10^6
1,0x10^6
8,0x10^5
6,0x10^5
4,0x10^5
2,0x10^5
0,0
F0 - F (a.u.)
0
5
10
15
20
25
cCuCl2(phen) (μmol L-1)
One site binding model
Kb = 1.30 (± 0.17) x 10^6 M-1

Figure 7:

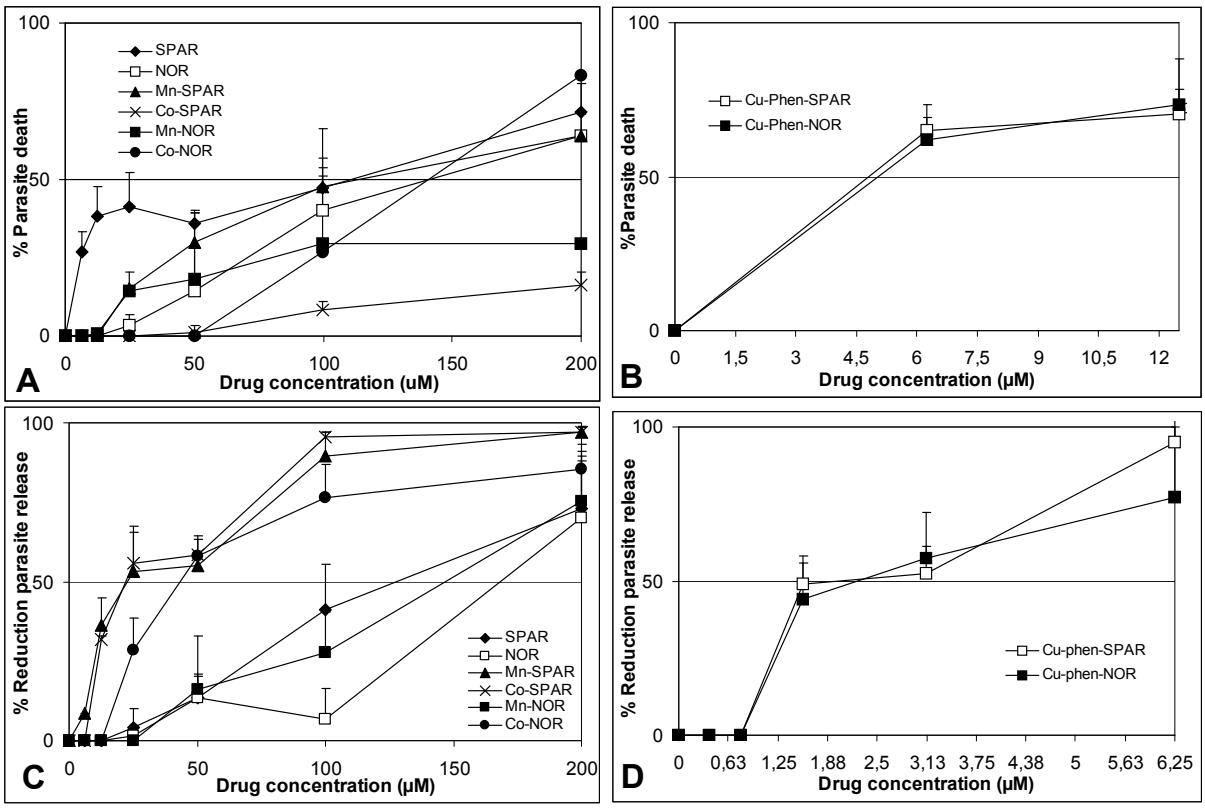

A
% Parasite death
Drug concentration (uM)
SPAR
NOR
Mn-SPAR
Co-SPAR
Mn-NOR
Co-NOR
B
%Parasite death
Drug concentration (µM)
Cu-Phen-SPAR
Cu-Phen-NOR
C
% Reduction parasite release
Drug concentration (µM)
D
% Reduction parasite release
Drug concentration (µM)
Cu-phen-SPAR
Cu-phen-NOR

Table 1: Chemical characteristics of the compounds

Ligands/complex	Abreviation	MM(g mol^{-1})	Solubility
Fluoroquinolones			
Sparfloxacin	SPAR	394.41	DMSO[1]
Norfloxacin	NOR	319.33	DMSO
$[Mn(SPAR)Cl_2]$	Mn-SPAR	520.26	Water
$[Co(SPAR)Cl_2]$	CO-SPAR	524.25	Water
$[Mn(NOR)Cl_2]$	Mn-NOR	445.17	Water
$[Co(NOR)Cl_2]$	Co-NOR	449.17	Water
[Cu(phen)(SPAR)Cl]Cl	Cu-phen-SPAR	671.61	Water
[Cu(phen)(NOR)Cl]Cl	Cu-phen-NOR	598.51	Water

DMSO[1] = Dimethylsulfoxide

Table 2: The activity and selectivity index (SI) of the compounds and of Benznidazole (Bz) upon bloodstream trypomastigotes and intracellular forms of *T. cruzi in vitro*

Compounds	**BT**		**Intracellular forms**	
	IC_{50} (µM)	SI	IC_{50} (µM)	SI
SPAR	114.12±20.36	>1.75	129.6±42.95	>1.55
NOR	126.84±30.19	>1.57	172.5±17.36	>1.16
Mn-SPAR	114.67±33.04	>1.75	32.95±22.49	*>6.06*
Co-SPAR	>200	>1	24.61±6.98	*>8.12*
Mn-NOR	>200	>1	144.31±0	*>1.38*
Co-NOR	139.1±0.79	>1.43	41.89±2.36	*>4.77*
Cu-phen-SPAR	4.36±1.44	2.86	2.24±1.01	*2.57*
Cu-phen-NOR	4.65±0.12	2.68	1.62±0.16	*2.66*
Bz	10.92	*nd*	*nd*	*nd*

*SI: Selective Index: Ratio between LC_{50}/IC_{50} values. For bloodstream and intracellular forms the corresponding LC_{50} values were related to 24 and 72 h of drug incubation, respectively.

**nd: not done

Research Highlights

- Microanalyses suggest the formation compounds
- The molar conductivity data indicate that all complexes are non electrolytes.
- The potency of fluorquinolones justifies trypanocidal screening.

Artigo#02: Aceito à publicação na revista Polyhedron 2010.

Título: **"Manganese(II) complexes with N^4-methyl-4-nitrobenzaldehyde, N^4-methyl-4-nitroacetofenone, and N^4-methyl-4-nitrobenzophenone thiosemicarbazone: Investigation of *in vitro* activity agaisnt *Trypanosoma cruzi*"**

Estado do conhecimento quando da concepção do trabalho:

- ✓ Tiossemicarbazonas são representantes de uma classe que apresenta um amplo espectro de ação biológica incluindo propriedades antiinflamatórias, anti-tumorais, e microbicidas sobre vírus, bactérias e protozoários, incluindo o *T. cruzi*.

- ➢ **Questões propostas:**

1. Avaliar a atividade antiparasitária, toxicidade e a seletividade *in vitro* sobre tripomastigotas de sangue e formas intracelulares do *Trypanosoma cruzi* de 3 tiossemicarbazonas ($H4NO_2Fo4M$, $H4NO_2Ac4M$ e $H4NO_2Bz4M$) e de seus complexos metálicos (Manganês)
2. Correlacionar atividade tripanocida com a composição química dos compostos.

Seguem 7 páginas

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Manganese(II) complexes with N^4-methyl-4-nitrobenzaldehyde, N^4-methyl-4-nitroacetofenone, and N^4-methyl-4-nitrobenzophenone thiosemicarbazone: Investigation of *in vitro* activity against *Trypanosoma cruzi*

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ABSTRACT

Thiosemicarbazones are known to be active against different pathogenic microorganisms including *Trypanosoma cruzi*, the etiological agent of Chagas disease. In the search for new therapeutic drugs against this illness, the complexes $[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**), $[Mn(H4NO_2Ac4M)_2Cl_2]$ (**2**) and $[Mn(H4NO_2Bz4M)_2Cl_2]$ (**3**) of N^4-methyl-4-nitrobenzaldehyde thiosemicarbazone ($H4NO_2Fo4M$), N^4-methyl-4-nitroacetophenone thiosemicarbazone ($H4NO_2Ac4M$) and N^4-methyl-4-nitrobenzophenone thiosemicarbazone ($H4NO_2Bz4M$) were obtained and screened *in vitro* against bloodstream and intracellular forms of *T. cruzi*. $H4NO_2Fo4M$, $H4NO_2Ac4M$ and their Mn(II) complexes displayed poor effect on bloodstream trypomastigotes, with IC_{50} values ranging from 68 to >200 μM. However, although $H4NO_2Bz4M$ was also not active, its corresponding Mn(II) complex presented high effect on this *T. cruzi* form, with an IC_{50} value of 19 μM. The effect of complex (**3**), against trypomastigotes of *T. cruzi* supports further *in vitro* as well as *in vivo* studies.

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1. Introduction

Chagas' disease is a tropical neglected illness that is the leading cause of heart disease in Latin America where it affects approximately 12 million people living in very poor social conditions [1]. In addition, the globalization of Chagas' disease is now acknowledged as it can also be found throughout the world due to international immigration [2,3]. Thus, this anthropozoonosis, discovered a century ago by the Brazilian physician Carlos Chagas, still represents a serious Public Health problem in the affected areas claiming for care and resolution of its current challenges, including the imperative need to sustain public policies related to the transmission control and the urgent requirement for new chemotherapic agents [4,5]. In fact, since the existing therapy, based on the nitroheterocycles nifurtimox (3-methyl-4-[(5-nitrofurfurylidene) amino]thiomorpholine-1,1-dioxide) and benznidazole (2-nitro-*N*-(phenylmethyl)-1*H*-imidazole-1-acetamide), developed empirically over three decades ago leads to severe side effects, requires long term treatment and is of limited efficacy especially in its chronic phase, identification of new drugs for Chagas disease is indisputable [6–8].

Thiosemicarbazones and their metal complexes represent an interesting class of compounds with a wide range of pharmacological applications [9]. Many examples of this class of small molecules have been evaluated over the last 50 years as having antitumor [10–12], antibacterial [13,14], antifungal [13], antiviral [15–17] and antiprotozoal activities [18,19]. Additionally, some studies demonstrated that novel thiosemicarbazones exhibit significant *in vitro* activity against trypanosomatids such as *Leishmania* sp. [20], *Trypanosoma brucei* [19], and *Trypanosoma cruzi* [21–23].

Some of us recently started an investigation on the pharmacological profile of 4-nitroacetophenone-derived thiosemicarbazones and their metal complexes. It has been shown that these thiosemicarbazones and their Cu(II) complexes present significant *in vitro* anti-trypanosomal activity, the complexes resulting to be at least 5 times more active than the free ligands [18]. Recently, Ru(II) complexes with N^4-methyl-4-nitrobenzaldehyde thiosemicarbazone ($H4NO_2Fo4M$) and N^4-methyl-4-nitroacetophenone thiosemicarbazone ($H4NO_2Ac4M$) containing 1,4-bis(diphenylphospine) butane (dppb) and 2,2′-bipyridine (bipy), 4,4′-dimethyl-2,2′-bipyridine (Mebipy) or 1,10-phenanthroline (phen) as co-ligands have

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been developed [24]. All complexes revealed to be at least 10 times more active than their corresponding free thiosemicarbazones [24].

In the present study, Mn(II) complexes with N^4-methyl-4-nitrobenzaldehyde thiosemicarbazone ($H4NO_2Fo4M$), N^4-methyl-4-nitroacetophenone thiosemicarbazone ($H4NO_2Ac4M$) and N^4-methyl-4-nitrobenzophenone thiosemicarbazone ($H4NO_2Bz4M$) (Fig. 1) were obtained and their cytotoxicity, trypanocidal efficacy and selectivity were evaluated *in vitro* against both bloodstream trypomastigotes and intracellular amastigotes of *T. cruzi*.

2. Experimental

2.1. Physical measurements

Elemental analyses were performed on a Fison equipment, model EA 1108. A Radiometer Copenhagen Meter Lab., model CDM 230 was employed for molar conductivity measurements. Infrared spectra (KBr pellets) were obtained using a BOMEM MICHELSON instrument, model 102. Magnetic susceptibility measurements were carried out on a Johnson Matthey MSB/AUTO balance. X-band electron paramagnetic resonance (EPR) spectra were obtained with a Bruker ESP300E equipment with modulation frequency of 100 kHz and modulation amplitude of 0.1 or 1 mT. Samples in the solid state were measured in glass capillaries at room temperature; frozen ethanol solutions were measured in Teflon® tubes of 3 mm of internal diameter immersed in liquid N_2 (77 K).

The electrochemical experiments were carried out at room temperature in dichloromethane containing 0.1 mol L^{-1} tetrabutylammonium perchlorate (TBAP) (Fluka Purum) using an electrochemical analyzer from Bioanalytical Systems Inc. (BAS), model 100BW. The working and auxiliary electrodes were stationary Pt foils, and the reference electrode was Ag/AgCl, a medium in which ferrocene is oxidized at 0.48 V (Fc^+/Fc).

Luminescence studies were made on a Perkin–Elmer LS 55 spectrofluorimeter using 10 nm spectral bandpass. Fluorescence measurements were performed in 1 cm quartz cells using solutions prepared either in CH_2Cl_2 or in dimethylformamide (DMF). In solid state, fluorescence was made using a front solid surface apparatus (Perkin–Elmer) and a cell with a quartz window. For phosphorescence measurements, the front solid surface apparatus was also employed and the signals were measured directly from the surface of dry low background cellulose substrates [25] using both detector delay and detector gate times set at 3 ms. Measurements were made in an oxygen free environment established by the use of dried nitrogen gas. Solutions (5 μL) of the free thiosemicarbazones and their metal complexes, prepared in CH_2Cl_2, were deposited on the center of the cellulose substrates. These substrates were previously spotted with 5 μL of heavy atom salt solutions – $Pb(NO_3)_2$, $CdCl_2$, $TlNO_3$, $AgNO_3$, $HgCl_2$ – which were used as potential phosphorescence inducers. Measurements were made after the substrates were dried under vacuum (2 h).

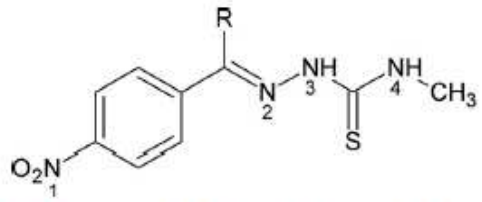

Fig. 1. Structures of N^4-methyl-4-nitrobenzaldehyde thiosemicarbazone (R = H, $H4NO_2Fo4M$), N^4-methyl-4-nitroacetophenone thiosemicarbazone (R = CH_3, $H4NO_2Ac4M$) and N^4-methyl-4-nitrobenzophenone thiosemicarbazone (R = C_6H_5, $H4NO_2Bz4M$).

2.2. Drugs

N^4-methyl-4-nitrobenzaldehyde thiosemicarbazone ($H4NO_2Fo4M$), N^4-methyl 4-nitroacetophenone thiosemicarbazone ($H4NO_2Ac4M$) and N^4-methyl-4-nitrobenzophenone thiosemicarbazone ($H4NO_2Bz4M$) were prepared as described in the literature [18,26]. Briefly, equimolar amounts of 4-nitrobenzaldehyde, 4-nitroacetophenone and 4-nitrobenzophenone (6.6×10^{-3} mol) were mixed with N^4-methyl thiosemicarbazide in absolute ethanol (40 mL) with addition of 2–4 drops of concentrated sulfuric acid as catalyst. The reaction mixture was kept under reflux for 6–7 h. The precipitated material were filtered, washed with diethylether and dried under vacuum.

The complexes were obtained by dissolving the desired thiosemicarbazone (0.40 mmol) in ethanol (20 mL) with gentle heating and stirring. After cooling, the solution to room temperature, $MnCl_2{\cdot}4H_2O$ (0.20 mmol) was added. The reaction was stirred at room temperature for 24 h. The solids which precipitated were filtered and washed with diethyl ether and dried.

2.2.1. $[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**)

Orange solid; yield: 78%. *Anal.* Calc. ($C_{18}H_{20}Cl_2MnN_8O_4S_2$): C, 35.89; H, 3.35; N, 18.60. Found: C, 35.79; H, 3.33; N, 18.58. IR (KBr, cm^{-1}): ν(C=C) + ν(C=N) 1561, $\nu_{ass}NO_2$ 1509, ν_sNO_2 1335, ν(C=S) 824. Molar conductivity (1×10^{-3} mol L^{-1} acetone): 1.70 μS cm^{-1}. μ_{eff}: 1.89 BM.

2.2.2. $[Mn(H4NO_2Ac4M)_2Cl_2]$ (**2**)

Yellow solid; yield: 72%. *Anal.* Calc. ($C_{20}H_{24}Cl_2MnN_8O_4S_2$): C, 38.10; H, 3.84; N, 17.77. Found: C, 38.10; H, 3.83; N, 17.76%. IR (KBr, cm^{-1}): ν(C=C) + ν(C=N) 1547, $\nu_{ass}NO_2$ 1507, ν_sNO_2 1339, ν(C=S) 847. Molar conductivity (1×10^{-3} mol L^{-1} acetone): 3.41 μS cm^{-1}. μ_{eff}: 1.86 BM.

2.2.3. $[Mn(H4NO_2Bz4M)_2Cl_2]$ (**3**)

Orange solid; yield: 88%. *Anal.* Calc. ($C_{30}H_{28}Cl_2MnN_8O_4S_2$): C, 47.75; H, 3.74; N, 14.85. Found: C, 47.69; H, 3.72; N, 14.81%. IR (KBr, cm^{-1}): ν(C=C) + ν(C=N) 1545, $\nu_{ass}NO_2$ 1513, ν_sNO_2 1338, ν(C=S) 825. Molar conductivity (1×10^{-3} mol L^{-1} acetone): 2.76 μS cm^{-1}. μ_{eff}: 1.97 BM.

Stock solutions of the thiosemicarbazones and their Mn(II) complexes (50 mM) were prepared in dimethylsufoxide (DMSO) and dimethylformamide (DMF). Fresh dilutions were prepared immediately before use, with the final solvent content never exceeding 0.4%, which led to no toxicity to the parasite or the mammalian host cells.

2.3. Parasites

Y strain of *T. cruzi* was used throughout the experiments. Bloodstream forms were harvested by heart puncture from *T. cruzi*-infected Swiss mice at the peak of parasitemia [27].

2.4. Mammalian cell cultures and toxicity assays

For both drug toxicity and infection assays, primary cultures of embryonic cardiomyocytes (CM) were obtained following the previously described method [28]. After purification, the CM were seeded at a density of 0.1×10^6 cells/well into 24-well culture plates, or 0.05×10^6 cell/well into 96-well microplates, containing gelatin-coated cover slips and sustained 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). All the cultures were maintained at 37 °C in an atmosphere of 5% CO_2, and the assays were run at least three times in duplicate.

In order to rule out toxic effects of the compounds on host cells, uninfected CM were incubated for 24 and 72 h at 37 °C in presence or absence of the compounds (up to 200 μM) diluted in DMEM. Then their morphology was evaluated by light microscopy and loss of cellular viability measured by the MTT colorimetric assay [29]. The absorbance was measured at 490 nm with a spectrophotometer (VERSAmax tunable, Molecular Devices, USA) allowing the determination of LC_{50} values (drug concentration that reduces 50% of cellular viability) and the respective selective indexes ($SI = LC_{50}/IC_{50}$).

2.5. Trypanocidal analysis

For the *in vitro* analysis on bloodstream trypomastigotes, the parasites were incubated at 37 °C for 24 h in the presence of increasing doses (0–200 μM) of each compound diluted in Dulbecco's modified medium supplemented with 5% fetal bovine serum and 1 mM L-glutamine (DMES) [30]. The incubation of parasites with benznidazole (0–125 μM) or with the Mn(II) salt (0–400 μM) alone, under the same conditions, was also performed. After drug incubation, death rates were determined by light microscopy through the direct quantification of live parasites using a Neubauer chamber, and the IC_{50} values (drug concentration that reduces 50% of the number of the parasites) were then calculated [30].

For the analysis of intracellular parasites, after the initial 24 h of host cell-parasite contact (using a CM: trypomastigotes ratio of 1:10), the cultures were washed to remove free parasites followed by the treatment with the compounds (0.2 mL of the drugs, at crescent concentrations up to 200 μM). Infected and untreated CM were used as controls. All cultures were maintained at 37 °C in an atmosphere of 5% CO_2 and air, and the medium, with or without the drugs, was replaced everyday. After 72 h of drug exposure, supernatants of untreated and treated cultures were recovered, the number of released parasites quantified by light microscopy using a Neubauer chamber [31,32], and IC_{50} values calculated.

All procedures were carried out in accordance with guidelines established by FIOCRUZ Committee of Ethics for the Use of Animals (approved protocol number L08/09).

3. Results and discussion

3.1. Microanalyses, molar conductivity and magnetic susceptibility studies

Microanalyses suggest the formation of $[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**), $[Mn(H4NO_2Ac4M)_2Cl_2]$ (**2**), $[Mn(H4NO_2Bz4M)_2Cl_2]$ (**3**) in which the thiosemicarbazones coordinate as neutral ligands. The molar conductivity data reveal that the complexes are non-electrolytes, in accordance with the proposed formulations. The values of magnetic moments, in the 1.86–1.97 BM range, are close to the calculated value of 1.73 BM, characteristic of the presence of one unpaired electron as in low spin Mn(II) complexes.

3.2. Infrared spectral studies

The ν(C=C) + ν(C=N) composed mode observed at 1583–1590 cm^{-1} in the spectra of the thiosemicarbazones shifts to 1545–1561 cm^{-1} in the spectra of the complexes, indicating coordination of the azomethine nitrogen N(2) [18,26].

The ν(C=S) absorption at 824–832 cm^{-1} in the spectra of the uncomplexed thiosemicarbazones is observed at 825–847 cm^{-1} in those of the complexes, in accordance with coordination through a thione sulfur [33–35]. The 7–19 cm^{-1} shift observed upon complexation is compatible with coordination of a neutral thiosemicarbazone in all cases [33–35]. Hence the infrared spectra indicate coordination through the N–S chelating system.

3.3. EPR spectral studies

The X-band EPR spectra of complexes (**1**)–(**3**) obtained at room temperature for the solid state samples, and at 77 K for the ethanol solutions are presented in Fig. 2. The EPR spectra of the powder samples show a broad signal with g value close to 2.01. In polycrystalline samples at room temperature Mn(II) complexes give very broad signals, which may be due to dipolar interactions and enhanced spin lattice relaxation.

Mn(II) presents a $3d^5$ electronic structure. If the ligand-field splitting of the *d* orbitals is not too large the ions will present a high spin state, $S = 5/2$. For a large ligand-field splitting, spin pairing leads to the low spin state, $S = 1/2$. Even in the high spin case, the effective spin is usually 1/2, because the distorted ligand-field symmetry displaces the $m_S = \pm 3/2$ and $\pm 5/2$ levels to higher energy values and only transitions between the levels $m_S = \pm 1/2$ are induced by the microwave. However, in this case, the effective g factor is very anisotropic. In the low spin case the g values are around the free-electron value ($g = 2.0023$) [36]. The ^{55}Mn nucleus presents a nuclear spin $I = 5/2$, so that the EPR spectrum of a Mn(II) solution at room temperature exhibits a characteristic six component hyperfine splitting. If the symmetry around Mn(II) is distorted due to complexation, the resonances become anisotropic and a randomly oriented sample may exhibit such a broad line that detection becomes difficult.

In the case of the solid state complexes (**1**)–(**3**), the EPR spectra are broad and isotropic (Fig. 2a). The hyperfine lines are not resolved. The *g* factor, 2.009, is very close to the free-electron value, suggesting a low spin Mn(II) ion, coming from a strong ligand-field. Complex (**3**), however, presented a much broader line (50 mT) than the others (20 mT). This is unexpected since the bulky ligand $H4NO_2Bz4M$ would weaken the magnetic dipolar interactions that broaden the lines. In general, dilution leads to line narrowing and better resolved spectra. In these complexes this does not occur, since both 1 and 0.1 mM ethanol frozen solutions presented the same 20 mT linewidth as the powder spectrum at room temperature (Fig. 2b). This suggests that line broadening was caused by the Mn(II)–ligand association with a low-symmetry ligand-field, not to the magnetic dipolar interaction between Mn(II) ions.

3.4. Electrochemical studies

The voltammograms of the thiosemicarbazones show a quasi-reversible process between −0.93 and −1.02 V, which has been attributed to the formation of the $Ar–NO_2^{\cdot-}$ radical. The second process between −1.11 and −1.34 V has been assigned to the formation of the Ar–NHOH species and the irreversible oxidation at 0.17–0.21 V to the formation of Ar–NO. The observed processes correspond to the well known mechanism for the nitro aromatic reduction, as previously proposed [18,26,37–40] (Fig. 3a).

The voltammograms of complexes (**1–3**) show all the events observed in the voltammograms of the free ligands (Fig. 3b). For complexes (**1**) and (**2**) the cyclic voltammograms show an irreversible process around −1.0 V attributed to the formation of the $Ar–NO_2^{\cdot-}$ radical. For complex (**3**), this process is quasi-reversible and the reduction with the formation of the $Ar–NO_2^{\cdot-}$ radical is observed at −1.00 V followed by its successive oxidation at −0.87 V. A second quasi-reversible reduction attributed to the formation of the Ar–NHOH species occurs between −1.26 and −1.22 V, followed by its respectively oxidation (−1.13 to −1.11), and an irreversible oxidation attributed to the formation of the Ar–NO species is

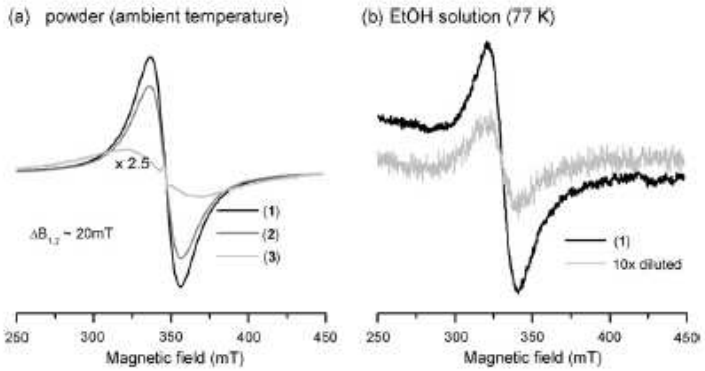

Fig. 2. (a) EPR spectra of complexes (**1**)–(**3**) in the solid state (powder samples) at room temperature. (b) EPR spectra of 1.0 and 0.1 mmol L^{-1} ethanol solutions of complex (**1**). Complexes (**2**) and (**3**) presented similar spectra.

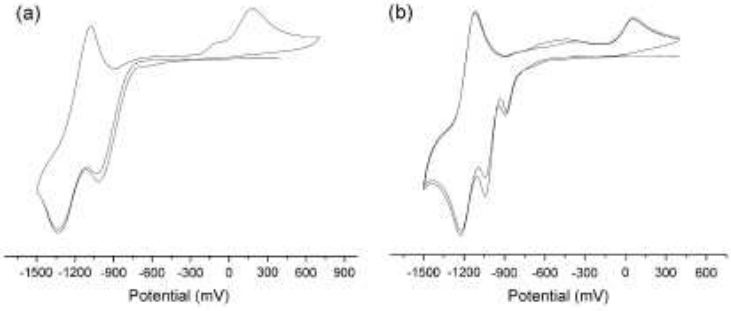

Fig. 3. Cyclic voltammograms of (a) $H4NO_2Fo4M$ and (b) $[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**) (CH_2Cl_2, 0.1 mol L^{-1} TBAP).

observed in the 0.48–0.83 V range for complexes (**1–3**). For all complexes the remaining processes are attributed to the irreversible reduction Mn(II)/Mn(I) (Table 1).

Experiments carried out on the main drugs used for the Chagas' disease treatment (nirfutimox and benznidazole) suggest that intracellular nitro-moiety reduction followed by redox cycling yielding reactive oxygen species may be their major mode of action against *T. cruzi* [22].

In the present work the quasi-reversible process between −1.00 and −1.08 V has been attributed to the formation of the $Ar–NO_2^{\bullet-}$ radical. The reported value for the same process in nirfutimox [22] falls into this same range. Interestingly, complexes (**1–3**) have adequate NO_2-reduction potential to act against *T. cruzi via* a redox cycling process.

3.5. Luminescence studies

Fluorescence studies in solution and in solid state as well as fosforescence using cellulose substrates have been measured for the N(4)-methyl nitrotiosemicarbazones and their Mn(II) complexes.

In the solid state, $H4NO_2Fo4M$, $H4NO_2Ac4M$ and $H4NO_2Bz4M$ present similar fluorescence spectra as indicated in Table 2. The maximum wavelength of excitation (λ_{exc}) and the maximum wavelength of emission (λ_{em}) appeared respectively at the 211–213 nm range and at the 372–373 nm range. Fluorescence from these compounds comes from the aromatic system; however, its low intensity can be explained by the presence of the NO_2 electron-withdrawing substituent that decreases the molecule fluorescence quantum yield by introducing a low lying n, π^* state. The long chain substitutions in the aromatic system also contribute with the decrease of fluorescence by favoring energy loss by radiationless processes.

The Mn(II) complexes present the characteristic fluorescence bands of the free thiossemicarbazones, indicating that metal coordination did not induce any relevant spectral changes. In the case of complex (**1**), a small fluorescence increasing after complexation has been observed. On the other hand, for complex (**3**) a decreasing

Table 1
Cyclic voltammetry data (V) for complexes (**1–3**) (0.100 V s^{-1}, CH_2Cl_2, 0.1 mol L^{-1}, TBAP).

Complex	$Ar–NO_2^{\bullet-}$		Ar–NHOH		Ar–NO	Mn^{II}/Mn^{I}
	E_{pc}	E_{pa}	E_{pc}	E_{pa}	E_{pa}	
$[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**)	−1.04	–	−1.22	−1.11	0.58	−0.89
$[Mn(H4NO_2Ac4M)_2Cl_2]$ (**2**)	−1.08	–	−1.25	−1.14	0.83	−0.91
$[Mn(H4NO_2Bz4M)_2Cl_2]$ (**3**)	−1.00	−0.87	−1.26	−1.13	0.48	−0.81

Table 2
Wavelengths of excitation (λ_{exc}, nm) and emission (λ_{em}, nm) and intensity of the fluorescence absorptions for the thiosemicarbazones and their Mn(II) complexes in the solid state.

Compound	λ_{exc} (nm)	λ_{em} (nm)	Intensity
$H4NO_2Fo4M$	211	373	102
$[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**)	211	374	147
$H4NO_2Ac4M$	213	373	115
$[Mn(H4NO_2Ac4M)_2Cl_2]$ (**2**)	213	374	111
$H4NO_2Bz4M$	212	372	188
$[Mn(H4NO_2Bz4M)_2Cl_2]$ (**3**)	212	373	109

of fluorescence was observed in comparison to the fluorescence from the free ligand. Finally, complex (**2**) and the free thiosemicarbazone presented identical spectra in terms of spectral position and signal intensity.

In CH_2Cl_2 solution, the $H4NO_2Fo4M$, $H4NO_2Ac4M$ and $H4NO_2Bz4M$ fluorescence spectra are very similar with λ_{exc} in the 214–216 nm range and λ_{em} in the 293–295 nm range. Comparative to the ones observed in solid state, the λ_{em} of the complexes in CH_2Cl_2 appeared in a significantly different spectral region (hypsochromic shift) indicating that solvation played an important role in excited state stabilization for these thiosemicarbazone and their complexes. In fact, when a more polar solvent (DMF) was employed, total fluorescence quenching was observed. Interaction between the withdrawing group (NO_2), which has great influence on the luminescence properties of these molecules, and the solvent (either polar of non-polar) is probably a major factor explaining these significant spectral changes. In Fig. 4, the fluorescence spectrum from $H4NO_2Fo4M$ in CH_2Cl_2 solution is shown.

Complexes (**1**)–(**3**) could not be dissolved in CH_2Cl_2, therefore, it was not possible to obtain fluorescence spectra from them in such medium. The use of a more polar solvent (DMF) enables the dissolution of these complexes; however, no fluorescence was observed from these solutions, possibly indicating either a dynamic quenching caused by DMF or an effective stabilization of the excited state bringing it to close to the fundamental state and therefore favoring radiationless energy deactivation.

These studies indicated that natural fluorescence from the thiosemicarbazones and their Mn(II) complexes may be used to identify and monitor these substances in biological systems.

The thiosemicarbazones and their complexes (**1**)–(**3**) have been deposited on a cellulose substrate previously treated with solutions of salts of Tl(I), Hg(II), Pb(II), Cd(II) or Ag(I) which are traditionally used as phosphorescence inducers from organic compounds. However, no phosphorescence was observed under these conditions.

3.6. In vitro anti-trypanosomal activity

Results showed that $H4NO_2Fo4M$, $H4NO_2Bz4M$, and complexes (**1**) and (**2**) were not active against bloodstream trypomastigotes, showing IC_{50} values higher that 142 μM (Fig. 5a, Table 3), while $H4NO_2Ac4M$ was moderately active (IC_{50} = 68 μM). However, complex (**3**) presented interesting trypanocidal activity, with IC_{50} = 19 μM and reaching 95% of parasite death at 50 μM (Fig. 5a, Table 3). It is important to notice, that the later complex showed quite similar *in vitro* trypanocidal effect upon bloodstream forms as compared to benznidazole, the reference drug for Chagas disease (Table 3). The effect of Mn(II) chloride salt was evaluated and the determined IC_{50} value of 340 μM indicates that Mn(II) does not possess anti-*T. cruzi* activity *per se* (Table 3).

The toxicity of the studied compounds towards mammalian host cells was also evaluated by employing primary cultures of cardiac cells. The results demonstrated that none of the thiosemicarbazone reduced the cellular viability even when higher doses (200 μM) and longer periods of incubation (72 h) were used.

The effect of the compounds was also evaluated in *T. cruzi*-infected cardiac culture assays aiming to further analyze their activity against the intracellular forms of the parasite. However, the thiosemicarbazones and their Mn(II) complexes did not present trypanocidal effect against intracellular parasites, exhibiting IC_{50} values between 111 and >200 μM (Fig. 5b, Table 3).

Further analysis of the selective index (SI) demonstrated that complex (**3**) was the most selective compound, being 10 times more active against bloodstream trypomastigotes than against mammalian cells (Table 3).

The lower activity of complex (**3**) against amastigotes (IC_{50} = 111 μM), a highly intracellular proliferative stage found within mammalian cells, as compared to its activity against bloodstream trypomastigotes may be explained by reduced ability of the compound to get into the host cells and/or by the fact that this compound may act on different targets according to the parasite stage.

Different efficacy was also noticed when Pt(II) complexes of 3-(5-nitrofuryl)acroleine thiosemicarbazones were assayed by other authors against both epimastigote and trypomastigotes of *T. cruzi*

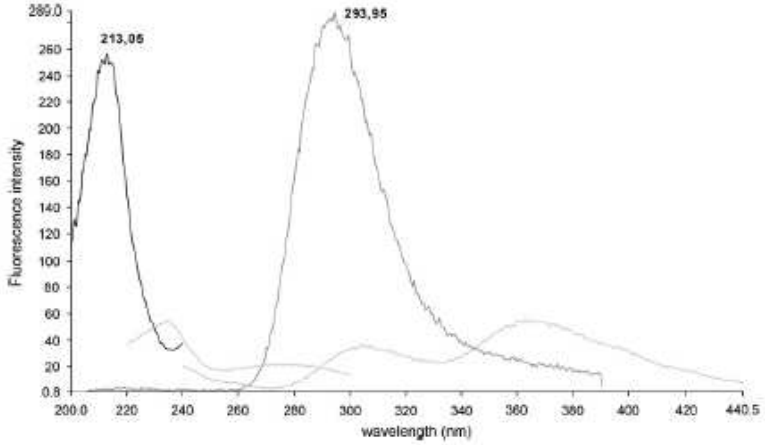

Fig. 4. Emission and excitation fluorescence spectra of $H4NO_2Fo4M$ in CH_2Cl_2 solution.

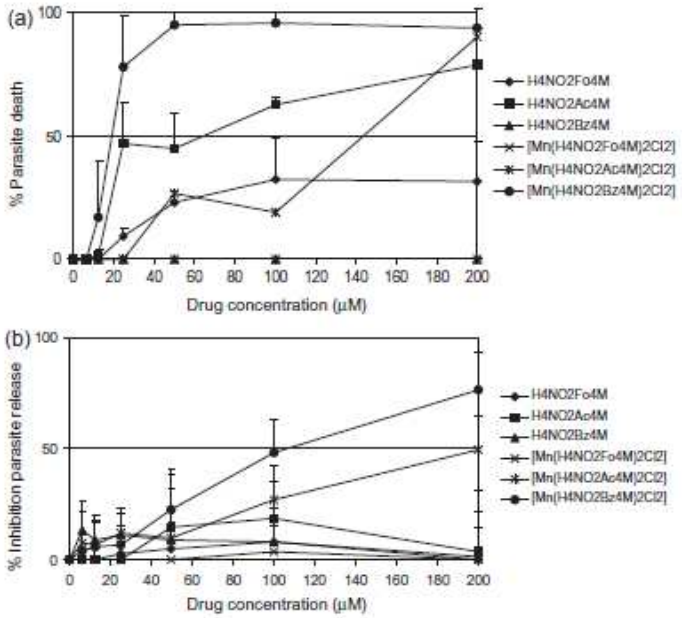

Fig. 5. Activity of $H4NO_2Fo4M$, $H4NO_2Ac4M$, $H4NO_2Bz4M$, $[Mn(H4NO_2Fo4M)_2Cl_2]$ (**1**), $[Mn(H4NO_2Ac4M)_2Cl_2]$ (**2**) and $[Mn(H4NO_2Bz4M)_2Cl_2]$ (**3**), upon bloodstream (a) and intracellular (b) forms of *T. cruzi in vitro*.* The effect upon parasites was evaluated during the treatment at 37 °C with the drugs diluted in culture medium. The percentage of dead bloodstream parasites was measured after 24 h of treatment (a) and the effect upon intracellular forms was evaluated by the quantification of parasite release in supernatant of untreated and drug-treated infected cultures after 72 h of treatment (b). Data are expressed as mean ± SD of three independent experiments.

Table 3
IC_{50} and respective SI* values for the thiosemicarbazones and their Mn(II) complexes upon bloodstream and intracellular forms of *Trypanosoma cruzi*.

Compound	BT*		Intracellular forms	
	IC_{50} (μM)	SI	IC_{50} (μM)	SI
$H4NO_2Fo4M$	>200	>1	>200	>1
$[Mn(H4NO_2Fo4M)_2Cl_2]$	>200	>1	>200	>1
$H4NO_2Ac4M$	68 ± 44	>3	>200	>1
$[Mn(H4NO_2Ac4M)_2Cl_2]$	142.5 ± 7	>1.4	200	>1
$H4NO_2Bz4M$	>200	>1	>200	>1
$[Mn(H4NO_2Bz4M)_2Cl_2]$	19.21 ± 0.1	>10	111 ± 29	>1.8
$MnCl_2{\cdot}4H_2O$	340 ± 83		nd	
Benznidazole [55]	10.92		nd	

* SI: selective index: ratio between LC_{50}/IC_{50} values. For bloodstream and intracellular forms the corresponding LC_{50} values were related to 24 and 72 h of drug incubation, respectively.
* Bloodstream trypomastigotes, nd: not done.

[41]. The activity of these complexes was superior against trypomastigotes as compared to epimastigotes [41]. As mentioned by these authors, it is important to consider the morphologic changes that occur during transformation between forms along the biological cycle of the parasite, which may imply important metabolic and macromolecular content changes that may alter sensibility to drugs [41].

In fact, the detailed mechanism of action of thiosemicarbazones is not fully understood and a bulk of data suggests that it may be mediated by multiple targets [19,21,42,43]. It has been shown that thiosemicarbazones inhibit ribonucleotide reductase, an enzyme essential for DNA synthesis [44–47]; also impairing dihydrofolate reductase [48,49]. There are also evidences that metal complexes of thiosemicarbazones may induce oxidative stress [22,50] and inhibit cysteine proteases [19]. Regarding *T. cruzi* although we presently did not explore the mechanisms of action of these nitro-thiosemicarbazones and their Mn(II) complexes, literature data showed that some active thiosemicarbazone derivatives hamper cruzain (aka cruzipain), which is the major cysteine protease of the parasite [43]. Cruzain is expressed in all evolutive stages of the parasite, playing a role in invasion, replication and differentiation steps [51–54]. Currently, cruzain inhibition is one of the most advanced and widely studied strategies in the design of new chemotherapeutic agents for the treatment of Chagas disease [51]. However, other *in vitro* studies also suggested that 5-nitrofuryl containing thiosemicarbazones that display higher trypanocidal activity than nifurtimox, could act both through the inhibition of cruzain and also by promoting oxidative stress in *T. cruzi* [50]. Thus, it is possible that these novel metal complexes of TS presently assayed may be also operating on these cellular targets of the parasite although others targets cannot be excluded justifying biochemical and electron microscopy studies that are underway to clarify this subject.

4. Conclusion

Despite the fact that many different classes of compounds have been assayed against *T. cruzi* both *in vitro* and *in vivo*, except for few of them, such as allopurinol, itraconazole and fluconazole, none moved to clinical trials [7]. Therefore, although clearly not of immediate clinical use, the non-peptide nature of the thiosemicarbazone group (i), its relative small size (ii), its extremely low cost of production (iii), its capacity to form the $Ar–NO_2^{\cdot-}$ radical in a potential range similar to that of nitro-containing anti-trypanosomal drugs (iv) and its trypanocidal efficacy (v) are positive arguments in favor that they represent promissory agents for the

development of much-needed new anti-trypanosomal drugs especially when coordinated to metal ions.

Complex (**3**) besides displaying higher efficacy than its corresponding thiosemicarbazone ligand upon bloodstream trypomastigotes, was 18-fold more effective than the Mn(II) salt. In addition, complex(**3**) showed the highest selective index, being as much as 10-fold more active against the trypanosomes than against the cardiac cells. Thus, the present study supports previous data reporting high trypanocidal effect induced by some metal complexes of thiosemicarbazones upon *T. cruzi* [18,24].

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Artigo # 03: Aceito à publicação na revista Parasitology 2010.

Título: **"Biological, ultrastructural effect and sub-cellular localization of aromatic diamidines in *Trypanosoma cruzi"***

Estado do conhecimento quando da concepção do trabalho:

- ✓ Diamidinas aromáticas são compostos aromáticos dicatiônicos que apresentam um amplo espectro de ação microbicida, sendo utilizadas na clínica para tratamento de patologias como a doença do sono (e.g. pentamidina).
- ✓ No entanto, apesar de sua excelente atividade, apresentam efeitos colaterais indesejáveis além de baixa biodisponibilidade oral, estimulando vários grupos de química medicinal a sintetizar análogos que mantenham sua excelente ação, mas que apresentem menor toxicidade e que possam ser administrados via oral.

- ➢ **Questões propostas:**

1. Avaliar a atividade antiparasitária, toxicidade e a seletividade in vitro sobre tripomastigotas de sangue e formas intracelulares do *Trypanosoma cruzi* de 06 diamidinas aromáticas - DB1582, DB1627, DB1645, DB1646, DB1651 e DB1670,
2. Correlacionar atividade tripanocida com a composição química dos compostos.
3. Verificar a localização, distribuição citoplasmática e alvos celulares dos compostos sobre formas tripomastigotas de sangue e intracelulares de *Trypanosoma cruzi* através de microscopia de fluorescência e de microscopia eletrônica de transmissão.

Seguem 9 páginas

Biological, ultrastructural effect and subcellular localization of aromatic diamidines in *Trypanosoma cruzi*

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SUMMARY

No vaccines or safe chemotherapy are available for Chagas disease. Pentamidine and related di-cations are DNA minor groove-binders with broad-spectrum anti-protozoal activity. Therefore our aim was to evaluate the *in vitro* efficacy of di-cationic compounds – DB1645, DB1582, DB1651, DB1646, DB1670 and DB1627 – against bloodstream trypomastigotes (BT) and intracellular forms of *Trypanosoma cruzi*. Cellular targets of these compounds in treated parasites were also analysed by fluorescence and transmission electron microscopy (TEM). DB1645, DB1582 and DB1651 were the most active against BT showing IC_{50} values ranging between 0·15 and 6·9 μM. All compounds displayed low toxicity towards mammalian cells and DB1645, DB1582 and DB1651 were also the most effective against intracellular parasites, with IC_{50} values ranging between 7·3 and 13·3 μM. All compounds localized in parasite nuclei and kDNA (with greater intensity in the latter structure), and DB1582 and DB1651 also concentrated in non-DNA-containing cytoplasmic organelles possibly acidocalcisomes. TEM revealed alterations in mitochondria and kinetoplasts, as well as important disorganization of microtubules. Our data provide further information regarding the activity of this class of compounds upon *T. cruzi* which should aid future design and synthesis of agents that could be used for Chagas disease therapy.

Key words: *Trypanosoma cruzi*, Chagas disease, chemotherapy, aromatic diamidines.

INTRODUCTION

Chagas disease, a tropical parasitic disease caused by the flagellate protozoan *Trypanosoma cruzi*, which was discovered by Carlos Chagas exactly a century ago (Chagas, 1909). Currently, there are approximately 12–14 million infected individuals in endemic areas of Latin America and many reports also point to the occurrence in non-endemic geographical areas such as the United States and Europe, mainly attributed to population movement of infected people (Dias, 2007; Gascón *et al.* 2007; Rodriguez-Morales *et al.* 2008; Guerri-Guttenberg *et al.* 2008; Milei *et al.* 2009). Clinically, Chagas disease has 2 phases: the acute, which appears shortly after the infection, and the chronic phase, which can develop in about one-third of the infected individuals, after a silent period of years or decades called the indeterminate phase (Cunha-Neto *et al.* 2006). The main clinical manifestations of Chagas' disease include both cardiac and/or digestive alterations, the former being the most common (Marin-Neto *et al.* 1999; Teixeira *et al.* 2006; Rocha *et al.* 2007). Pathogenesis of chronic chagasic cardiopathy is still not clearly understood but growing evidence shows that the pathogenesis is a consequence of a sustained inflammatory process, with anti-parasitic and/or anti-self-immune response, associated with a low-grade persistent parasite presence (Higuchi *et al.* 2003; Rocha *et al.* 2007; Marin-Neto *et al.* 2008).

Up to now, current therapy of chagasic patients includes nifurtimox (5-nitrofuran-(3-methyl-4-(5'-nitrofurfurylideneamine) tetrahydro-4H-1,4-tiazine-1,1-dioxide – Bayer 2502), and benznidazole (2-nitroimidazole (N-benzyl-2-nitroimidazole acetamide – RO 7-1051). Both compounds were developed empirically over 3 decades ago and present variable results depending on the disease phase (effective in the acute and recent chronic phases of the infection), dose and duration of the treatment, natural susceptibility of *T. cruzi* isolates, in addition both show undesirable side effects (Filardi and Brener, 1987; Coura and De Castro, 2002; Soeiro and De Castro, 2009). Heterocyclic di-cations, such as furamidine (DB75) and analogues, are DNA minor groove-binding ligands that recognize sequences of at least 4 AT base pairs, and present striking

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broad-spectrum antimicrobial effects (Wilson *et al.* 2008; Checchi and Barrett, 2008; Soeiro *et al.* 2008). However, due to their poor oral bioavailability and unfavourable side-effects, numerous new di-cationic analogues have been synthesized with the goal to improve on these deficiencies (Werbovetz, 2006). Previous studies reported the efficacy of diamidines such as DB75, DB569(*N*-phenyl-substituted analogue of DB75) and DB1362 (a diarylthiophene diamidine), as well as arylimidamides, including DB889, DB786 and DB702 upon *T. cruzi in vitro* (De Souza *et al.* 2004; Silva *et al.* 2007*a*, *b*, 2008). Additional studies performed with murine experimental models demonstrated the effect of these cationic compounds *in vivo*, leading to considerable protection against mice mortality with an important decrease in cardiac parasitism and inflammation. Furthermore, they reversed the electrocardiography alterations due to parasite infection (De Souza *et al.* 2006, 2007; Silva *et al.* 2008), justifying further studies with such aromatic dications.

Our present aim was to evaluate the *in vitro* activity of 6 aromatic diamidines upon bloodstream trypomastigotes and intracellular forms of *T. cruzi*. Moreover, fluorescence and transmission electron microscopy analyses were also performed in order to characterize the intracellular localization and cytoplasmatic distribution of the compounds as well as to evaluate their subcellular targets in the treated parasites.

MATERIALS AND METHODS

Drugs

The syntheses of the aromatic diamidines DB1582, DB1627, DB1645, DB1646, DB1651 and DB1670 (Fig. 1) were performed using standard procedures (Ismail *et al.* 2008). Stock solutions (5 mM) of the compounds were prepared in dimethyl sulfoxide (DMSO) with the final concentration of the latter in the experiments never exceeding 0·6%, which did not exert any toxicity towards the parasite or mammalian host cells (data not shown).

Parasites

The Y strain of *T. cruzi* was used throughout the experiments. Bloodstream trypomastigote forms were obtained from infected albino Swiss mice at the peak of parasitaemia as previously reported (Meirelles *et al.* 1986).

Mammalian cell cultures

For both drug toxicity and infection assays, primary cultures of embryonic cardiomyocytes (CM) were obtained following the previously described method (Meirelles *et al.* 1986). After purification, the CM

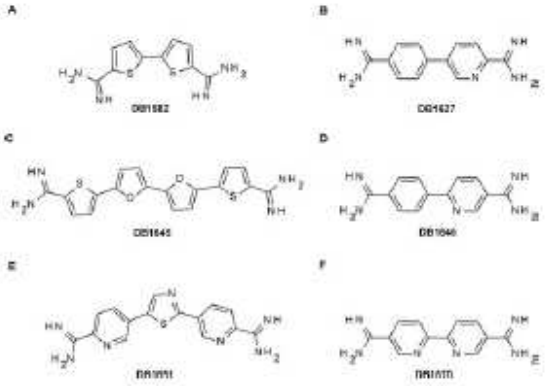


Fig. 1. Chemical structure of the six aromatic diamidines used in the present study.

were seeded at a density of $0{\cdot}1 \times 10^6$ cells/well into 24-well culture plates, or $0{\cdot}05 \times 10^6$ cell/well into 96-well microplates, containing gelatin-coated coverslips and sustained 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). All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA 0099/01). All the cell cultures were maintained at 37 °C in an atmosphere of 5% CO_2, and the assays were run at least 3 times in duplicate.

Cytotoxicity tests

In order to rule out toxic effects of the compounds on host cells, uninfected cardiac cell cultures were incubated for 24 and 72 h at 37 °C in the presence or absence of the diamidines (10·6–96 μM) diluted in DMEM and then their morphology evaluated by light microscopy and the cell death rates measured by the MTT colorimetric assay (Mosmann, 1983). The absorbance was measured at 490 nm wavelength with a spectrophotometer (VERSAmax tunable, Molecular Devices, USA) allowing the determination of LC_{50} values (drug concentration that reduces 50% of cellular viability).

Trypanocidal analysis

Bloodstream trypomastigotes (5×10^6 per ml) were incubated for 24 h at 37 °C in RPMI 1640 medium (Roswell Park Memorial Institute – Sigma Aldrich, USA) supplemented with 10% of fetal bovine serum, in the presence or absence of serial dilutions of the compounds (0·04–32 μM). This short incubation time-period (24 h) is a standard protocol to avoid loss of cellular viability of these extracellular non-dividing parasite forms. In addition, the potential applicability of these compounds for blood bank

prophylaxis was also evaluated by observation of the direct effect of the compounds on trypomastigotes at 4 °C maintained in freshly isolated mouse blood in the presence or absence of serial dilutions of the diamidines (up to 32 μM). After drug incubation, the parasite death rates were determined by light microscopy through the direct quantification of the number of live parasites using a Neubauer chamber, and the IC_{50} (drug concentration that reduces 50% of the number of the treated parasites) was then calculated. For analysis of the effect on intracellular parasites, after 24 h of parasite-host cell interaction (10:1 parasite : cardiac cell ratios), the parasitized cultures were washed to remove free parasites and then maintained at 37 °C in the presence or not of the compounds (0·04–32 μM), replacing the medium (with or without the respective drug) every 24 h. After 72 h of treatment that corresponds to 96 h of parasite infection, the supernatant of the infected cultures was recovered, the number of released parasites was quantified by light microscopy using a Neubauer chamber, and the corresponding IC_{50} values were averaged for at least 3 determinations done in duplicate.

Transmission electron microscopy (TEM) analysis

For TEM analysis, bloodstream trypomastigotes were treated for 2–24 h at 37 °C with the compounds at the concentration of their IC_{50} values, rinsed with PBS and fixed for 60 min at 4 °C with 2·5% glutaraldehyde and 2·5 mM $CaCl_2$ diluted in 0·1 M cacodylate buffer, pH 7·2, and post-fixed for 1 h at 4 °C with 1% OsO_4, 0·8% potassium ferricyanide and 2·5 mM $CaCl_2$ using the same buffer. The parasites were dehydrated in a graded series of acetone and finally embedded in Epon. Sections were stained with uranyl acetate and lead citrate and examined in a Jeol JEM-1011 electron microscope.

Fluorescence microscopy analysis

For fluorescence analysis, bloodstream trypomastigotes (3×10^6 cells/ml) and infected cardiac cell cultures (24 h of infection) were treated for 1 h with 10 μg/ml of each compound. Subsequently, the parasites and infected cultures were washed with PBS, fixed with 4% paraformaldehyde, mounted with 2·5% 1.4-diazabicyclo-(2.2.2)octane (DABCO) and fluorescence analysed with a x63 oil objective in a Zeiss photomicroscope equipped with epifluorescence (Zeiss Inc, Thornwood, New York), using a filter set for UV excited probes. Images were captured using the software AnalySIS OPT1.

RESULTS

The studies conducted to evaluate the efficacy of 6 cationic compounds upon bloodstream trypomastigotes of *T. cruzi* showed that DB1645, DB1582 and DB1651 present a time-dependent trypanocidal effect, reaching sub- and micromolar IC_{50} values of 0·15, 6 and 6·9 μM, respectively, after 24 h of incubation at 37 °C (Fig. 2A–C, Table 1). On the other hand, DB1646 and DB1670 showed only modest activity against bloodstream parasites with IC_{50} values of 31 and 32 μM, respectively. DB1627 did not exert any trypanocidal effect, displaying an IC_{50} value higher than 32 μM (Fig. 2D–F, Table 1). However, further analysis of the trypanocidal effect upon bloodstream forms incubated in the presence of mouse blood, at 4 °C, resulted in a significant reduction of the activity of all compounds, giving IC_{50} values $\geqslant 32$ μM (Fig. 2G).

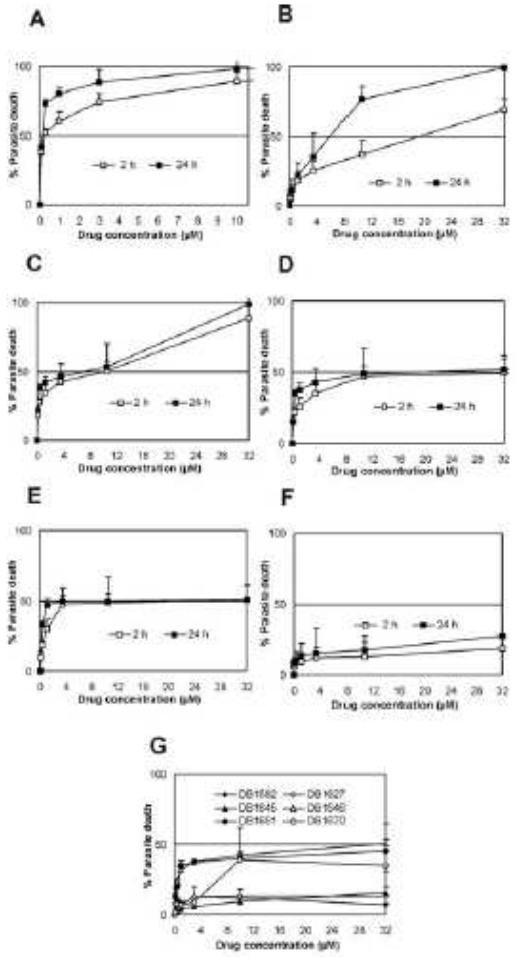


Fig. 2. Activity of the aromatic diamidines upon bloodstream trypomastigotes of *Trypanosoma cruzi in vitro*. (A) DB1645, (B) DB1582, (C) DB1651, (D) DB1646, (E) DB1670 and (F) DB1627. Effect upon the parasites evaluated during the treatment at 37 °C with the drugs diluted in the culture medium. (G) Effect upon the parasites evaluated during the treatment at 4 °C with the drugs diluted in mouse blood. The percentage of dead parasites was measured after 2 and 24 h of treatment.

Table 1. IC_{50} and SI values for the effect of the aromatic diamidines on *T. cruzi*

Compounds	Bloodstream trypomastigotes[1]		Intracellular parasites[2]	
	IC_{50} (μM)	SI[3]	IC_{50} (μM)	SI[3]
DB1582	6·0	15·9	11·8	8·1
DB1627	>32	3	>32	3
DB1645	0·15	640	7·3	13
DB1646	31	3	23·3	4·1
DB1651	6·9	13·9	13·3	7·2
DB1670	32	3	>32	3

SI = Selectivity index corresponds to the ratio LC_{50}/IC_{50}.
[1] Direct effect of the aromatic diamidines on bloodstream trypomastigotes performed after incubation for 24 h at 37 °C in RPMI.
[2] Effect on intracellular parasites measured by the determination of trypomastigotes released into the supernatant culture medium (96 h of infection) performed after 72 h of treatment at 37 °C.
[3] LC_{50} >96 μM.

Next, to assess possible toxic effects towards mammalian host cells, uninfected cardiac cultures were treated for 24–72 h/37 °C with increasing doses (up to 96 μM) of each di-cation and their morphology and viability were evaluated. The data demonstrated that after 24 h of incubation with 96 μM of all compounds induced less than a 20% loss in cellular viability (data not shown). Following treatment for 72 h, DB1627, DB1645, DB1651 and DB1670 resulted in 36, 21, 45 and 22% loss of cellular viability at 96 μM drug concentration, while both DB1582 and DB1646 gave a <20% reduction (data not shown).

The assessment of the anti-parasitic activity of the 6 compounds against the intracellular forms was performed by incubating the *T. cruzi*-infected cultures (24 h of parasite contact) with selected non-toxic doses (up to 32 μM) and the number of parasites released into the supernatant was quantified after 96 h of infection (corresponding to 72 h of drug treatment). A dose-dependent effect was observed, resulting in a decline in the number of parasites released, with IC_{50} values of 7·3, 11·8 and 13·3 μM for DB1645, DB1582, and DB1651, respectively (Fig. 3A–C, Table 1). While DB1646 showed a moderate effect (IC_{50} 23·3 μM) (Fig. 3D), both DB1670 and DB1627 did not show trypanocidal activity against intracellular parasites similar to the results found with bloodstream extracellular parasites (Fig. 3E–F).

The IC_{50} and LC_{50} findings allowed determination of the selectivity index (SI) for each compound tested. The results demonstrated that the most active compounds also displayed the highest SI values for both extracellular bloodstream trypomastigotes as well as for intracellular forms, as can be seen for DB1645, DB1582 and DB1651 which were 640, 15·9 and 13·9-fold, respectively (Table 1). Similarly, high SI values (13, 8·1 and 7·2), for DB1645, DB1582 and DB1651, respectively, in intracellular parasites (Table 1) were found.

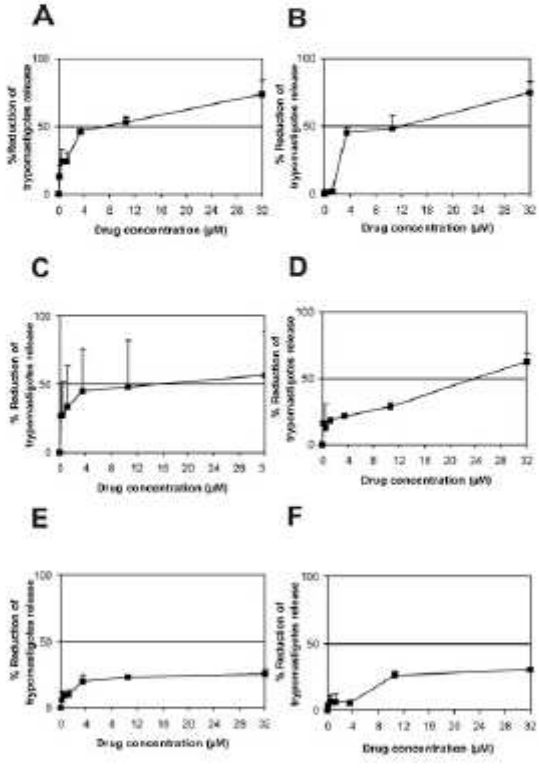


Fig. 3. Effect of the compounds upon intracellular parasites localized in *Trypanosoma cruzi*-infected cardiac cell cultures through the quantification of the number of parasites released into the supernatant of untreated and drug-treated infected cultures after 72 h of treatment at 37 °C. (A) DB1645, (B) DB1582, (C) DB1651, (D) DB1646, (E) DB1670 and (F) DB1627.

Due to the characteristics of the tested compounds, blue fluorescence is emitted when excited by UV light, the intracellular localization and distribution of these heterocyclic compounds in both

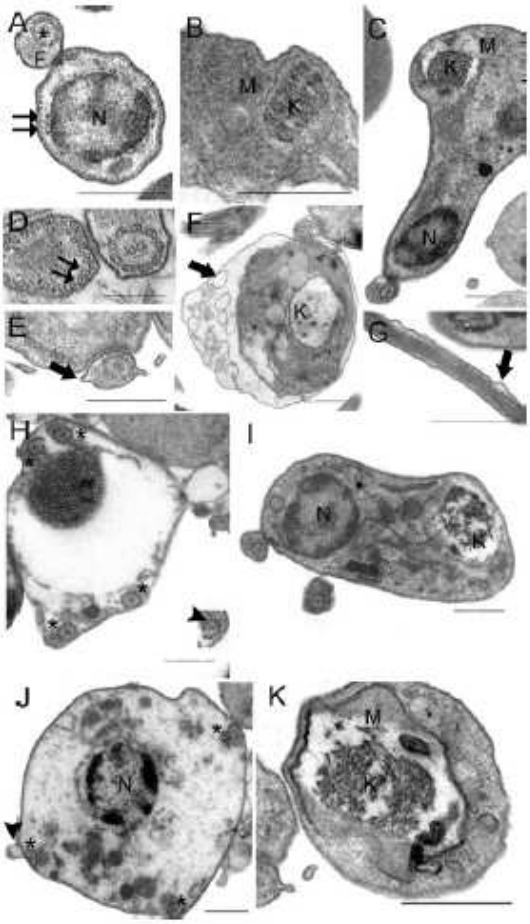


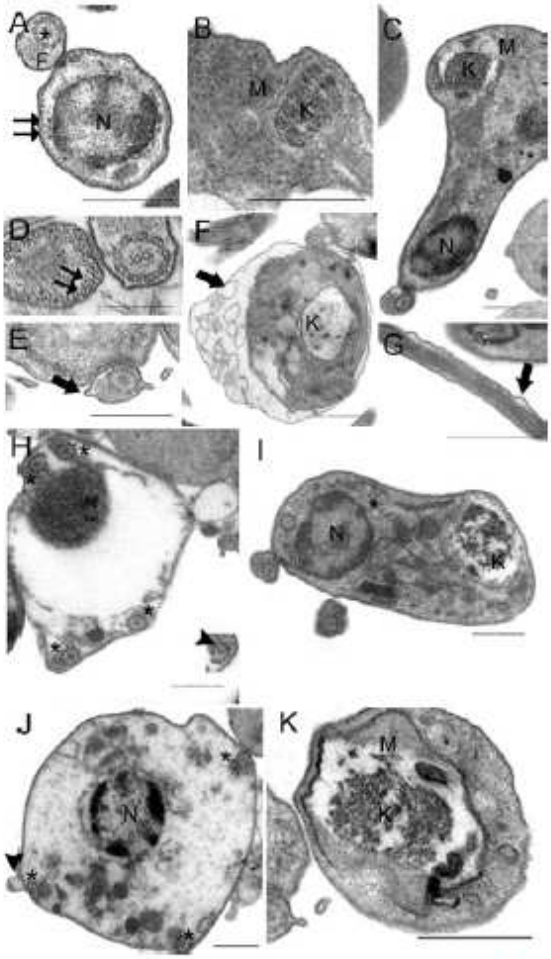
Fig. 5. Transmission electron micrographs of untreated bloodstream trypomastigote forms of *Trypanosoma cruzi* (A–B) and treated with the heterocyclic compounds for 2 (E–F, I and K) and 24 h (C–D, G–H and J). (C) DB1646, (D–F and K) DB1645, (G) DB1670, (H, J) DB1582 and (I) DB1651. (A–B) Untreated parasites showed characteristic nucleus (N), mitochondria (M), kinetoplast (K), subpellicular (double arrow) and axonemal (asterisks) microtubules. The compounds induced alterations in subpellicular (D, double arrow) and axonemal microtubules (H, inset, thin black arrow), detachment of plasma membrane (thick black arrow) of parasite body (F) and flagellum (E and G) and bleb formation (J, arrowhead), dramatic damage in the complex mitochondria-kinetoplast with kDNA disorganization and fragmentation (C, F, I and K) and multiple axoneme profiles (H–J, asterisks). Flagellum = F. A, C, E, F and H–J Scale bar = 0,5 μm. B, D and G Scale bar = 1 μm.

2009). The difference in efficacies of the diamidines and the arylimidamides is likely to be related to the significant differences in their physical properties. The amidines are highly basic molecules with pK_a values near 11, whereas the arylimidamides pK_a values are near 7. Consequently, at physiological pH amidines are protonated and thus cationic molecules and the arylimidamides are essentially neutral. This large difference in properties will significantly affect absorption and distribution and likely plays an important role in the differences in activity of the 2 classes of compounds.

Our present study also showed differences regarding drug susceptibility among intracellular parasites and bloodstream trypomastigotes, the latter being more sensitive. This disparity could possibly reflect differences in compound uptake and/or active extrusion, and/or different mechanisms of action upon non-dividing trypomastigotes and the highly multiplicative intracellular stages of the parasite, which are localized in the cytoplasm of infected host cells. In fact, although the precise mechanism of action of these cationic heterocyclic compounds has not been fully elucidated, it is likely that multiple modes of action are operative and then their transport represents a fundamental step in their action and contributing, in part, to their selectivity (Wilson *et al.* 2008; de Koning, 2001). Therefore it is possible that non-viable transport of the drugs to the cytoplasmic milieu (of both host cells and *T. cruzi*) could account for the lower susceptibility found in the intracellular parasites. However, the fact that these di-cationic compounds are localized in the nuclei of cardiac cells as well as within the kinetoplast and nuclei of amastigotes confirms their ability to cross the host cell plasmatic membrane reaching the intracellular parasites. Similar observations have been reported for other diamidines such as DB569 (De Souza *et al.* 2004). While several potential transporters that effectively carry diamidines have been studied in other parasites, including African trypanosomes, *Leishmania* species and *Plasmodium falciparum* (Carter *et al.* 1995; Bray *et al.* 2003; Barrett and Gilbert, 2006), the mechanisms of uptake of diamidines by *T. cruzi* is still unknown and deserves further investigation in order to understand the different profiles of susceptibility among the different evolutive forms of this protozoan.

The efficacy of these heterocyclic diamidines upon bloodstream forms was also evaluated in the presence of mouse blood, at 4 °C, considering the possible application of these compounds for prophylaxis of banked blood. Our present results show that all compounds display reduced activity in the presence of blood possibly due to their propensity to bind serum proteins as previously reported for other compounds or possibly due to drug instability or metabolism in the presence of blood constituents (Santa-Rita *et al.* 2006; Silva *et al.* 2008).

The fluorescence of many of these heterocyclic compounds makes it possible to follow their distribution in *T. cruzi* as was previously performed with African trypanosomes (Mathis *et al.* 2006, 2007; Wilson *et al.* 2008). As found with African trypanosomes incubated with the heterocyclic diamidines



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The fluorescence of many of these heterocyclic compounds makes it possible to follow their distribution in *T. cruzi* as was previously performed with African trypanosomes (Mathis *et al.* 2006, 2007; Wilson *et al.* 2008). As found with African trypanosomes incubated with the heterocyclic diamidines

DB75 and DB820 (Mathis *et al.* 2006, 2007), we also observed that all compounds display very strong fluorescence in the kinetoplast and, with less intensity, in the parasite nucleus. DB1651 and DB1582 also accumulated in non-DNA-containing punctuated organelles preferentially localized in the anterior portion of bloodstream trypomastigotes and near the nuclei and kinetoplast regions of amastigotes of *T. cruzi*. According to this intracellular distribution and morphology, and as suggested by previous studies with African trypanosomes (Mathis *et al.* 2007), these organelles are possibly acidocalcisomes, although other non-DNA-containing organelles can not be excluded. Acidocalcisomes are acidic calcium-storage organelles found in a diverse range of organisms, being first described in trypanosomes (Vercesi *et al.* 1994; Docampo *et al.* 1995). It is possible, as suggested for African trypanosomes, that the localization of these compounds within these acidic organelles of *T. cruzi* could also play a role in their mechanism of action and/or act as storage sites (Mathis *et al.* 2006, 2007). Previous studies performed with *T. brucei* reported that the localization of DB75 and DB820 (and their analogues) within non-DNA compartments was a time-dependent event (Mathis *et al.* 2006). However, in the studies performed here only 1 h of drug incubation was involved. Therefore, future studies are planned to further investigate whether longer periods of treatment (with DB1627, DB1645, DB1646 and DB1670) would lead to their localization in these cytoplasmatic organelles.

Since alterations in the fine structure of parasites evaluated by transmission electron microscopy provide insights into the nature of drug-induced lesions, allowing deduction of possible modes of action (Rodrigues and de Souza, 2008; de Souza, 2008), ultrastructural aspects of *T. cruzi* treated with the 6 compounds was investigated. The most prominent and usual alterations noticed with all compounds were related to changes in mitochondrial structure and the disorganization of the kDNA, as has been previously detected in *T. cruzi* treated with other diamidines (De Souza *et al.* 2004; Silva *et al.* 2008) as well as arylimidamides (Silva *et al.* 2007*b*). Other interesting findings were the dramatic alterations of microtubule organization induced by these heterocyclic compounds. In bloodstream trypomastigotes, the non-proliferative stage of *T. cruzi*, we observed that DB1582 induced disruption of microtubules axonemal organization and an unusual organization of multiple flagella without any evidence of flagellum duplication, as also previously reported for arylimidamides (Silva *et al.* 2007*b*). Some of the parasites treated with DB1645 also presented alterations in the structural organization of sub-pellicular microtubules in addition to modifications in the basic structure of (9 + 2) axonemal microtubules, showing more than 2 central microtubules. Microtubules are dynamic and very stable structures that play a role in several biological processes of protozoal parasites including cellular structural maintenance (subpellicular microtubules); motility (flagellar microtubules); and proliferation (basal body and mitotic spindles) (Menna-Barreto *et al.* 2009). Although different studies have been conducted using drugs that target microtubules such as taxol, colchicine and vinblastine, no major alterations were noticed in both subpellicular and flagellar microtubules of *T. cruzi* possibly due to the high content of acetylated tubulin and/or poly-glutamylation of tubulin (Souto-Padron *et al.* 1993; Dantas *et al.* 2003). Since these structures in trypanosomatids are very resistant to microtubule disrupters compared to those in mammalian cells they may represent interesting targets for drug development. Further investigations are needed to better understand the effect, if any, of these heterocyclic dicationic compounds upon *T. cruzi* microtubules.

Despite their high activity against a broad spectrum of microorganisms, a major concern for diamidines and related compounds is their selectivity (Soeiro *et al.* 2005). Therefore it is quite promising that these compounds show low toxicity towards mammalian cells. The most effective compound, DB1645, which gave excellent IC_{50} values against bloodstream trypomastigotes and intracellular parasites, exerted very low toxicity even after 72 h of treatment of cardiac cell cultures, leading to high selectivity indices (640 and 13, respectively). The identification of effective and selective compounds is a crucial element in drug development and the results reported herein justify further study of this class of compounds in experimental models of *T. cruzi* infection.

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Artigo # 04: Aceito à publicação na revista Antimicrobial Agents and Chemotherapy 2010

Título: **"Arylimidamide DB766: A potential chemotherapic candidate for Chagas disease treatment"**

Estado do conhecimento quando da concepção do trabalho:

- ✓ Estas limitações justificam a urgente necessidade de se desenvolver e selecionar novos compostos tripanocidas que possam substituir estes dois medicamentos introduzidos na clínica desde a década de 60,
- ✓ Arilimidamidas (AIA) representam uma nova classe de agentes tripanocidas derivados das amidinas reversas e que tem demonstrado excelente atividade sobre *Trypanosoma cruzi* em ensaios *in vitro* e *in vivo*.

- ➢ **Questões propostas:**

1. Avaliar a atividade antiparasitária, toxicidade e a seletividade *in vitro* da DB766 sobre formas intracelulares e tripomastigotas de sangue representantes de diferentes cepas e linhagens (cepa Y, YuYu, CL Brener e Colombiana), e de distintas regiões geográficas e ciclos de transmissão (peridomicilio e silvestre – isolados 762, 855, 875, 956, 958, 960, MR153 e RB vii), comparando com atividade de drogas de referência (Benznidazol violeta de genciana),
2. Verificar a localização, distribuição citoplasmática e alvos celulares desta AIA sobre formas tripomastigotas de sangue e intracelulares de *Trypanosoma cruzi* através de microscopia de fluorescência e de microscopia eletrônica de transmissão.
3. Investigar *in vivo* a toxicidade aguda e atividade tripanocida da DB766 sobre diferentes modelos experimentais de infecção aguda causada pelo *T. cruzi*, utilizando diferentes doses e esquemas de tratamento, e avaliando parâmetros parasitológicos (parasitemia e mortalidade), histopatológicos (carga parasitária e inflamação), clínicos (curva ponderal e eletrocardiograma), bioquímicos (dosagem de CK e GPT) e de cura parasitológica (Hemocultivo e PCR).

Seguem 12 páginas

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Arylimidamide DB766, a Potential Chemotherapeutic Candidate for Chagas' Disease Treatment[▽]

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Chagas' disease, a neglected tropical illness for which current therapy is unsatisfactory, is caused by the intracellular parasite *Trypanosoma cruzi*. The goal of this work is to investigate the *in vitro* and *in vivo* effects of the arylimidamide (AIA) DB766 against *T. cruzi*. This arylimidamide exhibits strong trypanocidal activity and excellent selectivity for bloodstream trypomastigotes and intracellular amastigotes (Y strain), giving IC_{50}s (drug concentrations that reduce 50% of the number of the treated parasites) of 60 and 25 nM, respectively. DB766 also exerts striking effects upon different parasite stocks, including those naturally resistant to benznidazole, and displays higher activity *in vitro* than the reference drugs. By fluorescent and transmission electron microscopy analyses, we found that this AIA localizes in DNA-enriched compartments and induces considerable damage to the mitochondria. DB766 effectively reduces the parasite load in the blood and cardiac tissue and presents efficacy similar to that of benznidazole in mouse models of *T. cruzi* infection employing the Y and Colombian strains, using oral and intraperitoneal doses of up to 100 mg/kg/day that were given after the establishment of parasite infection. This AIA ameliorates electrocardiographic alterations, reduces hepatic and heart lesions induced by the infection, and provides 90 to 100% protection against mortality, which is similar to that provided by benznidazole. Our data clearly show the trypanocidal efficacy of DB766, suggesting that this AIA may represent a new lead compound candidate to Chagas' disease treatment.

Chagas' disease (CD) is a neglected tropical illness that affects at least 12 million people in areas of disease endemicity in Latin America, where 50 million people are at risk of infection (22–24). In addition, many reports show that the occurrence of CD in areas where the disease is not endemic, such as the United States and Europe, is attributed mainly to the migration of infected people (30, 31, 50). The etiological agent is the intracellular obligatory parasite *Trypanosoma cruzi*, a hemoflagellate protozoan that presents a complex life cycle with distinct morphological stages in its obligatory passage through vertebrate and invertebrate hosts (63). CD can be transmitted by Triatominae insect feces, blood transfusion, organ transplantation, laboratory accidents, and oral and congenital routes (42, 66). It has the following two successive phases: a short acute phase characterized by a patent parasitemia, followed by a long progressive chronic phase that included mainly cardiac and/or digestive alterations (7, 61). The current therapy for CD comprised of nifurtimox (Nif) (5-nitrofurane, Bayer 2502; Bayer, Germany) and benznidazole (Bz) (2-nitroimidazole; Laboratório Farmacêutico do Estado de Pernambuco [LAFEPE], Brazil) has limitations, including long treatment periods, occurrence of side effects, variable results, and low efficacy during the chronic phase, which justify the search for new drugs (60, 69). Although Bz is not an ideal drug, it arrests electrocardiographic alterations, reduces progression of the disease, and protects the patients against deterioration of the clinical condition (70).

Aromatic diamidines (AD) and analogues target the minor groove of DNA and represent a promising class of antiparasitic agents (58, 65, 72). AD and their congeners present broad-spectrum activity against pathogenic microorganisms, and some of them have veterinary and human clinical uses (e.g., pentamidine isethionate [1,5-bis(4-amidino-phenoxy)pentane] (Pentacarinat; Rhodia)) (45, 57, 72). However, these compounds often cause considerable side effects and require parenteral routes of administration, which stimulates the synthesis and screening of new analogs and prodrugs to overcome these limitations (19, 34, 56, 59). The effect of several AD analogues was demonstrated against *T. cruzi*, and among them, significant *in vitro* activity was found for arylimidamides (AIAs), including DB889, DB786, and DB702 (46, 54, 55). Also, diamidines protect against mortality in mouse experimental models of acute *T. cruzi* infection (15, 19).

In the present work, our goal was to evaluate *in vitro* and *in vivo* the activity of a new synthesized AIA, DB766, against *T.*

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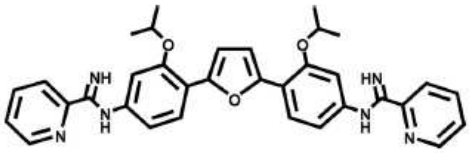

FIG. 1. Chemical structure of DB766.

cruzi. Our data showed the excellent trypanocidal effect of DB766 upon different forms and strains of the parasite, displaying efficacy similar to that of benznidazole in mouse experimental models of acute infection. Our results suggest that this AIA as well as other compounds of this class represent very promising candidates for Chagas' disease treatment.

MATERIALS AND METHODS

Drug. The synthesis of the AIA DB766 (Fig. 1) was performed, according to methodology previously reported by us (64). Stock solutions were prepared in dimethyl sulfoxide (DMSO), with the final concentration in the *in vitro* experiments never exceeding 0.6%, which did not exert any toxicity toward the parasite or mammalian host cells (data not shown). Bz (Rochagan; Roche) and gentian violet (Sigma-Aldrich) were used as reference drugs.

Parasites. The 12 strains of *T. cruzi* used in the present study are described in Table 1. Bloodstream trypomastigote forms (BT) were obtained from *T. cruzi*-infected Swiss mice at the peak of parasitemia (39). The Colombian strain was maintained by serial passages in C3H/He mice (25). In some assays, following parasite isolation from the different hosts (Table 1), epimastigote forms were maintained in liver infusion tryptose (LIT) medium, with weekly passages, and then were used in the *in vitro* studies (16). Intracellular amastigotes lodged within cardiac cell cultures were employed, as previously reported (18).

Samples collection and parasite isolation. The strains 762, 855, 875, 956, 958, and 960 of *T. cruzi* were isolated from *Triatoma brasiliensis* captured in peridomiciles in a rural area of Russas in the Vale do Jaguaribe (Ceará, Brazil). The RBviii and MS1523 strains were isolated from *Rhodnius brethesi* (Hemiptera; Reduviidae) and *Didelphis marsupialis* (Marsupialia; Didelphidae), respectively, captured in the Amazon forest (Rio Negro, Amazonas, Brazil). Parasite isolation through xenodiagnosis and hemoculture followed procedures previously described (27). The isolated parasites were transferred to LIT medium supplemented with 10% fetal calf serum by weekly passages (11). The cultures were maintained under controlled conditions (28°C) for the trypanocidal assays.

Mammalian cell cultures. For both drug toxicity and infection assays, primary cultures of embryonic cardiomyocytes (CM) were obtained from Swiss mice as previously reported (39). After purification, the CM were seeded at a density of 1.0×10^5 cells/well into 24-well culture plates or 0.5×10^5 cells/well into 96-well microplates containing gelatin-coated coverslips. The cultures were then sustained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum (FBS), 2.5 mM $CaCl_2$, 1 mM L-glutamine, and 2% chicken embryo extract. The cell cultures were maintained at 37°C in an atmosphere of 5% CO_2, and the assays were performed at least three times in duplicate.

Cytotoxicity tests. In order to rule out the toxic effects of DB766 on host cells *in vitro*, uninfected CM were incubated for 24 and 72 h at 37°C in the presence or absence of DB766 (0 to 96 μM) diluted in DMEM. The CM morphology and spontaneous contractibility were evaluated by light microscopy. The cell death rates were measured by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] colorimetric assay (41). The absorbance was measured at a wavelength of 490 nm with a spectrophotometer (VersaMax tunable microplate reader; Molecular Devices), which allows for the determination of LC_{50}s (drug concentrations that reduce 50% of cellular viability). Additionally, cellular viability was monitored through creatine kinase cardiac isotype (CK-MB) levels released into the supernatant of untreated and DB766-treated CM, as reported previously (62).

Trypanocidal analysis. BT (5.0×10^6 cells per ml) were incubated for 24 h at 37°C in RPMI 1640 medium (Sigma-Aldrich) supplemented with 5% FBS, in the presence or absence of serial dilutions of the compounds (0 to 32 μM). According to protocols already established by our group (M. N. Correia Soeiro and A. J. Romanha), experiments were also performed at 4°C, with BT maintained in freshly isolated mouse blood (96% and 50%) in the presence or absence of serial dilutions of the compound (up to 32 μM). After drug incubation, the parasite death rates were determined by light microscopy using a Neubauer chamber that allows the direct visualization and quantification of the number of motile and live parasites, and then IC_{50}s (drug concentrations that reduce 50% of the number of the treated parasites) were calculated. For the analysis of the effect on intracellular parasites, after 24 h of parasite-host cell interaction (10:1 parasite/CM ratio), the infected cultures were washed to remove free parasites and then incubated at 37°C with increasing but nontoxic doses of the test drug (up to 10.6 μM). The CM were maintained at 37°C in an atmosphere of 5% CO_2 and air, and the medium was replaced every 24 h. After drug exposure, the untreated and treated infected CM were fixed for 20 min at 4°C with 4% paraformaldehyde (PFA) and stained with 10 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for DNA staining to enable visualization of parasites and host cell nuclei and direct quantification of the infection levels. Finally, the samples were mounted with 2.5% 1.4-diazabicyclo-(2.2.2)-octane (DABCO) and examined using a Zeiss photomicroscope equipped with epifluorescence (Zeiss, Thornwood, NY). The mean number of infected host cells was then determined using at least 400 host cells for three independent experiments run in duplicate. Only characteristic parasite nuclei and kinetoplasts were counted as surviving parasites, since irregular structures could mean parasites undergoing death (18). The drug activity was estimated by calculating the inhibition levels of the endocytic index (EI; the percentage of infected cells versus the mean number of parasites per infected cell) (15). The IC_{50}s were averaged from at least three determinations done in duplicate.

For the analysis of the effect on epimastigotes, 5×10^6 parasites/ml were maintained as described above, harvested during exponential phase of growth (5-day-old culture forms), and incubated at 28°C for up to 96 h in LIT medium in the presence or absence (control samples) of increasing doses of DB766 (0 to 32 μM) and Bz (0 to 250 μM) (40). After daily quantification using a Neubauer

TABLE 1. *Trypanosoma cruzi* strains presently used[a]

T. cruzi strain	Origin	Host	Susceptibility to Bz[b]	*T. cruzi* lineage type (DTU)	Parasite forms
Y	São Paulo, Brazil	Acute human case	MR	II	BT and amastigotes
Colombian	Colombia	Chronic human case	R	I	BT
YuYu	Bahia, Brazil	*Triatoma infestans*	R	II	BT
CL Brener	RGS, Brazil	*T. infestans*	S		BT
762	Ceará, Brazil	*T. brasiliensis*	ND	I	Epimastigotes
855	Ceará, Brazil	*T. brasiliensis*	ND	I	Epimastigotes
875	Ceará, Brazil	*T. brasiliensis*	ND	I	Epimastigotes
956	Ceará, Brazil	*T. brasiliensis*	ND	I	Epimastigotes
958	Ceará, Brazil	*T. brasiliensis*	ND	I	Epimastigotes
960	Ceará, Brazil	*T. brasiliensis*	ND	I	Epimastigotes
MS1523	Amazonas, Brazil	*D. marsupialis*	ND	I	Epimastigotes
RBVIII	Amazonas, Brazil	*R. brethesi*	ND	Zymodeme III	Epimastigotes

[a] ND, not defined; DTU, discrete-type unit; BT, bloodstream trypomastigotes; RGS, Rio Grande do Sul.
[b] R, resistant; S, susceptible; MR, moderately resistant (*in vivo* drug susceptibility as described by Filardi and Brener [28]).

chamber, the IC_{50}s were averaged from at least three determinations done in duplicate.

Transmission electron microscopy (TEM) analysis. For TEM analysis, BT were treated for 2 to 24 h at 37°C with DB766 at the concentration of its IC_{50}, rinsed with phosphate-buffered saline (PBS), fixed for 60 min at 4°C with 2.5% glutaraldehyde diluted in 0.1 M cacodylate buffer, pH 7.2, and postfixed for 1 h at 4°C with 1% OsO_4 and 0.8% potassium ferricyanide using the same buffer. The parasites were dehydrated in a graded series of acetone and finally embedded in Epon. Sections were stained with uranyl acetate and lead citrate and examined using a Jeol JEM-1011 electron microscope (15).

Fluorescence microscopy analysis. For fluorescence analysis, BT (3×10^6 cells/ml) were incubated with 10 μg/ml DB766. Subsequently, the parasites were washed and mounted with 2.5% DABCO, and the fluorescence was analyzed with a Zeiss photomicroscope equipped with epifluorescence (Zeiss Inc., Thornwood, NY). The fluorescence intensity was determined using the program ImageJ 1.41 (NIH, Bethesda, MD) by the sum of the fluorescent pixel values in the selections (nuclear DNA [nDNA] and kinetoplast DNA [kDNA]). The results were expressed as kDNA/nDNA ratios that reflect the partition of kDNA and nDNA fluorescence measurements of at least 50 individual parasites (14).

Mouse infection and treatment schemes. Five- to seven-week-old female and male C3H/He (*H-2*^{k}) mice and eight-week-old male Swiss mice were obtained from the animal facilities of Fundação Oswaldo Cruz (CECAL; Rio de Janeiro, Brazil). All animal procedures were previously approved and carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA L-028/09, L-371/07, and L-486/08).

Mice were housed at a maximum of 8 per cage and kept in a specific-pathogen-free (SPF) room at 20 to 24°C under a 12-h light/12-h dark cycle and provided with sterilized water and chow *ad libitum*. Infection was performed by intraperitoneal (i.p.) injection of 10^2 or 5×10^3 bloodstream forms of the Colombian strain in C3H/He (*H-2*^{k}) mice, a model that reproduces features of the CCC strain (35) and of 10^4 Y strains in Swiss mice (19).

The animals were divided into the following groups: uninfected (noninfected and nontreated); untreated (infected but nontreated); and treated (infected and treated with up to 100 mg/kg/day DB766 or with 100 mg/kg/day Bz [15, 20]). Swiss and C3H/He (*H-2*^{k}) mice received 0.2 and 0.1 ml by i.p. dosing, respectively, or 0.1 ml by the oral route (gavage) of DB766 at 5 and 8 days postinfection (dpi) (Y strain), at the parasitemia onset (21 dpi and 5 dpi for the Colombian and Y strains, respectively), and then daily or every other day (e.o.d.) for 10 treatments. For the Bz treatment, the infected mice received 0.1 ml by gavage by following the same therapeutic schemes as described above. In all assays, only mice with positive parasitemia were used to compose the experimental groups ($n \geq 6$ animals per group).

Mouse acute toxicity. In order to determine the maximum tolerated dose (MTD) of DB766 for *in vivo* testing against *T. cruzi*, 8- to 10-week-old female and male Swiss Webster and C3H/He (*H-2*^{k}) mice were used. On day 1, each animal was i.p. injected with DB766 every 2 h with an increasing dose of the compound, starting at 5 mg/kg. After 2 h, an additional 15 mg/kg was applied (resulting in a cumulative dose of 20 mg/kg). After a further 2 h, 30 mg/kg was applied (cumulative dose = 50 mg/kg), after another 2 h, 50 mg/kg was applied (cumulative dose = 100 mg/kg), and finally, after another 2 h, 100 mg/kg was applied (cumulative dose = 200 mg/kg). On days 2 and 3, mice were inspected for toxic and subtoxic symptoms, according to OECD (Organisation for Economic Cooperation and Development) guidelines. Forty-eight hours after drug injection, the MTD was determined. The MTD of Bz was also evaluated as described above.

Parasitemia, mortality rate, and ponderal curve analyses. The level of parasitemia was checked by using the Pizzi-Brener method (8). Body weight was evaluated at 0 to 120 dpi, and the mortality was checked daily and expressed as a percentage of cumulative mortality (percent CM) (15, 20).

Electrocardiography (ECG). ECG recording and analysis were performed as previously described (15, 19). Briefly, mice were placed under stable sedation with diazepam (20 mg/kg i.p.) and fixed in the supine position, and eight-lead ECGs were recorded from 18-gauge needle electrodes subcutaneously implanted in each limb and two electrodes at precordial position lead II. The electrocardiographic tracings were obtained with a standard lead (dipolar lead DII), recording with amplitude set to give 2 mV/1 s. ECG was recorded by using band-pass filtering (Bio Amp; AD Instruments, Hastings, United Kingdom) between 0.1 and 100 Hz. Supplemental amplification and analog-digital conversion was performed with a PowerLab 16S instrument (AD Instruments, Hastings, United Kingdom). Digital recordings (16 bit, 4 kHz/channel) were analyzed with the Scope (version 3.6.10) program (AD Instruments). The signal-averaged ECG (SAECG) was calculated by using the mouse SAECG extension (version 1.2) program (AD Instruments) and a template-matching algorithm. The ECG parameter was evaluated using the following standard criteria: the heart rate was monitored by beats per minute.

Histopathological analysis. Heart tissue was removed, cut longitudinally, rinsed in ice-cold PBS, and fixed in Millonig-Rosman solution (10% formaldehyde in PBS). The tissue was dehydrated and embedded in paraffin. Sections (3 μm) were stained by hematoxylin and eosin and analyzed by light microscopy. The numbers of amastigote nests and inflammatory infiltrates (more than 10 mononuclear cells) were determined with at least 100 fields (total magnification, 4×) for each slide. The mean number of amastigote nests or inflammatory infiltrates per field was determined from at least three mice per group, with three sections from each mouse.

Biochemical analysis. At different times of infection, mouse blood was collected and immediately submitted for determination of glutamate pyruvate transaminase (GPT) and creatine kinase (CK) using the Reflotron system (Roche Diagnostics, F. Hoffmann-La Roche Ltd., Basel, Switzerland) (53).

Cure assessment. Cure criteria were based on the following two parasitological methods: PCR and hemoculture assays, which detect parasite kDNA minicircle-specific sequences or the parasite itself, respectively. Animals presenting negative results by both tests were considered cured. Briefly, 60 days after drug treatment, 1,000 μl of blood was collected from the hearts of anesthetized mice, and then 500, 200, and 250 μl were used for PCR, hemoculture, and biochemical analyses, respectively. For PCR, the blood was diluted in a 1:3 volume of guanidine solution (guanidine-HCl [6 M]/EDTA [0.2 M]) and heated for 90 s in boiling water in order to cleave the parasite kDNA network (10). The PCR was performed using primers (5′AAATAATGTACGGG[T/G]GAGATGCATGA3′ and 5′GGTTCGATTGGGGTTGGTGTAATATA3′), which amplify a 330-bp sequence from the kinetoplast DNA (approximately 120,000 copies/parasite), as previously described by Wincker et al. (1994) (73). The PCR was carried out using GeneAmp PCR system 9700 (Applied Biosystems) as follows: one step at 94°C for 3 min (to activate the *Taq* DNA polymerase), 2 cycles at 98°C for 1 min and 64°C for 2 min, 38 cycles at 94°C for 1 min and 64°C for 1 min, followed by a final extension at 72°C for 10 min. The amplification products were detected on a 1.50% agarose gel electrophoresis following staining with ethidium bromide (5 mg/ml). For hemoculture, 200 μl of blood was added to 5 ml LIT medium, incubated at 28°C for 60 days, and weekly examined by light microscopy to detect epimastigote forms (28). Only negative hemoculture samples were further screened by PCR analysis.

Statistical analysis. Statistical analysis was carried out using Student's *t* test, with the level of significance set at P values of <0.05. The data represent means ± standard deviations from 3 experiments run in duplicate.

RESULTS

The incubation of BT (Y strain) for 2 h in the presence (4°C) or absence (37°C) of 96% mouse blood resulted in a dose-dependent effect, yielding IC_{50}s of 1.14 ± 0.05 and 0.56 ± 0.03 μM, respectively (Fig. 2A and B). After 24 h of treatment, DB766 was highly effective, giving IC_{50}s of 0.11 ± 0.07 μM and 0.060 ± 0.004 μM after incubation at 4°C and 37°C, respectively (Fig. 2A and B). The activity of this AIA and that of furamidine (DB75) against BT were further studied in another set of assays employing parasite strains with different patterns of natural resistance to Bz and Nif, including a CL-susceptible pattern and Colombian- and YuYu-resistant patterns, which were compared to those of parasites from the moderately resistant Y strain (28). The incubation at 4°C with the compounds diluted in 50% mouse blood showed that no significant effect was found with DB75 ($IC_{50} > 32$ μM) (Table 2). However, treatment with DB766 resulted in striking activity, regardless of the drug resistance phenotype of the strain. The IC_{50}s of DB766 yielded values of ≤1 μM (Table 2), which were about 30-fold lower than that of gentian violet (30.6 μM) (data not shown). Also, DB766 was equally active upon epimastigote forms from eight different *T. cruzi* stocks obtained from peridomiciliar and sylvatic triatomines and from a sylvatic mammalian host (*D. marsupialis*) (Table 3). DB766 was more active than Bz against all parasite stocks (Table 3). It is worth noting

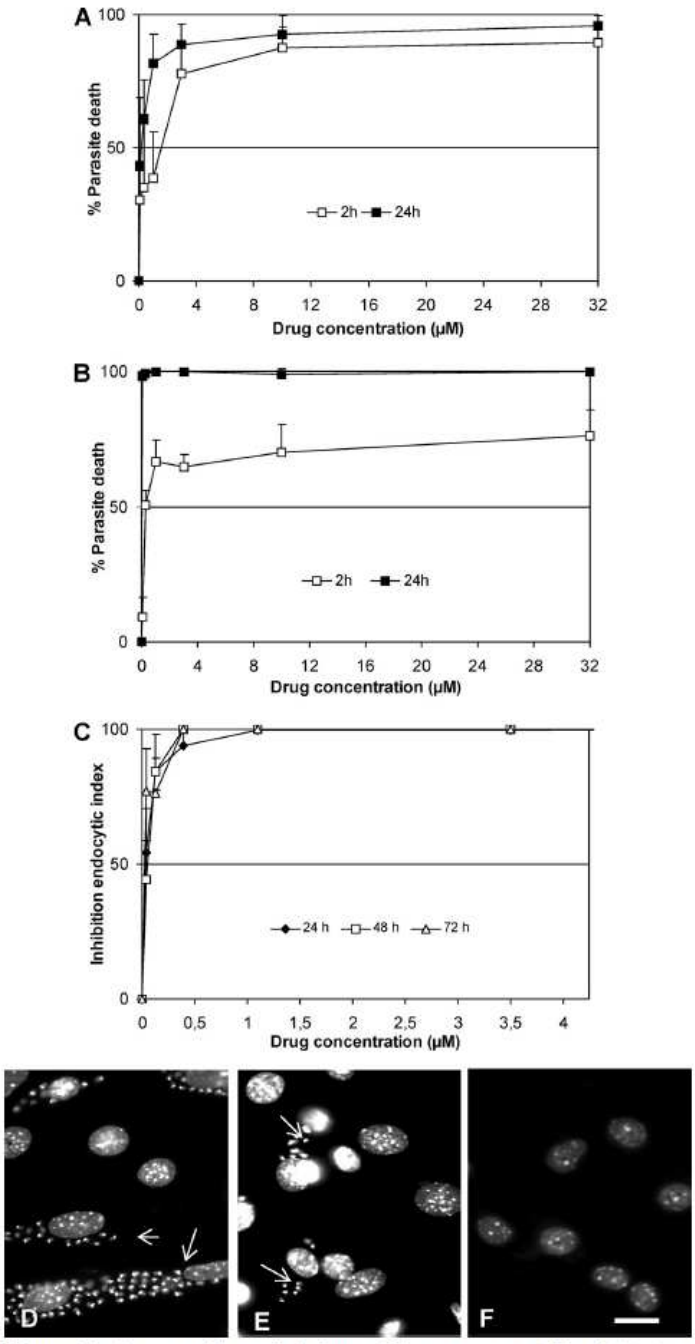

FIG. 2. Activity of DB766 upon bloodstream and intracellular forms of *Trypanosoma cruzi* (Y strain) *in vitro*. (A, B) Activity of DB766 upon bloodstream trypomastigotes during the treatment at 4°C with the drug diluted with 96% of mouse blood (A) and during the treatment at 37°C with the drug diluted in the culture medium (B). The percentages of dead parasites were measured after 2 h and 24 h of treatment. (C to F) Activity of DB766 on *T. cruzi*-infected cardiomyocytes (C), with illustrations showing untreated cultures (D) and cultures treated for 48 h with 0.04 μM (E) and 10.6 μM (F) doses of DB766. Arrows indicate intracellular forms. Bar = 1 μM.

TABLE 2. *In vitro* activity of DB766 and DB75 upon bloodstream trypomastigotes from different *T. cruzi* stocks

Compound	IC_{50} (μM)			
	YuYu	Colombian	Y	CL
DB75	>32	>32	>32	>32
DB766	0.82	1	0.71	0.71

that while treatment of the 855 and 875 strains with DB766 gave IC_{90} values of 0.33 ± 0.03 μM and 0.24 ± 0.09 μM, the treatment of those with Bz gave values of 225.6 ± 9.41 μM and 195.7 ± 73.8 μM, respectively (Table 3).

Prior to assessing the activity of DB766 on *T. cruzi*-infected cardiomyocytes, its threshold for toxicity on mammalian cells was determined by MTT. LC_{50}s were higher than 32 and 16.42 μM after 24 and 72 h of DB766 incubation, respectively. Morphological and biochemical findings (dosage of CK-MB) confirmed the toxicity of DB766 only in doses greater than 10.6 μM (data not shown). This value became the highest drug concentration used in the tests with *T. cruzi*-infected CM. DB766 also proved to be extremely potent against intracellular forms (Y strain), yielding an IC_{50} of 0.025 ± 0.006 μM after 72 h of drug treatment, which corresponds to 96 h of infection (Fig. 2C to F).

Due to the characteristic fluorescence of DB766, it was possible to follow its subcellular distribution in *T. cruzi*. This arylimidamide accumulated in the nuclei and kDNA of the parasites (Fig. 3A). Since consistently higher labeling was found in the kDNA compared to that found in the parasite nuclei, the accumulation of 10 μg/ml DB766 was evaluated in both of the DNA-enriched structures over a period of time, and the results were expressed as kDNA/nDNA ratios (14). Our data showed a statistically significant ($P = 0.003$) time-dependent increase in kDNA binding compared to that in the nucleus, reaching ratio values of 2.1 ± 0.5 after 60 min of drug exposure (Fig. 3A, inset).

To further explore the parasite targets of DB766, TEM studies were performed with BT treated for 2 and 24 h. Our findings showed that even after short periods of incubation, the main and highly consistent ultrastructural alteration was related to the parasite nucleus and, importantly, to the mitochondria, with considerable mitochondrial swelling and crista dilation (Fig. 3D and E).

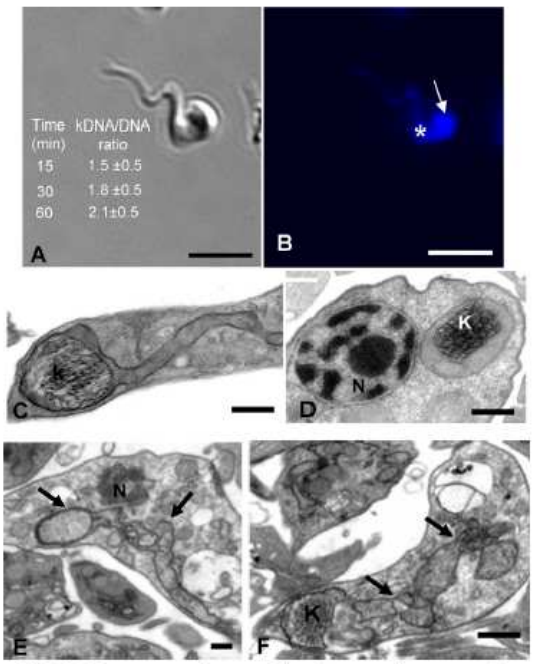

FIG. 3. Fluorescent and transmission electron microscopy analyses of the effect of DB766 on trypomastigotes of *T. cruzi*. Differential interference contrast (A) and fluorescent (B) analyses showing intracellular localization within bloodstream trypomastigotes of *T. cruzi* after treatment for 1 h at 37°C with 10 μg/ml DB766. Note the localization of this arylimidamide within the kDNA (arrow) and nuclei (asterisk). (C to F) Transmission electron microscopy showing mitochondrial alteration, with swollen cristae (arrows) in bloodstream-treated parasites (E, F), while untreated parasites display characteristic morphology (C, D). N, nuclei; K, kDNA. Bar = 0.5 μM.

Our first *in vivo* approach evaluated DB766 and Bz acute toxicity in C3H/He ($H\text{-}2^k$) mice and female and male Swiss mice. The administration of increasing doses of both drugs up

TABLE 3. *In vitro* activity of DB766 and Bz upon epimastigotes from different *T. cruzi* stocks

Parasite stock	Inhibitory concn (μM) of[a]:			
	Bz		DB766	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Y	7.65 ± 3.8	86.56 ± 38.18	0.07 ± 0.006	0.22 ± 0.07
762	ND	ND	0.06 ± 0.007	0.18 ± 0.10
855	12.57 ± 3.13	225.58 ± 9.41	0.09 ± 0.01	0.33 ± 0.03
875	7.9 ± 2.29	195.74 ± 73.76	0.06 ± 0.02	0.24 ± 0.09
956	3.96 ± 1.04	20.42 ± 6.08	0.16 ± 0.11	0.32 ± 0.05
958	13.70 ± 4.17	58.33 ± 7.22	ND	ND
960	6.44 ± 0.75	38.17 ± 16.73	0.09 ± 0.02	0.28 ± 0.07
MS1523	5.30 ± 0.14	30.59 ± 35.08	0.17 ± 0.10	0.41 ± 0.042
RBVIII	5.06 ± 3.53	35.68 ± 1.51	0.18 ± 0.14	0.44 ± 0.08

[a] ND = not done.

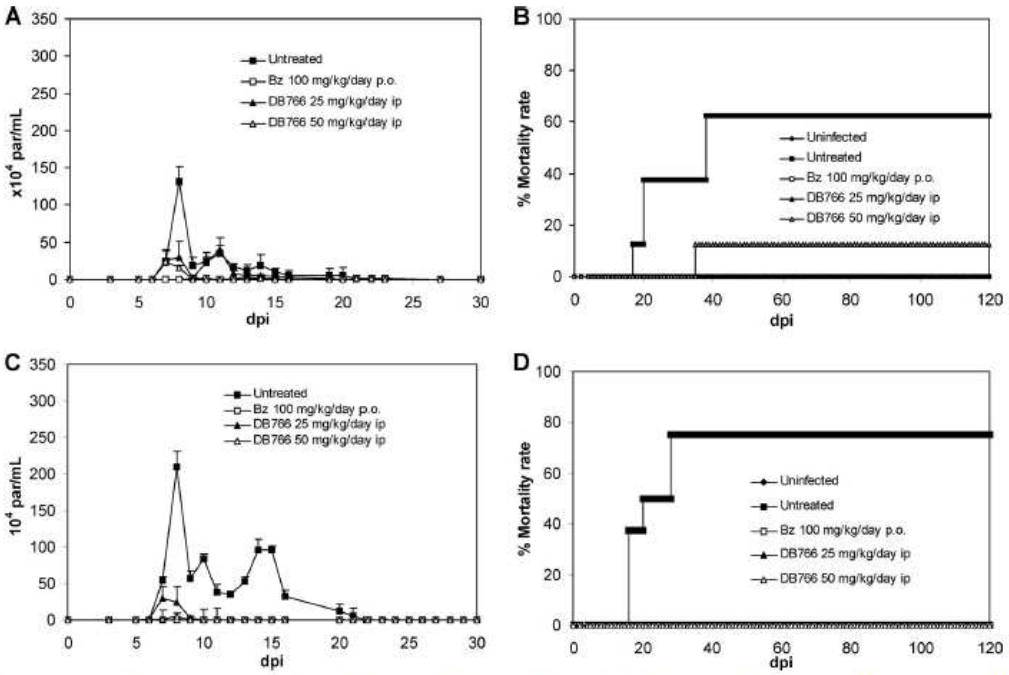

FIG. 4. Effect of DB766 in *T. cruzi* infection (Y strain) *in vivo*. The activity of 25 and 50 mg/kg/day DB766 (i.p.) and 100 mg/kg/day benznidazole (by p.o. gavage) was evaluated upon *T. cruzi* infection in male Swiss mice inoculated with 10^4 bloodstream trypomastigotes, using therapy regimens of 2 doses (5 and 8 dpi) (A, B) and 10 daily doses (starting at 5 dpi) (C, D). Parasitemia curves (A to C) and mortality rates (B to D) are presented. par, parasites.

to 200 mg/kg induced neither mortality nor major detectable toxic side effects up to 48 h after drug injection, except weight loss that reached about 10% and 7% in male Swiss mice treated with DB766 and Bz, respectively (data not shown).

DB766 activity was first assayed in Swiss mice inoculated with 10^4 bloodstream parasites (Y strain) using two doses (25 and 50 mg/kg/day i.p.) at the onset (5 dpi) and at the parasitemia peak (8 dpi). In parallel, a control group was orally (p.o.) treated with Bz (100 mg/kg/day) as the reference drug. DB766 was highly active *in vivo*. When infected mice were treated for only 2 days with 25 and 50 mg/kg/day, the peak load of circulating parasites (at 8 dpi) was reduced by 78% and 88%, respectively, showing animal survival rates (at 120 dpi) of 100 and 88%, respectively, in comparison with a survival rate of 37.5% in the infected and untreated group (Fig. 4A and B). As this treatment regimen did not result in a parasitological cure, as evaluated by both hemocultive and PCR assays of all surviving mice, including Bz-treated animals (data not shown), we extended the treatment to a 10-day regimen, starting at the parasitemia onset (5 dpi) and continuing for 10 consecutive daily doses. DB766 at 25 and 50 mg/kg/day led to a reduction of the levels of parasitemia at the peak (8 dpi) by 88% and 98%, respectively (Fig. 4C). Furthermore, the survival rate was 100% at 120 dpi (Fig. 4D). The analysis of body weight showed that the animals treated with both doses of DB766 recovered the body weight loss induced by the parasite infection at a lower rate than with Bz treatment (data not shown).

Next, the later dosing scheme was adopted for mice infected with 10^2 and 10^3 bloodstream trypomastigotes of the Colombian strain, starting treatment at the onset of parasitemia (21 dpi) (Fig. 5). A striking decrease in parasitemia occurred in mice infected with 10^2 organisms when both doses were tested by an i.p. route, reaching about 73% reduction in the mean number of parasites using the 25 mg/kg/day dose (parasitemia peaked at 44 dpi) compared to that in untreated mice, which displayed $468 \pm 219 \times 10^4$ parasites/ml at the parasitemia peak (44 dpi) (Fig. 5A). Mice treated with 100 mg/kg/day Bz or 50 mg/kg/day DB766 presented a delayed parasitemia peak (59 dpi), reaching maximum levels of 54 ± 34 and $33 \pm 36 \times 10^4$ parasites/ml, respectively (Fig. 5A). At 120 dpi, Bz-treated mice present increased GPT levels, while neither doses of DB766 caused hepatic alterations (data not shown) Similar to the finding observed during the infection with the Y strain, animals of both dosage groups of DB766 did not recover the body weight loss induced by the Colombian infection at the same rate as those treated with Bz (data not shown).

The i.p. administration of DB766 at 25 and 50 mg/kg/day for 10 consecutive days also proved effective for controlling parasitemia in mice infected with 10^3 trypomastigotes of the Colombian strain, leading to a profound reduction in the mean

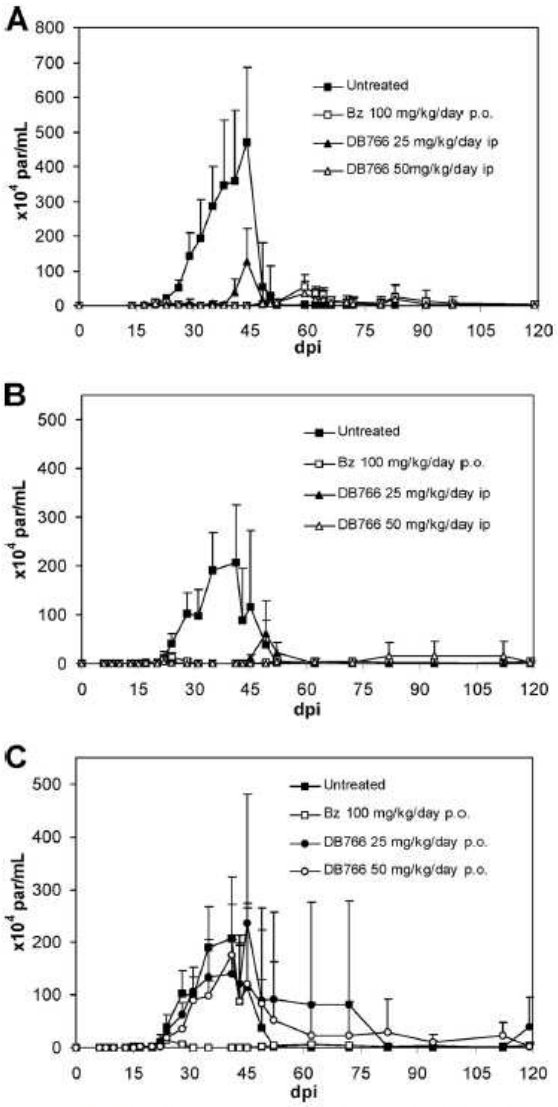

FIG. 5. Effect of DB766 on *T. cruzi* infection (Colombian strain) *in vivo*. Activity of 25 and 50 mg/kg/day DB766 and 100 mg/kg/day benznidazole upon parasitemia curves of *T. cruzi* infection in female C3H mice inoculated with 10^2 (A) and 10^3 (B, C) bloodstream trypomastigotes using a regimen of 10 daily doses (starting at parasitemia onset). DB766 was administrated by i.p. (A, B) and p.o. (C) routes, while Bz (A to C) was given only by gavage.

number of circulating parasites (Fig. 5B). At 41 dpi (parasitemia peak), 50 mg/kg/day DB766 showed efficacy similar to that of Bz (Fig. 5B). However, when Colombian strain-infected mice were orally treated with DB766, neither dose reduced parasitemia (Fig. 5C). Next, oral administration of DB766 at a higher dose (100 mg/kg/day) was given for 10 days, for comparison with the i.p. administration. At this dose, oral administration of DB766 presented activity similar to that of Bz. At 31 dpi, a strong reduction of parasitemia—98% for DB766 p.o. and 99% for Bz—was observed, resulting in 100% survival, while all the infected and untreated mice were dead at day 120 (Fig. 6A and B). Treatment with DB766 by the i.p. route, although showing an impressive parasitemia reduction (>99%), led to the survival of only 60% of the animals (Fig. 6A and B). Additionally, i.p. administration of 100 mg/kg/day DB766 profoundly decreased cardiac parasitism and inflammation at 43 dpi, showing 95%- and 75%-higher reduction in both parameters, respectively, compared to that of the infected and Bz-treated mouse groups (Fig. 6C and D). Interestingly, while Bz-treated mice displayed an increase of 68% in CK, treatment with DB766 by both routes showed enzyme levels similar to those of the uninfected group (Fig. 6E). ECG analysis also showed that DB766, by both routes, but not Bz, yielded heart rate levels similar to those of the uninfected group (Fig. 6F). In addition, dosing of DB766 by both routes did not significantly alter GPT plasma levels compared to that of the uninfected group, while the reference drug showed an enhancement of 54% (Fig. 6G). The mice treated with DB766, by both routes did not recover the weight loss induced by the parasite infection at the same rates as those treated with Bz (Fig. 6H).

Since i.p. treatment with 100 mg/kg/day DB766 led to a reduction in circulating and cardiac parasitism superior to that of Bz, but it failed to provide 100% protection against mouse mortality, and this AIA yields long half-lives and large volumes of distribution (M. Z. Wang, unpublished data), our next step was to evaluate whether DB766 administration on every other day (e.o.d.; total of 5 doses, with each dose given every 48 h) could retain the efficacy and improve animal survival by reducing possible drug toxicity. Our data showed that administration (i.p.) of 100 mg/kg/day DB766 for 10 alternated days resulted in a strong reduction in parasitemia levels (Y strain), reaching >98% of inhibition while Bz provided only a 79.4% reduction (Fig. 7A). Histopathological analysis performed at 14 dpi confirmed the high efficacy of DB766 that importantly reduced cardiac parasitism (94%) and inflammation (78%), giving results similar to those for Bz use (Fig. 7C and D). Biochemical analysis of CK and GPT levels at 14 dpi showed that under this therapy scheme, DB766 and Bz were able to protect against muscle (Fig. 7E) and hepatic (Fig. 7F) lesions induced by parasite infection. Although this scheme of treatment also led to complete recovery of the body weight loss induced by the infection (Fig. 7G), DB766 treatment protected 80% of the animals against mortality, whereas Bz protected 100% of them (Fig. 7B).

Finally, although DB766 and Bz presented remarkable efficacy in reducing parasitemia and mortality rates even against the naturally Bz-resistant Colombian strain, in all schemes (i.p. and p.o.; daily and e.o.d. dosing), both drugs failed to induce parasitological cure, as evaluated by light microscopy, hemoculture, and PCR analyses (data not shown).

DISCUSSION

It has been suggested that the ideal drug for CD etiological treatment should fulfill the following requirements: (i) be orally effective against the acute and chronic phases of infec-

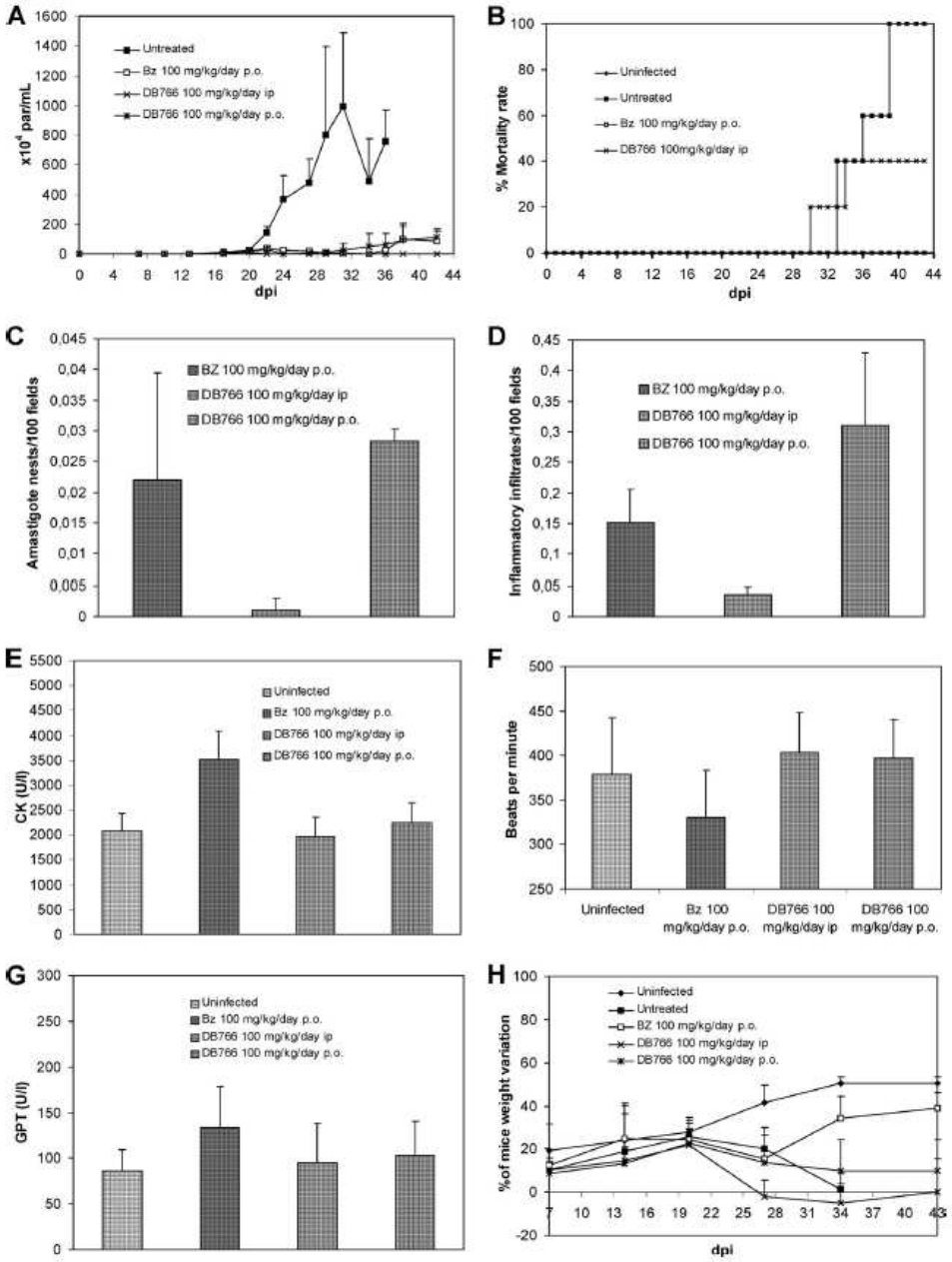

FIG. 6. Effect of daily administration of DB766 on *T. cruzi* infection *in vivo*. Activity of 100 mg/kg/day DB766 (i.p. and p.o.) and benznidazole (p.o.) upon *T. cruzi* infection in male C3H mice inoculated with 5×10^3 bloodstream trypomastigotes (Colombian strain) using 10 daily doses, starting at the parasitemia onset. (A) Parasitemia curve; (B) mortality rates; (C) cardiac parasitism; (D) cardiac inflammation; (E) plasma CK levels; (F) cardiac frequency; (G) plasma GPT levels; (H) ponderal curve. U/l, units/liter.

tion, using a single or few doses (treatment regimen under 60 days); (ii) exert no toxicity to the patients; (iii) provide pediatric formulation; (iv) be active against a broad panel of *T. cruzi* stocks and lineages; and (v) be effective against bloodstream trypomastigotes, inhibiting the invasion of new cells, and upon intracellularly dividing amastigotes, preventing the release of new infective parasites (6, 38, 51). In the present study, experimental assays were designed in order to address

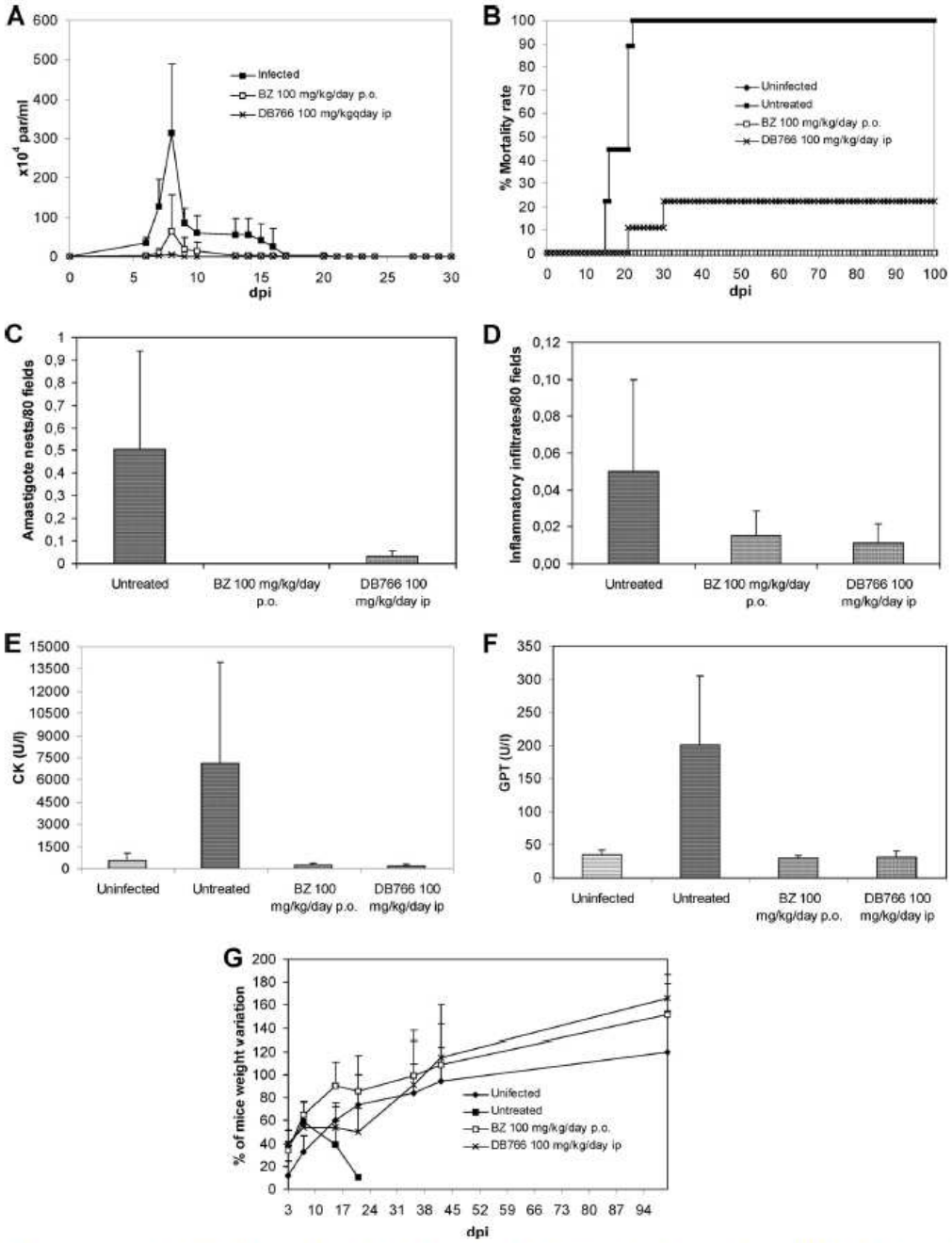

FIG. 7. Effect of e.o.d. treatment of DB766 on *T. cruzi* infection *in vivo*. Activity of 100 mg/kg/day DB766 (i.p.) and benznidazole (p.o.) upon *T. cruzi* infection in male Swiss mice inoculated with 10^4 bloodstream trypomastigotes (Y strain) and treated e.o.d., starting at the parasitemia onset. (A) Parasitemia curve; (B) mortality rates; (C) cardiac parasitism; (D) cardiac inflammation; (E) plasma CK levels; (F) plasma GPT levels; (G) ponderal curve.

these requirements, considering the complexity of the parasite biology. Therefore, we investigated the DB766 activity through *in vitro* and *in vivo* assays using highly stringent protocols, evaluating the toxicity on mammalian cells and the efficacy against amastigote and trypomastigote forms, on different parasite strains (including Bz-susceptible and -resistant stocks), and through short schemes and routes of treatment *in vivo*. With the goal of establishing a head-to-head comparison of efficacy and toxicity between Bz and DB766, similar therapeutic schemes were employed.

Our analysis confirmed previous reports regarding the excellent activity of AIAs *in vitro* against *T. cruzi* (46, 54, 55) and *Leishmania* spp. (52), being superior to that of aromatic diamidines (18, 52). As observed in this study with DB766, AIAs display low toxicity on mammalian cells and are highly active at nanomolar doses against both BT and amastigotes (Y strain) as little as 2 h after drug treatment. AIAs also retain the activity at 4°C in the presence of blood constituents, thus having potential use in blood banks (55). In this regard, DB766 showed an activity that was 30.6-fold higher than that of gentian violet, a reference drug for blood therapy in CD. In addition, DB766 was active against parasite isolates that circulate in peridomiciliar and sylvatic ecotopes in the following two different regions in Brazil: (i) the northeast (Jaguaribe Valley, Ceará state), which represents an important area for CD surveillance, where high rates of natural triatomine infection are observed (mostly *T. cruzi* type I lineage) (M. M. Lima, unpublished data) and vectorial control still requires effort to avoid new cases of human transmission (2, 21), and (ii) the Amazon region, which presents an important new epidemiologic challenge due to the increasing reports of human acute cases, mainly by oral contamination as well as by wild triatomine vectorial transmission (1).

Since several strains of *T. cruzi* exhibit different levels of resistance to Nif and Bz, and this variability may partially explain the observed differences in effectiveness of chemotherapy involving these two compounds (9, 51, 60), further *in vitro* studies were performed comparing the activities of DB766 and BT on *T. cruzi* strains that present naturally Bz-susceptible (CL), -moderate (Y), and -resistant (Colombian and YuYu) profiles. Here we have documented the high trypanocidal activity of DB766, regardless of the drug resistance profile. The significant activity of DB766 upon several parasite strains represents a very important finding, since *T. cruzi* comprises numerous clonal populations with distinct characteristics, such as different sensitivity to Nf and Bz, diverse biological parameters, and enzymatic diversity (26, 28, 43, 47–49). In addition, strain heterogeneity may also be related to the different clinical manifestations and outcomes in CD (3, 38).

Interestingly, DB766 presented higher efficacy than DB75, confirming that the activity of AIAs is superior to that of AD on *T. cruzi* (46, 55). This improved activity of AIAs compared to that of AD may be due to differences in their physical properties, as follows: while AD are highly basic molecules, with a pK_a of near 11, the AIA pK_a is near 7. Therefore, at physiological pH, amidines are protonated and, thus, cationic molecules, while the AIAs are essentially neutral molecules, enabling their passive diffusion through membranes of both parasite and host cells. This large difference in physical properties probably affects absorption and distribution and likely plays an important role in the differences in activity of these two classes of compounds (5).

The fluorescent and transmission electron microscopy studies showed that DB766 is localized in the parasite nucleus and in kDNA, accumulating in larger amounts in the latter structure. The AIAs promoted ultrastructural alterations in the mitochondrial cristae. Our data corroborate with previous studies with AD and AIAs performed with *Leishmania* spp. (13, 29, 33), *T. cruzi* (15, 18, 46, 54), and *Trypanosoma brucei* (37). These results suggest that DB766 may act on the mitochondrion-kinetoplast complex, although other cellular targets cannot be excluded and further biochemical studies are needed.

Our *in vitro* results show the excellent activity of DB766 against different *T. cruzi* strains, with efficacy superior to those of Bz and gentian violet. The outstanding selectivity index, ranging between >533 (for BT, Y strain) and 714 (for amastigotes, Y strain), is at least 10-fold higher than the 50-fold index proposed by Nwaka and Hudson (44). All together, these data justify further studies *in vivo* employing different models of experimental *T. cruzi* infection. Since no major acute toxicity was noted with uninfected mice treated by DB766, we moved to using models of experimental *T. cruzi* infection. In these studies, the stringency level was increased after initially evaluating the effect upon the Y strain and then advancing to the Colombian strain experimental model. The evaluation of the effects of DB766 was performed using a combination of parasitological, biochemical, clinical, and histopathological tests, which allowed for a thorough investigation of its activity and toxicity.

Previous results demonstrated that AD such as DB569 (20) and DB1362 (15), although not affecting the parasitemia, reduced the cardiac parasitism and inflammation and downmodulated the expression of $CD8^+$ T cells in heart tissue (19). While both of the AD showed lower trypanocidal efficacy than Bz, DB766 presented activity similar or even superior to that of this reference drug, resulting in 100% protection against mortality, depending on the therapy scheme used. This protection in DB766-treated mice is likely derived from the reduction of tissular lesions (e.g., cardiac and hepatic), resulting from a decrease in the parasite load itself and/or the lower inflammation levels (36). In fact, reduced plasma levels of CK and GPT were consistently found in DB766-treated mice. Furthermore, DB766 also reversed the ECG alterations induced by the parasite infection, corroborating previous data on diamidines (15, 19).

Since *T. cruzi* is an obligatory intracellular parasite and reservoirs of amastigotes can be found in quite distinct organs and tissues, the ability of AIAs such as DB766 to traverse host cell membranes possibly by passive diffusion and or use of transporters (4, 17), along with their extensive tissue binding in the liver, spleen, and heart (71), makes this class of compounds very attractive for CD treatment. In fact, DB766 proved to be quite effective against intracellular amastigotes, which are considered the main stage for drug targeting (38). The effectiveness of AIAs like DB766 in eradicating intracellular parasites probably is related to their pharmacokinetic properties, including long half-lives and large volumes of distribution (71). These pharmacokinetic characteristics of DB766 are especially relevant, since the poor activity of the nitroheterocyclic com-

pounds during the chronic stages of CD may be related to their unfavorable pharmacokinetic properties, such as relatively short half-lives and limited tissue penetration (69).

Oral administration of DB766 at 100 mg/kg/day showed good efficacy, with reduced circulating and cardiac parasitism, decreased CK and GPT levels, prevention of electric abnormalities induced by the parasite infection, and significantly reduced mouse mortality without major side effects. These data suggest that sufficient quantities of this AIA were absorbed from the mouse gastrointestinal tract, thus effectively delivering DB766 molecules across the gut mucosa, similar to the results reported for the AD prodrug DB289 (32, 67, 68).

The only overt adverse effect noted for DB766 was moderate body weight loss, which may be the cause of the lower animal survival rate (about 60%) found during the administration of 100 mg/kg/day DB766 by the i.p. route. Nevertheless, this dosing resulted in almost undetectable parasite levels in blood and heart and trypanocidal efficacy superior to that of Bz. With the goal of maintaining this superior efficacy but reducing possible drug toxicity, as well as taking into consideration the extensive tissue binding and the relatively long half-life of DB766, we evaluated the effect of 100 mg/kg/day of this AIA through i.p. administration every other day. We found that in addition to keeping the high trypanocidal efficacy, mouse weight recovery at levels similar to those of Bz and uninfected mouse groups was achieved. Using the e.o.d. therapy of DB766, the mouse survival rates improved to 80%. However, the survival rates with this dosage scheme did not reach the same levels as those of Bz and DB766 given orally. Despite these encouraging results, DB766 (using both i.p. and p.o. routes) and Bz did not provide a parasitological cure, as evaluated by the hemocultive and PCR methods. This therapeutic failure may be due to the highly stringent protocols used, which employed treatment periods of only up to 10 days. Many studies of mouse models of acute CD use treatment regimens of at least 20 days (38). A recent study demonstrated that to achieve parasitological cure with Bz, *T. cruzi*-infected mice were submitted to two treatment regimens (the first during the acute phase and the second during the chronic phase), employing 20 days of drug administration each (12). The encouraging results with DB766 via i.p. administration of up to 50 mg/kg/day and p.o. administration of 100 mg/kg/day merit further studies using longer periods of e.o.d. treatment or combination therapy with Bz, which are now under way.

In conclusion, our *in vitro* and *in vivo* data validate the further exploration of DB766 and other AIAs as new potential candidates for CD therapy. As for any new drug, extensive pharmacological and safety studies are required before advancing to clinical trials. Synthesis and evaluation of new AIAs, together with critical information regarding pK/metabolism, will hopefully lead to the identification of improved candidates for the treatment of Chagas' disease.

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Artigo # 05: Submetido à publicação na revista PLOS One

Título: **"Combined Treatment of Heterocyclic dications and Benznidazole upon *Trypanosoma cruzi in vivo*"**

Estado do conhecimento quando da concepção do trabalho:

- ✓ A terapia combinada representa uma abordagem interessante, pois permite a aplicação de pelo menos dois compostos que podem atuar sobre diferentes alvos celulares e vias metabólicas, possibilitando reduzir concentrações e número de doses, levando a diminuição de efeitos colaterais e do risco de resistência a drogas.

- ➢ **Questões propostas:**

1. Verificar o efeito *in vivo* do co-tratamento da diamidina aromática (DB289) associada ao Beznidazol durante a infecção experimental aguda de camundongos pelo *Trypanosoma cruzi* (cepa Y), analisando distintos parâmetros parasitológicos (parasitemia e mortalidade), clínicos (curva ponderal), e de cura parasitológica (Hemocultivo e PCR).
2. Verificar o efeito *in vivo* do co-tratamento da arilimidamida (DB766) associada ao Beznidazol durante a infecção experimental aguda de camundongos pelo *T. cruzi* (cepa Y), analisando distintos parâmetros parasitológicos (parasitemia e mortalidade), histopatológicos (carga parasitária e inflamação), clínicos (curva ponderal e eletrocardiograma), bioquímicos (dosagem de CK e GPT) e de cura parasitológica (Hemocultivo e PCR).

Seguem 32 páginas

Combined Treatment of Heterocyclic Analogues and Benznidazole upon Trypanosoma cruzi in vivo

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Abstract

Chagas disease caused by *Trypanosoma cruzi* is an important cause of mortality and morbidity in Latin America but no vaccines or safe chemotherapeutic agents are available. Combined therapy is envisioned as an ideal approach since it may enhance efficacy by acting upon different cellular targets, may reduce toxicity and minimize the risk of drug resistance. Therefore, we investigated the activity of benznidazole (Bz) in combination with the diamidine prodrug DB289 and in combination with the arylimidamide DB766 upon *T. cruzi* infection *in vivo*. The oral treatment of *T.cruzi*-infected mice with DB289 and Benznidazole (Bz) alone reduced the number of circulating parasites compared with untreated mice by about 70% and 90%, respectively. However, the combination of these two compounds decreased the parasitemia by 99% and protected against animal mortality by 100%, but without providing a parasitological cure. When Bz (p.o) was combined with DB766 (via ip route), at least a 99.5% decrease in parasitemia levels was observed. DB766+Bz also provided 100% protection against mice mortality while Bz alone provided about 87% protection. This combined therapy also reduced the tissular lesions induced by *T. cruzi* infection: Bz alone reduced GPT and CK plasma levels by about 12% and 78% compared to untreated mice group, the combination of Bz with DB766 resulted in a reduction of GPT and CK plasma levels of 56% and 91%. Cure assessment through hemocultive and PCR approaches showed that Bz did not provide a parasitological cure, however, DB766 alone or associated with Bz cured ≥13% of surviving animals.

Keywords: Chagas disease, combined therapy, Benznidazole, DB289; DB766

1. Introduction

Discovered by the Brazilian physician Carlos Chagas one century ago, Chagas disease (CD) is a zoonosis caused by kinetoplastid flagellated *Trypanosoma cruzi* [1]. It is well known that CD is an endemic illness in poor areas of 15 developing countries of Latin America, affecting about 12 to 14 million people. Less well known is that CD is becoming a health problem in non-endemic areas such as Europe and United States largely due to the migration of infected people to these regions [2-4]. Due to the lack of an efficient therapy, mainly for chronic chagasic patients, and since it has been considered by many pharmaceutical industries to have limited economical potential, CD has been designated a neglected tropical disease [5].

The main route of transmission of *T. cruzi* infection to humans is through the feces of blood-sucking triatomine insects but other routes also exist including blood transfusion, transplacentally, organ transplantation, laboratory accidents and oral ingestion of contaminated food [6-12]. CD is the leading cause of infectious myocarditis worldwide, which is one of its most serious and frequent clinical manifestations observed during the chronic phase of the disease that appears in about 20-40% of infected individuals years or decades after the acute infection [13-15]. The available treatment is based on two nitroheterocycles: nifurtimox (4-[(5-nitrofurfurylidene)-amino]-3-methylthio morpholine-1,1-dioxide), a nitrofuran produced only in El Salvador by WHO-Bayer, and benznidazole (*N*-benzyl-2-nitro-1-imidazoleacetamide), a nitroimidazol produced by LAFEPE-Brazil. Both are indicated for the treatment of all acute and early chronic cases, exhibiting about 60-80% efficacy [16]. Nevertheless, neither compound is highly effective against the late chronic phase (about 20% cures), both require long-term therapy in addition to

displaying side-effects that can lead to interruption of the treatment. These deficiencies justify the search for new chemotherapeutic options [5,17,18].

Many studies have demonstrated the excellent activity of aromatic diamidines (AD), pentamidine and related compounds, against many pathogens, such as bacteria, fungi and protozoa [19]. Although the exact mechanism of action is still not precisely known, it has been proposed that the binding of these cationic molecules in the DNA minor groove, mainly at AT-rich regions, contributes, at least in part, to their effect upon trypanosomatids [16,20-21]. We have previously reported the *in vitro* and *in vivo* activity of AD and analogues such as arylimidamides (AIA) upon *T.cruzi* [22-25]. A recent study demonstrated that the AIA, DB766 shows superior efficacy than Bz upon different parasite strains, including those naturally resistant to Bz [26].

Combination therapy represents a promising approach for the enhancement of drug efficacy since it (i) allows the use of at least two compounds that may act upon different cellular elements and metabolic pathways (ii) may reduce drug concentrations and number of doses thus contributing to the lowering of toxic effects, and (iii) may minimize the risk of drug resistance [27]. For the reasons stated above, the combination of different trypanocidal compounds merits exploration [16,27-28]. Our present goal is to evaluate *in vivo* the combined effect of Bz with the prodrug DB289 and with the arylimidamide DB766, to determine if a scheme of therapy with these drugs could reduce toxicity and improve efficacy in an animal model for *Trypanosoma cruzi*-infection.

2. Material and methods

2.1. Compounds

The aromatic diamidine DB289 (pafuramidine maleate; 2,5-bis[4-(N-methoxyamidino)phenyl]furan monomaleate) and the arylimidamide DB766 (Fig. 1) were synthesized according to methodology previously reported by us [29-31]. A DB289 stock solution was made in a solvent consisting of sterile distilled water (99.4%), Tween 80 (0.1%), and ethanol, which was freshly prepared immediately before use each day. The route of administration used was oral gavage. DB289 and DB766 were dissolved in DMSO and then freshly diluted with sterile distilled water before use by intraperitoneal (ip.) or p.o. routes. The stock solution of Benznidazole (*N*-benzyl-2-nitroimidazol acetamide, Rochagan, Roche) was prepared in sterile distilled water with 3%Tween 80, and before use was diluted in sterile distilled water for p.o. administration.

2.2. Parasites

Bloodstream trypomastigotes (BT) of the Y strain were used throughout and were harvested by heart puncture from *T. cruzi*-infected Swiss mice on the day of peak parasitemia, as described [32].

2.3. Mice infection

Male Swiss mice (20-24 g) were obtained from the animal facilities of the Oswaldo Cruz Foundation (CECAL, Rio de Janeiro, Brazil). Mice were housed at a maximum of 8 per cage and kept in a specific pathogen free (SPF) room at 20-24°C under a 12/12 h light/dark cycle and provided with sterilized water and chow ad *libitum*. The animals were allowed to acclimate for 7 days before starting the experiments. Infection was performed by ip injection of 10^4 BT. Age-matched non-infected mice were maintained under identical conditions.

2.4. Treatment schemes

The animals were divided into the following groups (Table 2): uninfected (non-infected and non-treated); untreated (infected but non-treated); and treated (infected

and treated with the different compounds combined or not with Bz) [23-24]. Drug therapy was performed by 20 daily consecutive doses (ip. and p.o., Table 2), starting at the onset of the parasitemia (5 dpi). In all assays, only mice with positive parasitemia were used in the infected groups.

2.5. Parasitemia, mortality rates and ponderal curve analysis

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 [33]. The mortality rates were checked daily until 60 dpi and expressed as cumulative mortality (% CM). Body weight was evaluated from 0 up to 60 dpi, and expressed as percentage of weight variation [23-24].

2.6. Histopathological analysis

At 14 dpi (peak of cardiac parasitism and inflammation in this experimental model as described in de Souza et al., 2006 [23], the heart tissues were removed, cut longitudinally, rinsed in ice-cold phosphate buffered saline (PBS), and fixed in Millonig-Rosman solution (10% formaldehyde in PBS). The tissues were dehydrated and embedded in paraffin. Sections (3 µm) were then stained with hematoxylin-eosin and analyzed by light microscopy. The number of amastigote nests was determined in at least 60 fields (total magnification, 40X) for each slide. The mean number of amastigotes' nests per field was obtained from at least three mice per group, with three sections from each mouse.

2.7. Biochemical analysis

At 14 day post infection (dpi), mice blood was collected and immediately submitted to analysis for biochemical determination of plasma tissular markers including glutamate pyruvate transaminase (GPT) and total creatine kinase (CK) using the Reflotron System (Roche Diagnostics, F. Hoffmann-La Roche Ltd, Basel, Switzerland) [34].

2.8. Cure assessment.

Cure criteria were based on two parasitological methods: polymerase chain reaction (PCR) and hemoculture assays which detect kDNA minicircle specific sequences or the parasite itself, respectively. Animals presenting negative results by both tests were considered cured. Briefly, after 40 days of drug treatment, about 700 µL of blood were collected from the heart of anesthetized mice and then 500 µL and 200 µL were used for PCR and hemoculture analysis, respectively. For PCR, the blood was diluted in 1:3 volume of guanidine solution (6M guanidine-HCl/0.2M EDTA), and heated for 90 s in boiling water in order to cleave the parasite kDNA network [35] and the PCR performed using the primers: (5´AAATAATGTACGGG(T/G)GAGATGCATGA3´) and (5´GGTTCGATTGGGGTTGGTGTAATATA3´), which amplify a 330 bp sequence from the kinetoplast DNA (aprox 120 000 copies per parasite), as previously described by Wincker et al. (1994) [36]. The PCR was carried out using a GeneAmp® PCR System 9700 (Applied Biosystems) as follows: one step at 94°C for 3 min (to activate the Taq DNA polymerase), 2 cycles at 98°C for 1 min and 64°C for 2 min, 38 cycles at 94°C for 1 min and 64°C for 1 min, followed by a final extension at 72°C for 10 min. The amplification products were detected on a 1.5 % agarose gel electrophoresis following staining with ethidium bromide (5 mg/mL). For hemoculture, 200 µL of blood was added to 5 mL LIT medium and incubated at 28°C for 60 days, being weekly examined by light microscopy to detect epimastigote forms [37].

All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (protocol approved - CEUA 0099/01).

3. Results

Since (i) in a previous study we found that a phenyl-substituted analogue of furamidine gave a trypanocidal effect upon a *T. cruzi* infection *in vivo* [22], and (ii) oral administration of only two doses at onset (5 dpi) and at parasitemia peak (8 dpi) of 25 and 50 mg/kg/day of the furamidine prodrug (DB289) resulted in about a 60% decrease of parasitemia (Fig. 2A), we evaluated the combination of DB289 with Bz to determine if an enhancement of efficacy against the parasite was observed. Our results show that although treatment with 25 mg/kg/day of DB289 or 50 mg/kg/day of Bz, both alone, lowered the parasitemia peak levels by about 70% and 90%, the combined treatment reduced the number of circulating parasites at 8 dpi by more than 99% (Fig. 2B; Table 1). Mice survival rates of about 85% and 40% were found for DB289 treated and untreated mice groups, respectively. The combination of the prodrug DB289 with Bz resulted in 100% animal survival (Fig. 2C). At three weeks post infection when the highest body weight lose is observed in this *T. cruzi* experimental model [26], both DB289 alone and Bz + DB289 showed considerable lose of mice body weight similar to or even as high as that for the untreated animals (Fig. 2D). At 60 dpi, the group treated with only DB289 still showed high rates of weight loss, even more than the untreated mice group (Fig. 2D). Cure assessment evaluated by both hemoculture and PCR did not reveal a parasitological cure in any mice groups (Table 2).

Since our recent study demonstrated the high efficacy of DB766 upon *T. cruzi* infection *in vivo* and *in vitro* [26], its combination with Bz was evaluated. Using the same therapy scheme as above, we found that the treatment of infected mice with 50 mg/kg/day of Bz or DB766 (ip.) or with DB766 (ip.) plus Bz (50 mg/kg/day each) resulted in decreases in mice parasitemia by about 90%, >96% and 100%, respectively, as compared to the untreated mice group (Fig. 3A, Table 1). The analysis of cumulative mortality revealed that while DB766 treated, Bz treated and

untreated groups resulted in 50, 12.5 and 100% of death, the combined therapy of Bz plus DB766 resulted in 100% of protection, avoiding animal death (Fig. 3B). We found at 20 dpi, that the Bz treated and the combined therapy group showed partial restoration of the mice body weight compared to uninfected mice, however, the group treated with DB766 alone displayed even higher body weight loss than that of the untreated mice group (Fig. 3C).

In these experimental groups, at the 14 dpi, we found that the *T. cruzi* infection led to an increase in biochemical markers, resulting in a rise of 2- and 29-fold for GPT and CK levels, respectively (Fig. 3 D-E). Bz treatment alone did not reverse the hepatic damage induced by parasite infection [34], however, DB766 alone or in combination with Bz produced a decrease of about 69% and 57% in GPT levels when compared to untreated control (Fig. 3E). Muscle lesions, as evaluated by CK plasma levels were decreased with Bz treatment by about 70%. Treatment with DB766 alone or combined with Bz resulted in reductions greater than 91% when compared to untreated control (Fig. 3D). Histopathological assays confirmed the high efficacy of DB766 combined with Bz which resulted in 100% reduction in cardiac parasitism as compared to untreated animals (data not shown). Cure assessment also revealed that the administration of 50 mg/kg/day DB766 (ip.) plus 50 mg/kg/day Bz (p.o.) resulted in a 13% parasitological cure, as evaluated by both hemocultive and PCR analysis (Table 2).

Since DB766 shows high trypanocidal efficacy against *T. cruzi* in *vivo* on oral administration at 100 mg/kg dose [26], we evaluated another combination dosing regimen using sub-optimum oral doses of both DB766 (50 mg/kg/day by p.o.) and Bz (50 mg/kg/day by p.o.). DB766 (p.o.) alone reduced parasitemia by only about 20% (Fig. 4A) and the mortality rates were reduced by about 25% (data not shown). The administration of Bz plus DB766 (both p.o) decreased the parasitemia by 54% (Fig.

4A, Table 1), and gave a 100% mice survival rate similar to that of Bz alone (data not shown). At 20 dpi, both Bz alone and the combined therapy provide a partial recovery of mice body weight, however DB766 alone displayed similar body weight loss to that of untreated animals (Fig. 4B). In the oral treatment studies, the only parasitological cure noted by the hemoculture and PCR methods was for the group treated with 50 mg/kg/day DB766 (p.o.) (Table 2).

4. Discussion

Since the introduction of Nifurtimox and Bz (1960-1970), despite the urgent need for new CD therapies, only allopurinol and a few azoles have moved to clinical trials, possibly due to (i) limited investment in this research field, (ii) an earlier mistaken concept that during the later phase of CD parasitism was absent and thus was not relevant to disease outcome and pathogenesis, and (iii) the absence of universal standardized protocols for drug screening [16]. The current profile for development of new drug candidates for CD includes 1) efficacy against different stocks, 2) efficacy against parasite forms relevant to the infection of mammalian hosts for both the acute and chronic phases, 3) oral administration in a minimum number of doses, 4) reduced toxicity, 5) yield high levels of tissue accumulation and long terminal half lives and 6) low costs [16,38-39].

In this study using a murine model of *T. cruzi* acute infection, the trypanocidal efficacy of pafuramidine (DB289) and the arylimidamide DB766 either alone or in combination with benznidazole was evaluated over a relatively short period of treatment (20 days) employing both intraperitoneal and oral administration.

Diamidine-containing compounds such as pentamidine and furamidine are DNA minor groove binders with broad-spectrum activity upon different species of human and veterinary pathogens [19]. DB289 is the orally active prodrug of DB75 (furamidine) that exerts microbicidal effects upon different pathogens including

Trypanosoma brucei, *Pneumocystis jiroveci* and *Plasmodium falciparum*, [40-44]. Interestingly, DB289 showed good oral efficacy in murine models of human African trypanosomes (HAT) suggesting that sufficient quantities are absorbed from the mouse gastrointestinal tract, delivering this dicationic molecule across the gut mucosa [42,45]. Similar to the therapy of HAT [42], oral efficacy is a desirable feature for treatment of CD. However, in contrast to the studies performed with *T. brucei* [42], DB289 alone was not very effective against *T. cruzi.* This difference may be due to the fact that in contrast to the African trypanosomes*, T. cruzi* has intracellular stages living inside the cytoplasm of host cells, which represents an additional obstacle for drug access and delivery. We have found that the combination of Bz with DB289 improved the efficacy of the diamidine by reducing parasitemia and resulted in protection against mortality. In addition, this combined therapy provided a 9-fold enhancement of activity compared to that of Bz alone. Despite showing efficacy in Phase III clinical trials against HAT and initial indications of low toxicity in African, Asian, Caucasian and Hispanic populations, further studies with DB289 revealed considerable side effects leading to its withdrawal from advanced human trials [16,42]. In fact, when higher doses of DB289 (≥100 mg/kg/day) were evaluated against *T. cruzi* infection *in vivo* (unpublished data of DGJB), higher numbers of circulating parasites and mortality rates were noticed as compared to untreated mice, perhaps a consequence of compound toxicity .

A previous study by our group demonstrated the beneficial effect of DB766 upon *T.cruzi in vivo*: a ten-day regimen of treatment reduced both blood and cardiac tissue parasitism, resulting in 90-100% protection against death even with an infection with naturally resistant *T.cruzi* strain (Colombian) to benznidazole [26]. Also, this AIA ameliorated electric heart alterations and reduced hepatic and heart lesions induced by the parasite infection [26]. Despite the promising trypanocidal effects of this AIA

via ip (up to 50 mg/kg/day) and by p.o (100 mg/kg/day) routes which showed efficacy similar to Bz (100 mg/kg/day), DB766 (as well as Bz) failed to provide a parasitological cure [26]. This result may be a consequence of the highly stringent protocol employed (maximum of 10 days of drug administration). In fact, previous studies performed in *T. cruzi*-infected murine models with Bz and azoles reported high rates of parasitological cure only when dosing was continued for longer periods (≥40 days) [46-48], which

supports using longer periods of therapy for our combination studies.

Our studies show that AIA are more active against *T.cruzi* than diamidine compounds [25,49]. The greater activity may be related to differences in their physical properties since AD are highly basic molecules with pK_a values near 11 while AIA pK_a values are near 7. At physiological pH, AD are protonated and thus cationic molecules while AIA are essentially neutral molecules enabling their passive diffusion through the plasma membranes of both parasites and host cells. This large difference in properties likely affects absorption and distribution and may play an important role in the different activity of these two classes of compounds.

Our data showed that while DB766 alone reduced parasitemia giving a superior result to that of Bz, the combination of Bz and DB766 leads to undetectable parasitism, thus improving the efficacy of both compounds, especially Bz, whose potency was increased at least 20-fold. The improved activity of the combined therapy may reflect different targets (still incompletely understood for both) and/or effects upon different parasite forms. As reported, intracellular parasites must be considered the main parasite stage for drug targeting in CD since *T. cruzi is* an obligatory intracellular parasite [27]. In fact, although Bz has high activity upon BT, it probably presents more limited effectiveness upon intracellular parasites due to low tissular accumulation [39,50]. On the other hand, DB766 has a long half-life and a

large volume of distribution [31], perhaps providing effective concentrations of drug for action against the intracellular forms. Alternatively, the combination of both drugs could help to reduce the deleterious and exacerbated acute phase response induced by the parasite (e.g. effect of Bz on TNF and thus upon mice cachexia)[51]. Importantly, the superior efficacy of the combined therapy which leads to a 13% parasitological cure possibly is attributed to reduced tissular lesions (e.g. cardiac and hepatic) due to parasite load control itself and/or to a drop in exacerbated inflammation levels which has been previously demonstrated with DB766 [26]. A sustained unbalanced inflammatory process associated with the parasite presence plays a fundamental role in triggering pathology [52-53].

As previously reported, DB766 displayed oral efficacy against an experimental *T. cruzi* infection when high but non-toxic doses (100 mg/kg/day) are employed [26]. However, when we evaluated the p.o. treatment with Bz and DB766 using sub-effective doses of both compounds (50 mg/kg/day each), the combined therapy only enhanced the activity of the AIA by 1.8 fold. The combined therapy showed a lower effect on parasitemia (but not on mortality rates) as compared to Bz treatment alone, suggesting an antagonistic effect that deserves to be further explored. One out of three surviving mice treated with DB766 by p.o. was cured as assessed by both hemocultive and PCR analysis. Although we did not find a considerable reduction in the mean parasitemia in this mice group, the cured animal was the one that displayed the lowest level of circulating parasitism, reaching undetectable parasitism (by light microscopy counting) after 23 dpi.

Although no visible adverse effects were noticed for DB289 and DB766, when they were used alone, both increased the cachexia induced by the parasite infection. This effect raises the possibility of drug toxicity and/or up-regulation of inflammatory mediator levels such as TNF-alpha that is implicated in loss of mice weight during *T.*

cruzi acute infection [51]. Although no detectable acute toxicity was observed in mice treated up to a cumulative dose of 200 mg/kg/day of DB766 [26] and 100 mg/kg/day DB289 [42], and our data showed reduced hepatic and muscle lesions during the treatment of infected mice with DB766, a detailed biochemical and histopathological analysis is needed to clarify this matter.

The measurement of pro and anti-inflammatory cytokines in the plasma of infected and treated mice would contribute to the understanding of the possible role of these mediators upon drug toxicity and efficacy. Although no data is available for AIA, some studies suggest a regulatory effect by pentamidine upon pro-inflammatory cytokines [55-56]. Additionally, Bz down-regulates the synthesis of TNF-alpha by murine stimulated macrophages [57], ameliorates LPS-induced inflammatory response in mice by decreasing peak levels of this serum cytokine [58] and markedly reduces the production of pro-inflammatory cytokines and NO-derived metabolites in experimentally *T. cruzi*-infected rats [59]. These data may explain the weight recovery found in Bz-infected treated mice as compared to untreated mice since this pro-inflammatory mediator is strongly expressed in *T. cruzi*-infected mice [59-60], and is thought to be related to mice weight loss [51]. In our studies, we found a correlation between mice cachexia and mortality rates, including in the DB766 groups (ip. and p.o.) that may explain the lower protection against animal mortality in the animal groups that only received the AIA.

In conclusion, this study has shown that DB766 is much more potent in this mouse experimental model of *T. cruzi* infection than DB289 and that the trypanocidal activity is improved by combination therapy of both AD and AIA with Bz. Our data support additional studies with other diamidines and AIAs alone or in combination with other drugs with the goal of identification of new candidate therapies for the treatment of Chagas disease.

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Figure legends

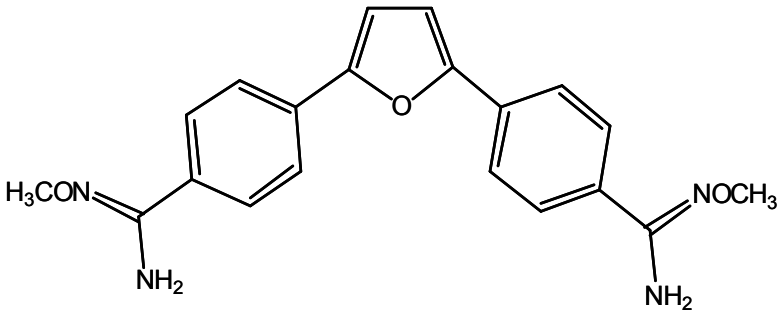
Figure 1: Chemical structures of DB289 (A) and DB766 (B).

Figure 2: Activity of DB289 alone or in combination with benznidazole (Bz) upon *T. cruzi*-infection in mice. (A): Parasitemia curve of infected mice that were either not treated or treated at 5 and 8 dpi with 25 and 50 mg/kg/day DB289 and 100 mg/kg/day Bz. (B): Parasitemia curve, (C) Cumulative Mortality and (D) Ponderal Curve of infected mice treated or not for twenty daily doses with 25 mg/kg/day DB289 combined or not with 50 mg/kg/day.

Figure 3: Activity of DB766 (ip.) combined or not with benznidazole (Bz) upon *T. cruzi*-infection in mice. (A): Parasitemia curve, (B) Cumulative Mortality and (D) Ponderal Curve of infected mice treated or not for twenty daily doses with 50 mg/kg/day DB766 combined or not with 50 mg/kg/day Bz. Plasma levels of (D) Glutamate pyruvate transaminase (GPT) and of (E) Creatinine kinase (CK) at 14dpi from uninfected, untreated, DB766, Bz and combined therapy treated mice.

Figure 4: Activity of DB766 (p.o.) combined or not with benznidazole (Bz) upon *T. cruzi*-infection in mice. (A): Parasitemia curve, and (B) Ponderal Curve of infected mice treated or not for twenty daily doses with 50 mg/kg/day DB766 (per oral) combined or not with 50 mg/kg/day Bz.

Figure 1:



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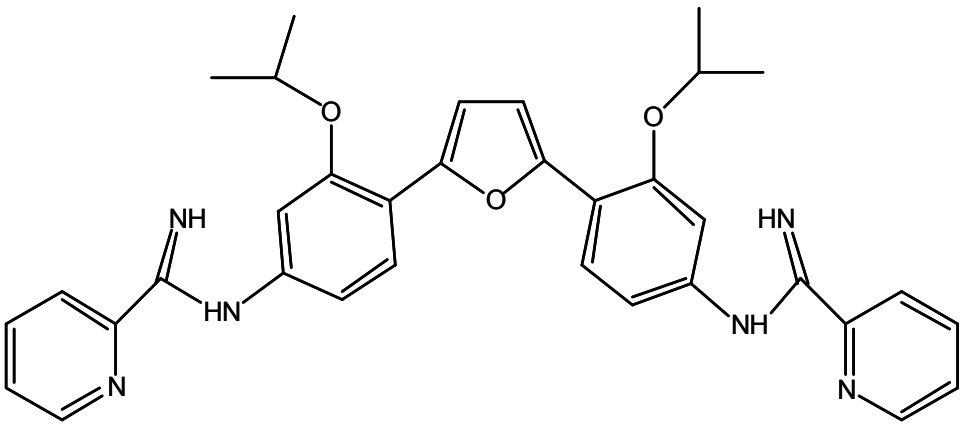


B

Figure 2:

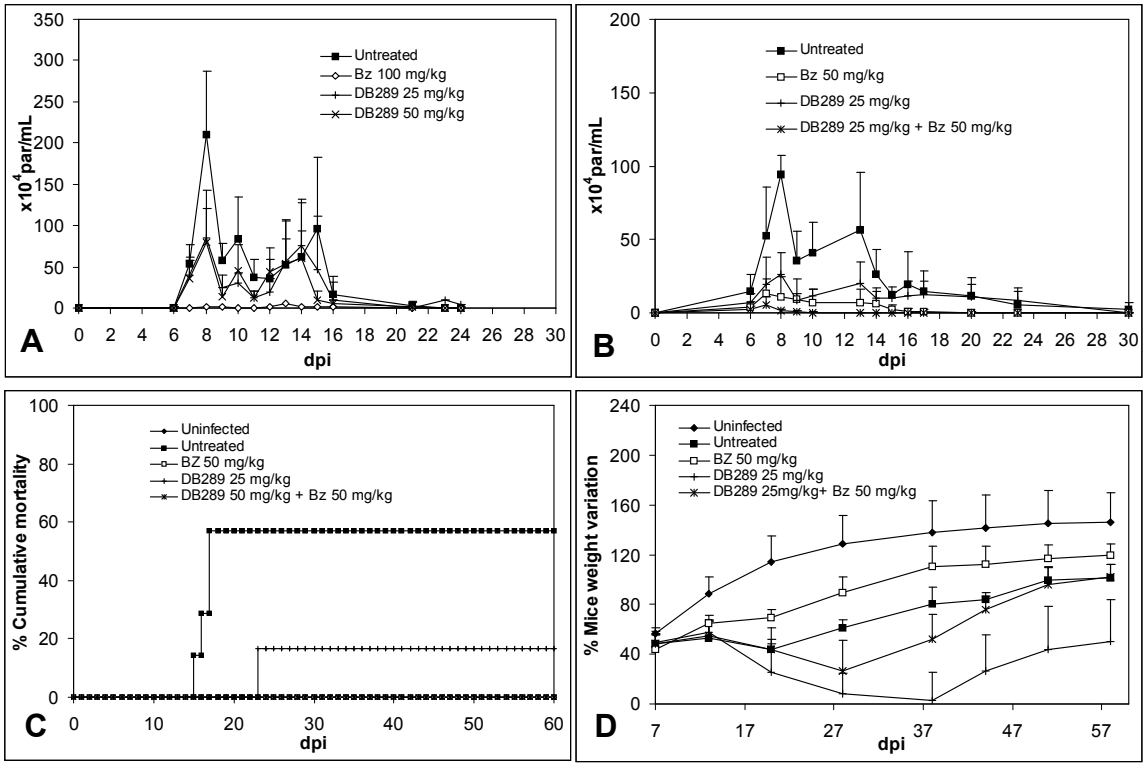

A
x10^4par/mL
dpi
Untreated
Bz 100 mg/kg
DB289 25 mg/kg
DB289 50 mg/kg
B
x10^4par/mL
dpi
Untreated
Bz 50 mg/kg
DB289 25 mg/kg
DB289 25 mg/kg + Bz 50 mg/kg
C
% Cumulative mortality
dpi
Uninfected
Untreated
BZ 50 mg/kg
DB289 25 mg/kg
DB289 50 mg/kg + Bz 50 mg/kg
D
% Mice weight variation
dpi
Uninfected
Untreated
BZ 50 mg/kg
DB289 25 mg/kg
DB289 25mg/kg+ Bz 50 mg/kg

Figure 3:

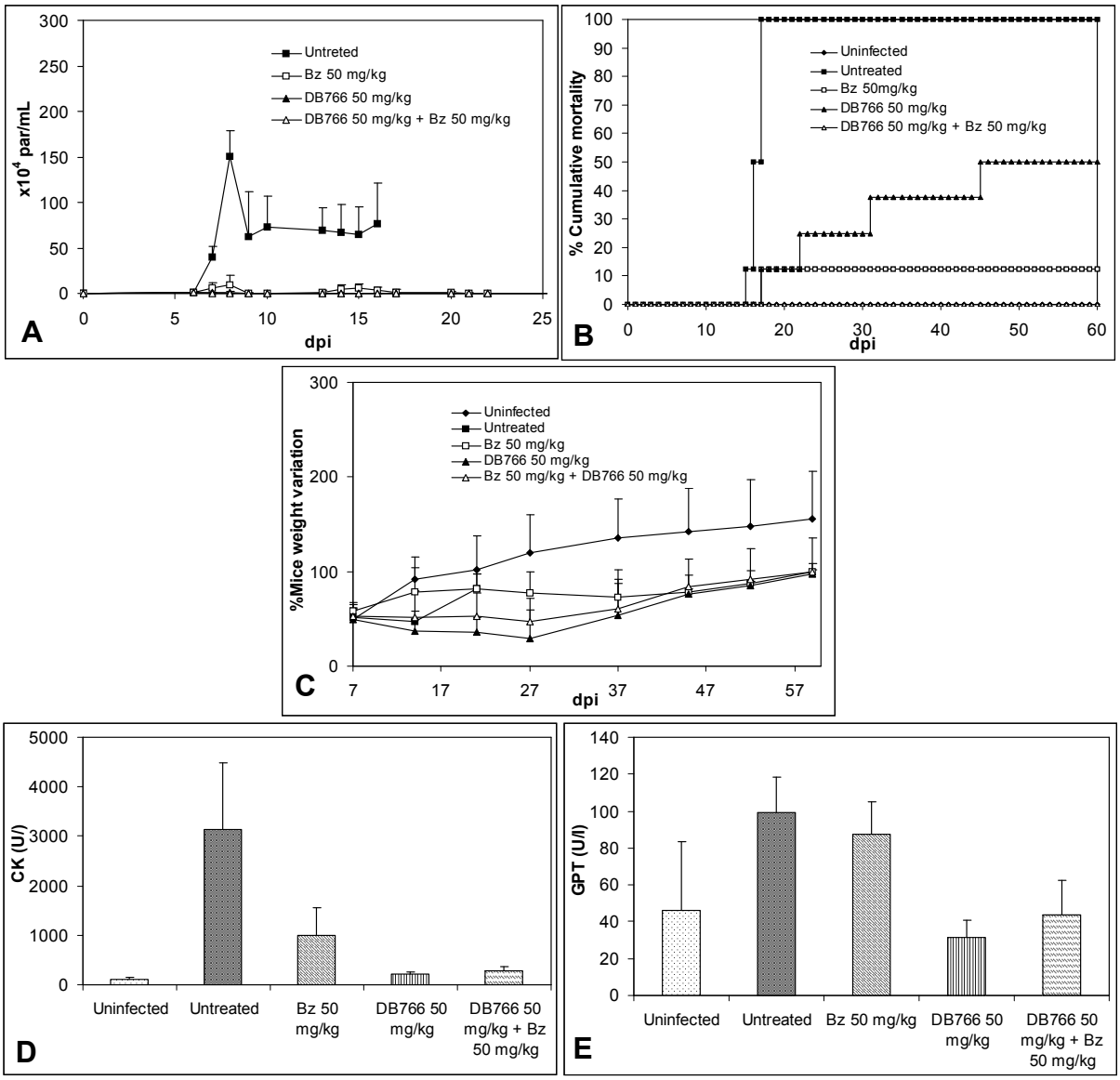
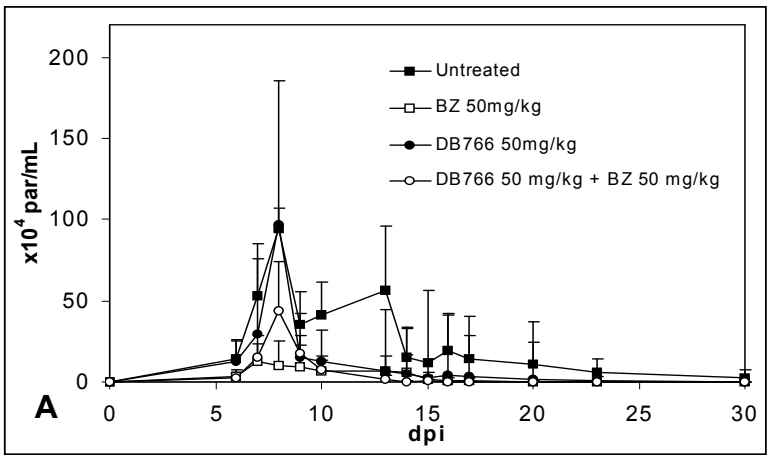

A
x10^4 par/mL
dpi
Untreted
Bz 50 mg/kg
DB766 50 mg/kg
DB766 50 mg/kg + Bz 50 mg/kg
B
% Cumulative mortality
dpi
Uninfected
Untreated
Bz 50mg/kg
DB766 50 mg/kg
DB766 50 mg/kg + Bz 50 mg/kg
C
%Mice weight variation
dpi
Uninfected
Untreated
Bz 50 mg/kg
DB766 50 mg/kg
Bz 50 mg/kg + DB766 50 mg/kg
D
CK (U/l)
Uninfected
Untreated
Bz 50 mg/kg
DB766 50 mg/kg
DB766 50 mg/kg + Bz 50 mg/kg
E
GPT (U/l)
Uninfected
Untreated
Bz 50 mg/kg
DB766 50 mg/kg
DB766 50 mg/kg + Bz 50 mg/kg

Figure 4:


200
150
100
50
0
x10^4 par/mL
Untreated
BZ 50mg/kg
DB766 50mg/kg
DB766 50 mg/kg + BZ 50 mg/kg
A
0
5
10
15
20
25
30
dpi

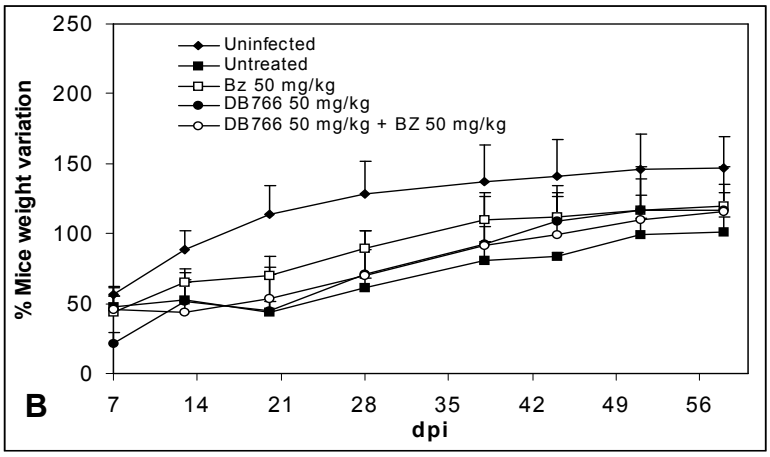

250
200
150
100
50
0
% Mice weight variation
Uninfected
Untreated
Bz 50 mg/kg
DB766 50 mg/kg
DB766 50 mg/kg + BZ 50 mg/kg
B
7
14
21
28
35
42
49
56
dpi

Table 1: Parasitemia peak of *T. cruzi*-infected mice treated or not with DB289 and DB766 associated or not with Benznidazole (Bz)

Experimental group	Therapy route	Parasitemia peak (x10^4 par/mL) and (% parasite reduction)
Untreated [1]	-	150.98±27.3
Bz 50 mg/kg[1]	p.o.	9.6±10.2 (93%)
DB766 50 mg/kg[1]	ip	0.9±1.1 (99.4%)
DB766 50 mg/kg + Bz 50 mg/kg[1]	ip + p.o.	0.0±0.0 (100%)
Untreated [2]	-	95±12.8
Bz 50 mg/kg[2]	p.o.	10.5±14.4 (89%)
DB766 50 mg/kg[2]	p.o.	78±88 (17%)
DB766 50 mg/kg + Bz 50 mg/kg[2]	p.o. + p.o.	43.6±31 (54%)
DB766 50 mg/kg[2]	ip	4±3 (96%)
DB766 50 mg/kg + Bz 50 mg/kg[2]	ip + p.o.	0.5±1 (99.5%)
DB289 25 mg/kg[2]	p.o.	26.4±14.4 (72%)
DB289 25mg/kg +Bz 50 mg/kg[2]	p.o. + p.o.	1.2±1 (99%)

1 and 2 = two independent representative studies

Table 2: Cure assessment of DB289 and DB766 combined or not with benznidazole (Bz) in murine model of acute *T. cruzi*-infection[1]

Experimental groups	Therapy route [2,3]	Number of surviving/ total number of animals	Assays performed after 60 days post treatment	
			Number of negative hemoculture samples/number of mice	Number of negative blood PCR samples/number of mice
Uninfected		13/13	-	-
Untreated	-	4/15	0/4	0/4
Bz 50 mg/kg/day	p.o.	14/15	11/14	0/14
DB289 25 mg/kg	p.o.	5/6	2/5	0/5
DB289 25 mg/kg + Bz 50 mg/kg	p.o. + p.o.	6/6	1/6	0/6
DB766 50 mg/kg	ip	6/15	0/6	0/6
DB766 50 mg/kg+ Bz 50 mg/kg	ip + p.o.	15/15	3/15	2/15
DB766 50 mg/kg	p.o.	3/7	1/3	1/3
DB766 50 mg/kg + Bz 50 mg/kg	p.o. + p.o.	8/8	0/8	0/8

[1] Swiss male mice weight 20 to 24 g inoculated with 10^4 blood trypomastigotes (Y strain). Treatment was initiated at 5º dpi followed by 20 daily doses.
[2] Intraperitoneal – ip
[3] per oral – p.o.

DISCUSSÃO

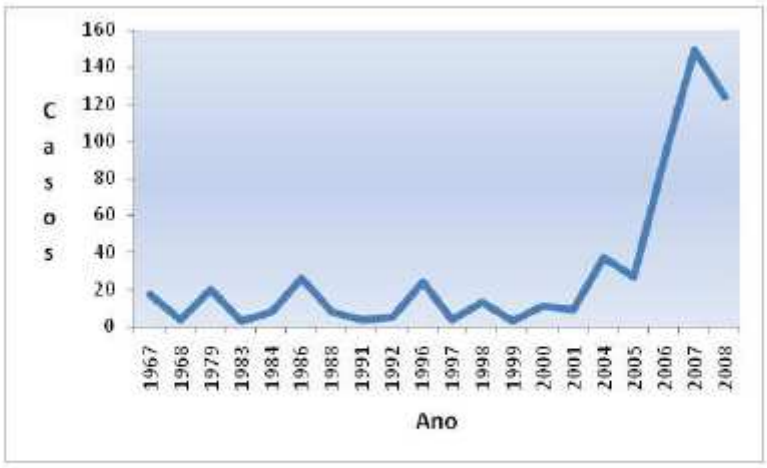
4. DISCUSSÃO

O controle da transmissão da doença de Chagas (DC) é realizado utilizando diferentes estratégias, tais como o uso de agentes químicos destinados ao controle do vetor presente nas áreas domiciliares e peridomiciliares, pela vigilância entomológica, pela busca sorológica ativa e compulsória (em áreas endêmicas) em bancos de sangue e centros de transplante de órgãos, pelo diagnóstico e tratamento da transmissão congênita, pelo controle sanitário da fabricação e comercialização de alimentos e outros insumos minimizando o risco de contaminação oral, além do tratamento de todos indivíduos na fase aguda da doença, incluindo crianças e jovens até 15 anos (Moncayo e Silveira, 2009).

Mesmo após cem anos de descoberta da doença de Chagas, esta parasitose não poderá ser prontamente eliminada. Isto em decorrência das múltiplas características de transmissão desta doença e da complexidade da biologia do parasito, de seus hospedeiros e respectivos ciclos evolutivos (domiciliar, peridomiciliar e silvestre). Assim, vários desafios continuam presentes incluindo (i) a necessária manutenção das políticas de educação, informação e comunicação a sociedade, profissionais, pacientes e familiares, (ii) a constante vigilância epidemiológica e controle da transmissão em caráter homogêneo e universal, em especial nas áreas endêmicas (conhecidas como áreas “hot-spot”, como a região do Gran Chaco na Argentina além de vários distritos na Bolívia, como Cochabamba) e que ainda concentram um grande número de casos agudo, (iii) identificação e padronização de ferramentas de diagnóstico e mesmo de marcadores de evolução clínica, (iv) e desenvolvimento e seleção de novos compostos tripanocidas que possam substituir os dois medicamentos introduzidos na clínica desde a década de 60, o NF e o BZ, e que apresentam baixa eficácia sobretudo na fase crônica, além de consideráveis efeitos colaterais e necessidade de longos períodos de tratamento, o que muitas vezes resulta no abandono da terapia (Torrico et al., 2004).

Segundo o Ministério da Saúde (MS), casos de DC aguda por provável transmissão vetorial, vertical e por transfusão de sangue ou transplantes de órgãos têm ocorrido em diversos estados brasileiros. Ademais, surtos relacionados à ingestão de alimentos contaminados (caldo de cana, açaí, bacaba e outros) ocorreram nos estados do AP, AM, BA, CE, PA e SC. De fato, apesar do decréscimo acentuado no número de novos casos agudos nas últimas décadas em decorrência das políticas de controle (vetorial e transfusional) adotadas pelos países do Cone Sul (dos quais o Brasil faz parte), nos últimos 5 anos observa-se uma

crescente ocorrência de novas infecções (Figura 9), sendo notificado mais de 500 casos e cerca de 90% deles na Amazônia Legal, sendo 75% somente no Pará (MS, 2009).



Fonte: IEC/MS e SVS/MS

Figura 9: Casos confirmados de DC aguda por todas as formas de transmissão, segundo UF, Brasil, no período de 1967 a 2008 (593 casos).

Neste contexto, a síntese de novos compostos que sejam eficazes e menos tóxicos e que possam ser aplicados no tratamento desta patologia negligenciada se faz urgente (Soeiro et al., 2008; Soeiro e De Castro, 2009). De todos os compostos avaliados em ensaios pré-clínicos desde a introdução do Bz e do NF, apenas o alopurinol e alguns azóis passaram para os ensaios clínicos, isto possivelmente devido à (i) falta de investimentos em pesquisas nessa área, principalmente por indústrias farmacêuticas haja vista a ausência de um mercado seguro e de um retorno comercial para este investimento longo e de alto custo, (ii) conceito anterior equivocado de que, na fase crônica da doença de Chagas, o parasitismo era ausente e, portanto, não estava relacionado com a evolução da doença e patogênese, e (iii) ausência de protocolos universais padronizados para os testes de drogas (Soeiro et al., 2009).

Consensualmente, devido às limitações das drogas disponíveis a OMS, o DND*i* e diferentes autores sugerem que a droga ideal para tratamento etiológico doença de Chagas deva cumprir minimamente os seguintes requisitos: (i) ser eficaz na fase aguda, crônica indeterminada e em indivíduos que apresentem recidivas

(imunocomprometidos); (ii) ser ativa contra diferentes populações (cepas) de *T. cruzi* e respectivas linhagens (Tc I, Tc II e Tc III); (iii) ter atividade sobre formas tripomastigotas livres inibindo a invasão de novas células, e sobre formas intracelulares impedindo a liberação de novos parasitos infectantes; (iv) ter formulação para todas as faixas etárias, incluindo para uso pediátrico; (v) ter capacidade de cura, que deve ser confirmada por testes parasitológicos e sorológicos, ≥ 70% dos indivíduos infectados na fase crônica e > 95% dos pacientes com recidivas; (vi) ter ação sobre cepas de *T. cruzi* que apresentem resistência natural as drogas de referência (nitrofuranos e nitroimidazóis); (vii) apresentar segurança superior ao BZ, não requerendo monitoramento clínico ou laboratorial durante a vigência do tratamento; (viii) não apresentar contra indicação para mulheres grávidas e lactantes assim como ausência de efeito inotrópico negativo (diminuem a carga de trabalho do coração reduzindo a frequência e a força dos batimentos cardíacos, e.g. beta-bloqueadores), prolongamento do intervalo QT, genotoxicidade e teratogenicidade; (ix) ausência de interações medicamentosa sobretudo com anti-hipertensivos, anti-arrítmicos e anti-coagulantes; (x) apresentar formulação oral; (xii) ter estabilidade de 5 anos em zona climática IV (regiões tropicais); e (xii) ter regime de tratamento em dose única ou poucas doses (≤ 5) (Coura e Castro, 2002; McKerrow et al., 2009; Bettiol et al., 2009; Nwaka e Hudson, 2006; Urbina, 2009; Soeiro et al., 2009; DNDi, 2009; WHO, 2009).

A partir dos dados acima descritos, no presente estudo tivemos por objetivo avaliar a ação tripanocida de novos compostos sintéticos que possam ser eficazes e seguros para o tratamento dos milhões de pacientes chagásicos. Neste sentido, a triagem de análogos/derivados de distintas classes de compostos microbicidas sintetizados por diferentes grupos de química medicinal, em especial pelos laboratórios do Dr. David W. Boykin (Universidade de Atlanta, EUA) e da Dra. Letícia R. Teixeira (Universidade de Minas Gerais, Brasil), foi avaliada através de ensaios *in vitro* e *in vivo*. Assim, como a identificação de novos compostos baseia-se, principalmente (a) no desenho racional de novas moléculas específicas e seletivas para determinantes metabólicos/vias exclusivas do patógeno a ser investigado, e (b) na análise de fármacos já utilizadas na clínica para outras patologias (Soeiro e Castro, 2009), derivados de fluorquinolonas, tiossemicarbazonas e diamidinas aromáticas foram estudados por serem classes de compostos já aplicados na terapia de infecções causadas por vírus, bactérias, fungos e mesmo protozoários. Entre seus diversos alvos celulares descritos, todas apresentam ação direta e/ou

indireta sobre a molécula do DNA e têm sido consideradas moléculas promissoras para terapia em infecções causadas por parasitos de relevância médica, como, por exemplo, pelo *T. cruzi* (De Souza et al., 2004; Silva et al., 2007a, Gonzales-Perdomo, 1990, Soares, 2007).

4.1. Fluorquinolonas

No artigo#01 a síntese e a atividade biológica de duas fluorquinolonas, a esparfloxacina e a norfloxacina, e seus complexos metálicos (Mn, Co e Cu-fen) foi realizada sobre formas tripomastigotas de sangue e parasitos intracelulares de *T. cruzi in vitro*, por serem consideradas as formas relevantes para a infecção de mamíferos (McKerrow et al., 2009, Urbina, 2009). Ademais, avaliamos a possível toxicidade e relativa seletividade destes compostos sobre células de mamíferos empregando culturas primárias de células cardíacas, por serem consideradas importantes alvos de infecção e de inflamação nesta parasitose.

O uso de antibióticos sintéticos quinolônicos se iniciou com a descoberta inadvertida do ácido nalidíxico em 1962, durante a síntese da cloroquina, um agente antimalárico (Appelbaum e Hunter, 2000) Apesar do ácido nalidíxico não ter tido bom desempenho no tratamento de infecções bacterianas além de causar efeitos adversos, serviu de base para a síntese de análogos com importantes propriedades terapêuticas sobre uma ampla gama de bactérias, menor toxicidade além de apresentar melhores características farmacocinéticas e de tolerabilidade (revisto em Tasso, 2008). Diferentes esquemas de classificação têm sido utilizados para descrever a evolução da classe das quinolonas sob a ótica cronológica: primeira geração (ácido nalidíxico), segunda geração (ácido oxolínico, cinoxacina e norfloxacina): terceira geração (ciprofloxacina, esparfloxacina, levofloxacina e ofloxacina) e quarta geração (moxilfoxacina e gatifloxacina) (Oliphant e Green, 2002; Blondeau, 2004).

Nos últimos trinta anos, fluorquinolonas, como a norfloxacina, têm sido amplamente utilizadas na clínica para o tratamento de patógenos intracelulares, incluindo o *Mycobacterium tuberculosis*, *Clostridium difficile*, *Legionella pneumophila, Mycoplasma pneumonia*, entre outros (Furet e Pechère, 1991; Riccardi et al., 2009; Coia, 2009). No entanto, apesar destes agentes serem utilizados no tratamento de infecções bacterianas graves e mesmo fatais, estudos tem relatado a resistência de algumas cepas a estes fármacos (Anquetin et al., 2006). Além disso, há fortes restrições associadas a toxicidade das FQ mais

antigas, principalmente relacionadas a alterações no sistema nervoso, digestivo, urinário, tegumentar-esquelético, cardiovascular, além de danos no sistema imunológico e indução de efeitos teratogênicos (Sepcić et al., 2009). Essas limitações têm estimulado a síntese e avaliação de novas FQ menos tóxicas que possam ser utilizadas em infecções bacterianas e mesmo em outras patologias (Furet e Pechère, 1991).

Há evidências de que FQ além de agirem sobre um amplo espectro de bactérias, também apresentam atividade anti-tumoral (Mondal et al., 2004), anti-HIV (Ohmine et al., 2002), e algumas delas, em especial a ciprofloxacina, a gatifloxacina e a norfloxacina são capazes de induzir a produção de citocinas pró-inflamatórias frente a estímulo por LPS (Ogino et al., 2009). Além disso, o efeito *in vitro* de FQ contra outros patógenos também foi relatado, incluindo sobre cepas de *Plasmodium falciparum* sensíveis e resistentes a cloroquina (Divo et al., 1988; Tripathi et al., 1993; Anquetin et al., 2006), sobre o *Trypanosoma cruzi* (Gonzales-Perdomo, 1990), *Trypanosoma brucei* (Nenortas et al., 1999, 2003; Chollet et al., 2009), *Leishmania spp* (Romero et al., 2005; Cortázar et al., 2007), e *Toxoplasma gondii* (Anquetin et al., 2006).

Tendo em vista as propriedades microbicidas desta classe de compostos, nos propusemos a investigar a possível ação de novos complexos metálicos de FQ ([$MnCl_2$(HNOR)(H_2O)$_2$] (**1**), [$MnCl_2$(HSPAR)(H_2O)$_2$] (**2**), [$CoCl_2$(HNOR)(H_2O)$_2$] (**3**) [$CoCl_2$(HSPAR)(H_2O)$_2$] (**4**), [$CuCl_2$(fen)(HNOR)] (**5**) e [$CuCl_2$(fen)(HSPAR)] (**6**)) sobre o *T. cruzi* comparando com a atividade das FQ parentais (NOR e SPAR).

Nossos dados mostram que os derivados **5** e **6** que apresentam complexos de Cu(II)-fen foram os mais ativos sobre ambas as formas evolutivas do parasito, com atividade tripanocida na faixa micromolar. A maior atividade (cerca de 80 vezes) dos compostos em relação ao NOR e SPAR, pode estar relacionada a presença dos íons metálicos induzindo a um aumento nos níveis de captação destes compostos pelos parasitos e/ou pela ação sobre diferentes alvos celulares (por exemplo, no DNA nuclear e/ou mitocondrial). De fato, o acúmulo intracelular de quinolonas e FQ é essencial para sua atividade microbiana e tem sido demonstrado ocorrer por difusão simples e/ou por canais porínicos presentes na membrana celular de bactérias (Bryan et al., 1989; Piddock et al., 1999). Estes agentes inibem a DNA girase bacteriana (DNA topoisomerase II-TOPOII) e as enzimas topoisomerase IV, alterando assim a replicação do DNA e sua transcrição (Luzzaro, 2008; Tossa, 2008). Vale ainda destacar que há vários exemplos na literatura demonstrando a

maior eficácia de complexos metálicos que seus respectivos ligantes livres e que sendo a fenantrolina um grupo apolar, esta propriedade pode facilitar o transporte passivo do fármaco através da membrana celular do parasito (Accorsi et al., 2009). Vale ressaltar que estes dois compostos (**5** e **6**) também exerceram moderada toxicidade sobre células de mamíferos, apresentando Índices de seletividade de >2.6 em relação as células cardíacas (artigo#01, Tabela 3). Apesar de modesto, este índice seletivo é encorajador e pode ser melhorado na próxima geração de compostos derivados destes complexos de FQ, visto que modificações químicas podem aumentar consideravelmente a potência e o espectro de atividade microbicida de FQ (Anquetin et al., 2006; Chollet et al., 2009).

Nossos resultados confirmam dados anteriores em tripanossomatídeos que demonstraram a atividade leishmanicida e tripanocida de FQ. Estudos recentes mostraram que a segunda geração de FQ (enoxacina e ciprofloxacina) foi ativa contra formas amastigotas e promastigotas de *Leishmania (Viannia) panamensis* (Romero et al., 2005; Cortázar et al., 2007). Os autores sugerem que o efeito leishmanicida poderia ser relacionado, pelo menos em parte, a inibição da topoisomerase II nuclear (Cortazar et al., 2007). Novas FQ também foram testadas contra formas sanguíneas de *Trypanosoma brucei brucei in vitro*, sendo os análogos tetracíclicos os mais potentes sobre estes tripanosomas (Nenortas et al., 1999). Neste estudo sugeriu-se que um dos alvos celulares destes compostos seja a TOPO II, através da demonstração da direta correlação entre a formação de complexos covalentes proteína-DNA e a atividade antiparasitária das FQs (Nenortas et al., 1999).

Em outro estudo, FQ com substituições pirrolidinil foram testadas contra *Trypanosoma bruce*i e sobre células de mamíferos e os resultados mostraram que os substituintes em C-7 ou no anel tiazolidina aumentam atividade e seletividade dos compostos (Nenortas et al., 2003). Também foi relatado que a atividade destes derivados de FQ está relacionada a inibição da biossíntese de ácidos nucléicos pela ação sobre a TOPO II destes parasitos (Nenortas et al., 2003). Dados mais recentes reportaram a síntese de uma série de compostos contendo um sistema conjugado de melamina a diferentes FQ objetivando incrementar a atividade tripanocida sobre o *T. brucei* através do aumento da captação seletiva destes compostos via o transportador P2 (Chollet et al., 2009). Os autores constataram que, embora haja uma atividade moderada após o tratamento com NOR e SPAR, alguns dos novos conjugados (e.g. melamina conjugada a norfloxacina)

apresentaram superiores efeitos tripanocidas, sendo cerca de oito vezes mais ativos que a NOR livre (Chollet et al., 2009). Os experimentos de microscopia de fluorescência mostraram que o derivado mais potente, mas não a NOR sozinha, induziu a formação de múltiplos kDNAS nos parasitos tratados, sugerindo que o efeito desses conjugados não esteja associado à inibição da replicação do DNA (e sobre toposoimerases), mas pela ação sobre outros alvos celulares (Chollet et al., 2009).

Nossos estudos também confirmam dados anteriores que relataram efeito tripanocida induzido pela ofloxacina, bem como por outros inibidores de TOPO II (e.g. ácido nalidíxico e novobiocina) sobre *T. cruzi* (Gonzales-Perdomo, 1990). A microscopia eletrônica de transmissão destes parasitos tratados mostrou que as principais alterações foram relacionadas ao cinetoplasto das formas sanguíneas e de amastigotas intracelulares, sugerindo que esta estrutura possa representar um importante alvo (Gonzales-Perdomo, 1990). Assim, a identificação de alvos celulares em *T. cruzi* representa um interessante aspecto e que será futuramente analisado pelo nosso grupo.

Em resumo, os nossos resultados, somados as características farmacocinéticas e farmacodinâmicas (como boa biodisponibilidade oral, excelente penetração na maioria dos tecidos e fluídos corporais, além de meia via longa (revisto em Tossa et al., 2008) desta classe de compostos e que são relevantes para o desenho de novos fármacos voltados para patologias crônicas causadas por parasitos intracelulares como *o T. cruzi,* justificam a continuidade de estudos com FQ visando o futuro desenvolvimento de novas drogas tripanocidas.

4.2. Tiossemicarbazonas

No artigo#02 a atividade de três tiossemicarbazonas (N^4-metil-4-nitrobenzaldeido tiossemicarbazona - $H4NO_2Fo4M$, N^4-metil-4-nitroacetofenona tiosemicarbazona - $H4NO_2Ac4M$ e N^4-metil-4-nitrobenzofenona tiossemicarbazona - $H4NO_2Bz4M$) e de seus respectivos complexos [$(Mn(H4NO_2Fo4M)_2Cl_2$] (**1**), [$Mn(H4NO_2Ac4M)_2Cl_2$] (**2**) e [$Mn(H4NO_2Bz4M)_2Cl_2$] (**3**)) foi testada *in vitro* sobre formas tripomastigotas de sangue e parasitos intracelulares de *T. cruzi*, avaliando ainda *in vitro* a toxicidade e seletividade destes compostos sobre células de mamíferos (células cardíacas).

As tiossemicarbazonas exibem um amplo espectro de ação biológica apresentando propriedades antiinflamatórias, anti-tumorais, e microbicidas sobre

vírus, bactérias e protozoários, incluindo o *T. cruzi* (Revisto em Soares, 2007; Sebastian et al., 2008; Vieites et al., 2008, 2009; Sen e Chaudhuri, 2009). Como sua síntese é geralmente muito simples, proporcionando elevados rendimentos, as TS têm também sido amplamente empregadas como moléculas intermediárias na síntese de outros compostos (Beraldo et al., 2004).

Nossos dados mostraram que somente o derivado **3** apresentou considerável atividade tripanocida sobre as formas sanguíneas, sendo cerca de 10 vezes superior ao composto parental ($H4NO_2Bz4M$). Por outro lado, nenhum dos compostos avaliados foi efetivo sobre as formas intracelulares resultando em valores de IC_{50} superiores a 111 μM. A ausência de atividade do derivado **3** sobre estes parasitas intracelulares em relação aos tripomastigotas pode ser justificada: (i) pela menor exposição dos parasitos intracelulares ao composto **3** frente a baixa captação pelas células hospedeiras e/ou sua inativação no citoplasma das células hospedeiras, (ii) maior capacidade de extrusão do composto pelas formas proliferativas e/ou (iii) pela possível atuação sobre distintos alvos celulares, sendo as formas sanguíneas, as mais sensíveis/susceptíveis a sua ação.

Diferenças quanto a eficácia de derivados de TS também foram relatadas sobre formas tripomastigotas e epimastigotas de *T. cruzi,* estas últimas, formas também proliferativas encontradas no hospedeiro invertebrado (Vieites et al., 2009). Os autores descrevem que o derivado metálico de TS (platina (II) 3-(5-nitrofurila) acroleína tiossemicarbazona) apresentou superior atividade tripanocida sobre as formas tripomastigotas em relação aos epimastigotas. Conforme discutido por esses autores, é importante considerar as mudanças morfológicas que ocorrem durante a transformação entre as formas evolutivas do parasito ao longo do seu ciclo biológico, e que podem implicar em importantes alterações metabólicas e de conteúdos macromoleculares, levando a diferenças na sensibilidade à fármacos (Vieittes et al., 2009). De fato, o exato mecanismo de ação de tiossemicarbazonas não é totalmente elucidado, havendo evidências sobre a ação sobre múltiplos alvos (Du et al., 2002; Greenbaum et al., 2004; Vieites et al., 2008).

Tem sido demonstrado que tiossemicarbazonas tridentadas (com três átomos capazes de efetuar ligações coordenadas) são capazes de inibir a ribonucleotídeo redutase, uma enzima essencial para a síntese de DNA (Finch et al., 2000; Li et al., 2001), além de também alterar a atividade de dihidrofolato redutase (Foye et al., 1990; Choi et al., 2002). Há também evidências de que os complexos metálicos de

TS possam induzir estresse oxidativo, além de inibir proteinases de distintos protozoários (Aguirre et al., 2004; Greenbaum et al., 2004; Otero et al., 2006).

Alguns derivados ativos de TS atuam sobre cisteínas proteinases de diferentes patógenos incluindo a cruzaína (aka cruzipaína) do *T. cruzi* (Du et al., 2002). A cruzaína é a principal cisteína protease do *T. cruzi* sendo expressa em todas as suas formas evolutivas, atuando sobre as etapas de invasão, replicação e diferenciação do parasito (Bonaldo et al., 1981; Meirelles et al., 1992; Harth et al., 1993; McKerrow et al., 2009). O uso de fármacos que tenham como alvo a cruzaína representa uma importante estratégia experimental no desenho de novos agentes quimioterápicos para o tratamento da doença de Chagas (McKerrow et al., 2009). Por outro lado, estudos *in vitro* sugeriram que alguns derivados de TS (contendo grupos 5-nitrofurila) que apresentam superior atividade tripanocida que o Nifurtimox, podem não somente atuar sobre cruzaína mas também promover estresse oxidativo no *T. cruzi* (Aguirre et al., 2004). Além disso, quando uma série de segunda geração de TS foi sintetizada e testada *in vitro* contra o *T. cruzi*, alguns deles (em especial aqueles com substituintes nas posições 3'-ou 4'- no anel aromático) foram ativos tanto sobre a enzima purificada (cruzaína) como sobre os parasitos. Por outro lado, outros (como os derivados de TS-piridina) embora não tenham sido capazes de inibir a enzima alvo, apresentaram excelente atividade tripanocida sobre o parasito, sugerindo que estes compostos possam estar agindo através da inibição da cisteína protease e/ou por outros mecanismos independentes desta enzima (Greenbaum et al., 2004).

A eficácia de novos derivados metálicos de TS (3 - (5-nitrofurila) tiossemicarbazonas acroleína complexados a platina) foi avaliada *in vitro* sobre *T. cruzi* e os resultados também sugeriram que alguns dos compostos mais ativos atuam sobre pelo menos dois alvos celulares: através da geração de radicais livres e pela interação com o DNA (Vieites et al., 2008). Em outro estudo, a atividade de outra série de complexos metálicos de TS (4-nitroacetofenona-tiossemicarbazonas e seus derivados de cobre) também foi demonstrada sobre formas epimastigotas do *T. cruzi*, com alguns deles exibindo superior eficácia que o BZ e o NF (Pérez-Rebolledo et al., 2008).

Nossos dados também revelaram a baixa toxicidade sobre células de mamíferos dos complexos de TS avaliados. O composto **3** (Mn-Bz4M) além ser o mais efetivo sobre os parasitos sanguíneos foi também o que apresentou o maior índice de seletividade, sendo cerca de 10 vezes mais ativo contra os parasitos em relação as

células cardíacas. Este índice é encorajador e certamente poderá ser melhorado na próxima geração de derivados de TS (síntese em curso pela Dra Letícia R. Texeira) visando aumentar a potência tripanocida e reduzir ainda mais a toxicidade sobre as células hospedeiras.

Em resumo deste bloco, podemos sugerir que embora não seja de uso clínico imediato, (i) a natureza não peptídica do grupo TS, (ii) o relativo pequeno tamanho molecular, (iii) baixo custo de síntese, (iv) capacidade de gerar radicais Ar-NO2•- semelhantemente aos compostos nitroderivados usados na clínica para DC (como apontado na discussão do artigo 2), somados (v) a atividade tripanocida que identificamos do composto **3,** são relevantes características que apontam para o promissor efeito de TS sobre o *T. cruzi* justificando a continuidade destes estudos.

4.3. Diamidinas aromáticas e arilimidamida

No artigo#03, estudos foram conduzidos para verificar a atividade *in vitro* de 06 diamidinas aromáticas (DB1582, DB1627, DB1645, DB1646, DB1651 e DB1670) sobre formas tripomastigotas sanguíneas e intracelulares de *T. cruzi*, sua possível toxicidade sobre células de mamíferos, além de avaliar por ensaios de fluorescência e por microscopia eletrônica de transmissão (MET) os potenciais alvos dos compostos. Os 06 compostos estudados apresentam como características estruturais: moléculas curvas com o mesmo tamanho ou maiores (DB1645, DB1651) que a furamidina (DB75), molécula curva menor que a DB75 (DB1582) e moléculas lineares menores que a DB75 (DB1627, DB1646, DB1670). Nossos dados revelaram que as diamidinas pertencentes aos dois primeiros grupos (DB1645, DB1651 e DB1582) foram ativas sobre o parasito (apresentando atividade tempo e dose dependente, na escala micromolar), enquanto as moléculas lineares não foram eficazes. Estudos sobre a relação entre atividade e estrutura molecular destes compostos revelam que em geral os mais ativos desta classe são os que apresentam maior associação ao DNA (e.g. ao kDNA de tripanosomas como o *T. brucei*) e que a maioria dos compostos lineares apresentam menor associação e consequente inferior atividade em relação as moléculas curvas (como a pentamidina e a DB75), sugerindo a importância desta característica quanto ao melhor encaixe de análogos relacionados a pentamidina e a DB75 à fenda menor do DNA que apresenta característica convexa (Wilson et al., 2008).

Nosso estudo também mostrou diferenças quanto à suscetibilidade entre os parasitos intracelulares e os tripomastigotas durante o tratamento com estas

diamidinas, sendo as formas sanguíneas as mais sensíveis. Estes dados podem refletir diferenças na captação e/ou extrusão dos compostos e/ou ainda ação sobre distintos alvos celulares sobre as formas não-proliferativas (tripomastigotas) e as formas intracelulares (altamente multiplicativas) do parasito. De fato, embora o exato mecanismo de ação desses compostos catiônicos aromáticos não esteja totalmente esclarecido e tenha sido principalmente relacionado à sua associação a fenda menor do kDNA em sequências de bases AT, outros mecanismos de ação tem sido também propostos incluindo a inibição de toposoimerases, de cisteínas proteinases além de interferências na síntese de poliaminas (Soeiro et al., 2008). Além disso, como as formas intracelulares se encontram no citoplasma de suas células hospedeiras e, portanto não diretamente expostas aos compostos, outra possibilidade para justificar as diferenças encontradas seria a de que o transporte destas diamidinas, através das membranas das células hospedeiras, não estivesse ocorrendo. No entanto, como estes compostos apresentam características fluorescentes, foi possível acompanhar a sua distribuição no *T. cruzi* e observar sua localização no cinetoplasto e núcleo de parasitos intracelulares assim como no núcleo de cardiomiócitos (artigo#03 Fig. 5F) (Soeiro et al., 2005). Assim demonstrando a efetiva captação destes compostos pelas células hospedeiras e consequente acesso aos parasitos intracelulares. De fato, apesar de caracterizados em *Trypanosoma brucei*, *Plasmodium falciparum* e *Leishmania sp*, os transportadores/mecanismos envolvidos na captação de diamidinas por *T. cruzi* ainda são desconhecidos e merecem ser identificados a fim de compreender melhor os distintos perfis de sensibilidade observados entre as diferentes formas evolutivas deste parasito (Carter et al., 1995; Barrett et al., 2003; Bray et al., 2003).

Visando avaliar a possível aplicação em bancos de sangue, a eficiência destas diamidinas sobre formas sanguíneas também foi avaliada a 4ºC na presença de sangue de camundongos. Nossos resultados demonstraram que todos os compostos tiveram redução de atividade nestas condições experimentais. Isto possivelmente devido a associação destes compostos à proteínas plasmáticas, como a albumina, como já sugerido para outros compostos e/ou ainda, devido à instabilidade e/ou metabolismo destas diamidinas na presença de sangue, e portanto, não apresentam características relevantes para uso profilático em bancos de sangue (Santa-Rita et al., 2006; Silva et al., 2007a; Pacheco et al., 2009).

Como discutido anteriormente, os compostos estudados apresentam fluorescência intrínseca. Assim foi possível avaliar a sua distribuição em duas

formas do *T. cruzi.* Observamos que como anteriormente relatado em tripanosomas africanos e no próprio *T. cruzi* todos os compostos estudados se localizaram em estruturas que contêm DNA, núcleo e cinetoplasto, apresentando consistentemente maiores níveis no kDNA (De Souza et al., 2004; Mathis et al., 2006, 2007). Além disso, as diamidinas DB1651 e DB1582 também foram detectadas em compartimentos intracelulares preferencialmente localizados na porção anterior de formas tripomastigotas de sangue, e próximos ao núcleo e cinetoplasto de amastigotas de *T. cruzi*. Com base nesta distribuição intracelular e morfologia destas estruturas somada aos dados prévios descritos em *Trypanosoma brucei*, sugerimos que estes compartimentos sejam acidocalcisomas, embora outras organelas desprovidas de DNA não possam ser totalmente excluídas (Mathis et al., 2006, 2007).

Estudos bioquímicos realizados em *T. cruzi* e *Trypanosoma brucei* demonstraram que acidocalcisomas apresentam pH ácido, isto em decorrência da ação de uma próton ATPase do tipo vacuolar e de uma Ca^{2+}-ATPase (De Souza, 2009). Estas organelas contêm cálcio, magnésio, sódio, zinco e fósforo presente em pirofosfatos e polifosfatos, e podem ser observadas ao microscópio óptico através do uso de corantes, como por exemplo, a diamidina 4,6-diamidino-2-fenilindole (conhecido como DAPI) (Miranda et al., 2004; Moreno e Docampo, 2009). Suas potenciais funções incluem o estoque de cálcio e fósforo, participação no metabolismo do pirofosfato inorgânico (PP(i)), homeostasia do cálcio, manutenção da homeostasia do pH intracelular, e osmoregulação (Vercesi et al., 1994; Docampo et al., 1995). Além de serem consideradas importantes alvos para o desenho de drogas anti-*T. cruzi* (Docampo e Moreno, 2008).

Como demonstrado para *Trypanosoma brucei* (Mathis et al., 2006, 2007), a localização das diamidinas em acidocalcisomas do *T. cruzi* sugere que estas organelas sejam alvos e/ou sítios de estoque destes compostos aromáticos, o que será futuramente avaliado pelo nosso grupo. Ademais, como estudos anteriores em *Trypanosoma brucei* relataram que a localização da DB75 e da DB820 (e seus análogos) em compartimentos desprovidos de DNA seja um evento tempo-dependente (Mathis et al., 2006), e em nossos estudos apenas avaliamos um tempo fixo de incubação (uma hora), não podemos descartar a possibilidade de que as outras diamidinas (DB1627, DB1645, DB1646 e DB1670) também se acumulem nestas estruturas e assim, ensaios utilizando uma cinética de tratamento serão conduzidos.

Visto que a análise por microscopia eletrônica de transmissão (MET) de alterações morfológicas induzidas por drogas possibilita inferir sobre possíveis mecanismos de ação, aspectos ultraestruturais do *T. cruzi* após o tratamento com estas 06 diamidinas foram investigados (Rodrigues e De Souza, 2008; De Souza, 2008). A alteração mais importante e frequentemente observada com todos os compostos foi relacionada a alterações na morfologia da mitocôndria além da desorganização estrutural do kDNA, corroborando dados ultraestruturais anteriores que apontam para estas estruturas como importantes alvos no *T. cruzi* e *Leishmania spp.* de compostos da classe de diamidinas (Fusai et al., 1997; De Souza et al., 2004; Silva et al., 2008).

Outros resultados interessantes também observados foram as alterações na organização de microtúbulos em formas tripomastigotas de sangue. Observamos que nesta forma não proliferativa do *T. cruzi*, a DB1582 induziu uma organização incomum de múltiplos axonemas sem evidências de duplicação do flagelo, como também já foi previamente descrito durante o tratamento destas formas evolutivas com derivados de diamidinas, as arilimidamidas (Silva et al., 2007b). Em formas tripomastigotas, a DB1645 também induziu (i) alterações na estrutura e organização de microtúbulos sub-peliculares e (ii) modificações na estrutura básica (9+2) do axonema, exibindo mais de 1 par central de microtúbulos. Sabe-se que microtúbulos são estruturas dinâmicas, mas extremamente estáveis e que desempenham relevantes papeis em diversos processos biológicos. Em protozoários o microtúbulos estão principalmente relacionados a manutenção da estrutura, estabilidade celular e controle de internalização de moléculas e partículas (microtúbulos sub-pelicular); motilidade (microtúbulos no flagelo) e proliferação (corpúsculo basal e fusos mitóticos) (Menna-Barreto et al., 2009).

De fato, embora diferentes estudos tenham sido realizados sobre tripanosomatídeos, incluindo o *T. cruzi,* utilizando drogas que interferem na estabilidade de microtúbulos, como o taxol, colchicina e vinblastina, não há relatos de alterações nestas estruturas do parasito (microtúbulos sub-pelicular e flagelar), isto possivelmente devido ao alto teor de tubulina acetilada (Souto-Padron e col., 1993; Dantas et al., 2003). Assim, como diferentemente de células de mamíferos, estas estruturas em tripanosomatídeos são altamente resistentes a ação da grande maioria de agentes desestabilizadores de microtúbulos conhecidos, a ação destes compostos sobre microtúbulos do *T. cruzi* abre uma interessante perspectiva de trabalho que merece ser aprofundada e sugere ainda que estas estruturas possam

ser alvos para estas drogas o que é interessante do ponto de vista de desenvolvimento de novos fármacos mais seletivos.

Apesar da sua alta atividade contra um amplo espectro de microorganismos, uma grande preocupação relacionada as diamidinas atualmente utilizadas na clínica é sua toxicidade, o que tem estimulados vários grupos de química medicinal a sintetizarem compostos dicatiônicos aromáticos mais seletivos (Soeiro et al., 2005). Neste contexto, o composto mais eficaz sobre ambas as formas do *T. cruzi*, a DB1645, foi também o que apresentou menor toxicidade mesmo após 72 horas de tratamento das culturas de células cardíacas, levando aos melhores índices de seletividade, justificando, portanto outros estudos *in vitro* e *in vivo* com esta classe de compostos. No entanto, a DB1645 não se revelou mais potente sobre este parasito em relação a outros derivados de diamidinas, as amidinas reversas hoje denominadas de arilimidamidas (AIA), e que exibem excelente efeito tripanocida, em doses nanomolares (Silva et al., 2007a, b; Pacheco et al., 2009).

Assim, dando sequência ao presente estudo, nos artigos #04 e #05, ensaios *in vitro* e *in vivo* foram conduzidos com uma arilimidamida, a DB766, visando explorar o seu potencial efeito tripanocida sobre *T. cruzi*. Nestes estudos utilizamos protocolos de alta estringência, investigando (Artigo#04) a eficácia e seletividade *in vitro* sobre ambas as formas evolutivas do *T. cruzi* relevantes para a infecção humana (tripomastigota sanguínea e amastigota intracelular), considerando também a análise sobre diferentes populações de parasitos com distintos perfis de suscetibilidade a nitroderivados (BZ e NF) e que circulam em distintos ciclos de transmissão (domiciliar, peridomiciliar e silvestre). Analisamos (Artigo#04) também por ensaios de microscopia de fluorescência e MET a localização e alvos celulares desta AIA sobre o parasito. Por fim, avaliamos (Artigos# 04 e 05) em diferentes modelos experimentais de infecção aguda e através de diferentes protocolos de tratamento e vias de administração, o efeito desta AIA sozinha ou associada ao BZ em doses previamente selecionadas a partir dos dados obtidos em ensaios de toxicidade aguda.

No quarto artigo, descrevemos a excelente atividade da DB766 sobre formas sanguíneas e amastigotas intracelulares do *T. cruzi*, alcançando sobre a cepa Y em ensaios a 37°C, valores de IC_{50} 60 e 25 nanomolares após incubação por 24 e 72 horas, respectivamente. Nossa análise confirma dados anteriores sobre a excelente atividade de AIAs sobre este parasito (Souza et al., 2004; Silva et al., 2007a,b; Silva

et al., 2008; Pacheco et al., 2009), assim como sobre *Leishmania spp.* e *Toxoplasma gondii* (Rosypal et al., 2008; Leepen et al., 2008).

A atividade desta AIA foi evidente mesmo após curtos períodos de tratamento dos tripomastigotas e das células cardíacas infectadas (2 e 24 horas respectivamente), e mesmo a 4°C na presença de sangue (96 e 50%), mostrando seu potencial uso em bancos de sangue como já descrito também para outras AIAs como a DB889 e DB702 (Silva et al., 2007a). Neste sentido, a DB766 mostrou superior atividade (30,6 vezes maior) em relação à violeta genciana, uma droga de referência para o tratamento de sangue. Além disso, DB766 foi igualmente ativa sobre cepas de *T. cruzi* de distintas áreas geográficas e que circulam em distintos ciclos de transmissão: domiciliares (Y, no Brasil), peridomiciliares (762, 855, 875, 956, 958 e 960) e silvestres (MS1523 e RBVIII). Vale ressaltar que contra todas as cepas, a DB766 apresentou sempre superior eficácia que o BZ (e.g. DB766 cerca de 140 vezes mais ativa sobre a cepa 855 que o BZ).

A triagem de drogas tripanocidas sobre distintas cepas representantes das diferentes linhagens do parasito, incluindo aquelas naturalmente resistentes a nitroderivados, representa uma etapa fundamental na descoberta de compostos ideais para o futuro desenvolvimento de novas terapias para doença Chagas (Soeiro e De Castro, 2009; Ribeiro et al., 2009). O estudo das cepas provenientes de vetores peridomicliares da região Nordeste (Vale do Jaguaribe, Estado do Ceará) apresenta ainda um relevante caráter epidemiológico em estudos de triagem de fármacos anti-*T. cruzi*, pois esta é uma importante área de vigilância entomológica para a doença de Chagas no Brasil, visto que altos índices de infecção natural de triatomíneos tem sido observado (Dr. MM Lima-IOC/Fiocruz, dados não publicados) e assim a manutenção do controle vetorial ainda demanda atenção, visando evitar novos casos de transmissão humana (Alencar, 1987; Dias, 2007). Igualmente é relevante a análise sobre cepas que circulam na região Amazônica, pois ela representa um atual e importante desafio epidemiológico devido ao grande número de relatos de casos agudos, principalmente via transmissão oral (Aguillar et al., 2007).

Análise de atividade tripanocida de novos compostos sobre cepas de *T. cruzi* que apresentam diferentes níveis de resistência à NF e BZ se faz importante, pois esta variabilidade somada ao perfil genético do hospedeiro, pode, em parte, explicar as diferenças entre os diversos índices de cura observados em distintas áreas geográficas frente a terapia com ambos compostos (Postan et al., 1984; Dvorak,

1984; Filardi e Brener, 1987; Coura e de Castro, 2002; Yun et al., 2009; Pena et al., 2009). Ademais, a heterogeneidade das cepas também pode estar relacionada às diferentes manifestações clínicas na doença de Chagas (Andrade et al., 1999; McKerrow et al., 2009;). Nossos dados mostram que a DB766 foi uniformemente ativa sobre cepas de *T. cruzi* que apresentam naturalmente baixa (CL), moderada (Y) e alta resistência (Colombiana e YuYu) ao tratamento *in vivo* com BZ, alcançando valores de IC_{50} na faixa sub-micromolar, sendo cerca de 30X inferiores a violeta de genciana. Essa atividade notável da DB766 sobre várias linhagens do parasito revela outro aspecto bastante promissor do mesmo.

A DB766 também se revelou mais ativa que a DB75, confirmando dados da literatura que demonstraram em *T. cruzi* a superior atividade tripanocida de AIAs em relação a diamidinas (Silva et al., 2007a, Pacheco et al., 2009). É possível que esta superior eficácia tenha relação com propriedades físicas destas moléculas. As diamidinas são moléculas básicas com valores de pK_a próximos a 11, enquanto as AIAs tem valores próximos a 7, consequentemente em pH fisiológico, as DAs são protonadas e assim, catiônicas enquanto as arilimidamidas são essencialmente moléculas neutras, o que facilita seu transporte passivo através de membranas biológicas (da célula hospedeira e do parasito). Esta diferença pode assim afetar significativamente a absorção e distribuição dos compostos e possivelmente contribuir para as diferenças nos níveis de atividade reportados entre AIAs e DAs.

A semelhança do que foi observado com as DAs no artigo#03, os ensaios de microscopia de fluorescência e MET nos permitiram analisar a localização e alvos celulares desta AIA sobre o *T.cruzi*. Em nossos estudos identificamos nas formas tripomastigotas sanguíneas a DB766 no núcleo e no kDNA, havendo um maior acúmulo, tempo-dependente, na última. Esta AIA também promoveu alterações ultraestruturais no núcleo e na mitocôndria, causando importantes danos (intumescimento) nas cristas mitocondriais. Novamente nossos dados confirmam estudos anteriores com DAs e outras AIAs realizados em *Leishmania spp.* (Croft e Brasil, 1982; Hentzer e Kobayasi, 1977; Fusai et al., 1997) e *T. cruzi* (De Souza et al., 2004; Silva et al., 2007b, 2008; Batista et al., 2009) e *T. brucei* (Mathis et al., 2007), que sugerem o complexo mitocôndria/cinetoplasto como alvo de ação destes compostos aromáticos, embora outros mecanismos não possam ser excluídos e merecem ser melhor aprofundados.

Em resumo, nossos resultados *in vitro* mostraram a excelente atividade da DB766 sobre diferentes cepas de *T. cruzi* com eficácia superior ao BZ e a violeta

genciana, exibindo um excelente índice seletivo (> 533 e 714 para tripomastigotas e amastigotas da cepa Y, respectivamente), que é pelo menos 10 vezes maior que o índice mínimo proposto na literatura para promover a análise de compostos tripanocidas de *in vitro* para *in vivo* (Nwaka e Hudson, 2006). Esses dados em conjunto motivaram as análises *in vivo.* Inicialmente avaliamos a toxicidade aguda sobre camundongos (Suíço, Balb/c e C3H, machos e fêmeas) e como até a dose de 200 mg/kg/dia não foi observado (i) mortalidade, (ii) alterações comportamentais e (iii) bioquímicas (análise no sangue após 48 h de tratamento dos níveis de CK, GPT e uréia), nosso próximo passo foi avaliar a atividade da DB766 em diferentes modelos de infecção aguda experimental (cepas Y e colombiana) pelo *T. cruzi*. A análise do efeito DB766 *in vivo* foi realizada usando uma combinação de parâmetros parasitológicos, moleculares, bioquímicos, clínicos e histopatológicos que permitiram uma detalhada investigação sobre a eficácia da DB766 sobre este patógeno.

Resultados anteriores demonstraram que DAs, como a DB569 (De Souza et al., 2006) e a DB1362 (Silva et al., 2008), embora não sejam capazes de reduzir significantemente a parasitemia circulante, levam a um acentuado decréscimo no parasitismo (acima de 90%) e na inflamação cardíaca, além de modular negativamente a expressão de células T $CD8^+$ no coração dos camundongos infectados e tratados (De Souza et al., 2007). Nossos dados mostram que ao contrário das diamidinas previamente avaliadas *in vivo*, a administração da DB766 via ip reduziu a parasitemia e o parasitismo cardíaco, apresentando atividade sobre a cepa Y e Colombiana ≥ que o BZ, e resultando (dependendo do esquema de tratamento – doses de até 50 mg/kg/dia, por 10 dias consecutivos, iniciando-se tratamento no início da parasitemia e somente avaliando animais positivos) em 100% de sobrevida. Esta proteção contra a mortalidade possivelmente é decorrente dos menores níveis de lesões teciduais (e.g. muscular e hepático), resultante da redução da carga parasitária e/ou pela queda nos níveis de inflamação que, também, observamos. Tendo em vista os dados da literatura, o processo inflamatório associado à presença do parasito (mesmo em níveis sub-patentes no caso de infecções crônicas) exerce fundamental papel para geração das lesões e desencadeamento da patologia induzida por esta infecção (Higuchi et al., 2003; Marin-Neto et al., 2007). De fato, os níveis plasmáticos de CK e GPT foram consistentemente menores em camundongos tratados com DB766 em relação ao grupo tratado com BZ o não tratado. Além disso, os dados de ECG mostram que a DB766 foi capaz de reverter as alterações elétricas induzidas pela infecção

confirmando dados anteriores referentes ao tratamento com diamidinas (De Souza et al., 2007; Silva et al., 2008).

É ainda importante considerar que como a DB766 (i) é capaz de atravessar membranas celulares possivelmente por difusão passiva e/ou mesmo via transportadores de membranas, e (ii) apresenta uma meia vida longa além de exibir grandes volumes de distribuição, acumulando-se em órgãos como fígado, baço e coração (comunicação pessoal, Dr. Michael Z. Wang), esta classe de compostos apresenta importantes características para a terapia da doença de Chagas haja vista o *T. cruzi* é um parasito intracelular obrigatório e ninhos de amastigotas podem ser encontrados em todos os tecidos e órgãos. Outro importante resultado é referente á excelente atividade da DB766 sobre formas amastigotas intracelulares (IC_{50} = 25 nM), que são consideradas como principal alvo para o desenvolvimento de drogas anti-*T.cruzi* (McKerrow et al., 2009), em especial para o tratamento da fase crônica, na qual se observam somente cerca de 10-20% de cura frente ao tratamento com BZ e NF (Coura e De Castro, 2002).

Embora a DB766 não seja uma pró-droga e em geral DAs apresentam baixa biodisponibilidade oral (Soeiro et al., 2009), quando administrada por via oral em doses semelhantes ao BZ (100 mg/kg/dia), esta AIA apresentou sobre a infecção pela cepa Y e Colombiana, excelente eficácia (similar ao BZ), resultando (i) na importante redução no número de parasitos circulantes e do parasitismo cardíaco, (ii) diminuição dos níveis de CK e GPT, (iii) na prevenção contra alterações elétricas cardíacas induzidas pela infecção, e (iv) em 100% de proteção contra mortalidade. Estes dados sugerem que quantidades suficientes desta AIA foram absorvidas via trato gastro-intestinal dos animais tratados, sendo distribuída ao plasma e tecidos como descrito para DB289 (Tidwell et al., 1990; Hall e cols. 1998; Thuita et al., 2008). Dados ainda não publicados do Dr. Wang realizados via administração oral da DB766, confirmam sua boa biodisponibilidade e acúmulo em tecidos de camundongos. A eficácia oral é um dado interessante já que representa a via desejável para tratar pacientes com doença de Chagas, bem como para outras doenças parasitárias negligenciadas, não requerendo internação do paciente (Thuita et al., 2008). A atividade oral de diamidinas foi anteriormente relatada para o berenil (aceturato diminazeno) em pacientes com tripanosomíase africana (Abaru et al., 1984), embora este medicamento seja de uso veterinário, não estando registrado para uso humano (Soeiro et al., 2005).

Por outro lado, embora importantes efeitos adversos não tenham sido detectados pelas técnicas que utilizamos em animais não infectados e tratados com a DB766, a não recuperação e o agravamento da caquexia induzida pela infecção pode estar relacionada com a menor proteção contra a mortalidade (de somente 60%) dos animais infectados e tratados por 10 dias, via ip, com 100 mg/kg/dia DB766. Apesar deste esquema de tratamento resultar em níveis quase indetectáveis de parasitismo (sangue e no coração) teve maior eficácia tripanocida com o BZ.

Considerando-se as propriedades farmacocinéticas e farmacodinâmicas desta AIA (e.g. extenso acúmulo no tecido e sua longa meia-vida) e com os objetivos de manter essa superior eficácia e reduzir a possível toxicidade da droga, analisamos em outro esquema terapêutico, o efeito da DB766 utilizando doses intercaladas (administradas a cada 48 horas) de 100 mg/kg/dia, via ip. Observamos que além de manter a alta eficácia tripanocida e proteger contra a caquexia dos animais infectados em níveis semelhantes aos animais tratados com BZ e ao grupo de não infectados, a administração de DB766 via ip em dias intercalados resultou em melhores taxas de sobrevivência dos camundongos (índices de 80% de proteção). Entretanto, apesar dos excelentes resultados, o tratamento com DB766 (ip e p.o.) por até 10 dias não resultou em cura parasitológica, avaliada pelo hemocultivo e PCR, que também não foi observado no tratamento com a droga de referência.

Neste sentido, como usamos rigorosos protocolos de tratamento de até 10 dias, e (i) a maioria dos ensaios de drogas em modelos murino utiliza regimes de pelo menos 20 dias de administração (McKerrow et al., 2009), (ii) estudos tem demonstrado que, para alcançar altos índices de cura parasitológica na infecção experimental pelo *T. cruzi* longos períodos de tratamentos são necessários (> 20 dias) mesmo utilizando o BZ (Bustamante et al., 2008) e triazoles como o TAQ187 (Urbina et al., 2003), e (iii) notável efeito foi observado com a DB766 via ip (até 50 mg/kg/dia) e *gavage* (100 mg/kg/dia), estudos adicionais por períodos mais longos de tratamento com esta AIA associada ou não ao BZ foram conduzidos e apresentados no artigo#05.

No artigo#05 realizamos ensaios *in vivo* a fim de avaliar a possível eficácia da associação entre o BZ e a DB766. O uso combinado de compostos representa uma interessante abordagem terapêutica que permite atuar sobre diferentes elementos celulares e vias metabólicas, propiciando assim, distintos mecanismos de ação. Esta abordagem permite ainda reduzir a concentração de cada composto contribuindo assim para a diminuição dos efeitos tóxicos, além de minimizar o risco de resistência

às drogas (McKerrow et al., 2009). Neste contexto, avaliamos a eficácia da DB766 e de uma pafuramidina, a pró-droga DB289, em esquemas de terapia combinada ao BZ, empregando 20 dias de tratamento, através de diferentes vias (p.o. e ip).

A DB289 (dicloridrato de furamidina; 2,5-bis (4-dicloridrato de furano guanilfenil) é uma pró-droga da furamidina, e quando administrada oralmente apresenta efeito microbicida sobre diferentes patógenos, incluindo *Trypanosoma brucei*, *Pneumocystis jiroveci* e *Plasmodium falciparum* (Yeramian et al., 2005; Chen et al., 2007; Wenzler et al., 2009; Nyunt et al., 2009;). Esta pró-droga foi avaliada (via oral) em ensaios clínicos para tratamento da tripanosomíase africana, sendo este estudo recentemente interrompido na fase III, pois embora indicações iniciais de baixa toxicidade nas populações africanas, asiáticas, caucasianas e hispânicas tenham sido observadas, estudos adicionais realizados na África do Sul revelaram efeitos hepáticos e renais secundários (Thuita et al., 2008; Soeiro et al., 2009). De modo semelhante ao tratamento da DC, para a tripanossomíase humana africana, outra relevante doença negligenciada e que afeta população pobres de países em desenvolvimento, a eficácia oral é um requisito para o desenvolvimento de novos quimioterápicos (Thuita et al., 2008).

Ao contrário da excelente atividade sobre *Trypansoma brucei in vivo* (Thuita et al., 2008), a DB289 sozinha revelou somente uma moderada ação *in vivo* contra o *T. cruzi*, possivelmente devido a diferenças na biologia destes parasitos como por exemplo, as formas tripomastigotas sanguíneas que não se replicam, enquanto no *T. cruzi* há uma forma intracelular proliferativa, o que pode representar um obstáculo adicional para o acesso e distribuição de compostos. Contudo, a combinação de BZ com DB289 induziu importante declínio da parasitemia e 100% de sobrevida, potencializando em cerca de nove vezes a atividade tripanocida de BZ sozinho (dose sub-ótima de 50 mg/kg/dia). Por outro lado, quando maiores doses de DB289 (100 mg/kg/dia) foram testadas contra a infecção por *T. cruzi in vivo* (dados não publicados), observamos um maior número de parasitos circulantes e superiores taxas de mortalidade em relação aos camundongos não tratados, que pode ser um reflexo de toxicidade do composto.

Semelhante ao observado no artigo#04, quando administrada por 20 dias (via ip), a DB766 (50 mg/kg/dia) apresentou excelente ação tripanocida, sendo ainda mais efetiva que o BZ (50 mg/kg/dia) pois reduziu os níveis parasitêmicos e, mesmo, parasitismo e inflamação cardíaca. Entretanto, neste esquema de tratamento, esta AIA induziu menor proteção contra a mortalidade em relação ao

BZ, utilizando mesmos tratamento e dose da AIA por 10 dias como realizado no artigo#04. Esta inferior proteção na sobrevida dos animais pode estar associada a não recuperação e mesmo agravamento da caquexia induzida pela infecção, haja vista que observamos uma correlação entre morte e menor peso dos animais. Por outro lado, a combinação de BZ (oral) e DB766 (ip) resultou em parasitemia não detectável, sobrevida de 100% e recuperação de caquexia. Esta recuperação na curva ponderal pode estar relacionada ao efeito modulatório do BZ sobre o TNF-alfa, que é uma citocina pro-inflamatória importante na infecção pelo *T. cruzi* estando envolvida na caquexia induzida na infecção experimental por este parasito (Tarleton, 1988). Assim, embora os ensaios de toxicidade aguda não tenham revelado efeitos adversos até o uso de 200 mg/kg/dia de DB766 (Batista et al., 2009) e que esta AIA seja capaz de reduzir lesões hepáticas e musculares induzidas pela infecção, se faz necessária uma análise toxicológica mais detalhada (ensaios bioquímicos e histopatológicos) para esclarecer esta questão.

Outro ponto importante a ser explorado é relacionado ao perfil imune de resposta celular e humoral (plasma e tecidos como coração) incluindo mediadores pro- e anti-inflamatórios frente ao tratamento ou não com a AIA e o BZ sozinhos, comparando com o co-tratamento com ambos. Este estudo poderá compreender melhor as possíveis interferências entre a resposta imune sobre a toxicidade versus eficácia da AIA sozinha e/ou combinada. É importante destacar que alguns estudos mostram o efeito regulador da pentamidina sobre citocinas pró-inflamatórias (Quay et al., 1993; Van Wauwe et al., 1996) e dados prévios do nosso grupo demonstram a regulação do perfil celular inflamatório no coração de camundongos tratados com a diamidina DB569 (De Souza et al., 2007). Além disso, BZ regula a síntese de TNF-alfa em macrófagos de camundongos estimulados *in vivo* (Revelli et al., 1999), regula a resposta inflamatória induzida pelo LPS, diminui os níveis plasmáticos desta citocina (Pascutti et al., 2004), e reduz acentuadamente a produção de citocinas pró-inflamatórias e metabólitos derivados de NO em camundongos experimentalmente infectados pelo *T. cruzi* (Manarin et al., 2008). A modulação negativa de TNF-alfa nos nossos camundongos infectados e tratados com BZ poderia justificar a recuperação da curva ponderal nestes animais quando comparados aos camundongos infectados não tratados e a não recuperação da caquexia no grupo infectado e tratado com a DB766. De fato, como acima discutido, em nossos estudos, encontramos uma correlação entre caquexia de camundongos e as taxas de mortalidade, inclusive nos grupos DB766 (via ip e p.o.).

Em relação à análise de cura parasitológica avaliada por hemocultivo e PCR, observamos que embora em nenhum dos protocolos utilizados houve cura parasitológica com o BZ (até 20 dias de tratamento), a terapia combinada de DB766+BZ resultou na cura de 2 animais dos quinze sobreviventes (13%). Este tratamento combinado resultou em reduções superiores a 99.5% na parasitemia e parasitismo cardíaco dos animais, e de 60 e 90% nos níveis de marcadores de lesões teciduais (hepático e cardíaco, respectivamente). Este último dado possivelmente se deu pelo controle de carga parasitaria e/ou da queda na inflamação. De fato, dados demonstram que um processo inflamatório exacerbado/desequilibrado sustentado pela presença do parasito desempenha papel fundamental no desencadeamento da patologia chagásica (Higuchi et al., 2003; Marin-Neto et al., 2007).

Como relatado no artigo#04, embora não seja uma pró-droga como a DB289, a DB766 na dose sub-ótima de 50 mg/kg/dia apresentou moderada atividade sobre a infecção aguda experimental por *T. cruzi* quando administrada oralmente por 20 dias, sendo menos efetiva em relação ao protocolo anterior (artigo#04) quando administrada por 10 dias consecutivos com a dose de 100 mg/kg/dia. Verificou-se que um dos três animais sobreviventes tratados com DB766 (50 mg/kg/dia, p.o.) revelou-se curado pelos parâmetros de hemocultivo e PCR. A análise individual da curva parasitêmica de cada animal permitiu identificar que este animal curado foi o que exibiu parasitismo não detectável através de contagem pela microscopia óptica ainda durante o curso da terapia.

Quando avaliamos o co-tratamento de BZ+AIA utilizando doses sub-ótimas dos citados compostos (50 mg/kg/dia) por via oral, observamos um aumento de 1,8 vezes na atividade da AIA sozinha. No entanto, a terapia combinada quando comparada com o tratamento realizado com BZ sozinho revelou um efeito antagônico na redução dos níveis parasitêmicos, que merece ser mais explorado. Vale ressaltar que todos os animais tratados com esta combinação sobreviveram e que a terapia combinada, atingiu ainda que, em níveis discretos, cura parasitológica. Estes dados estimulam a continuidade de estudos com esta nova classe de compostos (AIA) isoladamente ou associada a outros fármacos licenciados, como os medicamentos de referência NF e BZ, objetivando a identificação de novos candidatos para o tratamento da doença de Chagas.

Resumindo, os nossos dados *in vitro* e *in vivo* revelam a superior eficácia da AIA sobre as outras classes de fármacos avaliadas neste trabalho, validando assim,

futuras abordagens pré-clinicas com a DB766 por via oral, por períodos menores de 60 dias e com a administração em dias alternados. Tendo em vista a excelente atividade *in vitro* e *in vivo* sobre o *T. cruzi* são válidos também futuros testes com outras AIAs. Como para qualquer novo fármaco, estudos farmacológicos mais detalhados e de tolerabilidade devem ser explorados, visando a identificação de novas AIAs que apresentem potencial para o tratamento da doença de Chagas.

CONCLUSÕES

1. A atividade *in vitro* dos antibióticos sintéticos quinolônicos - esparfloxacina (SPAR) e norfloxacina (NOR), e de seus complexos metálicos (manganês, cobalto e cobre-fenantrolina) avaliada sobre formas tripomastigotas sanguíneas e intracelulares do *T. cruzi* (cepa Y) revelou que embora SPAR e NOR e seus complexos de Mn e Co não tenham sido ativos, os derivados **5** e **6**, complexos de $CuCl_2$(fen)(NOR) e $CuCl_2$(fen)(SPAR), apresentaram boa atividade tripanocida (IC_{50} de 1,6 a 4,6 μM) sobre ambas as formas do parasito, sendo cerca de 80 vezes mais ativos que os ligantes livres. A superior ação destes compostos pode estar relacionada ao aumento nos níveis de sua captação pelos parasitos e/ou pela ação sobre diferentes alvos celulares. Nossos resultados corroboram dados anteriores em tripanosomatídeos que demonstraram a atividade leishmanicida e tripanocida de fluoroquinolonas.
2. A atividade *in vitro* de tiossemicarbazonas e seus derivados metálicos de manganês avaliada sobre formas tripomastigotas sanguíneas e intracelulares do *T. cruzi* (cepa Y) revelou que somente o derivado **3** ($Mn(H4NO_2Bz4M)_2Cl_2$) apresentou boa atividade tripanocida (IC_{50} = 19 μM) sobre formas sanguíneas, sendo cerca de 10 vezes mais ativo contra os parasitos em relação as células hospedeiras. A promissora atividade do composto **3** confirma dados prévios da literatura que apontam sobre o efeito tripanocida e leishmanicida de derivados metálicos de tiossemicarbazonas *in vitro*, estimulando a síntese e análise de outros derivados desta classe de compostos sobre o *T. cruzi*.
3. A atividade *in vitro* de diamidinas aromáticas avaliada sobre formas tripomastigotas sanguíneas e intracelulares do *T. cruzi* (cepa Y), revelou que embora todas tenham exercido baixa toxicidade sobre células hospedeiras, somente três (DB1645, DB1582 e DB1651) foram ativas contra ambas formas do parasito alcançando valores de IC_{50} entre 0,15 a 13,3 μM. O efeito tripanocida das DAs apresentou correlação direta com estrutura química dos compostos, estando relacionada a curvatura das moléculas, sendo as lineares (DB1627, DB1646 e DB1670) as não ativas.
4. A análise ultraestrutural revelou que as DAs induziram alterações mitocondriais, no kDNA e em microtúbulos. Ensaios de fluorescência revelaram que todas se localizaram no núcleo e no kDNA, com maior acúmulo na última estrutura. As DB1582 e DB1651 também se acumularam em organelas desprovidas de DNA, possivelmente acidocalcisomas, podendo, como sugerido

para tripanosomas africanos, representar alvo e/ou sítios de estoque dos compostos.

5. A eficácia (e.g. DB1645 com IS de 640) *in vitro* das DAs confirma dados prévios sobre a ação desta classe de compostos aromáticos contra tripanosomatídeos, justificando a continuidade dos estudos pré-clínicos sobre o *T. cruzi* com estes ligantes de DNA.
6. A atividade e seletividade *in vitro* da arilimidamida DB766 avaliadas sobre formas tripomastigotas sanguíneas e intracelulares do *T. cruzi* mostraram excelente ação sobre formas sanguíneas (60 nM) e intracelulares (25 nM) da cepa Y, mantendo ótima ação na presença de sangue a 4°C, sugerindo potencial uso profilático em bancos de sangue.
7. DB766 apresentou superior eficácia que as drogas de referência (BZ e violeta de genciana) sobre diferentes cepas e linhagens do parasito, incluindo isolados de vetores e outros hospedeiros presentes nos ciclos peridomiciliar e silvestre, além importante efeito tripanocida sobre cepas naturalmente resistentes ao BZ, como YuYu e Colombiana.
8. A localização desta AIA em compartimentos ricos em DNA, somados aos danos ultraestruturais na mitocôndria sugere esta organela como alvo, embora outros não possam ser excluídos.
9. Frente ao excelente índice de seletividade (>533 e 714), ensaios foram conduzidos frente infecção aguda experimental por *T. cruzi (*cepas Y e Colombiana), e os dados mostram que DB766 (25 e 50 mg/kg/dia ip e 100 mg/kg/dia p.o.) reduziu (>90%) carga parasitária (sangue e tecido cardíaco) e protegeu 90-100% contra mortalidade, com semelhante eficácia que BZ. DB766 reverteu a inflamação, as alterações elétricas cardíacas e protegeu contra lesões hepáticas e musculares induzidas pela infecção. Embora DB766 e BZ não tenham induzido cura parasitológica, possivelmente devido aos protocolos de alta estringência utilizados (tratamento no início da parasitemia e por até 20 dias), nossos dados confirmam a promissora atividade das AIAs como a DB766, que demonstrou ≥ eficácia *in vivo* que a droga de referência utilizada na clínica para doença de Chagas
10. Na terapia combinada a utilização de doses sub-ótimas de BZ (p.o.) associado a DB289 (p.o.) ou a DB766 (ip) resultaram em ≥99% redução de parasitemia e 100% de proteção sobre mortalidade. BZ (p.o.)+DB766 (ip) resultou em cura parasitológica (2 em 15 animais) avaliada por hemocultivo e PCR, estimulando

a continuidade de ensaios pré-clínicos com outras AIAs visando identificação de novos fármacos para a doença de Chagas.

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