Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ

Pós-Graduação em Biologia Celular e Molecular

ATIVIDADE, SELETIVIDADE E MECANISMOS DE AÇÃO DE DIAMIDINAS AROMÁTICAS E ANÁLOGOS SOBRE *Trypanosoma cruzi*: UM ENFOQUE SOBRE O kDNA.

Anissa Daliry

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

Orientadoras: Dra. Solange Lisboa de Castro
Dra. Maria de Nazaré Correia Soeiro

RIO DE JANEIRO
2011

Daliry, A.
Atividade, seletividade e mecanismos de ação de diamidinas aromáticas e análogos sobre *Trypanosoma cruzi*: um enfoque sobre o kDNA / Anissa Daliry. – Rio de Janeiro: 2011, XX p.; il.

Tese (Doutorado) – Instituto Oswaldo Cruz, Biologia Celular e Molecular, 2011.

1. *Trypanosoma cruzi* 2. Diamidinas aromáticas 3. kDNA 4. Quimioterapia

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Rio de Janeiro, 06 de janeiro de 2011

Agradecimentos

Primeiro de tudo pelo Sopro Divino, por me conferir a existência e permitir a vida, com tudo de bom que ela tem a oferecer.

Sou profundamente grata ao sempre pronto e guerreiro apoio das minhas queridas orientadoras, Solange Lisboa de Castro e Nazaré Soeiro, que estiveram sempre dispostas a me incentivar em todas as minhas vontades e necessidades. Por terem me recebido de braços abertos em seus laboratórios e ter dado todo o apoio para a realização de uma etapa muito importante da minha formação, a ida aos Estados Unidos.

Ao amor da minha vida, Bruno, que incondicionalmente esteve ao meu lado, sempre com muito cuidado, ternura e amor. Suas constantes e eloquentes "palestras" abordando desde aspectos banais até as questões mais importantes da vida são e serão sempre importantes para o meu crescimento.

À minha amada família, minha mãe, Sima Daliry, meu pai Sina Daliry e meu irmão Sobhan Daliry, que sempre me deram apoio e se orgulharam de cada etapa cumprida e cada vitória alcançada. Além de propiciar todo o amor, conforto material e psicológico para que eu vá em busca dos meus objetivos, com perseverança e dignidade.

À Raquel Pacheco, que muito entusiasmada com as questões do interessantíssimo kDNA foi uma grande incentivadora do projeto, e me recebeu em seu laboratório, dando todo o apoio para a extração tão trabalhosa e árdua do kDNA.

Ao meu querido e também orientador Dr. David Wilson, que me recebeu em seu laboratório e junto com sua amável esposa, Carol Wilson, me acolheram em Atlanta como se fosse uma filha, além de todo o incentivo científico e maravilhosos frutos dessa nossa colaboração. À todos do Dr. Wilson´s lab, em especial: Caterina, Michael, Rupesh and Shuo. E à minha amiga e roomate bagunceira: Qing Li.

Às amigas de laboratório e de vida, Elen Mello, Kellyta Salomão e Patrícia

Bernardino (apesar desta última não me considerar como tal), com as quais tive a agradável companhia diária durante esses quatro anos de LBC. Registro meu desejo aqui, de que essa amizade perdure por toda a vida, independente de onde estivermos.

À extensão da família Wagner Guimarães (sogrinho), Márcia Cosentino (sogrinha) e Renato Cosentino (cunhadinho) por estarem sempre presentes e apoioando e torcendo por mim.

À Denise da Gama que foi a minha tutora inicial quando da minha chegada ao laboratório, me inteirando das metodologias e logística dos ensaios realizados em nosso laboratório. À minha parceira em vários trabalhos, Cristiane França, que jura que eu sei fazer microscopia de fluorescência.

Aos amigos por cuja amizade e presença nesse mundo eu sou muito grata: Bianca Bello, Aimeé DuBois, André Kano, Patrícia Fampa, Giselle Assafin, Tahereh Sherafat, Vahid Sherafat, Thaís Ferraz, Fernanda Menelau e Karla Cristine. E as minhas filhinhas de coração e musas inspiradoras da minha vida Renata Bahrampour, Carol Bahrampour e Soraya Ardestani: não se importem com a ordem eu amo as três igualmente!

Aos participantes da minha banca de defesa, pela aceitação de contribuir com essa etapa em uma época de férias e festas, Natal e Ano Novo. Com certeza as contribuições serão muito importantes para mim e para pesquisas futuras do nosso grupo.

Ao Trypanosoma. cruzi, por ser meu objeto de estudo e o ser que eu tive mais contato durante esses 4 anos e pelo qual eu tenho profunda admiração, principalmente pela sua desenvoltura no processo de replicação do kDNA e edição de RNA.

Obrigada, obrigada e obrigada!!!!!!

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Atividade, seletividade e mecanismos de ação de diamidinas aromáticas e análogos sobre *Trypanosoma cruzi*: um enfoque sobre o kDNA

RESUMO

Anissa Daliry

A doença de Chagas, causada pelo *Trypanosoma cruzi,* é endêmica na América Latina, afetando mais de 15 milhões de pessoas. Como seu tratamento apresenta eficácia limitada e considerável toxicidade novas drogas são necessárias. Neste contexto, com a colaboração de grupos de química medicinal, nosso objetivo é a investigação da atividade tripanocida, seletividade, alvos celulares e mecanismos de ação de diamidinas aromáticas (DAs) e análogos. Inicialmente avaliamos o efeito tripanocida de onze DAs sobre as formas tripomastigotas e amastigotas intracelulares, assim como sua toxicidade para células de mamíferos e localização intracelular no parasito. Entre estes onze compostos, **2**, **5** e **7** foram os mais ativos, com valores de IC_{50} na faixa micromolar e um alto índice de seletividade sobre as duas formas de *T. cruzi*. Através de microscopia de fluorescência (MF) foi possível localizar todos os compostos em organelas ricas em DNA, núcleo e mitocôndria (kDNA) e a análise ultraestrutural utilizando os compostos **5** e **7** revelou que estes compostos levam a danos mitocondriais, incluindo desorganização do kDNA em formas tripomastigotas. O acúmulo das diamidinas foi maior no kDNA do que no núcleo, porém tal acúmulo não está correlacionado a uma maior atividade tripanocida. A seguir, visando um melhor entendimento do mecanismo de ação de diamidinas e análogos, investigamos uma possível correlação entre as propriedades de ligação ao kDNA de treze compostos com a atividade tripanocida, através de estudos de desnaturação térmica (T_m) e dicroísmo circular (DiC). Nossos resultados mostram tanto com kDNA purificado de epimastigotas como com uma sequência conservada de 22-mer presente em minicírculos de *T. cruzi*, que a forte interação de amidinas ao kDNA não é o fator determinante para desencadear sua atividade tripanocida. Nosso próximo passo foi a avaliação de alterações topológicas induzidas por dez compostos aromáticos sobre o kDNA de *T. cruzi* através de alteração da mobilidade em gel e MF. Os estudos eletroforéticos foram conduzidos pela incubação de fragmentos de kDNA, obtidos pela digestão com as endonucleases *EcoRI* e *CvQI*, com os compostos por análise em gel de poliacrilamida. As diamidinas DB889 e DB185 induziram consideráveis alterações na mobilidade dos fragmentos. Além disso, incubando a rede intacta e purificada de kDNA com a DB75 e monitorando o efeito por MF, obervarmos a capacidade desta DA de induzir um grande aumento da área da rede. Em resumo, nossos resultados revelam que diamidinas e congêneres são capazes de induzir profundas alterações na topologia do kDNA do *T. cruzi* sugerindo que esta estrutura pode ser um dos possíveis alvos destes compostos. Entretanto, a localização preferencial dos compostos no kDNA, assim como sua afinidade à esta estrutura e capacidade de alterar a mobilidade dos fragmentos em géis de poliacrilamida não está correlacionada com sua atividade tripanocida. Estes dados sugerem fortemente que outros fatores podem estar envolvidos no mecanismo de ação destes compostos, operando de modo primário ou secundário a interação composto:kDNA. Outros estudos serão necessários para melhor identificar os mecanismos envolvidos na ação destes compostos, visando contribuir para o desenho racional de compostos líderes para o tratamento da doença de Chagas.

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Activity, selectivity and mechanisms of action of aromatic diamidines and analogues upon *Trypanosoma cruzi*: focusing on kDNA

ABSTRACT

Anissa Daliry

Chagas disease, caused by *Trypanosoma cruzi*, is endemic in Latin America, affecting more de 15 million people. Due to the limited efficacy and considerable toxicity, the therapy for Chagas disease is far from being considerable ideal and thus alternative drugs are urgently needed. In this context, with the collaborations of medicinal chemistry groups, our aim was to investigate the trypanocidal activity, selectivity, cellular targets and mechanisms of action of aromatic diamidines (ADs) and analogues. First, we tested the trypanocidal effect of eleven ADs upon intracellular amastigotes and trypomastigotes, their toxicity towards mammalian cells and their sub-cellular localization. Compounds **2**, **5** and **7** were the most active, presenting IC_{50} values on the micromolar range and displaying high selectivity indexes for both *T. cruzi* forms. Using fluorescence microscopy (FM), all the compounds were localized in organelles rich in DNA, nucleus and mitochondrion (kDNA) and in bloodstream forms treated with the compounds 5 and 7 we observed ultrastructural damages on mitochondrial organelle, including kDNA disorganization. The accumulation of the diamidines was higher in the kDNA than in the nucleus, but such accumulation could not be correlated with a higher trypanocidal activity. Next, aiming to better understand the mechanism of action of diamidines and analogues, we investigated the possible correlation between kDNA binding properties of thirteen compounds with their anti-*T. cruzi* effect, through thermal denaturation (T_m) and circular dichroism (CiD) studies. Our data demonstrated using the purified kDNA of epimastigotes or a conserved synthetic parasite sequence of 22-mer present in *T. cruzi* minicircles, that the strong interaction of the amidines with the kDNA is not a determinant factor to the triggering of the trypanocidal activity. Our next step was the evaluation of topological changes induced by ten aromatic compounds on *T. cruzi* kDNA, through gel mobility shifts and FM. The eletrophoretic studies were conducted by the incubation of kDNA fragments obtained by digestion with the endonucleases *EcoRI* e *CvQI* with the compounds and analysis by poliacrylamide gel. The diamidines DB889 and DB185 induced substantial mobility shifts in the fragmented bands. Additionally, by FM of whole kDNA network incubated with DB75 we observed the ability of this diamidine to cause a striking expansion of the kDNA network area. Taken together our results suggest that diamidines and related compounds provoke profound alterations in the normal topology of *T. cruzi* kDNA suggesting that this structure may represent one of the potential targets of these compounds. However, the compound preferential accumulation in kDNA, as well as their affinity and capability of inducing topological changes in that structure is not correlated with their anti-*T. cruzi* activity. These findings strongly suggest that other molecular mechanisms may be also operating primarily or secondarily to the drug:KDNA interaction. Further studies are needed to better identify the mechanisms involved in the activity of this class of chemical, aiming to contribute to the rational design of lead compounds for the treatment of Chagas disease.

ÍNDICE

I. INTRODUÇÃO

1. Doença de Chagas: desafio que persiste um século após sua descoberta

Após mais de 100 anos da sua descoberta por Carlos Chagas (1909) a doença de Chagas (DC) ainda representa um desafio no cenário da saúde pública de vários países da América Latina (Lannes-Vieira et al., 2010). Acredita-se que a relação entre os seres humanos e o *T. cruzi* seja bastante antiga uma vez que a presença de DNA de *Trypanosoma cruzi*, seu agente etiológico, foi detectada em múmias datadas de ~9.000 anos atrás (Aufderheide et al., 2004). A DC está dispersa em 18 países na América Latina (Rocha et al., 2007), afetando aproximadamente 15 milhões de pessoas (Coura & Dias, 2009). Esta parasitose apresenta duas fases clínicas consecutivas: aguda e crônica. Na fase crônica, existe uma grande variedade regional na sua morbidade incluindo desde formas indeterminadas assintomáticas até formas cardíacas e/ou digestivas severas (20-40%)(Coura & Borges Pereira, 2010). No Brasil, dados do Ministério da Saúde apontam para 760 casos de DC aguda durante os anos de 2005 a 2010, com 20 óbitos (www.saude.gov.br).

A distribuição da DC vai desde o sul dos Estados Unidos até o sul da Argentina, porém novos casos estão sendo detectados em outros países como Espanha, Portugal, Canadá, Japão e Austrália (Fig. 1.1). Nestes países onde a DC é ainda emergente a sua principal causa decorre de imigrações de indivíduos infectados de regiões endêmicas para essas áreas (Schmunis, 2007; Clayton, 2010; Coura & Viñas, 2010).

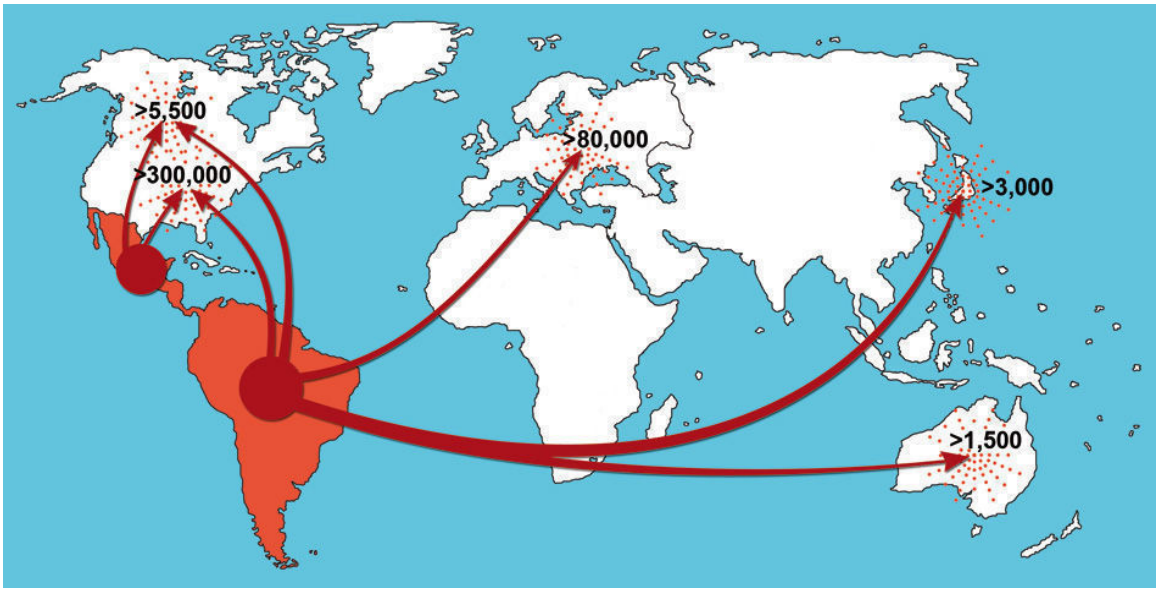


Fig. 1.1. Rotas de imigração (vermelho escuro) a partir da América Latina para outros países e número estimado de casos em países onde a doença é emergente. Países endêmicos estão mostrados em vermelho claro. Fonte: Coura & Viñas, 2010.

1.1. O parasito e o seu ciclo de vida

O Trypanosoma cruzi é um protozoário flagelado pertencente à ordem Kinetoplastida, família Trypanosomatidae e gênero *Trypanosoma*, sendo um parasito heteroxênico, alterando seu ciclo entre um hospedeiro invertebrado e outro vertebrado (Deane et al., 1984). O hospedeiro invertebrado, também conhecido como barbeiro, é um inseto pertencente à ordem Hemiptera, família Reduviidae e subfamília Triatominae. Dentre os gêneros mais importantes envolvidos na transmissão de DC estão o *Triatoma, Panstrongylus* e *Rhodnius.* Mais de 130 diferentes espécies já foram demonstradas como potenciais vetores da DC, sendo o *Triatoma infestans* e o *Rhodnius prolixus* os mais importantes (Lent & Wygodzinky, 1979).

O ciclo de vida do parasito está representado na Fig. 1.2 e se inicia quando as formas infectivas (tripomastigotas metacíclicos) presentes no intestino posterior do inseto vetor são eliminadas junto com as fezes e urina duranto o repasto sanguíneo (Brack, 1968; Brener & Alvarenga, 1976).

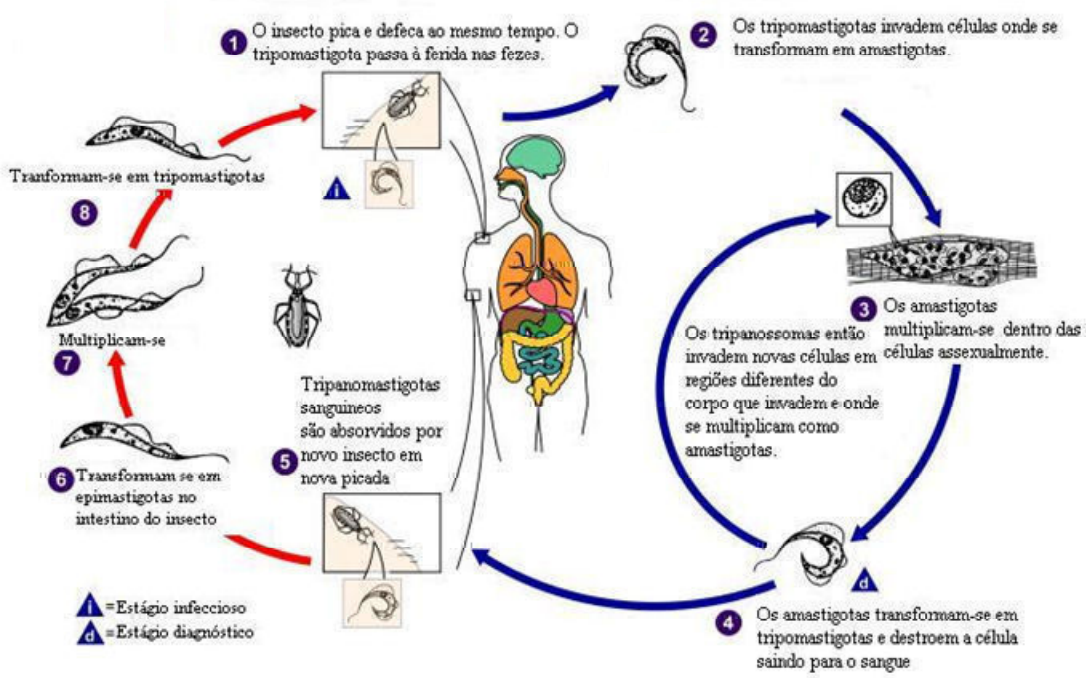


Fig.1.2. Ciclo de vida do *Trypanosoma cruzi*, agente causador da doença de Chagas. Adaptado de CDC (Center of Disease Control and Prevention, Atlanta, GA, USA; www.dpd.cdc.gov/dpdx).

No local de picada do inseto, as formas metacíclicas podem ganhar acesso a diferentes tipos celulares, sofrendo, no citoplasma das células hospedeiras, diferenciação para amastigotas que por sua vez irão se proliferar por sucessivas divisões binárias. Decorridos vários ciclos de divisão ocorre a diferenciação para formas tripomastigotas que são liberadas após ruptura da célula para o espaço extracelular, podendo, através da corrente sanguínea, infectar novas células e/ou atingir outros órgãos e tecidos.

Neste estágio, um barbeiro não infectado pode adquirir as formas tripomastigotas presentes no sangue do hospedeiro vertebrado. No lúmen do intestino médio do inseto, o parasito se diferencia para epimastigota, que é uma forma replicativa, que sofrerá sucessivas divisões binárias. Na porção final do intestino essas formas encontram um ambiente propício para sua diferenciação em tripomastigotas metacíclicos, num processo chamado metaciclogênese, reiniciando assim, o ciclo (Mortara et al., 2005).

As diferentes formas evolutivas de *T. cruzi* podem ser diferenciadas de acordo com a morfologia, a posição do flagelo em relação ao núcleo, e a característica do flagelo. Os tripomastigotas são alongados, com cinetoplasto arredondado localizado na região posterior ao núcleo e o flagelo emergindo da bolsa flagelar que se localiza lateralmente, na região posterior do parasito. O flagelo emerge e se adere ao longo

do corpo do parasito, tornando-se livre na região anterior. Os amastigotas apresentam forma ovalada, com cinetoplasto em forma de bastão, anterior ao núcleo, flagelo curto que emerge da bolsa flagelar. A forma epimastigota também apresenta o cinetoplasto em forma de bastão, porém é mais alongada e apresenta o flagelo emergindo da bolsa flagelar percorrendo o corpo do parasito e permanecendo livre na região anterior. O cinetoplasto é anterior ao núcleo (Fig. 1.3).

Fig. 1.3. Formas evolutivas de *Trypanosoma cruzi* encontradas no hospedeiro vertebrado (Tripomastigota e amastigota) e no hospedeiro invertebrado (epimastigota e tripomastigota). Fonte: Docampo et al., 2005.

1.2. Transmissão e controle

A transmissão de DC ocorre primariamente pela via vetorial (80-90%), porém pode ocorrer também por transfusão sanguínea e por via congênita (Moya & Moretti, 1997; Carlier, 2007; Buekens et al., 2008). Outras formas são acidentes de laboratório (Dias, 1990; Herwaldt, 2001) e transplante de órgãos (Atclas et al., 2008; Campos et al., 2008). Além disso, anualmente, vários surtos decorrentes de contaminação oral tem sido registrados principalmente no Norte e Nordeste do país (Silva et al., 1968; Shikanai-Yasuda et al., 1991), porém estados como Santa Catarina também foram afetados (Steindel et al., 2008). Embora de rara ocorrência, a amamentação pode ser outra via de transmissão da doença (Bittencourt, 1988; Rassi et al., 2004).

Na região da Amazônia brasileira os casos de transmissão via oral é a forma mais comum de infecção, com grande incidência de parasitos pertencentes ao grupo *T. cruzi* I (Coura et al., 2002; Coura & Dias, 2009). Os parasitos são provavelmente

originados de triatomíneos silvestres que eventualmente invadem a área doméstica e peridoméstica (Pinto et al., 2008). A iniciativa de vigilância epidemiológica da DC na região Amazônica teve início em 2004, após a detecção do aumento do número de casos agudos, principalmente devido à ingestão de suco de açaí (Moncayo & Silveira, 2009).

A transmissão vetorial inclui três ciclos: (i) o ciclo doméstico que é responsável pela manutenção da doença em humanos, ocorrendo principalmente em áreas urbanas e peri-urbanas, sendo humanos, cães e gatos os principais reservatórios do parasito; (ii) o ciclo silvestre, no qual triatomíneos silvestres infectam roedores, marsupiais e outros mamíferos e (iii) o ciclo peridomiciliar, que representa uma conexão entre o ciclo doméstico e o silvestre, no qual se observa o fluxo de mamíferos (roedores domésticos, marsupiais, gatos e cachorros principalmente) entre casas e áreas silvestres bem como a ocorrência de espécies de triatomíneos silvestres que acessam as casas infectando diretamente pessoas, animais e mesmo alimentos (Remme et al., 2006).

A globalização da DC é atualmente uma grande preocupação estando associada à migração de pessoas infectadas de regiões rurais para áreas urbanas assim como para países não endêmicos mudando o padrão epidemiológico da doença. Esta mudança tem sido apontada como a principal causa do aumento do número de infectados pela via transfusional documentados no Canadá, Estados Unidos e em alguns países da Europa (Schmunis, 2007; Grant et al., 1989; Kirchhoff et al., 1987; Nickerson et al., 1989). Em decorrência destes dados, a análise sorológica de bolsas de sangue e durante os exames pré-natais tem sido estimulada nestes países, além de ser obrigatória nas regiões endêmicas (Moncayo & Silveira, 2009).

Apesar das transmissões vetorial e transfusional terem sido fortemente reduzidas nos últimos 20 anos, alguns desafios ainda precisam ser enfrentados, incluindo a manutenção e sustentabilidade de políticas de saúde pública e de estratégias de controle do vetor além do investimento na educação das populações envolvidas, em geral de áreas rurais e pobres (Dias et al., 2008; Abad-Franch et al., 2010).

O controle da DC envolve principalmente a prevenção da transmissão humana pela: (i) eliminação do inseto vetor e controle da transmissão via ciclos domésticos e peridomésticos, através do uso de inseticidas de longa ação residual, (ii) seleção de doadores em bancos de sangue e/ou a esterilização do sangue

utilizado para transfusão; (iii) melhoria das moradias e indução de programas de educação sanitária (Dias, 2007; Dias et al., 2008). O programa de controle da doença, iniciado em 1991 em países do Cone Sul, foi muito bem sucedido reduzindo a incidência da doença. Assim, a transmissão vetorial de *T. cruzi* foi interrompida no Uruguai em 1997 e no Chile em 1999 (Salvatella, 2007). O Ministério da Saúde do Brasil recebeu em julho de 2006, a Certificação Internacional de Eliminação da Transmissão da Doença de Chagas conferida pela Organização Pan-Americana da Saúde e concedida pela OPAS/PAHO (Dias, 2007; OPAS, 2007).

1.3. Manifestações clínicas

O local da entrada do parasito pode ser identificado por uma lesão de pele denominada chagoma de inoculação. No caso da contaminação pela conjuntiva ocular, esta lesão é denominada sinal de Romaña (Coura & Dias, 2009). Logo após a infecção, observa-se um curto período de incubação, dando início à fase aguda da DC, com características clínicas oligossintomáticas, sendo reconhecidas em apenas 1-2% dos casos, e com duração de 6-8 semanas. Esta fase está relacionada a um parasitismo sanguíneo e tissular intensos, com presença de infiltrados inflamatórios em diferentes tecidos (Lopes & Chapadeiro, 1997). Nos casos mais graves, em especial em crianças menores de dois anos e em indivíduos imunossuprimidos, a ausência de tratamento pode levar a letalidade (5%), principalmente devido à miocardite ou meningoencefalite (Rassi Jr et al., 2009). A grande maioria dos casos agudos não tratados evolui para a forma indeterminada da fase crônica. Esta é uma fase de infecção sub-patente (revelada somente pela sorologia e/ou métodos parasitológicos indiretos) associada à ausência de sintomatologia evidente, avaliada por exames clínicos, eletrocardiográficos e radiológicos (coração, esôfago e cólon) (Ribeiro & Rocha, 1998; Bilate & Cunha Neto, 2008). Frente a uma resposta imunológica eficiente, a maioria dos indivíduos infectados permanece nesta forma assintomática por toda a vida, porém, de 10-20 anos após a infecção, cerca de 20-40% dos casos evoluem para a fase crônica sintomática, cujas principais manifestações são as alterações cardíacas (20-30%) e/ou digestivas (10%) (Rassi Jr et al., 2006; Moncayo & Ortiz-Yanine, 2006), sendo difícil prever quais indivíduos irão evoluir para cada forma da doença (Marin-Neto et al., 2009).

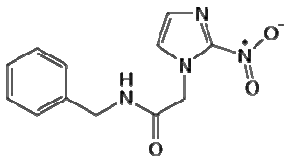
Na fase crônica, apesar da parasitemia muito baixa ou quase não detectável, observa-se intenso processo inflamatório afetando diversos órgãos, em especial o

coração (Campos de Carvalho et al., 2009). De fato, a grande maioria dos pacientes chagásicos crônicos cardiopatas apresenta dano cardíaco progressivo, caracterizado por aneurisma apical, arritmias, tromboembolismo, insuficiência cardíaca progressiva, podendo ocorrer ainda morte súbita (Higuchi et al., 2003; Rassi Jr et al., 2006). A cardiopatia chagásica crônica (CCC) é microscopicamente caracterizada pela presença de infiltrados inflamatórios multifocais a difusos, compostos principalmente por linfócitos $TCD8^+$. Dados da literatura sugerem que a persistência do parasito associada a uma resposta imune desbalanceada é fundamental para a evolução da CCC (Tarleton, 2003; Rocha et al., 2007; Marin-Neto et al., 2007). Outros 6-10% dos pacientes infectados apresentam comprometimento digestivo caracterizado por alterações da secreção, motilidade, absorção e, nos casos mais graves, pelo aparecimento de megavísceras (esôfago e cólon) (Köberle, 1956; Rezende, 1984; Meneghelli, 2004). Lesões neurológicas periféricas, principalmente meningoencefalites podem também ocorrer em indivíduos chagásicos (3%) (WHO, 1997; Higuchi et al., 2003; Rassi Jr et al., 2009).

1.4. Tratamento

Atualmente o tratamento da DC está restrito ao uso de duas drogas, introduzidas nas décadas de 60 e 70: Nifurtimox (Nf) e Benznidazol (Bz) (Fig. 1.4).

Benznidazol



Nifurtimox

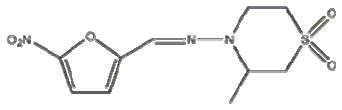


Fig. 1.4. Estruturas químicas do benznidazol (derivado nitroimidazólico) (LAFEPE) e do nifurtimox (nitrofurano) (Lampit®, Bayer HealthCare). Fonte: Grunberg et al., 1967.

Nif foi largamente utilizado na clínica, porém teve sua produção descontinuada na década de 80. Em 2000, a produção de Nif foi retomada em El Salvador. Esta retomada se deve ao seu uso combinado a eflornitina para o tratamento da tripanosomíase africana causada por *T. brucei gambiense* (Nussbaum et al., 2010). A distribuição gratuita de Nif tem sido realizada desde 2006, pela OMS/OPAS.

Com relação ao Bz, em 2005 a Roche interrompeu sua produção no Brasil, cedendo os direitos de patente deste medicamento ao governo brasileiro, que passou a se encarregar de sua fabricação, comercialização e possível exportação para outros países sul-americanos. O composto está sendo produzido pelo Laboratório Farmacêutico do Estado de Pernambuco (LAFEPE), e em colaboração com o DNDi (Drugs for Neglected Diseases initiative) estão sendo produzidas formulações pediátricas do benznidazol, sem custo para distribuição mundial (www.dndi.org.br).

Os resultados obtidos com Nif e Bz indicam que nenhum destes compostos é ideal, pois (i) não são ativos durante a fase crônica da doença e apresentam sérios efeitos colaterais; (ii) o período de tratamento é longo, e (iii) a ação de ambos varia muito de acordo com a susceptibilidade de isolados do parasito (Soeiro & De Castro, 2009). Em geral os resultados são considerados bons no tratamento de casos agudos e crônicos recentes da doença principalmente em crianças (<15 anos), estimando-se uma cura parasitológica entre 60-80% (Cançado, 1999, 2002). Assim, recomenda-se o tratamento de todos os pacientes em fase aguda, crônica recente e frente a quadros de reagudização (Andrade et al., 1996; Cançado, 1997; Sosa Estani et al., 1998; Rassi et al., 2000; Bern et al., 2007). Entretanto, estudos de longos períodos de acompanhamento de pacientes chagásicos crônicos tratados com benznidazol mostraram apenas 8% de cura parasitológica, avaliada por sorologia negativa (Cançado, 2002). Por outro lado, em outros estudos o índice de cura foi substancialmente maior (20%), quando avaliados pelo método de Reação em Cadeia da Polimerase (PCR) (Garcia et al., 2005; Martins et al., 2008; Fernandes et al., 2009). Com isso, o tratamento de pacientes na fase crônica da doença ainda é controverso apesar dos dados alcançados até o momento no projeto BENEFIT (Benznidazole Evaluation for Interrupting Trypanosomiasis) apontarem que o tratamento tenha um importante impacto sobre a evolução clínica da cardiomiopatia chagásica (Marin-Neto et al., 2009). De fato, apesar da maioria dos estudos revelar uma baixa eficiência destes fármacos durante a terapia de pacientes crônicos

tardios, outros demonstram que a terapia etiológica é capaz de reduzir o parasitismo, diminuindo a severidade e progressão da CCC (Garcia et al., 2005; Viotti et al., 2006).

Apesar das pesquisas na área de quimioterapia da DC serem bastante intensas as indústrias farmacêuticas têm pouco interesse no desenvolvimento de novos fármacos, devido ao alto custo dos investimentos e a falta de um mercado potencial e seguro, o que dificulta o lançamento de novas drogas no mercado. (Gutteridge, 1987; Coura & De Castro, 2002; Soeiro et al., 2009; Urbina, 2010).

O desenvolvimento de uma droga antiparasitária pode ser determinado por diferentes estratégicas, incluindo (i) estabelecimento de princípios ativos de produtos naturais utilizados na medicina popular ou a partir de compostos sintéticos que tenham reconhecida atividade para outras doenças, uma vez que eles já foram submetidos a ensaios clínicos muito dispendiosos, ou (ii) através de estudos racionais pela determinação de alvos específicos de vias metabólicas do parasito (Steverding & Tyler, 2005; Duschak & Couto, 2007; Soeiro e De Castro, 2009; Urbina, 2010).

Apesar da extensa lista de diferentes classes de compostos que apresentam atividade *in vitro* e *in vivo* sobre *T. cruzi*, desde a introdução de nifurtimox e benznidazol, somente alopurinol, cetoconazol, itraconazol e fluconazol (Brener et al., 1993; Solari et al., 1993; Tomimori-Yamashita et al., 1997; Apt et al., 2003, 2005) foram avaliados em ensaios clínicos. Esses últimos compostos anti-fúngicos reduzem, porém não induzem cura da infecção pelo *T. cruzi* (Urbina, 2002). No Chile, itraconazol está sendo usado como droga de segunda escolha para tratar adultos crônicos chagásicos (Apt et al., 2005).

Uma série de novos azóis, inibidores da biossíntese de ergosterol, ensaiado *in vivo* sobre *T. cruzi* se destaca entre os compostos mais promissores para futuros estudos clínicos, incluindo o albaconazol (UR-9825), TAK-187 (Takeda Chemical Compány) e posaconazol (SCH56592, Schering-Plouch). Estes inibidores induziram cura parasitológica de animais experimentais infectados na fase aguda (90-100% para as cepas Y e CL; 50% para a cepa Colombiana), aumentando ainda a sobrevida de animais na fase crônica (Molina et al., 2000; Urbina et al., 2004; Guedes et al., 2004; Corrales et al., 2005; Ferraz et al., 2007). Posaconazol age também em sinergia com amiodarona, um composto antiarrítmico freqüentemente prescrito para o tratamento da DC sintomática, levando à ruptura do equilíbrio de Ca^{2+} parasitário assim como ao bloqueio da síntese de ergosterol (Benaim et al.,

2006). Este protocolo representa uma nova esperança quanto ao uso combinado de ambas as drogas já utilizadas na clínica médica (Benaim et al., 2006). Ainda deve-se ressaltar a importância do desenvolvimento de uma terapia com o uso de células-tronco com capacidade de auto-replicação e de diferenciação em diversos tipos celulares, para o reparo de lesões em pacientes com doença crônica em estágio avançado (Campos de Carvalho et al., 2009).

Assim, após 101 anos de sua descoberta, a DC ainda representa, no Brasil e em vários países endêmicos, um atual desafio de saúde pública frente a sua epidemiologia particular, caracterizada por uma gama de fatores de risco (diversos vetores e reservatórios, diferentes formas de transmissão e de isolados de *T. cruzi*, presentes em ambientes domésticos, peridomésticos e silvestres) e ausência de medidas profiláticas e de esquemas efetivos de tratamento (Clayton, 2010; Dias 2007; Coura 2007; Coura & Viñas, 2010).

2. O DNA mitocondrial dos cinetoplastídeos: um importante alvo quimioterápico

2.1. A organização do kDNA

A organização e composição do DNA mitocondrial de organismos da ordem Kinetoplastida, o kDNA, são importantes características moleculares, sendo único em sua estrutura, função e modo de replicação. O kDNA é composto por milhares de moléculas circulares interligadas e concatenadas em uma grande rede (Englund et al., 1982; Hajduk et al., 1984; Simpson & Berliner, 1974; Stuart et al., 2005) (Fig. 1.5) que corresponde aproximadamente a 20-25% do total de material genético da célula (Englund et al., 1982). Esta estrutura localiza-se dentro da mitocôndria única destes organismos, e está conectada ao corpo basal através de um sistema de filamentos transmembranares recentemente descritos como *Tripartite attachment complex* (TCA) (Ogbadoyi et al., 2003).

A rede do kDNA é composta por dois tipos de moléculas circulares: os minicírculos e os maxicírculos (Hajduk et al., 1984; Shapiro & Enlgund, 1995; Stuart et al., 2005) (Fig. 1.5).

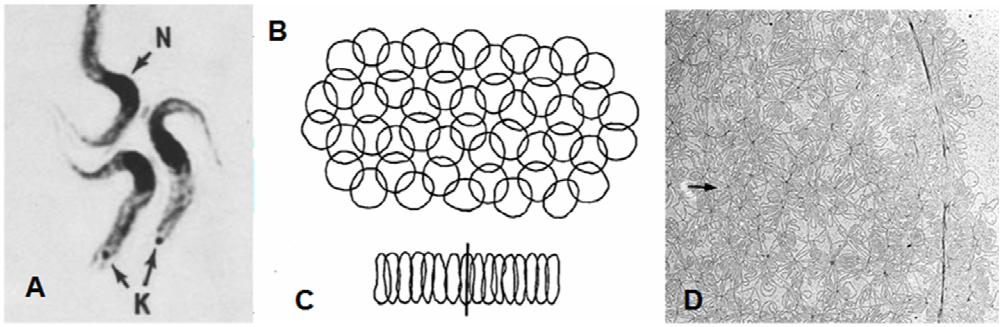


Fig. 1.5. Representações esquemáticas da organização da rede do kDNA. **A**: Coloração em Giemsa e análise ao microscópio óptico de *Trypanosoma equiperdum* revelando núcleo (N) e cineitoplasto (K) dos parasitos (Fonte: Renger & Wolstenholme, 1972); **B**: Esquema da representação de minicírculos em disposição planar, estando interligados a três outros minicírculos (Shapiro & Englund, 1995); **C**: Diagrama de uma seção transversal mostrando a disposição perpendicular dos minicírculos *in vivo* (Shapiro & Englund, 1995). **D:** Micrografias eletrônicas de rede de kDNA de *Crithidia fasciculata.* Os pequenos *loops* são os minicírculos (correspondem a 2.5 kb) e a seta indica parte de um maxicírculo (Fonte: Englund et al., 2005).

Os maxicírculos, componentes minoritários da rede (~50 cópias), são homólogos funcionais do DNA mitocondrial de outros organismos eucariotos, e variam de tamanho de acordo com a espécie (20 a ~40 kb). Foram inicialmente descritos em *C. fasciculata* e posteriormente detectados em outros tripanosomatídeos como *T. cruzi* (Leon et al., 1980). Na rede, os maxicírculos estão interligados entre si, assim como aos minicírculos, formando redes dentro de outras redes (Shapiro, 1993). Eles codificam RNAs ribossomais mitocondriais (9S e 12S) além de diversas proteínas envolvidas no metabolismo energético da mitocôndria, como, por exemplo, citocromo *b*, subunidades da citocromo oxidase, NADH desidrogenase e subunidade 6 da ATPase (Simpson et al., 1982; Johnson et al., 1982). A característica mais notável dos maxicírculos é que a maioria dos seus transcritos deve passar por um processo de edição para formar um RNA_m funcional (Benne et al., 1986; Benne, 1994; Simpson et al., 2004).

Os minicírculos, que perfazem um total de 90% da massa da rede, variam entre 0.5 a 2.9 Kb nas diferentes espécies (Shapiro & Englund, 1995) e apresentam entre 5.000 a 20.000 cópias por rede. Na maioria das espécies os minicírculos da rede são do mesmo tamanho, porém a sua composição pode variar em uma mesma espécie. Em *T. cruzi*, *Trypanosoma brucei* e *C. fasciculata,* por exemplo, observa-se uma grande heterogeneidade em suas sequências (Ntambi et al., 1984). Em *T. brucei*, mais de 250 diferentes sequências já foram descritas em uma mesma rede (Stuart & Gelvin, 1980; Steinert & Van Assel, 1980). Os minicírculos de *T. cruzi* apresentam 1.4 kb e contém quatro repetições internas de aproximadamente 200-300 pb (Morel et al., 1980; González, 1986).

A rede do kDNA isolada através de centrifugação diferenciada (com uso ou não de gradientes de cloreto de césio) pode ser vizualizada por microscopia eletrônica de transmissão (MET), de força atômica (AFM) e de fluorescência (MF) (Simpson & Berliner, 1974; Delain & Riou, 1970). A rede isolada de *C. fasciculata* apresenta cerca de 5.000 cópias de minicírculos organizados em uma rede elíptica de aproximadamente 15 µm (Pérez-Morga & Englund, 1993). Quando analisada por diferentes tipos de microscopia, como MET e MF, esta rede apresenta uma disposição planar na lâmina de vidro utilizada para vizualização da amostra, como representado na Fig. 1.5 B. Já nos parasitos íntegros, é possível observar que os minicírculos permanecem compactados de forma perpendicular ao plano do corpo do parasito (Renger & Wolstenholme, 1972).

2.2. O processo de replicação da rede

A replicação do DNA requer mecanismos de controle que possibilitem duplicar o genoma, contendo milhares de pares de bases, apenas uma vez a cada divisão celular. No *T. cruzi*, a fase S do ciclo celular (momento em que ocorre a replicação do DNA) ocorre por 2,4 h em um ciclo de 24 h. O processo de replicação ocorre de maneira ordenada na qual inicialmente há o reconhecimento da origem de replicação, sendo posteriormente realizada a iniciação e, por fim, a replicação semiconservativa do DNA (Machado C: www.fiocruz.br/chagas).

Com relação ao kDNA, sua duplicação envolve a replicação individual de cada minicírculo e maxicírculo da rede e sua distribuição igualitária nas duas células-filhas, sendo um processo bastante peculiar e interessante do ponto de vista biológico. A replicação se inicia durante a fase S, quando os minícirculos covalentemente fechados sofrem ação de uma topoisomerase do tipo II (Englund, 1979; Melendy et al., 1988) e são liberados em uma região denomidada zona cinetoflagelar (do inglês *Kinetoflagellar Zone*, KFZ) (Fig. 1.6). O disco do kDNA é flanqueado por dois sítios antipodais onde estão concentradas proteínas importantes para o processo de replicação, como DNA primases, polimerases e uma endonuclease estrutura específica (do inglês *structure-specific endonuclease* – SSE1) (Fig 1.6). Outras proteínas presentes nos sítios antipodais também descritas como a p38 e p93, desempenham papel importante na replicação dos minicírculos (Liu et al., 2006; Li et al., 2007).

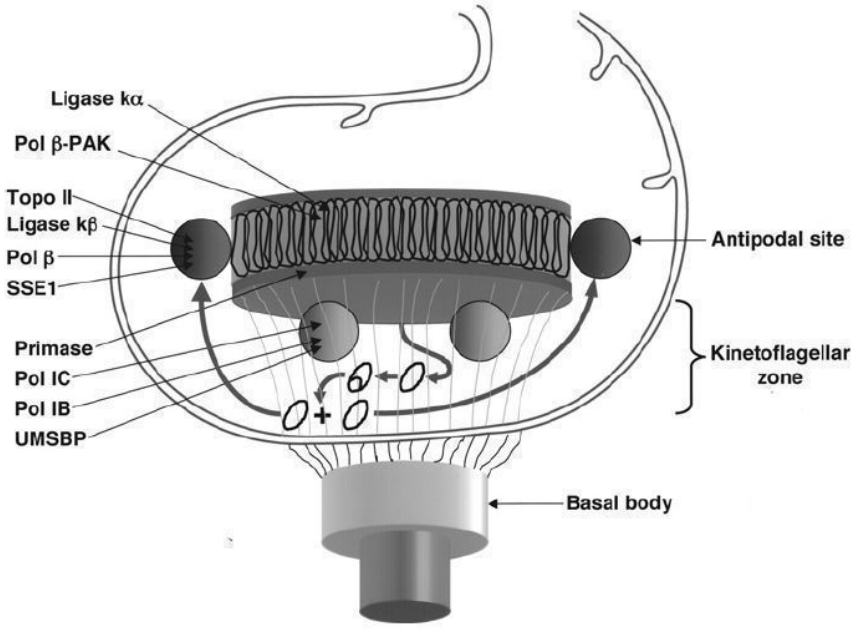


Figura 1.6. Modelo de replicação do kDNA mostrando a localização de algumas proteínas envolvidas na replicação e os intermediários da replicação de minicírculos. É possível observar também os filamentos que ligam o disco de kDNA (kDNA disk) ao corpo basal (do inglês *basal body*) do flagelo (Ogbadoyi et al., 2003). Abreviações: Pol, DNA polimerase; PAK, rica em prolina, alanina e Lisina; Topo, topoisomerase; SSE1, endonuclease estrutura-específica 1; UMSBP, proteína de ligação à sequência universal do minicírculo. Fonte: Liu et al., 2005.

Uma vez na KFZ, a proteína de ligação a sequência universal do minicírculo (do inglês *Universal Minicircle Sequence Binding Protein* - UMSBP) reconhece e se associa a sequências conservadas na origem de replicação (Milman et al., 2007). A mais conhecida delas é uma sequência de 12-*mer*, chamada de sequência universal do minicírculo (UMS) (Tzfati et al., 1992). A contínua liberação de minicírculos da região central da rede causa a formação de "buracos" claramente visíveis por microscopia eletrônica e de fluorescência. A replicação ocorre de forma unidirecional e os minicírculos são duplicados em estruturas em forma de teta (Θ) (Ferguson et al., 1992). Os minicírculos já duplicados apresentam um *nick* ou *gap* e são re-ligados à periferia da rede através da ação de uma outra topoisomerase do tipo II (Liu et al., 2006) localizada no disco do kDNA (Shlomai, 1994). Por conta de pequenas rotações do disco de kDNA presente em *C. fasciculata*, *T. cruzi* e *Leishmania donovani* (Pérez-Morga & Englund, 1993) os minicírculos recém sintetizados apresentam uma distribuição uniforme na periferia do kDNA enquanto que em *T. brucei* esta organização ocorre nos sítios antipodais de forma polarizada (Fig.1.7) (Ferguson et al., 1992, 1994).

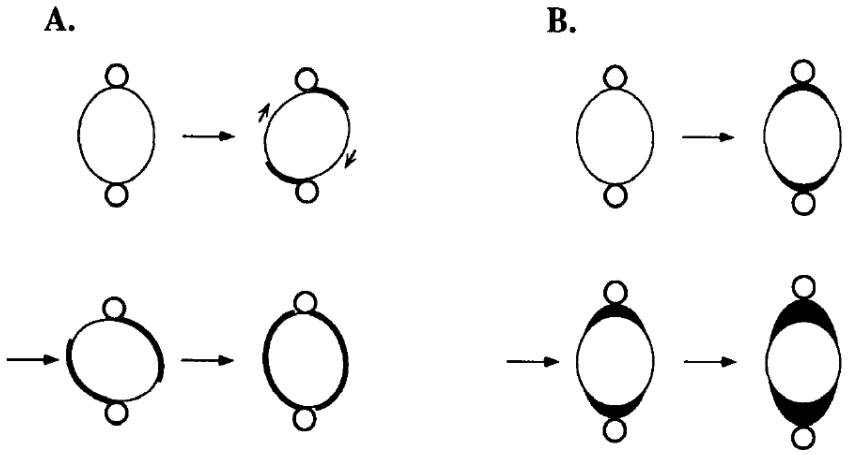


Fig. 1.7. Comparação da replicação da rede em *C. fasciculata*, *T. cruzi* e *Leishmania* sp. (**A**) e em *T. brucei* (**B**), mostrando com a rotação do disco de kDNA em (**A**) leva a formação de uma distrubuição uniforme dos minícirculo recém duplicados ao redor da rede. Fonte: Shapiro & Englund, 1995.

Ao retornar ao disco, os minicírculos são gradualmente reparados (Pérez-Morga & Englund, 1993) por DNA polimerases presentes nos sítios antipodais e a rede duplicada é dividida e segregada entre as duas células filhas durante a citocinese (Milman et al., 2007). Durante a citocinese, a associação ao corpo basal é necessária para a correta distribuição da rede nas células-filhas (Robinson & Gull, 1991).

Cavalcanti e colaboradores (2009) demostraram em *T. cruzi* a localização de proteínas associadas ao cinetoplasto. Por conta de sua natureza básica, pequeno tamanho e similaridade com proteínas associadas ao cinetoplasto (do inglês *Kinetoplas-associated proteins* – KAP´s) de *C. fasciculata* foi postulada a sua função como neutralizadores de carga elétrica de DNA além de participarem na compactação do mesmo.

A replicação dos maxicírculos foi pouco estudada até o momento, porém também ocorre unidirecionalmente em estruturas em forma de Θ (Hoeijmakers & Borst, 1982). Uma das principais diferenças entre a replicação dos minicírculos e de maxicírculos, e que estes últimos não são liberados da rede. Micrografias eletrônicas evidenciam que em *T. brucei* as redes em estágio final de duplicação apresentam os maxicículos localizados na região central das duas redes em formação, no ponto onde serão divididas após a citocinese (Hoeijmakers & Borst, 1982).

2.3. A edição de RNA

O processo de edição de RNA é baseado em inserções ou deleções de Uridinas (U) de alguns transcritos primários codificados por maxicírculos dando origem a RNA_m funcionais (Benne et al., 1986). A extensão da edição pode variar de 4 inserções de U´s na citocromo oxidase c II (COXII) a centenas de U´s adicionadas e outras dúzias deletadas no caso da citocromo oxidase c III (COXIII) de *T. brucei* (Ochsenreiter et al., 2007).

A especificidade dessa edição é determinada pelos chamados RNAs guia (RNAg), que são pequenos RNAs (~70 Kb) codificados majoritariamente pelos minicírculos (Sturm & Simpson, 1990; Blum et al., 1990). Essa especificidade é direcionada através da complementaridade dos RNAg´s à regiões a serem editadas (Panigrahi et al., 2006; Babbarwal et al., 2007). Os RNAg´s apresentam três diferentes regiões funcionais: (i) a região 5´ que é chamada de âncora e é complementar à região imediatamente 3´ do transcrito a ser editado; (ii) a porção central do RNAg que contem a informação a ser usada durante a edição, sendo complementar ao RNA maduro, ditando as bases que serão retiradas ou adicionadas e (iii) a porção 3´, que apresenta uma cauda poli-U adicionada pós-transcricionalmente e envolvida com a estabilidade do complexo RNAg/RNA (Blum & Simpson, 1990).

O processo de edição é catalizado por um grande complexo multiprotéico denominado editosomo (Amaro et al., 2008). A edição ocorre em três diferentes etapas enzimáticas: (i) clivagem endonucleolítica, (ii) adição/remoção de U´s e (iii) ligação do RNA, sendo todas as enzimas necessárias localizadas nos editosomos (Seiwert et al., 1996; Kable et al., 1996; Cruz-Reyes & Sollner-Webb, 1996) (Fig.1.8). Sabe-se que uma uridil transferase terminal (TUTase) é responsável pela adição de resíduos de U´s na região 3´ do fragmento 5´ clivado (Fig 1.8) (Aphasizheva et al., 2009). Por outro lado, a deleção é mediada por uma exonuclease também U-específica (3´ ExoUase), parcialmente purificada de *Leishmania tarentolae* (Aphasizhev & Simpson, 2001). A ligação dos fragmentos após a edição é feita por RNA ligases (Seiwert & Stuart, 1994).

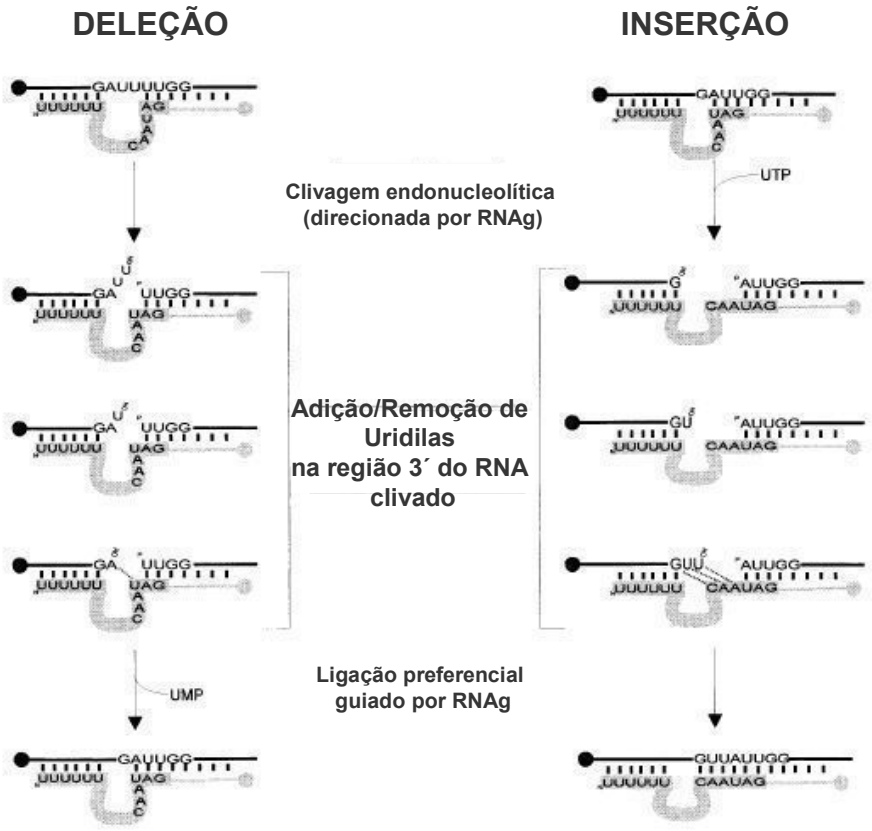


Fig.1.8. Desenho esquemático de edição de RNA em tripanosomatídeos (adaptado de Stuart et al., 1997).

2.4. kDNA como potencial alvo quimioterápico

Além das regiões ricas em sequências codificadores dos RNAg´s, os minicírculos da maioria das espécies apresentam uma região conservada de aproximadamente 100-200 bp (Ray, 1989). Os minicírculos de *T. brucei*, *L. tarentolae* possuem apenas uma dessas regiões (Chen & Donelson, 1980; Kidane et al., 1984), enquanto que *C. fasciculata* e *T. cruzi* (Fig. 1.9.) apresentam duas e quatro, respectivamente (Sugisaki & Ray, 1987; Degrave et al., 1988).

Fig. 1.9. Sequência nucleotídica de minicírculo de cepa Y de *T. cruzi* depositada no GenBank (Número de acesso: M18814) (Degrave et al., 1988). Sublinhados estão as regiões conservadas de aproximadamente 120 pb onde está localizada a origem de replicação (UMS) de 12 bp (duplamente sublinhado). Em caixas estão destacadas a sequência utilizada em nossas análises no artigo #3. Imagem: Anissa Daliry.

Nos minicírculos de *T. cruzi,* as quatro repetições estão localizadas a 90° uma em relação à outra (Degrave et al., 1988; González, 1986), tendo entre elas sequências variadas. Além disso, Ntambi & Englund (1985) descreveram pela primeira vez uma região de ~13 pb (GGG GTT GGT GTA A) conservada em diferentes espécies de tripanosomatídeos, hoje conhecida como sequência de minicírculo universal (do inglês *Universal Minicircle Sequence* - UMS) (Fig. 1.9), direta e comprovadamente associada ao início da replicação (Abu-Elneel et al., 1999; Onn et al., 2006).

Sequências conservadas estão geralmente relacionadas a importantes funções celulares e podem assim, representar, importantes alvos quimioterápicos para tratamento de doenças causadas por protozoários. As sequências conservadas dos minicírculos de *T. cruzi* são ainda mais promissoras, uma vez que são especialmente abundantes (4 repetições por minicírculo, ~ 20.000 minicírculos,

perfazendo um total de 80.000 repetições em cada célula) (Degrave et al., 1988). Esse dado nos motivou a estudar uma sequência localizada em regiões conservadas dos minicírculos como potencial alvo quimioterápico como descrito no **Trabalho #3**.

Outra característica relevante da maioria das espécies de tripanosomatídeos é a presença de regiões que apresentam curvatura intrínseca (*bending*) do DNA. Essas regiões estão associadas à presença de curtas seqüências com repetições de 5 a 6 bases Adenosina (A) localizadas a cada 10.5 pb. A soma das curvas de cada região resulta numa curvatura final substancial da hélice. Em tripanosomatídeos, a curvatura no DNA foi primeiramente identificada em um fragmento de minicírculo de *L. tarentolae*, que apresentava alterações em sua mobilidade eletroforética quando avaliado comparativamente em géis de agarose e de poliacrilamida (Marini et al., 1982). O fragmento estudado apresentava uma diminuição da mobilidade em géis de poliacrilamida: como os poros de géis de poliacrilamida são menores, a mobilidade do fragmento foi diminuída em decorrência de sua possível curvatura. Observou-se pela primeira vez que a curvatura devia-se unicamente a sequência de nucleotídeos do fragmento, e não dependia de outros fatores, com proteínas ou interações com outras moléculas. Interessantemente, enquanto esse fenômeno já foi observado em várias espécies como *C. fasciculata*, *T. equiperdum*, *T. brucei* e *Herpetomonas muscarum*, até o momento não foi detectado em *T. cruzi* (Ntambi et al., 1984). Em outros organismos eucarióticos, curvaturas intrínsecas ao DNA atuam no empacotamento do DNA e na regulação da transcrição (Ross et al., 1999), sendo uma característica comum de regiões promotoras (Garcia et al., 2007).

Além de curvaturas intrínsecas, alterações topológicas de sequências de DNA podem ser induzidas por diversos fatores como proteínas, RNA e fatores exógenos como fármacos, podendo desta forma, representar interessantes estratégias para o desenho de novos agentes quimioterápicos. Fatores que induzem alterações moleculares podem afetar importantes funções na célula, como a transcrição, replicação e/ou a edição de RNAs, levando à morte celular. De fato, alguns estudos mostram a capacidade de alguns compostos de induzirem a curvatura ou mesmo o “endireitamento” de fragmentos de DNA submetidos a diferentes tratamentos (Cons & Fox, 1990; Barcelo et al., 1991; Bailly & Chaires, 1998).

A inibição de proteínas importantes no processo de edição de RNA também é capaz de induzir a morte em tripanosomatídeos. Drozdz e colaboradores (2002) demostraram inibição de crescimento parasitário após silenciamento gênico da proteína TbMP81 de *T. brucei* presente no editosomo, inativando assim, a edição de RNA *in vivo*. O silenciamento de TbMP52, uma proteína que apresenta atividade de ligase e também está presente no editosomo, demostrou-se letal para formas sanguíneas de *T. brucei* (Schnaufer et al., 2001). Estes dois estudos comprovam a importância de proteínas essenciais no processo de edição de RNA, e sugerem seu uso como alvos de drogas.

Compostos aromáticos anti-turmorais e antiparasitários como diamidinas aromáticas e análogos também têm sido estudados quanto ao seu potencial de induzir alterações topológicas em fragmentos de DNA sintéticos que mimetizam sequências encontradas em tripanosomatídeos (Soeiro et al., 2005; Wilson et al., 2008). Como esses compostos apresentam uma forte afinidade por sequências ricas em AT´s (em especial AATT), o kDNA de tripanosomatídeos pode representar um promissor alvo de ação destes compostos haja vista que sua composição apresenta cerca de 60% de bases AT´s. De fato, Mathis e colaboradores (2007) demostraram que várias diamidinas aromáticas, incluindo a pentamidina e a furamidina (DB75) acumulam-se em grandes quantidades em organelas ricas em DNA, como núcleo e cinetoplasto. Além disso, o tratamento com alguns representantes desta classe de compostos (incluindo DB75 e DB569) induz importantes alterações ultraestruturais no kDNA, levando a uma visível desestruturação da rede, que em alguns casos desaparece (De Souza et al., 2004).

Em estudos realizados na década de 70, observou-se que parasitos vivos submetidos ao tratamento com brometo de etídio, um intercalante de DNA que também se acumula no kDNA de parasitos como *T. cruzi*, induzia importantes danos ao kDNA, com as redes fracionadas em numerosas esferas fibrilares (Riou & Delain, 1969). Além disso, demonstrou-se que parasitos cultivados na presença deste intercalante apresentavam moléculas não vistas em parasitos não tratados, como dímeros, trímeros e até pentâmeros circulares (Delain & Riou, 1970), visualizados por técnicas de coloração positiva para MET.

Alterações conformacionais induzidas por outras classes de compostos também já foram investigadas através da análise de alterações no perfil eletroforético das moléculas de DNA. Fox (1990) mostrou a perda da curvatura do

kDNA na presença de um agente anti-tumoral chamado nogalomicina. Além disso, Cons & Fox (1990) observaram que a distamicina, um ligante de fenda menor de DNA também usado como agente anti-turmoral, causa diminuição da curvatura de fragmentos ricos em A além do aumento da curvatura daqueles desprovidos destas regiões. A perda de curvatura intrínseca de fragmentos de kDNA de *C. fasciculata* também já foi demonstrada frente a incubação com distamicina (Barcelo et al., 1991).

Mais recentemente, Tevis e colaboradores (2009) demonstraram que diamidinas como a DB75 e alguns análogos apresentam um efeito sobre os ângulos de curvatura de fragmentos de DNA sintéticos ricos em A e em sequências ATATA. Os autores demonstraram que este efeito depende tanto da estrutura dos compostos como da natureza da sequência do DNA.

3. Diamidinas e seus análogos

Diamidinas aromáticas (DAs), incluindo pentamidina, propamidina e o berenil (aceturato de diminazeno), têm sido utilizadas com sucesso na clínica humana e veterinária. A pentamidina é usada há mais de 60 anos no tratamento da fase inicial da tripanosomíase africana e também na terapia da leishmaniose causada por *Leishmania guyanensis* e nos casos de resistência à antimoniais (Oliaro et al., 2005; Blum et al., 2004; Werbovetz, 2006). Diamidinas também apresentam boa atividade contra agentes causadores de outras patologias como *Pneumocystis jiroveci* (Tidwell et al., 1990), *Saccharomyces cerevisiae* (Lanteri et al., 2004); *Plasmodium falciparum*, *Leishmania amazonensis* (Bell et al., 1990; Macharia et al., 2004), *Toxoplasma gondii* (Lindsay et al., 1991), *Trichomonas vaginalis* (Crowell et al., 2004) e *Giardia lamblia* (Bell et al., 1993). Contudo, as duas principais limitações dessa classe de compostos são a baixa biodisponibilidade oral e a presença de efeitos colaterais. Visando contornar estes obstáculos, a síntese de novos análogos e de pró-drogas tem sido realizada por diferentes grupos de química medicinal (Wilson et al., 2008). Um destes exemplos é a diamidina DB289, uma pró-droga da furamidina (DB75), que apesar de ter demonstrado excelente atividade tripanocida e baixa citotoxicidade *in vitro* e *in vivo*, ensaios clínicos de fase III tiveram que ser interrompidos por conta de seus efeitos colaterais (Soeiro et al., 2008). Este dado reforça a importância da síntese de novas diamidinas e análogos, que mantenham a excelente atividade antiparasitária, mas que apresentem baixa toxicidade para o

paciente. De fato, novos compostos aromáticos têm sido sintetizados e avaliados tanto *in vitro* como *in vivo* (Wilson et al., 2008). Nos últimos anos, nosso laboratório (Laboratório de Biologia Celular do IOC/Fiocruz) tem investigado a eficácia e seletividade de novas DAs e análogos através de modelos *in vitro* e *in vivo* (Soeiro et al., 2009). Nossos resultados apontam para a atividade promissora de vários destes compostos sobre *T. cruzi*, apresentando ampla janela terapêutica (Silva et al. 2007a,b, 2008; Batista et al., 2010a,b; De Souza et al., 2010).

Um dos primeiros estudos *in vitro* identificou a ação da furamidina (DB75) e de seu análogo, a DB569, sobre diferentes formas e cepas do *T.cruzi* (De Souza et al., 2004). Ambas DAs induziram importantes alterações ultra-estruturais na mitocôndria e no núcleo (De Souza et al., 2004) e a análise mais detalhada, por citometria de fluxo, revelou que parte dos parasitos morre com características de morte celular programada do tipo I (apoptose), sendo que a DB569 além de ser mais ativa que a DB75, também induziu maiores níveis desta via de morte (De Souza et al., 2006b). Quando analisada *in vivo*, a DB569 foi capaz de reduzir a carga parasitária e modular negativamente a expressão de células T $CD8^+$ em tecido cardíaco, além de reverter alterações eletrocardiográficas (ECG) causadas pela infecção aguda, resultando ainda em uma maior sobrevida (De Souza et al., 2006a). Resultados semelhantes foram obtidos com animais crônicos que também apresentaram menores índices de alterações na freqüência cardíaca em decorrência do tratamento com a DB569 (De Souza et al., 2006a). Outra diamidina testada, a DB1362 revelou-se ativa sobre formas amastigotas e tripomastigotas de *T.cruzi,* com valores de IC_{50} na faixa submicromolar, apresentando ainda baixa toxicidade para a célula hospedeira (Silva et al., 2008). As alterações induzidas nos tripomastigotas foram correlacionadas à desorganização da estrutura do cinetoplasto e à interferência no potencial da membrana mitocondrial. Em animais experimentais, a administração de somente duas doses de 25 mg/kg da DB1362 no início e no pico da parasitemia, resultou na redução de 40% da parasitemia e sobrevida de 100%. Os animais tratados com a DB1362 também apresentaram redução do parasitismo cardíaco a níveis similares ao Benznidazol (Bz) (Silva et al., 2008).

Estudos recentes correlacionando atividade com estrutura química destes compostos revelaram que moléculas curvas (de mesmo tamanho, maiores ou menores que a DB75 como DB1645, DB1651 e DB1582, respectivamente) são mais ativas e seletivas sobre amastigotas e tripomastigotas sanguineas de *T.cruzi* em relação aos análogos lineares (Batista et al., 2010b). Estes dados sugerem a

relevância da curvatura quanto a eficácia destas moléculas aromáticas, possivelmente favorecendo uma superior associação à fenda menor do kDNA do parasito (Batista et al., 2010b). Resultados *in vitro* também têm demonstrado a relação entre a ação tripanocida destes compostos e a presença dos dois grupamentos catiônicos terminais característicos de DA, como pentamidina e a DB75. Também foi observado que o tipo de associação do grupo imino ao grupamento arila pode conferir diferenças na ação destas moléculas sobre o parasito (Pacheco et al., 2009; Silva et al., 2007a,b). Resultados *in vitro* e *in vivo* têm demonstrado a promissora ação de arilimidamidas (AIAs), anteriormente denominadas de amidinas reversas (Silva et al., 2007a,b; Pacheco et al., 2009; Batista et al., 2010a).

De fato, AIAs apresentam uma atividade excepcional sobre diferentes espécies de *Leishmania* (Stephens et al., 2003; Rosypal et al., 2007, 2008,) e *T. cruzi* (Silva et al., 2007a,b; Pacheco et al., 2009; Batista et al., 2010a,b; De Souza et al., 2010). Estes compostos diferem de outros diamidinas clássicas porque o grupamento amidina está ligado ao núcleo aromático central através de um átomo de nitrogênio em vez de carbono (Rosypal et al., 2008). Estudos recentes *in vitro* e *in vivo* com a AIA DB766 mostraram sua excelente atividade tripanocida e seletividade para *T. cruzi* com IC_{50} de 25 e 60 nM, sobre amastigotas intracelulares e tripomastigotas, respectivamente (Batista et al., 2010a). DB766 quando testado sobre um painel de diferentes cepas do parasito, incluindo algumas naturalmente resistentes ao Bz, apresentou atividade superior a este composto padrão e ao cristal violeta. Em ensaios *in vivo*, DB766 reduziu parasitemia e carga parasitária no tecido cardíaco com eficácia similar ao Bz tanto em animais infectados com a cepa Y como com a cepa Colombiana. Além disso, DB766 reduziu alterações de ECG e protegeu contra lesões cardíacas e hepáticas induzidas pela infecção, resultando ainda na sobrevivência de 90-100% dos animais tratados, semelhantemente ao Bz (Batista et al., 2010a). Estes resultados apontam para esta AIA como um novo potencial composto líder para estudos subsequentes visando uma nova droga para DC. DB766 também se mostrou bastante ativa em ensaios *in vivo* com *Leishmania donovani* (Wang et al., 2010). Além disso, esta AIA não induz mutagenicidade, apresenta baixa toxicidade aguda, boa biodisponibilidade oral, sendo distribuída em diferentes tecidos e órgãos como fígado, coração e baço, além de apresentar altos volumes de distribuição e uma meia vida em camundongos de cerca de 1 a 2 dias (Wang et al., 2010).

Em um trabalho recente, seis novos compostos entre AIAs e diamidinas foram testados *in vitro* sobre *T. cruzi* e também avaliados sobre culturas primárias de cardiomiócitos (De Souza et al., 2010). Todas as ADs foram ativas e a AIA DB1470 foi o composto mais efetivo, apresentando alto índice de seletividade, além de ser capaz de manter atividade tripanocida a 4°C na presença de sangue (De Souza et al., 2010). De fato é interessante que algumas AIAs, como DB1470 e DB766, apresentem atividade tripanocida na presença de sangue (Silva et al., 2007b; Batista et al., 2010b), representando potenciais compostos candidatos ao uso profilático em em áreas endêmicas onde não há screening de bancos de sangue.

Enquanto diferentes sistemas de transporte de diamidinas já foram descritos em *T. brucei*, *Leishmania* sp. e *Plasmodium falciparum* (Carter et al., 1995; Barret et al., 2003; Bray et al., 2003), muito pouco ainda se sabe sobre a captação deste compostos pelo *T. cruzi*. Devido à característica de fluorescência intrínseca de algumas destas moléculas, como a DB75, foi possível acompanhar a distribuição e localização destas moléculas no *T. cruzi* (De Souza et al., 2004). Como anteriormente reportado para tripanosomas africanos (Mathis et al., 2006, 2007; Wilson et al., 2008), algumas diamidinas como DB1582 e DB1651 além de se localizarem no núcleo e, em maior intensidade no kDNA, também foram identificadas em estruturas desprovidas de DNA localizadas na posição anterior de formas sanguíneas de tripomastigotas e próximas ao núcleo e ao cinetoplasto em amastigotas, com morfologia e distribuição intracelular compatível com acidocalcisomas. Tal localização sugere que estas organelas acídicas possam representar sítios de armazenamento, ou ainda que sejam alvos celulares destes compostos (Mathis et al., 2006, 2007) Quanto a outros potenciais alvos celulares, dados de MET revelaram que além de alterações nucleares e na mitocôndria (em especial no kDNA), algumas amidinas, como a DB786 e DB1582, causam importantes alterações na organização de microtúbulos, incluindo a formação de múltiplos axonemas em tripomastigotas (Silva et al. 2007b; Batista et al. 2010b).

O exato mecanismo de ação de diamidinas sobre outros tripanosomatídeos não foi ainda claramente estabelecido, tendo sido sugerida a ocorrência de múltiplos mecanismos (De Koning, 2001; Wilson et al. 2008). Um dos mecanismos que tem sido mais avaliado é referente à propriedade destes compostos em se associar a sequências ricas de AT presentes em minicírculos de kDNA, levando à fragmentação e mesmo desaparecimento do kDNA e morte do parasito (Wilson et al., 2005; Soeiro et al., 2005). Como o kDNA de cinetoplastídeos apresenta uma alto

teor de sítios AT nos seus minicírculos, é possível que estas estruturas sejam de fato alvos potenciais para diaminas e análogos (Wilson et al., 2008). Análises por citometria de fluxo demonstrando a interferência no potencial de membrana mitocondrial induzida por diferentes diamidinas corroboram a hipótese de que um dos alvos destes compostos seja o complexo mitocôndria/cinetoplasto de *T. cruzi* (Silva et al., 2007b, 2008). Outros alvos também propostos na literatura para outros tripanosomatídeos incluem a inibição de tirosil-DNA fosfodiesterase, topoisomerases, proteína quinase A, proteases e polimerases (Dykstra et al., 1994; Shapiro & Englund, 1995; Soeiro et al., 2008).

Por este levantamento podemos observar que a eficácia de diamidinas e análogos sobre *T. cruzi* estimula novos estudos de atividade e de mecanismo de ação visando o estabelecimento de uma nova quimioterapia para DC.

II. OBJETIVOS

Objetivo geral

Analisar atividade, seletividade, mecanismos de ação e alvos celulares de diamidinas (e análogos) sobre *Trypanosoma cruzi in vitro*.

Objetivos específicos

(a) Avaliar a atividade biológica *in vitro* de diamidinas aromáticas, e análogos, sobre formas tripomastigotas sanguíneas, amastigotas e epimastigotas intracelulares de *T. cruzi*.

(b) Determinar o limiar de toxicidade dos compostos sobre células de mamíferos, determinando seu índice de seletividade (IS).

(c) Identificar por microscopia de fluorescência e microscopia eletrônica de transmissão, a localização e os danos celulares induzidos no *T. cruzi* durante o tratamento com as diamidinas aromáticas e seus análogos.

(d) Avaliar através de estudos de termodinâmica a possível correlação entre atividade biológica de diamidinas, e análogos, e seu efeito sobre a topologia do kDNA de *T. cruzi*.

III. RESULTADOS

TRABALHO #1

Situação: Publicado

Referências:

Soeiro MNC, Dantas AP, Daliry A, Silva CF, Batista DG, de Souza EM, Oliveira GM, Salomão K, Batista MM, Pacheco MG, Silva PB, Santa-Rita RM, Barreto RF, Boykin DW, Castro SL. Experimental chemotherapy for Chagas disease: 15 years of research contributions from in vivo and in vitro studies. *Mem Inst Oswaldo Cruz* (2009); 104 (Suppl 1): 301-310.

Págs. 31 à 41

Experimental chemotherapy for Chagas disease: 15 years of research contributions from in vivo and in vitro studies

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Chagas disease, which is caused by the intracellular parasite Trypanosoma cruzi*, is a neglected illness with 12-14 million reported cases in endemic geographic regions of Latin America. While the disease still represents an important public health problem in these affected areas, the available therapy, which was introduced more than four decades ago, is far from ideal due to its substantial toxicity, its limited effects on different parasite stocks, and its poor activity during the chronic phase of the disease. For the past 15 years, our group, in collaboration with research groups focused on medicinal chemistry, has been working on experimental chemotherapies for Chagas disease, investigating the biological activity, toxicity, selectivity and cellular targets of different classes of compounds on* T. cruzi. *In this report, we present an overview of these in vitro and in vivo studies, focusing on the most promising classes of compounds with the aim of contributing to the current knowledge of the treatment of Chagas disease and aiding in the development of a new arsenal of candidates with anti-*T. cruzi *efficacy.*

Key words: *Trypanosoma cruzi* - Chagas disease - experimental chemotherapy - aromatic diamidines - propolis - N,N-dimethyl-propenamines - naphthoquinones

Chagas disease is a neglected illness caused by the intracellular obligatory parasite *Trypanosoma cruzi*, which is transmitted by haematophagous reduviid vectors. While it was described 100 years ago by Carlos Chagas (1909), this disease still represents an important health problem, and is broadly dispersed in 18 developing countries in South and Central Americas (Rocha et al. 2007). The overall prevalence of the disease is about 12-14 million cases, and its main clinical manifestations include cardiac and/or digestive alterations. In addition, Chagas disease is a major cause of cardiac infectious disease in endemic areas (Dias 2007, Anonymous 2006). Recently, Chagas disease has also been recognised as an opportunistic disease in HIV-infected individuals (Vaidian et al. 2004) and it is reported throughout the world due to international immigration (Schmunis 2007).

Chagas disease has two phases: the acute phase, which appears shortly after infection and whose symptoms range from flu-like symptoms to intense myocarditis (in about 10% of infected people) and the chronic symptomatic phase, which develops in about one-third of infected individuals following an asymptomatic period (indeterminate form) of years or decades (Chagas 1909, Bilate & Cunha Neto 2008). While its pathology is still poorly understood, growing evidence has shown that parasite persistence within the target organs as a result of an unregulated host immune response is involved in pathogenesis, disease progression and outcome (Higuchi et al. 2003, Marino et al. 2005). In addition, despite fruitful efforts in the control of vectorial and blood transmission, Chagas disease still presents many challenges, including (i) its peculiar epidemiology, characterised by a variety of risk factors (many potential vectors and reservoirs, different forms of transmission and diverse parasite isolates present in domiciliar, peridomiciliar and sylvatic environments) and (ii) the lack of prophylactic therapies and effective chemotherapeutic schemes (Coura & De Castro 2002, Coura 2007, Dias 2007). Nifurtimox (Nf) and benznidazole (Bz) are the drugs that are available for the treatment of chagasic patients. However, due to their well-known toxicity and limited effect towards different parasite isolates and disease phases (primarily patients in the later chronic phase), new drugs are urgently needed (Coura & De Castro 2002, Soeiro & De Castro 2009). While many trypanocidal compounds have been screened in the past few decades and some promising targets have been reported since the introduction of Nf and Bz (1960-1970), only allopurinol and a limited number of azoles, such as itraconazole, fluconazole and ketoconazole have moved to clinical trials (Brener et al. 1993, Solari et al. 1993, Apt et al. 2005). This situation may reflect (i) low investments in this area, primarily by the pharmaceutical industry, (ii) the misconception

Financial support: CNPq, Faperj, Fiocruz, CAPES, PAPES V
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Received 20 April 2009
Accepted 8 June 2009

that during the later stages of the disease the parasite is absent and thus does not correlate with disease outcome and pathogenesis, and (iii) the lack of standardised protocols for drug screening. Thus, based on the current knowledge of parasite and host biology, an ideal drug candidate for Chagas disease would have the following characteristics: (i) a high level of activity against the evolutive forms present in mammalian hosts and against different stocks of the parasite; (ii) efficacy against both acute and chronic infections; (iii) oral administration in few doses; (iv) low toxicity and improved safety (including in children and women of reproductive age); (v) low cost and high stability for a long shelf life in tropical temperatures and (vi) high levels of tissue accumulation and long terminal half lives (Nwaka & Hudson 2006).

In this context, an overall discussion is provided on the main advances made over the last 15 years from in vitro and in vivo studies related to anti-*T. cruzi* candidates screened at the Laboratório de Biologia Celular of the Instituto Oswaldo Cruz/Fiocruz in collaboration with different groups working in medicinal chemistry, including those headed by Drs. David W. Boykin (Georgia State University, USA), Richard Tidwell (University of North Carolina, USA), Nelson Duran (Unicamp, Brazil) and Antonio Ventura Pinto (UFRJ, Brazil). However, additional pharmacological and safety tests are needed in subsequent studies to identify and move promising compounds to clinical trials.

Diamidines and related compounds

Diamidine-containing compounds such as pentamidine, propamidine and diminazene aceturate (also known as Berenil, a drug commonly used against trypanosome infections in livestock) are DNA minor groove binders that have long been used in infectious disease chemotherapy.

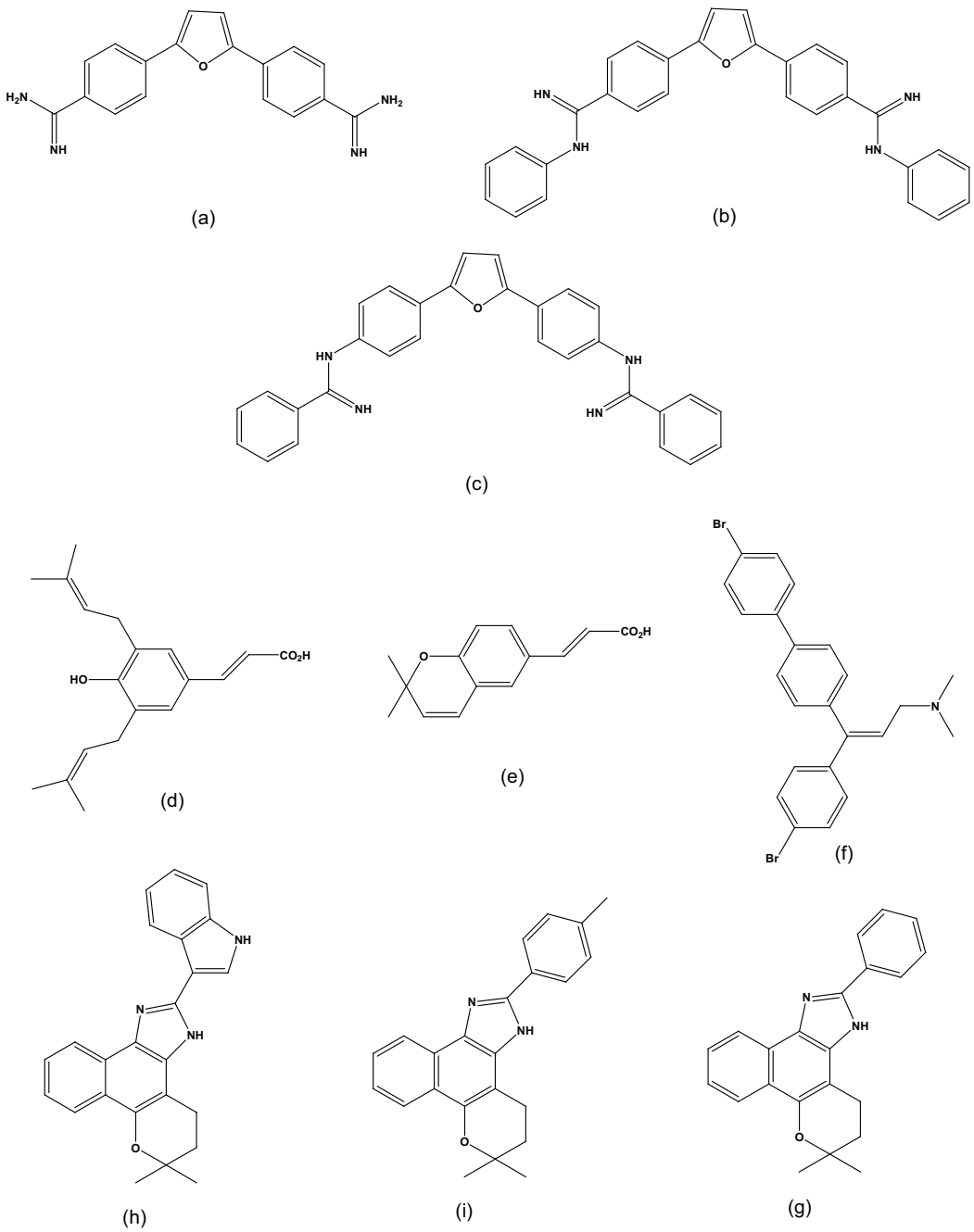
Pentamidine, employed for over 60 years against early-stage human African trypanosomiasis, is also used to treat cutaneous leishmaniasis caused by *Leishmania guyanensis* and antimony-resistant visceral cases (Blum et al. 2004, Oliaro et al. 2005, Werbovetz 2006).

However, while presenting such a high potency, pentamidine, as well as some other diamidines such as furamidine (DB75), display poor oral bioavailability and unfavorable side effects. In order to overcome these limitations, the synthesis and screening of new dications and their prodrugs have been performed (Werbovetz 2006). One such orally effective prodrug, DB75 (DB 89), was in Phase III clinical trials for the treatment of human African trypanosomiasis. Despite initial indications of low toxicity in African, Asian, Caucasian and Hispanic populations (Soeiro et al. 2008), recent results from studies using extended dosages led to a withdrawal of DB289 from further human trials.

While these dicationic compounds have been studied primarily against African trypanosomes, few have been assayed as anti-*T. cruzi* candidates (Wilson et al. 2008). In the past few years, our laboratory has been working on the anti-*T. cruzi* activity of novel dications using both in vitro and in vivo experimental models. The aim of this work has been to select and evaluate the effectiveness of furamidine analogues of different shapes, cationic centres and effective motifs as well as to test the assumed requirement of the dications. Our data clearly show a number of very promising candidates that display high therapeutic windows (De Souza et al. 2004, 2006a, b, 2007, Silva et al. 2007a, b, 2008). As a result of collaboration with the Drs. D. Boykin (Georgia State University, USA) and R. Tidwell (University of North Carolina, USA), we have obtained very exciting findings as both research groups have a large amount of experience with the molecules and a collective expertise in both anti-parasitic drug discovery and development on different parasitic infections.

Our data show that while furamidine and its N-phenyl substituted analogue (DB569) (Figure) display equivalent DNA binding properties, DB569 exhibited higher in vitro activity against different strains and stages of the parasite, displaying inhibitory values in the low-micromolar range. Due to the characteristic fluorescence of these compounds, it was possible to localise both in DNA enriched organelles, with kDNA being strongly labelled (De Souza et al. 2004, Soeiro et al. 2005). Importantly, flow cytometry and transmission electron microscopy (TEM) analysis have also demonstrated that both drugs disturb parasite mitochondria and nuclei, leading to morphological characteristics of programmed cell death, with DB569 achieving higher levels of apoptotic-like parasites (De Souza et al. 2004, 2006b). These findings stimulated further in vivo analysis with DB569, which reduced cardiac parasite load and down-modulated the expression of $CD8^+$ T cells in the heart tissues (De Souza et al. 2006a, 2007). DB569 also revert the electrocardiography (ECG) alterations in acutely treated *T. cruzi*-infected mice compared to those in untreated animals, leading to an increase in the survival rates of the former group (De Souza et al. 2007). The ECG protection provided by DB569 was also found during the chronic infection phase of experimental animals suggesting that the reversion to and the further maintenance of a normal ECG profile in the DB569-treated animals may be associated with reduced cardiac $CD8^+$ lymphocyte infiltration and parasitism (De Souza et al. 2006a, 2007).

Another study performed with a diarylthiophene diamidine (DB1362) (Figure) against both amastigotes and bloodstream trypomastigotes of *T. cruzi* showed good in vitro efficacy at submicromolar IC_{50} doses that did not induce host cytotoxicity (Silva et al. 2008). We also found a dose-dependent trypanocidal effect after 24 h of incubation in the presence of plasma constituents (mice blood), exhibiting similar IC_{50} values as those found in the absence of blood. This result argues for the possible application of blood banks in prophylactic treatment. Analysis of bloodstream parasites by TEM and flow cytometry confirmed that the most important alterations correlated to kinetoplast organisation and mitochondrial membrane potential (Silva et al. 2008). In view of the in vitro trypanocidal effect, we performed further in vivo studies in an acute *T. cruzi*-experimental mice model (male Swiss mice infected with the Y strain) us-



Chemical structures: a: furamidine; b: DB569; c: DB613A; d: 3,5-diprenyl-4-hydroxycinnamic acid derivative; e: 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran; f: 3-[4′-bromo-(1,1′-biphenyl)-4-yl]-N,N-dimethyl-3-(4-bromo-phenyl)-2-propen-1-amine; g: 4,5-dihydro-6,6-dimethyl-6H-2-(phenyl)-pyran[b-4,3]naphth[1,2-d]imidazole); h: 4,5-dihydro-6,6-dimethyl-6H-2-(3′-indolyl)-pyran[*b*-4,3]naphth[1,2-d] imidazole; i: 4,5-dihydro-6,6-dimethyl-6H-2-(4′-methylphenyl)-pyran[*b*-4,3]naphth[1,2-d]imidazole).

ing a two or a 10-day regime at doses that did not did not exert substantial side effects in treated animals. In parallel, we used a mouse group that received Bz (100 mg/kg by gavage) as the reference drug. Our findings show that the best results were obtained when the mice were treated with 25 mg/kg of DB1362 administrated at two intervals (1 at the onset and the 2nd at the parasitemia peak). While we found only a reduction of about 40% in the circulating parasitemia levels, the treatment induced 100% survival, which was largely due to reduced cardiac parasitism (similar levels as Bz) in addition to protecting against cardiac electric alterations due to the parasite infection (Silva et al. 2008).

In a recent unpublished study, the biological, ultrastructural effect and subcellular localisation of six diamidines was studied in *T. cruzi* (Batista et al. 2009). The

data confirmed the low toxicity of these diamidine compounds towards mammalian cells ($LC_{50} > 96$ μM). Additionally, the three dicationic compounds, with the exception of the smaller linear molecules (DB1627, DB1646 and DB1670), which were not effective, exerted a very high efficacy against both relevant forms of the parasite that are present in mammalian hosts, with IC_{50} values ranging from 0.15-13.3 μM (DGJ Batista et al., unpublished observations). 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, Barret et al. 2003, Bray et al. 2003), the mechanism of uptake of diamidines by *T. cruzi* remains unknown and deserves further investigation. However, due to the intrinsic fluorescent characteristics of these compounds, it is possible to follow their distribution in *T. cruzi* as was previously performed with African trypanosomes (Mathis et al. 2006, 2007, Wilson et al. 2008). We found that in addition to the known localisation of these compounds in the parasite nuclei and kDNA (with a greater intensity displayed in the later structure), two of the compounds (DB1582 and DB1651) were found within punctate, non-DNA-containing, cytoplasmic organelles. These organelles were preferentially localised in the anterior portion of bloodstream trypomastigotes and near the nuclei and kinetoplast regions of amastigotes and therefore, are possibly acidocalcisomes, which has been previously reported for *Trypanosoma brucei* (Mathis et al. 2006). As suggested for African trypanosomes, the localisation of these compounds within these acidic organelles could play a role in their mechanism of action; these organelles may also act as storage sites (Mathis et al. 2006, 2007) and additional studies are necessary to clear this matter. In the study by DGJ Batista et al. (unpublished observations) we also provided additional TEM data confirming that these diamidines caused striking alterations in the mitochondria and kinetoplast of the parasites. In addition, the compounds also induced disorganisation of microtubules leading to the formation of multiple axoneme structures in diamidine-treated bloodstream forms (Silva et al. 2007a, DGJ Batista, unpublished observations). To date, no major alterations have been reported in both sub-pellicular and flagellar microtubules of *T. cruzi* treated with microtubule-targeting drugs such as taxol, colchicine and vinblastine. This may be a consequence of the high content of acetylated tubulin and/or poly-glutamylation of tubulin that is present in these parasites (Souto-Padron et al. 1993, Dantas et al. 2003). Since these structures in trypanosomatids are very resistant to microtubule disrupters compared to the microtubule structures in mammalian cells, they may represent interesting targets for drug development and deserve further investigation.

In fact, the mechanism of action of diamidines and related compounds has not been fully elucidated and multiple modes of action have been proposed (Wilson et al. 2008). One of the long-hypothesised mechanisms of action of diamidines is their ability to bind to AT-rich regions of the DNA minor groove but other mechanisms such as inhibition of tyrosyl-DNA phosphodiesterase, topoisomerases, protein kinase A, proteases and polymerases have been suggested (Shapiro & Englund 1990, Dykstra et al. 1994, Soeiro et al. 2008). As the kDNA of kinetoplastid parasites possesses a high number of adenosine and thymine (AT) binding sites in thousands of repeated minicircles, these structures may represent potential cellular targets for diamidine and related compounds that have AT sequence binding specificity (Wilson et al. 2008). However, while diamidines are excellent minor groove DNA binders, this interaction itself cannot fully explain their biological activity. Recent reports suggest that their association with DNA is an initial step and that it is followed by topological changes that lead to molecule instability and destruction and/or modification of DNA-protein interactions; this, in turn leads to replication errors, DNA degradation and eventual parasite death (Singh & Dey 2007, Wilson et al. 2008). Interestingly, recent unpublished results from our group from the screening of 21 dicationic molecules against *T. cruzi* showed that while all compounds were localised to a greater extent within the kinetoplast than in the nucleus, no correlation could be found between compound activity and the level of kDNA accumulation (A Daliry et al., unpublished observations, CF Silva et al., unpublished observations). These results are consistent with previous data from *T. brucei* (Mathis et al. 2006).

Arylimidamides, previously known as reversed amidines, have extraordinary activity against both *Leishmania* (Stephens et al. 2003, Rosypal et al. 2007, 2008) and *T. cruzi* (Silva et al. 2007a, b, Pacheco et al. 2009, unpublished observations). They differ form other furan analogs because the amidine is bound to the central aromatic linker *via* a nitrogen atom rather than a carbon atom (Rosypal et al. 2008). In a recent study, the analysis of arylimidamides on promastigotes and amastigotes of *Leishmania* showed that most compounds (9 out of 10) from this class display a 250-fold and a 4.5-fold greater activity that pentamidine (Rosypal et al. 2008).

According to the *T. cruzi* data, we have demonstrated their potent in vitro dose-dependent activity (IC_{50} in the low-micromolar range), showing superior trypanocidal activity compared to diguanidino cationic groups (Silva et al. 2007a). TEM and flow cytometry data also showed primarily mitochondrial alterations in the amidine treated parasites (Silva et al. 2006).

In another recent study we reported the in vitro trypanocidal effect of several different heterocyclic cationic compounds, including diamidines, a monoamidine, an arylimidamide and a guanylhydrazone and found that all compounds exerted, at low micromolar doses and to various extents, a trypanocidal effect on both intracellular parasites and bloodstream trypomastigotes (Pacheco et al. 2009, unpublished observations). However, the potency and selectivity of DB613A, an arylimidamide (Figure), towards intracellular parasites (reaching a selective index of > 126), corroborates previous results that demonstrated the high activity of this class of compounds against this parasite. These results therefore justify further studies on experimental models of *T. cruzi* infection.

Propolis

Propolis is a product from bees that is extensively used in folk medicine for a wide spectrum of diseases (Bankova et al. 2000). Our laboratory is, and has been for several years, involved in the investigation of the trypanocidal effect of propolis (Higashi & De Castro 1994, De Castro & Higashi 1995, Cunha et al. 2004). This resinous material is collected from different plant exudates and presents a complex composition that is dependent on the plant sources accessible to the bees (De Castro 2001). In temperate zones, poplar trees (*Populus* spp.) are the main source of propolis, and its biological activity has been associated to the presence of flavonoids and caffeic acid derivatives (Hegazi et al. 2000, Prytzyk et al. 2003). In tropical regions, a variety of plant sources exist, which results in a number of samples with distinct compositions, a characteristic that is dependent on the local flora at the site of collection (Marcucci et al. 2001, Park et al. 2002, Trusheva et al. 2006).

Brazilian propolis has been the subject of intensive research over the past few decades and has been subdivided into four types based on the association of ethanol extracts of Brazilian samples with the levels of specific compounds (Marcucci 2000). In collaboration with Dr. Maria Cristina Marcucci (Uniban, São Paulo), who determined the composition of standardised ethanol extracts from Brazilian samples by high-performance liquid chromatography (Marcucci et al. 2001), we investigated the correlation between the presence of bioactive components with the activity against bloodstream trypomastigotes. Based on the predominant botanical origin in the region from which the samples were collected, the 10 extracts were separated into three groups: A (*Baccharis dracunculifolia* + *Araucaria* spp), B (*B. dracunculifolia*) and C (*Araucaria* spp). Analysis by multiple regression of all the extracts performed by Dr. Pedro Cabello (Fiocruz) showed a positive correlation with samples containing a higher concentration of the 3,5-diprenyl-4-hydroxycinnamic acid derivative 4 (DHCA4) and 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran (DCBEN) having a higher trypanocidal effect (Figure). Reliable statistical analysis on each of the three groups was not possible due to the small numbers of observations. However, an overall analysis revealed an association of anti-*T. cruzi* activity with dicaffeoylquinic acid 3 in group A and with caffeic acid in group B. No association was observed between the trypanocidal effect and any component of the extracts in group C. The present study reinforces the relevance of *para*-coumaric acid and its derivatives, particularly the prenylated and caffeolyquinic acid forms, on the biological activity of Brazilian propolis (Salomão et al. 2008).

In another line of research, we compared the activity and mode of action of propolis from temperate and tropical regions by determining the compositions and investigating the effects against *T. cruzi* of standard ethanolic extracts from a Bulgarian (Et-Blg) and a Brazilian sample (Et-Bra). Et-Blg possessed a high content of flavonoids and was active on epimastigotes and trypomastigotes. In the presence of blood, the activity of Et-Blg was similar to that of the standard drug crystal violet. A methanolic extract of the same sample displayed low activity on trypomastigotes, which was associated with a high concentration of mono and disaccharides and therefore resulting in a low concentration of potentially active compounds (Paulino et al. 2003, Prytzyk et al. 2003). The treatment of *T. cruzi*-infected skeletal muscle cells with Et-Blg led to a decrease of infection and an inhibition of intracellular proliferation of amastigotes with no toxicity to the host cell. Ultrastructural analysis revealed the mitochondrion and reservosomes to be the main targets of the extract in epimastigotes, while in trypomastigotes the mitochondrion-kinetoplast complex was the most susceptible structure (Dantas et al. 2006). In experimentally infected mice, treatment with 50 mg Et-Blg/kg body weight/day led to a decrease in parasitemia with no hepatic or renal toxic effect. Additionally, the extract induced a decrease in spleen mass and interfered with the basic properties of immune cells (Dantas et al. 2005). Et-Bra was obtained from a sample of Brazilian green propolis that was classified as a BRP1 (Marcucci 2000) because of the high content of the bioactive compounds DHCA, 2,2-dimethyl-8-prenyl-2H-1-benzopyran-6-propenoic acid, 3-prenyl-4-hydroxycinnamic acid, *para*-coumaric acid as well as the absence of DCBEN (Salomão et al. 2004). Data obtained from ultrastructural and flow cytometry analysis indicated that Et-Bra induced plasma membrane damage in trypomastigotes, reservosomes disorganisation and mitochondrial swelling in epimastigotes. Acutely infected mice were treated orally with Et-Bra and the parasitemia, mortality and GPT, GOT, CK and urea levels were monitored. The extract (25-300 mg/kg body weight/day for 10 days) reduced parasitemia, though not to significant levels; the extract also increased the survival of the animals and did not induce any hepatic, muscular lesion or renal toxicity. Since Et-Bra was not toxic to the animals, it could be assayed in combination with other drugs (Salomão et al. 2009, unpublished observations). While the results obtained with different samples all had established chemical compositions, the intrinsic complex characteristic of propolis and the in vivo results does not present propolis as an ideal trypanocidal agent for further investigation. However, since propolis from both temperate and tropical zones is active on different species of *Leishmania* (Machado et al. 2007), studies about the in vivo topical treatment of new formulations need to be performed in cutaneous models of leishmaniasis.

N,N-dimethyl-2-propen-1-amine derivatives

In collaboration with Dr. N. Duran (IQ/Unicamp) we developed a series of 3-[4′-bromo-(1,1′-biphenyl)-4-yl]-N,N-dimethyl-3-(4-X-phenyl)-2-propen-1-amine derivatives (X = H, OMe, NO_2, Me, SO_3H, Br, I and thienyl) and assayed their in vitro activity against *T. cruzi.* These compounds are characterised by the *para*-substitution on the phenyl moiety and were obtained as an isomeric mixture of Z:E (nearly 1:1) with high purity and good yields. These compounds can easily penetrate the plasma membrane and accumulate in the lysosomes, where they become trapped in the protonised form as a result

of the acid environment inside this organelle (Lüllmann et al. 1978). Barrett et al. (1982) have previously reported that the chloro derivative (X = Cl) tested as an E isomer salt is highly effective in suppressing mice infections induced by different stocks of *T. cruzi* and is more active than either Nf or Bz.

The IC_{50}/24 h values for the proliferative amastigote and epimastigote forms were in the range of 5-25 µM, while for trypomastigotes it was between 12-55 µM, which is in the same range as Nf (De Conti et al. 1996a, b, 1998, Souza et al. 2002, 2004). The unsubstituted and the chloro derivatives were the more active agents against all three forms of the parasite. The activity of the *cis* and *trans* isomers of both the unsubstituted and the bromo derivatives were evaluated, with the former being the most active on bloodstream trypomastigotes (Oliveira et al. 2005). The chromatographic analysis of epimastigotes treated with the unsubstituted 2-propen-1-amine showed a decrease in the ergosterol levels and an increase in the concentration of squalene. The 2-propen-1-amines can act to reduce the conversion of squalene to squalene epoxide, a reaction that is catalysed by squalene epoxidase. This enzymatic step is a target of allylamines in fungi and protozoa and represents an interesting target to be explored in drug discovery.

In experimentally infected mice, while the unsubstituted analogue (X = H) was ineffective, the corresponding bromo derivative (X = Br) (Figure) was very active. At a regimen of 5 mg/kg for nine consecutive days, the latter led to a consistent suppression of parasitemia combined with full protection against death at levels comparable to the treatment with Bz at 100 mg/kg (Pereira et al. 1998, Oliveira et al. 2005). This class of compounds deserves further investigation based on the excellent in vivo results obtained.

Naphthoquinones and derivatives

Naphthoquinones are compounds that are present in various families of plants; their molecular structures endow them with redox properties and therefore, they participate in different biological oxidative processes. In folk medicine and particularly among Indian populations, plants containing naphthoquinones have been employed for the treatment of different diseases such as cancer (Arenas 1987, Bastien et al. 1983). The biological redox cycle of quinones can be initiated by one electron reduction leading to the formation of semiquinones, unstable intermediates that react rapidly with molecular oxygen to generate free radicals. An alternative pathway is the reduction by two electrons, mediated by DT-diphorase, leading to the formation of hydroquinone. Lapachol, β-lapachone and α-lapachone isolated from the heartwood of trees of the Bignoniaceae family that are abundant in tropical rain forests. In Brazil, more than 46 types of such woods, popularly known by the name of *ipes* (*Tabebuia* sp.), have been described. These compounds act as inhibitors of electron transport, uncouplers of oxidative phosphorylation, DNA intercalating agents and as producers of reactive oxygen radicals (O´Brien 1991, Monks et al. 1992, Goulart et al. 2003). The activity of lapachol, β-lapachone and derivatives on viruses, *Schistosoma mansoni* and the pathogenic protozoan *Leishmania* sp, *Toxoplasma gondii* and *P. falciparum* has been examined (Pinto et al. 2007). Due to the easy access to natural sources of quinones from the Brazilian flora and the synthetic alternative routes previously developed by the group of Dr. Ventura Pinto (NPPN/UFRJ) (who are exploring the electrophilicity of 1,2-quinoidal carbonyls with reagents presenting heteroatom nucleophilic centres) (Pinto et al. 1982, 1985, 1997a, Lopes et al. 1984, Chaves et al. 1990), naphthoquinones were used as starting points in medicinal chemistry studies.

Since the 90´s we have been studying the anti-*T. cruzi* activity of this class of chemicals (De Castro et al. 1994). We screened 54 derivatives obtained by Dr. Ventura Pinto through the reaction of naphthoquinones with common reagents used in heterocyclic chemistry. This has led to the synthesis of 14 oxazolic, 30 imidazolic and 10 additional heterocyclic compounds, including a phenoxazine, a dihydro indol, six cyclopentenic and two pyranic. The analysis of these compounds, together with those of the original 9-naphthoquinones [lapachol, β-lapachone, α-lapachone, nor-β-lapachone, nor-lapachol, methoxy-β-lapachol, lawsone, O-allyl lawsone and C-allyl lawsone], were performed using infective bloodstream forms of the parasite that were treated for 24 h at 4°C in culture medium in the presence of 5% blood and using the IC_{50} value as the parameter as previously described (De Castro et al. 1994).

In comparing the activities of the original naphthoquinones, we concluded that minor structural features involved in increases in lipophilicity, such as the furane moiety or the presence of a methoxyl group or an aliphatic side chain led to an increase in the effect against *T. cruzi*. It is possible that a lipophylic character allows for better penetration of the compound through the plasma membrane of the parasite. The activity of the synthesised compounds against *T. cruzi* showed no uniform behaviour, at times showing higher, lower or similar activities to those of the original naphtoquinones from which they were obtained. For the naphthooxazoles assayed, there was no correlation between biological activity and the type of mono-oxygenated ring (pyrane vs. furane). As previously discussed for napthaquinones, the lipophylic character introduced by this appendage, as well as the presence of a methoxyl or a phenyl group, are factors that increase the trypanocidal activity. A characteristic of the synthesised naphthoimidazoles is that most of them present aromatic groups containing electron-releasing or electron-withdrawing groups attached to the imidazole ring (Pinto et al. 1997b, Neves-Pinto et al. 2000, 2002, Moura et al. 2001, 2004). The results suggested that electronic factors related to the phenyl group were not relevant for the biological effect. Among the investigated compounds, three naphthoimidazoles derived from β-lapachone and containing the aromatic moieties phenyl (N1), 3-indolyl (N2) and methyl-*p*-phenyl (N3) (Figure) showed the highest activity against the parasite and were selected for further studies on their activity on intracellular amastigote forms and their toxicity to mammalian cells (Menna-Barreto et al. 2005, 2007, 2009a). As mode of action of naphthoimidazoles on the parasite,

we can exclude damage caused by oxidative stress since unlike the original napthoquinones, these derivatives do not easily undergo redox reactions. It is important to note that several trypanocidal agents, such as Bz, contain basic imidazolic moieties (Winkelman et al. 1978, McCabe et al. 1983, Chabala et al. 1991, Nothenberg et al. 1991, Blandon et al. 1993, Sepulveda-Boza & Cassels 1996), which is consistent with the idea that trypanocidal activity is associated with the imidazolic skeleton.

The three naphthoimidazoles were active on trypomastigotes, intracellular amastigotes and epimastigotes, and presented low toxicity to the host cell. In epimastigotes, the compounds blocked the cell cycle, inhibited succinate cytochrome c reductase, metacyclogenesis and induced damage to the mitochondrion, Golgi complex and reservosomes. In treated trypomastigotes, alterations occurred in the kinetoplast, mitochondrion and induced plasma membrane blebbing and DNA fragmentation (Menna-Barreto et al. 2005, 2007). Investigation into their mode of action led to the identification of the mitochondrion, reservosomes and DNA as their main targets, and stimulated further studies on the death pathways. Ultrastructural analysis revealed both authophagic (autophagosomes) and apoptotic-like (membrane blebbing) phenotypes. Flow cytometry analysis showed that in N1 or N2-treated trypomastigotes, a small increase in phosphatidylserine exposure and a large increase in the percentage of necrosis were observed. These death phenotypes were not detected in treated epimastigotes. The strong increase in labelling of monodansyl cadaverine, the inhibition of the death process by wortmannin or 3-methyladenine, the overexpression of the *ATG* genes in treated epimastigotes, as well as the ultrastructural evidence point to autophagy as being the predominant phenotype induced by the naphthoimidazoles (Menna-Barreto et al. 2007, 2009a, c).

In another approach, new compounds were also prepared through molecular hybridisation. Exploring the electrophilicity of 1,2-quinoidal carbonyls, naphthoquinones were coupled to [1,2,3]-triazoles or arylamines resulting in a series of derivatives that were also assayed on bloodstream trypomastigotes. Substituted *ortho*-naphthofuranquinones (a non-substituted *para*-naphthofuranquinone), a new oxyrane and an azide were prepared from nor-lapachol while a new non-substituted *para*-naphthofuranquinone was prepared from α-lapachone. The most active compounds were three ortho naphthofuranquinones with trypanocidal activity higher than that of Bz, the standard drug (Silva et al. 2008b). The [1,2,3]-triazole derivatives of nor-β-lapachone were more active than the original quinones, with the apolar phenyl substituted triazole derivative being the most active compound (Silva et al. 2008a). Such hybrid molecules obtained from quinones plus a triazole or arylamino group endowed the quinines with redox properties. These strategies represent an interesting starting point for a medicinal chemistry program that is focused on the chemotherapy of Chagas disease.

Three new naphthofuranquinones were synthesised and assayed against *T. cruzi*. Two derivatives were obtained by the addition of iodine to C-allyl-lawsone (2-hydroxy-3-allyl-naphthoquinone), followed by cyclisation to generate a furan ring; a third compound was obtained through an acid-catalysed reaction by dissolution of the original quinone in sulfuric acid. These compounds were active against the trypomastigote, intracellular amastigote and epimastigote forms presented low toxicity toward host mammalian cells and their chemical structure endowed them with redox properties, thus stimulating further studies (Silva et al. 2006). Ultrastructural analysis of treated epimastigotes and trypomastigotes indicated potent effects of the three naphthofuranquinones in parasite mitochondria, which appeared drastically swollen and possessed a washed-out matrix profile. Fluorescence-activated cell sorting analysis of rhodamine 123-stained *T. cruzi* showed that they caused a potent dose-dependent collapse of mitochondrial membrane potential, especially in epimastigote forms. These compounds also specifically decreased mitochondrial complex I-III activities in both epimastigotes and trypomastigotes, which parallel the reduction in succinate-induced oxygen consumption. Mitochondrial hydrogen peroxide formation was also increased in epimastigotes after treatment with naphthofuranquinones. Our results indicate that the trypanocidal actions of the naphthofuranquinones are associated with mitochondrial dysfunction, leading to increased reactive oxygen species generation and parasite death (Menna-Barreto et al. 2009b, unpublished observations). The tractable synthesis route of these compounds in the laboratory opens the possibility for large-scale production with high yields that can be assayed in experimental mouse models.

In conclusion, a number of factors limit the utility of the existing drugs for Chagas disease primarily because of their low efficacy (mostly upon chronic patients), poor activity against many *T. cruzi* isolates circulating in different geographic areas and considerable side effects. Additionally, in the past few decades, few compounds have moved to clinical trials due to the minimal investments allocated in this area (as well as to other neglected diseases) and the lack of standardised protocols for drug screening. In addition, the identification of new trypanocidal candidates that could enter clinical studies requires integrated partnerships and interdisciplinary networks that involve expertise in a variety of fields such as molecular and cellular biology, chemistry and biochemistry, pharmacology and toxicology. Thus, with the advent of genomics, rapid DNA sequencing, bioinformatics, proteomics, combinatorial chemistry and automated high-throughput screening, extensive knowledge has be accumulated that provides new insight toward the discovery of more selective and successful compounds that could be applied to Chagas disease therapy.

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TRABALHO #2

Situação: Publicado

Referências:

Daliry A, Silva PB, Silva CF, Meuser MB, De Castro SL, Tidwell RR, Soeiro MNC (2009) *In vitro* analyses of the effect of aromatic diamidines upon *Trypanosoma cruzi*. *J Antimicrob Chemother* (2009):64: 747-750.

Pags. 44 à 50

Journal of Antimicrobial Chemotherapy (2009) **64**, 747–750
doi:10.1093/jac/dkp290
Advance Access publication 11 August 2009

JAC

In vitro analyses of the effect of aromatic diamidines upon *Trypanosoma cruzi*

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Received 7 May 2009; returned 19 June 2009; revised 29 June 2009; accepted 19 July 2009

***Objectives*: Aromatic diamidines (ADs) have been recognized as promising antiparasitic agents. Therefore, in the present work, the *in vitro* trypanocidal effect of 11 ADs upon the relevant clinical forms of *Trypanosoma cruzi* was evaluated, as well as determining their toxicity to mammalian cells and their subcellular localization.**

***Methods*: The trypanocidal effect upon trypomastigotes and amastigotes was evaluated by light microscopy through the determination of the IC_{50} values. The cytotoxicity was determined by the MTT colorimetric assay against mouse cardiomyocytes. For the subcellular localization, transmission electron microscopy and fluorescence approaches were used. The fluorescence intensity within the kinetoplast DNA (kDNA) and nuclear DNA (nDNA) of treated parasites was determined using the Image J program.**

***Results*: Compounds 2, 5 and 7 showed the lowest IC_{50} values (micromolar range) against intracellular amastigotes and trypomastigotes. In the presence of blood, all the tested ADs exhibited a reduction of their activity. The compounds did not exhibit toxicity to cardiac cells and the highest selectivity index (SI) was achieved by compound 5 with an SI of >137 for trypomastigotes and compound 7 with an SI of >107 for intracellular parasites. The subcellular effects upon bloodstream forms treated with compounds 5 and 7 were mainly on kDNA, leading to its disorganization. The higher accumulation in the kDNA observed for all tested ADs was not directly related to their efficacy.**

***Conclusions*: Our results show the high activity of this new series of ADs against both trypomastigote and amastigote forms, with excellent SIs, especially compound 7, which merits further *in vivo* evaluation.**

Keywords: chemotherapy, Chagas disease, kDNA, intracellular localization

Introduction

Chagas disease, caused by *Trypanosoma cruzi*, is endemic in Latin America.[1] The currently accepted drugs for its treatment are nifurtimox and benznidazole, and their efficacy for the chronic phase is still debatable; besides that they present side-effects frequently forcing the abandonment of the treatment.[2] Since the use of pentamidine for human African trypanosomiases, aromatic diamidines (ADs) have been recognized as promising antiparasitic agents, presenting striking broad-spectrum antimicrobial effects possibly related to their DNA binding properties.[3] However, due to their poor oral bioavailability and unfavourable side-effects, new analogues have been synthesized and assayed *in vitro* and *in vivo*.[4] Previous results from our laboratory showed high efficacy of ADs and analogues, such as reversed amidines or arylimidamides, against *T. cruzi* both *in vitro* and *in vivo*.[5,6] In the present work we evaluate the *in vitro* activity of 11 ADs against the relevant clinical forms of *T. cruzi* and their toxicity to mammalian cells. Additionally, mechanistic fluorescence studies and transmission electron microscopy analysis were performed in order to investigate their intracellular distribution.

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Methods

Drugs

All dicationic aromatic compounds tested in this study [Figure S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)] were synthesized according to published procedures.[4] Stock solutions of the drugs (5 mM) were prepared in DMSO.

Cell cultures and cytotoxicity assays

Primary cultures of embryonic cardiomyocytes (CMs) were purified following the method previously described.[7] In order to rule out toxic effects of the compounds on the host cell, uninfected CM cultures were incubated at 37°C with compounds 1–11, for 24 h (10.7–96 μM) and 72 h (10.7–32 μM). The cell death rates were measured by the MTT colorimetric assay allowing the determination of LC_{50} values (compound concentration that reduces 50% of cellular viability). 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).

In vitro antitrypanosomal activity

Bloodstream trypomastigote forms of the Y strain of *T. cruzi* were obtained from infected albino Swiss mice at the peak of parasitaemia as previously reported.[7] They were incubated for 24 h in the presence or not of serial dilutions of compounds 1–11 (0.04–32 μM). Alternatively, the treatment was performed with freshly isolated mouse blood at 4°C, with concentrations up to 96 μM. The parasite death rates were determined through direct analysis by light microscopy and calculating the IC_{50} values (compound concentration that reduces the number of parasites by 50%). For analysis of the effect on trypomastigote bursting, the infected cultures were maintained in the presence or not of the ADs (0.04–32 μM) for 72 h and the supernatants were recovered for the quantification of the number of released parasites and determination of the IC_{50} values.

Fluorescence microscopy

Trypomastigotes (3×10^6 cells/mL) and infected CMs (24 h of infection) were treated for 1 h with 10 μg/mL of each compound. Afterwards, the samples were routinely processed for fluorescence microscopy. The fluorescence intensity of at least 50 individual treated parasites was determined using the program Image J 1.41 (NHI, Bethesda, MD, USA).

Transmission electron microscopy analysis

Bloodstream trypomastigotes treated or not for 1 h at 37°C with the corresponding IC_{50} value (24 h) of the drug were routinely processed for transmission electron microscopy.[6]

Statistical analysis

Statistical analysis was carried out using Student's *t*-test, with the level of significance set at $P<0.05$. All the assays were run at least three times in duplicate.

Results

The direct effect of the ADs on bloodstream trypomastigotes was evaluated after 24 h of treatment at 37°C. The most active compounds were 2, 5, 7 and 9 with IC_{50}s between 0.7 and 2.7 μM (Table 1). All compounds exhibited a strong reduction of their trypanocidal activity in the presence of blood (Table 1), which for the most active compounds varied from 128- to higher than 210-fold. Against interiorized parasites in CMs, compounds 2, 5 and 7 were the most active (Table 1), resulting in a 100% decrease in the number of bursting parasites at a dose of

Table 1. IC_{50} and SI values of the tested ADs on *T. cruzi*

	Trypomastigotes[a] 24 h			Intracellular parasites[b] 72 h	
Compound	IC_{50} (μM), 4°C, whole blood	IC_{50} (μM), 37°C, medium	SI[c]	IC_{50} (μM)	SI[d]
1	91.6±20.2	>32	>3	>32	>1
2	>400	2.7±0.5	>35.5	1.7±0.8	>19
3	229.3±43.8	20.9±3.8	>4.6	5.1±2.7	>6.3
4	>400	19.0±2.8	>5	11±5.5	>3
5	128.6±18.3	0.7±0.1	>137	0.8±0.2	>40
6	>400	>32	>3	>32	>1
7	128.6±11.8	1.0±0.1	>96	0.3±0.2	>107
8	>400	>32	>3	>32	>1
9	>400	1.9±0.9	>50	16.4±6.3	>2
10	135.8±33.7	16.3±1.4	>6	8.3±1.4	>4
11	>400	>32	>3	16.0±6.0	>2

SI, selectivity index.
[a]Direct effect of the ADs on trypomastigotes performed after 24 h at 4°C in whole blood or at 37°C in RPMI.
[b]Effect on intracellular parasites measured by trypomastigote release into the supernatant of culture medium (96 h of infection) performed after 72 h of treatment at 37°C.
[c]$LC_{50}>96$ μM.
[d]$LC_{50}>32$ μM.

10.7 μM after 72 h (data not shown). After 24 or 72 h of incubation, none of the ADs was toxic to CMs, exhibiting values of LC_{50} of >32 μM. Regarding the selectivity index (LC_{50}/IC_{50} ratio), the compounds with the highest values were compounds 5 (>137) and 7 (>107), for trypomastigotes and intracellular parasites, respectively (Table 1).

Due to the high activity and selectivity of compounds 5 and 7, we next evaluated by transmission electron microscopy their main cellular targets in the treated bloodstream forms. The ultrastructural analysis showed that compounds 5 and 7 displayed similar effects, leading to the detachment of the nuclear envelope (Figure 1b, arrow) and plasmalemma (Figure 1c, arrows), besides inducing striking alterations in the mitochondria–kinetoplast complex (Figure 1b–d). Concerning the intracellular localization, we found that all drugs accumulated in the DNA-enriched structures: the nucleus and kinetoplast of trypomastigotes [Figure 1e and f and Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)] and intracellular amastigotes (Figure 1g), showing a kinetoplast DNA (kDNA)/nuclear DNA (nDNA) ratio of at least 1.5 for trypomastigotes (Table S1). However, the higher kDNA selectivity was not directly related to trypanocidal efficacy, since compound 5, although displaying the lower ratio (1.5), was the most active, and compound 6, which showed the highest accumulation in the kinetoplast (2.8), was the least active (Figure 1e and f, and Table S1).

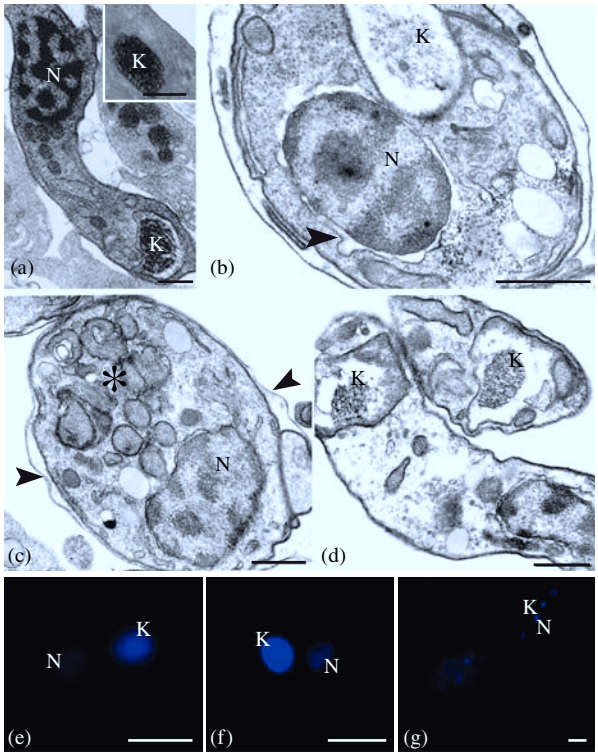

Figure 1. (a–d) Transmission electron micrographs of (a) untreated trypomastigote forms of *T. cruzi* and trypomastigotes treated for 1 h with (b and c) compound 5 and (d) compound 7. Inset: higher magnification of untreated *T. cruzi* kDNA. Note the damage to the mitochondria–kinetoplast complex (c, asterisk) with swollen mitochondrial cristae, (b and d) kDNA alterations and even (b) disappearance; bar=0.5 μm. (e–g) Intracellular localization of the ADs in *T. cruzi* after incubation for 1 h at a concentration of 10 μg/mL. Trypomastigotes treated with (e) compound 5 and (f) compound 6 and (g) amastigotes interiorized in CMs treated with compound 2. (e and f) Bar=0.5 μm. (g) Bar=2 μm. K, kinetoplast; N, nucleus. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Discussion

Pentamidine, berenil and other related aromatic compounds bind to AT minor groove sequences of trypanosomatid kinetoplasts resulting in excellent antiparasitic activity.[8] Our present results confirm the high activity of ADs, which displayed biological activity at the submicromolar level, and which did not display cytotoxicity to the mammalian host cells, as also found previously.[3,6] The potential applicability of these compounds in the prophylaxis of blood banks was evaluated by the direct effect on trypomastigotes at 4°C in the presence of whole blood. An important decrease in the trypanocidal effect was observed with all the ADs, which could be due to their association with serum components and/or to drug instability.[6] Since the requirements for a new anti-*T.cruzi* drug include the identification of drugs with SIs ≥ 50,[9] we found that compound 7 represents a very promising AD to be further assayed *in vivo*.

Our ultrastructural findings showed that the most active compounds, 5 and 7, induced detachments of the nuclear envelope and plasmalemma; however, the most frequent effect was upon the mitochondria–kinetoplast complex, corroborating previous reports.[5,6] Although the ADs presented stronger staining in the kinetoplast of bloodstream forms as already reported using other analogues for African trypanosomes[8,10] and *T. cruzi*,[3] it was not correlated to their trypanocidal activity. Interestingly, previous studies performed with bloodstream parasites of *Trypanosoma brucei* also failed to correlate AD overall intracellular accumulation and localization with compound activity.[10] However, this study represents the first report measuring the relative kDNA to nDNA accumulation of ADs in *T. cruzi* and the lack of correlation with their biological activity against this parasite. Although the exact mechanism of their action has not been clearly demonstrated, the data suggest that at least part of this activity may be related to the destruction of the kinetoplast leading to parasite death.[8,10] Therefore, the association of ADs with the DNA seems to be an initial step followed by topological changes that can lead to molecule instability and destruction.[10] Alternatively, these compounds could interfere with DNA-targeted enzymes and/or may act through direct inhibition of transcription, triggering cell death.[8,10]

Funding

The present study was supported by grants from Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ and Pensa Rio/FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), DECIT/SCTIE/MS and MCT by CNPq, PAPES V/FIOCRUZ, Coordenação de Aperfeiçoamento Pessoal do Ensino Superior (CAPES) and Consortium for Parasitic Drug Development (CPPD).

Transparency declarations

None to declare.

Supplementary data

Figure S1 and Table S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Supplementary data

Figure S1. Chemical structures of the ADs used in the present study.

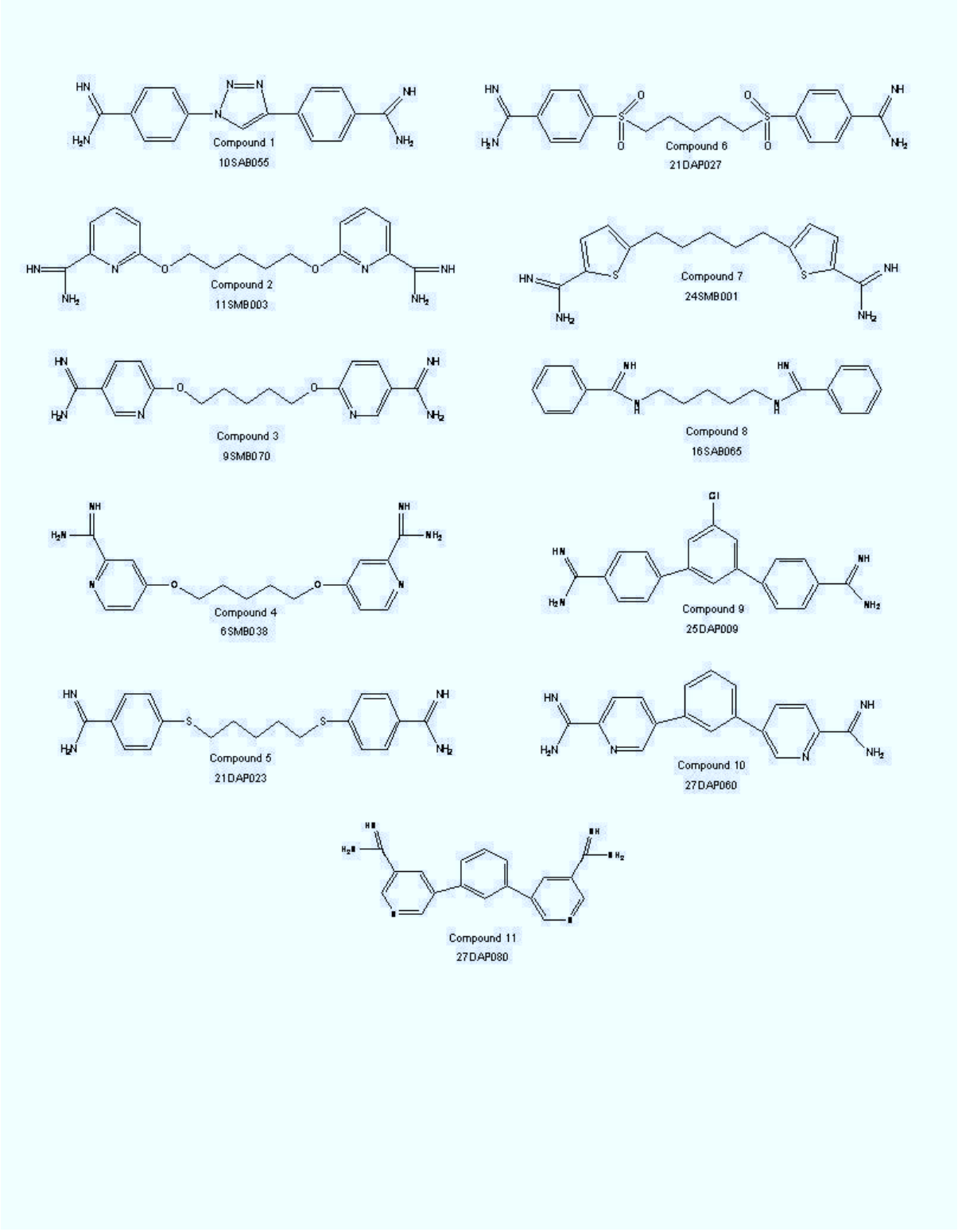

Table S1. Ratio of fluorescence intensity in the kinetoplast and nuclei of bloodstream trypomastigotes treated with the ADs

Compound	kDNA/nDNA ratio	Parasite death (%)[a]
1	2.4±0.4	10
2	2.0±0.2	7
3	2.3±0.4	4
4	1.8±0.2	10
5	1.5±0.2*	74
6	2.8±0.5*	11
7	1.9±0.3	51
8	2.3±0.6	9
9	2.0±0.3	47
10	2.1±0.4	18
11	2.2±0.5	10

[a]Trypomastigotes were treated for 1 h with 10 µg/mL AD.

*$P=2.3^{-20}$ - statistical analysis (Student's *t*-test) between values for compound 5 and compound 6.

TRABALHO #3

Situação: a ser submetido antes da defesa para a revista AAC

Referências:

Daliry A, Munde M, Pires MQ, Silva CF, Pacheco RS,Boykin DW, Wilson WD, De Castro SL, Soeiro MNC. The trypanocidal activity of amidine compounds does not correlated with their binding affinity to parasite KDNA.

Págs. 53 à 84

Trypanocidal activity of amidine compounds does not correlate with their binding affinity to *Trypanosoma cruzi* kDNA

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Keywords: *Trypanosoma cruzi*, Chagas disease, chemotherapy

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ABSTRACT

Due to the limited efficacy and considerable toxicity of the therapy for Chagas disease, the identification of new compounds for the treatment of this disease is desirable. Diamidines and related compounds, such as arylimidamides (AIAs), have a high activity against *T. cruzi*. To better understand the mechanism of action of these aromatic compounds, we investigated the possible correlation between the kDNA binding properties of thirteen amidines and their trypanocidal efficacy against *T. cruzi*. Four diamidines (DB75, DB569, DB1345 and DB829), eight AIAs (DB766, DB749, DB889, DB709, DB613A, DB1831, DB1852 and DB2002) and one guanylhydrazone (DB1080) were assayed in thermal denaturation (T_m) and circular dichroism (CiD) studies using whole purified *T. cruzi* kDNA and a conserved synthetic parasite sequence, comparing their binding properties with biological activity. Our findings showed that the overall CD spectrum using the whole kDNA was similar to that found for the conserved sequence and were consistent with minor groove binding characteristics, some of them causing changes in the DNA topology. However, while some active compounds such as DB766 induced profound alterations in kDNA topology, others such as DB1831, although effective, did not result in either altered T_m or CiD positive spectrum. Our data suggest that the strong interaction of amidines with kDNA may not be sufficient to generate and trigger their trypanocidal activity and that other mechanisms of action are likely involved in and/or associated with this activity.

1. Introduction

Protozoan parasites from the order Kinetoplastida are early diverging eukaryotes and important agents of neglected diseases, such as Chagas disease (*Trypanosoma cruzi*), human African trypanosomiasis (*Trypanosoma brucei* complex), and leishmaniasis (*Leishmania* sp.) (Morris et al., 2001). Half a billion people are at risk of contracting these diseases, and it is estimated that more than 20 million individuals are infected, resulting in more than 100,000 deaths per year (Stuart et al., 2008).

These parasites display a wide range of peculiarities, including polycistronic transcription, trans-splicing of precursor mRNAs, anchoring of surface proteins by glycosylphosphatidylinositol (GPI), antigenic variation and the presence of glycosomes, and this is likely due to their early divergence of the eukaryotic lineage (Estévez & Simpson, 1999). Mitochondrial DNA organization and the RNA editing process are remarkable features of kinetoplastids. These protozoa harbor a single mitochondrion enclosing a unique type of DNA organization called kinetoplast DNA (kDNA) that contains 20-25% of the total cellular DNA (Shapiro & Englund, 1995; Liu et al., 2005). The kDNA network consists of thousands of interlocked circular DNA molecules referred to as minicircles and maxicircles (Hajduk et al., 1984; Stuart et al., 2005). Minicircles comprise approximately 90% of the network mass, ranging from 0.5 to ~ 2.9 kb, while maxicircles are present in several dozen copies ranging from 20 to 40 kb (Shapiro & Englund, 1995). The major transcripts of maxicircles are mitochondrial ribosomal RNA and components of the mitochondrial oxidative phosphorylation system. Several maxicircle genes are modified by the RNA editing process (Simpson et al., 2004) through uridine (U) insertions or by removal from pre-mRNA transcripts, which is catalyzed by multiprotein complexes known as editosomes (Amaro et al., 2008). Editing specificity is directed by small RNAs called guide RNAs (gRNAs), which are mainly encoded by the minicircles and are complementary to the edited sequences in maxicircle pre-mRNAs (Simpson et al., 2004). RNA editing occurs by three different enzymatic reactions: endonucleolytic cleavage, U addition or removal and RNA ligation (Panigrahi et al., 2006, Babbarwal et al., 2007). During replication, individual covalently closed minicircles are released from the

network into the kinetoflagellar zone, where the universal minicircle sequence binding protein (UMSBP), primase and DNA polymerases are present (Liu et al., 2005). Unidirectional replication initiates from one of the minicircle replication origins, and the progeny minicircles are reattached to the network at peripheral locations by a type II topoisomerase (Liu et al., 2006). The double-sized network then splits in two, and the progeny networks segregate into the daughter cells during cytokinesis (Milman et al., 2007)

Although the kinetoplastid minicircles of most species are heterogeneous in sequence, a common feature of their nucleotide organization is the presence of a conserved region of approximately 100 to 200 base pairs (Ray, 1989). In *Crithidia fasciculata* and *Trypanosoma lewisi*, there are two copies of the conserved sequence oriented as direct repeats located 180° apart on the minicircle (Carnes et al., 2008; Ray, 1987), while in *T. cruzi*, there are four copies located 90° apart (Degrave et al., 1988). Within these conserved sequences, there is a 13-bp sequence (GGG GTT GGT GTA A) called the universal minicircle sequence (UMS) that is also present in minicircles from other trypanosomatids (Ntambi & Englund, 1985) and is associated with the process of replication initiation (Abu-Elneel et al., 1999; Onn et al., 2006). Within each of the four conserved sequences present in minicircles from *T. cruzi*, there is a ~21-mer sequence that is perfectly repeated in homologous blocks (Gonzalez, 1986). By comparing the minicircle sequences of different species of trypanosomatids, Ray DS (1989) found that a smaller sequence (AGG GGC GTT C) conserved inside the minirepeats of eight different trypanosomatids that could also be associated with the replication of minicircles. Conserved sequences are usually associated with important functional roles in the cellular system and could represent promising drug targets, as they are present in different species of disease-causing parasites.

Current drugs are unsuitable for the treatment of Chagas disease as they are often toxic, present variable efficacy, necessitate extensive courses of therapy and can lead to drug resistance. Therefore, new, effective, more selective and safer drugs are needed to treat diseases caused by kinetoplastids (Soeiro & de Castro, 2009; Urbina, 2010).

Aromatic diamidines (ADs) and related compounds such as arylimidamides (AIAs) are a promising group of heterocyclic compounds with a

remarkable activity against trypanosomatids both *in vitro* (De Souza et al., 2004, 2010; Silva et al. 2007a,b; Rosypal et al., 2008) and *in vivo* (De Souza et al., 2006a; Silva et al., 2008; Thuita et al., 2008; Mdachi et al., 2009; Batista et al., 2010). Many representatives of this class of compounds, including furamidine (DB75), accumulate in DNA-containing organelles, such as nuclei and, most notably, kinetoplasts (Mathis et al., 2006, 2007; Lanteri et al., 2008; Daliry et al., 2009; Silva et al., 2010). Additionally, many amidines cause important alterations in the kinetoplast structure, including kDNA disorganization and mitochondrial membrane swelling (De Souza et al., 2006b; Silva et al., 2007b; Daliry et al., 2009).

In the present work, our aim was evaluate the trypanocidal activity of thirteen structurally related amidines, exploring their interaction with parasite kDNA by thermal melting (T_m) studies and circular dichroism (CiD), using whole purified *T. cruzi* kDNA and a conserved synthetic parasite sequence. This study allowed the comparative analysis of the kDNA binding affinities of the various compounds with their chemical structure and trypanocidal activity.

2. Materials and Methods

2.1. Parasites

Epimastigotes of the Y strain of *T. cruzi* were grown in liver infusion tryptose (LIT) medium supplemented with 10% fetal calf serum, with weekly passages (Camargo, 1964). Bloodstream trypomastigotes (BT) from the Y strain were harvested by heart puncture of infected Swiss mice at the peak of parasitemia (Meirelles et al., 1982).

2.2. Compounds

The synthesis of compounds (Table 1) was performed according to our previously reported methodology. Stock solutions were prepared in dimethylsulfoxide (DMSO), with the final concentration of the solvent never exceeding 0.6%. This concentration was not toxic to the parasite or mammalian host cells (data not shown). Benznidazole (Rochagan, Roche) was used as a reference drug.

2.3. *In vitro* antitrypanosomal activity

For the analysis of the biological effect of the compounds, epimastigotes at the exponential phase of growth (5-day-old culture forms) were harvested, washed with phosphate-buffered saline (PBS) and resuspended in LIT medium at a concentration of 5 X 10^6 parasites/mL. The parasites were then incubated for 24 h at 28°C in the presence of increasing doses of each compound (0-1000 µM) or benznidazole (0-250 µM) (Batista et al., 2010). After quantification using a Neubauer chamber, the IC_{50} values (the compound concentration that reduces the number of parasites by 50%) were averaged from at least three determinations obtained in duplicate.

2.4. kDNA preparation

The methodology used for kDNA extraction was followed as previously reported by Morel et al. (1980) with minor modifications (Pacheco et al., 1990). Briefly, epimastigotes harvested during the exponential phase of growth were washed three times with centrifugation in cold SE buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0) and resuspended at a concentration of 2.5 X 10^8 cell/mL in SE. Next, the lysis was performed by the addition of 0.5 mg/mL of proteinase K and 3% sarkosyl (w/v), followed by an incubation for 3 h at 60^{o}C. The lysate was then passed through a #18 needle six times and centrifuged for 1 h at 4°C using a SW55 rotor (Beckman Coulter L-100XP Optima Ultracentrifuge, USA) at 33,000 rpm. The supernatant was discarded, and the pellet was resuspended in 5 mL of TE buffer (10 mM Tris-HCl, 0.1 M EDTA, pH 7.4) and centrifuged as before. The supernatant fluid was discarded, and the kDNA pellet was resuspended in 200 µL of TE, followed by the addition of RNase (4 µg/10^9 cells). After incubating for 1 h at 37°C, the DNA solution was extracted twice with chloroform-isoamyl alcohol (24:1) and centrifuged for 7 min at 13,000 rpm at room temperature. The aqueous phase was transferred to a clean tube, and 1 mL of ether was added. After vigorous shaking, the upper phase was discarded, and the procedure was repeated twice. The material was left to dry at room temperature, and the DNA was precipitated at -20°C overnight with 0.2 M NaCl and 3 volumes of absolute ethanol. Then the isolated kDNA was pelleted for 30 min at 13,000 rpm and resuspended in TE. DNA quantification

was performed reading the absorbance at 260 nm using a spectrophotometer (GeneQuant^{TM} Pro RNA/DNA calculator, GE Healthcare Life Sciences, USA).

2.5. Restriction endonuclease treatment

To confirm the purity of the isolated kDNA, the samples were subjected to restriction endonuclease pattern analysis. Briefly, purified kDNA was digested with three different enzymes: *EcoRI*, *HindIII* and *HinfI* (Promega, Madison, WI, USA). The reaction was performed in a total volume of 30 µL containing the following: 1X enzyme Buffer, 2 U of each enzyme, and 1 µg of DNA. The mixture was incubated for 3 h at 37ºC, and 15 µL (0.5 µg) was used for gel electrophoresis.

2.6. Polymerase Chain Reaction (PCR)

For the PCR amplification, 0.2 pg of purified kDNA were used in a total reaction volume of 25 µL containing the following: 1X PCR buffer, 0.2 µM of minicircle specific primers of our design (5´ TGG ATG GTT TTG GGA GGG GCG 3´ and 3´ CCA ACC CCA ATC GAA CCC CAC C 5´), 1.5 mM $MgCl_2$, 200 pmol dNTPs, and 0.5 U Taq polymerase. The samples were heated to 94°C for 2 min, followed by 35 cycles of 94°C for 15 sec, 60.5°C for 30 sec, and 72°C for 40 sec. A final extension at 72°C was carried out for 7 min. Finally, 10 µL of each PCR product was subjected to gel electrophoresis.

2.7. Gel electrophoresis

Agarose gels (0.8%) in 1X TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8.3) were used. Gels were stained with either ethidium bromide (0.5 mg/ml) for 1 h or SYBR Gold Nucleic Acid Gel Stain at the concentration and time recommended by the manufacturer (Invitrogen, Carlsbad, CA) (Tevis et al., 2009).

2.8. Conserved sequence of *T. cruzi* minicircles

The conserved sequence used in the CiD and thermal melting (T_m) experiments was designed based on the minicircle nucleotide sequences available in the GenBank database (Accession number: M18814) and was synthesized by Integrated DNA Technologies (IDT, Atlanta, USA) as follows:

5’ GTT TTG GGA GGG GCG TTC AAA T 3’

3’ CAA AAC CCT CCC CGC AAG TTT A 5’

2.9. Thermal melting analysis

The binding affinity of each compound toward either the whole kDNA or the conserved sequences of *T. cruzi* minicircles was calculated by thermal melting (T_m) approaches. The increase in T_m values due to compound complexes compared to uncomplexed DNA (ΔT_m) at specific DNA sequences provides a suitable method for ranking compound binding affinities. The experiments were conducted as previously reported (Arafa et al., 2005; Mathis et al., 2007). The ΔT_m values were determined by following the absorption change at 260 nm as a function of temperature and were calculated as follows:

$$\Delta T_m = T_{mc} - T_{mf} \text{ , where}$$

T_{mc} = temperature of melting of the complex compound-DNA

T_{mf} = temperature of melting of the free nucleic acid

The T_m values were determined from the first derivative plots. Experiments were done with 1.5 X 10^{-6} M DNA in 10 mM cacodylic acid buffer (pH 6.25) and 0.01 mM NaCl in 1-cm quartz cuvettes using a Cary 300 Bio spectrophotometer with the software supplied with the instrument.

2.10. Circular Dichroism analysis (CiD)

To further characterize the DNA-compound interactions, CiD analyses were performed as previously described, with minor modifications (Arafa et al., 2005; Liu et al., 2007). The CD spectra were obtained using a Jasco J-810 spectrophotometer (Walnut Creck, CA, USA) at a scan speed of 50 nm/min. The DNA samples were scanned from 220 to 500 nm in 1-cm quartz cuvettes in a buffer consisting of 10 mM cacodylic acid buffer (pH 6.25) and 0.01 mM NaCl, at 25°C. For the whole purified kDNA, a 0.2 ratio (compound:DNA) with a 15 X 10^{-6} M concentration of DNA was used. For the conserved synthetic sequence, aliquots of the compounds in concentrated stock solutions were titrated into the DNA at ratios indicated in the figures using the same DNA concentration mentioned above (Figs. 1A-H). The software supplied by Jasco provided

instrument control and data acquisition (Arafa et al., 2005). The data processing was performed with KaleidaGraph software (Liu et al., 2007).

2.11. Fluorescence microscopy of kDNA

To verify the quality of the purified preparations, spreading and analysis of the *T. cruzi* kDNA network was performed by fluorescent monitoring using the methodology previously reported, with minor modifications (Simpson & Berliner, 1974). Briefly, 1 µg of isolated kDNA was mixed with 1% bovine serum albumin and spread on a clean glass slide. Then the samples were fixed for 5 min in cold methanol, dried at room temperature and stained with 5 µg/mL of DAPI. Subsequently, the samples were mounted with 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO) and analyzed with a Zeiss photomicroscope equipped with epifluorescence (Zeiss Inc, NY, USA). Image J software (NIH, USA) was used for the network area calculations using measurements for at least 50 parasites in three different biological sample analyses.

2.12. Atomic Force Microscopy (AFM)

To further analyze the quality of the purified kDNA preparations, 0.1 µg/µL of isolated kDNA networks were diluted in the following buffer: 40 mM Hepes-Cl and 10 mM $MgCl_2$, pH 8.0. The solution was then spread on freshly cleaved mica and allowed to stand for 5 min at 25°C. It was then rinsed with deionized water (0.2–0.5 mL), air-dried and scanned on a Multimode V Microscope (Veeco, NY, USA). Nanoscope software was used for the calculation of minicircle circumferences.

3. Results

ADs and related compounds such as AIAs are potential anti-*T. cruzi* agents that have been intensively studied both *in vitro* and *in vivo* (Soeiro et al., 2009b). Although the parasite mitochondrion and its unique kDNA have been suggested to be probable targets of amidines, the exact mechanism of action of these compounds is still not completely understood (Soeiro et al., 2005; Wilson et al., 2008). Therefore, our aim was to investigate and correlate the putative

kDNA binding properties of ADs and congeners with their trypanocidal activity. Four ADs (DB75, DB569, DB1345 and DB829), eight AIAs (DB766, DB749, DB889, DB709, DB613A, DB1831, DB1852 and DB2002) and one guanylhydrazone (DB1080) were selected for this study based on previous *in vitro* studies performed using bloodstream trypomastigotes (BTs) (Table 1). However, due to difficulties in purifying large amounts of kDNA from the BT forms, the experiments herein were conducted using epimastigotes. All of the thirteen heterocyclics presented a dose dependent trypanocidal activity against BT´s and epimastigotes (data not shown). As observed with BTs, the most active compounds were the AIAs, especially DB766, with an IC_{50} of 0.08 ± 0.02 μM, which is similar to what was calculated in previous experiments with BTs (Tables 1 and 2). Interestingly, with the exception of DB569, all of the other tested diamidines were poorly active against epimastigotes, ranging in IC_{50} values from 725 ± 120 to 938 ± 25 μM (Table 2). As with BTs (De Souza et al., 2004), DB569 (a phenyl substitute of DB75) presented a higher efficacy than the parental compound against epimastigotes (Tables 1 and 2). With the exception of DB766 and DB2002, epimastigotes were less susceptible than BTs to all amidines, regardless of their class. For example, DB1080 was approximately 800 times less effective on epimastigotes compared to BTs. Similar data were also found when benznidazole was employed, reaching IC_{50} values of 12.94 and 168 for BTs and epimastigotes, respectively (Tables 1 and 2).

The relative binding affinities of synthetic and natural compounds for nucleic acid sequences may be assessed by evaluating modal changes in T_m values during DNA-drug complex formation (Wilson et al., 2008). We started the analysis by assessing the binding affinities of different amidines for a selected conserved 22-mer sequence from *T. cruzi* minicircles, using a ratio of 0.2 compound/base pair of DNA (Table 2). Interestingly, some of the compounds that exhibited the highest activity showed lower relative binding affinities. Our data demonstrated that with exception of DB613, the other studied AIAs, although very active against *T. cruzi*, were weak binders (ΔT_m ranging from 0 to 2°C) (Table 2). The interaction with DB766, DB709, DB1831 and DB1852 resulted in little or no change in the T_m measurements, suggesting that these AIAs are weak binders under these tested conditions (Table 2). For example,

DB1831, one of the most active compounds, had a 0°C ΔT_m value (Table 2). Overall, there was no correlation between relative binding affinities and trypanocidal activity. Although both DB75 and DB569 showed low activity, these compounds have a higher binding affinity compared to most of the AIAs, presenting ΔT_m values of 17 and 8°C, respectively (Table 2).

To further explore the effect of the compounds toward the DNA topology, their CiD spectra were analyzed. The conserved synthetic sequence present in the kDNA minicircles of *T. cruzi* was used to obtain additional information on the DNA-compound complexes (Fig. 1A-H). The spectrum analysis was performed using different ratios of compound to base pairs (0.5, 1, 2, and 4) and was monitored from 220 to 500 nm. Positive ratio-dependent CiD signals were observed for DB75, DB569, B766, DB709, DB613 and DB1852 (Fig. 1 A-G), which are characteristic of a compound being able to bind to the minor groove of DNA, as was observed for other diamidines. In contrast, DB1831 (Fig. 1F) and DB2002 (Fig. 1H), two AIAs that have a high and a low effect against *T. cruzi*, respectively, induced no significant signals, suggesting a weak binding to these minicircle sequences. All of the compounds that showed positive induced CiD changes also exhibited changes in the spectrum in the DNA region (260 nm) that could be attributed to a change in DNA conformation that was compound-DNA ratio dependent (Fig. 1 A, B, C, D, E and G). None of the compounds reached saturation, even when using the highest compound to DNA ratio (Fig. 1A-H).

Because some of the most active compounds do not strongly bind to this highly conserved sequence, we evaluated if they could associate to other sequences present in intact whole kDNA containing all of the possible minicircle sequences of the network through T_m and CiD assays (Figs. 2 and 3, Table 2).

After the kDNA isolation, the purity and integrity of the networks were assessed by the amplification of specific minicircle sequences, by restriction endonuclease patterns, fluorescence microscopy and AFM analyses (Fig. 2A-C). For the PCR amplification, specific primers (see Materials and Methods) that amplify a 230-bp fragment present in the minicircle sequence of *T. cruzi* were designed by our group (Accession number: M18814). After the PCR reaction, a band of the expected size (230 bp) was amplified and visualized in an agarose gel (data not shown), providing the first indication of the presence of

minicircles in the purified fraction. The incubation of the isolated kDNA fractions with different restriction endonucleases enzymes (*HindIII*, *EcoRI* and *HinfI*) resulted in a consistent pattern of restriction fragments characteristic of *T. cruzi* kDNA (Fig. 2A) (Simpson & Morel, 1980; Morel et al., 1980; Ntambi et al., 1984; Pacheco et al., 1998).

Through fluorescence microscopy (Fig. 2B) and AFM analyses (Fig. 2C), we could assess the integrity of the kDNA networks purified from epimastigotes. DAPI staining of intact parasites allows the identification of the nuclei and the kDNA (Fig. 2B inset). When the isolated kDNA was adhered to glass slides and stained with DAPI, rounded fluorescent molecules were observed (Fig. 2B), with the area of the networks ranging between 20-39 μm² (data not shown). AFM images captured by tapping mode revealed a kDNA network composed of minicircles consisting of small interconnected circles with sizes of ~0.5 μm (Fig. 2C). We also observed the presence of larger circles that were ~1 μm in size or bigger. These assays confirmed that the methodology employed for the kDNA purification was suitable for the next steps of the study.

Next, T_m (Table 2) and CiD (Fig. 3) studies were performed using intact whole *T. cruzi* kDNA. The overall CiD spectra of the whole kDNA were similar to those found for the conserved sequence, showing minor groove binding and some changes in the DNA region (Fig. 3A-M). In the assays using native kDNA, usually ADs and AIAs present different changes in the spectra in the DNA range with AIAs exhibiting greater induced conformational changes (Fig. 3E-I).

The T_m data demonstrated that except for DB709 and DB569, the incubation of the whole kDNA with the compounds resulted in similar binding patterns (Table 2), as was previously shown with the conserved sequences. In the case of DB569, we observed a higher binding affinity for the whole kDNA (ΔT_m =15°C), which suggests that it could be targeting sequences other than the conserved sequence used. Although not to the same extent, this was also the case for DB709, which showed a fourfold increase in ΔT_m values (Table 2).

Interestingly, in CiD experiments (Figs. 3J and L) and T_m studies (Table 2), the AIAs that did not bind to the conserved minicircle sequence, DB1831 and DB2002 (Table 2, Fig. 1F and H), did not associate with the whole kDNA. Our data suggest that despite its high biological activity, DB1831 does not bind

to any sequence present in the purified whole KDNA, as determined by both T_m (Table 2) and CiD experiments (Fig. 1F and Fig. 3J).

The lack of correlation between binding affinities and biological activity was confirmed using the whole kDNA. Some of the compounds that had the lowest activity, such as DB75, DB1345, DB829 and DB1080, showed the highest relative binding values (ΔT_m = 15, 14, 9, and 17°C, respectively). Likewise, other compounds that presented higher activity, such as DB766 and DB889, displayed a low binding affinity (ΔT_m = 3 and 5°C, respectively) (Table 2).

Small changes in the chemical structures of the compounds could be important for their binding to kDNA. This is observed when comparing DB766, an active compound that induced a positive CiD, with DB1831, a compound that is also active but displayed binding to neither the conserved sequence nor to the whole kDNA. The only difference between their structures is the substitution of a pyridine (DB766) for a pyrimidine group (DB1831) (Table 1).

4. Discussion

Despite the clinical usefulness of ADs such as pentamidine and berenil against agents of important human and veterinary infections, new, effective and less toxic oral drugs are needed (Wilson et al., 2008). Using peculiar characteristics of trypanosomatid parasites as targets is a fundamental approach for the design of novel drugs (Soeiro & De Castro, 2010). One of these peculiar characteristics is the DNA present in the mitochondria (kDNA) of trypanosomatids. In pre-clinical studies, it is essential to obtain knowledge of the mode action of novel compounds. Therefore, we evaluated the possible correlation between the anti-*T. cruzi* activity of thirteen structurally related amidines and their kDNA binding affinities through T_m and CiD studies.

We found that epimastigotes were less susceptible to the compounds compared to BTs, which corroborates previous data obtained with ADs (Batista et al., 2010). Interestingly, DB829 had very low activity against epimastigotes (IC_{50} = 938 μM) and trypomastigotes (IC_{50} = 437 μM) of *T. cruzi*, although it has been shown to be effective against *T. b. rhodesiense* (18.7 nM) (Ismail et al.,

2003). This difference in susceptibility among *T. brucei* and *T. cruzi* as well as between different parasites forms within the same specie likely involves a dissimilar mechanism of action between them. Pentamidine and other diamidines such as DB75 are actively transported into African trypanosomes via the P2, HAPT1, and LAPT1 system of transporters (Lateri et al., 2006), accumulating at very high concentrations in the parasite mitochondria (Mathis et al., 2006). However, until now, there has been no data regarding the internalization of amidines by *T. cruzi*. Thus, if the internalization of these compounds is also mediated by transporters in *T. cruzi*, the differences in susceptibility observed could be due to differences in the uptake of the compounds. Other explanations could include the existence of different cellular targets, different kinetics for the compound distribution and accumulation, and/or distinct modes of drug extrusion.

Another interesting finding is that the biological activity against epimastigotes confirmed previously published results in *Leishmania major*, *Leishmania tropica* (Rosypal et al., 2008) and BT and intracellular forms of *T. cruzi* (Stephens et al., 2003; Silva et al., 2007; Batista et al., 2010) that showed a greater effect with AIAs compared to other classes of amidines. Although they are not strong DNA binders, they cause striking alterations in kDNA topology as seen by CiD studies that could inhibit the access and connection of enzymes and other important molecules involved in DNA replication or transcription.

Because many amidines exhibit a preferential binding to minor grove DNA enriched in AT sequences (Wilson et al., 2008), we decided to investigate their interaction with the kDNA of *T. cruzi*. AT sequences are especially abundant in the minicircles (≥ 60% AT), providing potential targets for heterocyclic compounds that bind preferentially to AT sequences. Compounds that target the minicircles could impair essential steps in parasite replication, transcription and/or the RNA editing process. Removal or inhibition of some of the editosome proteins has been shown to lead to loss of its function and, consequently, parasite death (Wang et al., 2006; Salavati et al., 2006, Amaro et al., 2008).

To understand the interaction of amidines with kinetoplast DNA, ΔT_m and CiD studies were performed using a conserved minicircle sequence and whole purified kDNA networks of *T. cruzi*. As far as we know, this is the first study

conducted using the native purified structure of kDNA networks, which could lead to more objective results than synthetic homopolymers. Although there is no experimental evidence of a functional role for the minicircle sequence chosen for the present study, it comprises a conserved sequence (AGG GGC GTT C) that is present in different trypanosomes and overlaps the 5´ termini of newly synthesized minicircles of *C. fasciculata,* suggesting a potential role in replication initiation (Ray et al., 1989). The binding of the compounds to minicircle sequences involved in replication initiation could impair the synthesis of new minicircles. This impairment could induce important changes in gRNAs that are ultimately used for the RNA editing of maxicircles transcripts, leading to the loss of function of genes involved in the oxidative phosphorylation process. Additionally, the association of the compounds to any other sequences in the whole kDNA could also lead to interference in essential processes involved in parasite survival.

The thermodynamics analysis confirmed previous studies that demonstrated the strong binding characteristics of DB75, mainly at AT-rich sequences (Liu et al., 2007). This AD strongly bound the minicircle synthetic sequence (ΔT_m of 17°C and positive induced CiD) and the whole *T. cruzi* kDNA (ΔT_m of 15°C and positive induced CiD), although the binding was not as strong as that previously observed with an AATT sequence (ΔT_m = 25°C) (Mathis et al., 2007). This finding could be explained by the presence of sequences other than AATT that could be affecting the compound-kDNA interaction of the minicircle conserved sequence as well as in the whole kDNA.

Mathis et al. (2007) performed a series of studies with selected diphenyl furans and aza analogs of DB75 against trypanosomes of the *T. brucei* complex and found no correlation between activity and the increase in the melting temperature using poly(dA).poly(dT) oligomers and oligomer duplexes containing one single AATT site. In that study they found that DB244, a derivative of DB75 that has a cyclopentyl group added to each of the amidine groups, has a similar DNA binding affinity, thermodynamics and complex structure as that of DB75, however, it had a significant decrease in activity (10X) (Mathis el al., 2007).

The mechanism of action of ADs and congeners is still not understood. In trypanosomatids, one of the proposed mechanisms is related to compound

binding of parasite kDNA, leading to (a) interferences in the normal replication and/or transcription processes, (b) altered DNA topology interfering with its normal functions and/or (c) inhibition of proteins and/or topoisomerase access to the nucleic acid (Soeiro et al., 2005). However, recently published data by Mathis et al. (2007) and our group (Batista et al., 2010) suggest that other possible targets that are not involved in kDNA-compound interactions exist. It has been shown that in *T. cruzi*, as in *T. brucei*, diamidines accumulate in the nuclei, kDNA and DNA-free organelles such as acidocalcisomes (Batista et al., 2010).

Although DB75 and DB569 displayed different binding affinities to the conserved sequence (ΔTm = 17 and 8°C, respectively), further analysis with the whole purified kDNA network demonstrated similar binding capacities with these two ADs (ΔTm = 15°C). These data suggest that DB569 may be binding to a kDNA region different from the minicircle sequence selected in our studies. Although both diamidines presented the same affinity for the whole purified kDNA, the phenyl-substituted compound is approximately 100 times more active than DB75 (Table 2). These data suggests that the main mechanism of action of diamidines and AIAs on trypanosomatids may not be associated with strong binding affinities to parasite kDNA sequences. Additionally, although diamidines are localized within the nuclei and kDNA of *T. cruzi*, their higher accumulation in the latter structure does not predict compound efficacy *in vitro* (Daliry et al., 2009; Silva et al., 2010). Mathis et al. (2007) found that some potent compounds such as DB820 and DB75 in parasites from *T. brucei* complex accumulated to a lesser extent than the less active compounds DB244 and DB249. In fact, although AIAs, especially DB766, presented a greater biological effect compared to ADs (DB75, DB569, DB1345 and DB829), most of them are very weak binders (Table 2), further corroborating the hypothesis that the efficacy of amidines against *T. cruzi* may not be associated to the strength of their binding affinities but rather to their specificities. Our CiD studies demonstrated that although they are not strong kDNA binders, AIAs are able to induce profound alterations in parasite kDNA topology. One hypothesis for this observation is that the compounds are “chemical scissors” that act in a very specific but fast mode due to their ability to be rapidly released from the DNA. However, low DNA binding affinity is not necessarily associated with a high

trypanocidal effect because some amidines that presented low or an absence of kDNA binding, such as the mono-AIA DB2002, also displayed low activity (IC_{50} = 20.4 and 11 μM, for BTs and epimastigotes, respectively) .

It has been shown that the addition of nitrogen atoms to the aromatic groups of DB75, which originates DB829, changes the lipophilicity and polarity of the heterocyclic core, leading to a reduction in the binding affinities to a polymeric sequence containing AATT sites (the ΔT_m changed from 25 to 15.5) (Wilson et al., 2008). Our results corroborate these findings. We showed that there was a reduction in the ΔT_m (from 15 to 9°C) of DB75 in comparison with its aza analog DB829, which has two added nitrogen atoms.

In summary, our data suggest that the strong interaction of amidines with kDNA may be not sufficient to generate and trigger their trypanocidal activity which leads us to propose that mechanisms of action other than direct and strong kDNA binding may be operating in parasite death. The DNA binding may be an initial step that leads to unknown secondary effects that result in parasite death (Mathis el al., 2006). Thus, further molecular and biochemical studies are necessary to better understand these mechanisms occurring not only in *T. cruzi* but also in other parasites and mammalian cells. These studies are essential for the identification of novel, highly selective amidine compounds.

5. Acknowledgements

The present study was supported by grants from Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ and Pensa Rio/FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), DECIT/SCTIE/MS and MCT by CNPq, PAPES V/FIOCRUZ, Coordenação de Aperfeiçoamento Pessoal do Ensino Superior (CAPES) and Consortium for Parasitic Drug Development (CPPD). We are also gratefull for the technical support of Hiu Zhao from the Core Facility of Georgia State University.

6. Legends

Figure 1 A-H. CiD spectrum titration of the indicated amidine compounds with the kDNA conserved sequence (see Material and Methods for details). A spectrum for DNA without compound is shown in red and has no induced signal.

Figure 2 A-C. A: Agarose gel (0.8%) of isolated kDNA from epimastigote forms of *T. cruzi* subjected to restriction endonucleases. 1– Lambda *EcoRI*/*HindIII* molecular marker; 2– kDNA untreated; 3– kDNA treated with *HindIII*, 4– kDNA treated *EcorI*, 5- kDNA treated with *HinfI*. B: Fluorescence microscopy image of the purified kDNA network stained with DAPI. Inset: live epimastigotes stained with DAPI; arrow indicates the kDNA, and asterisks indicate the nuclei; Bar = 20 µm. C: AFM micrography of the kDNA purified network, Bar = 1 µm.

Figure 3 A-M. CiD spectrum of amidines incubated with the purified kDNA network of epimastigotes from *T. cruzi,* using a compound/kDNA base pair ratio of 0.2.

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Table 1. Anti-*T. cruzi* activity (IC_{50}/24h) against bloodstream trypomastigotes of Y strain of *T.cruzi.*

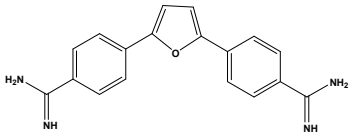
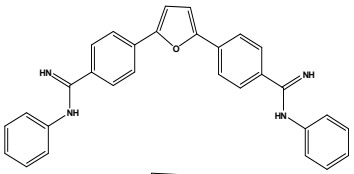
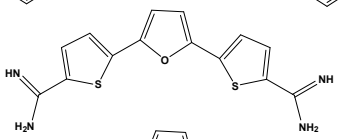
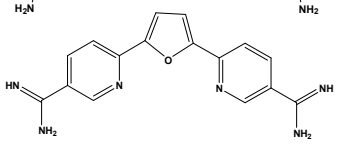
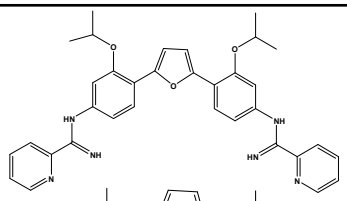
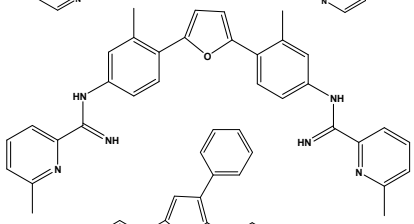
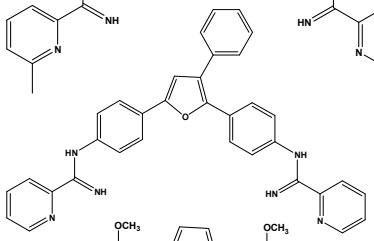
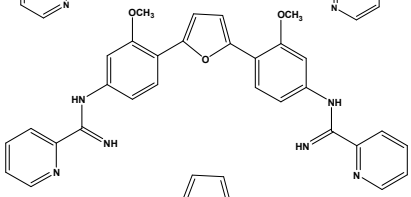
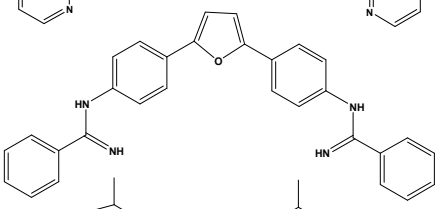
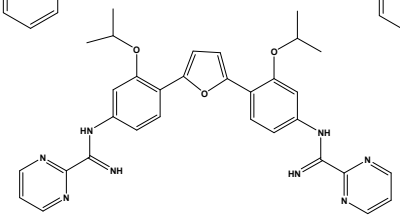
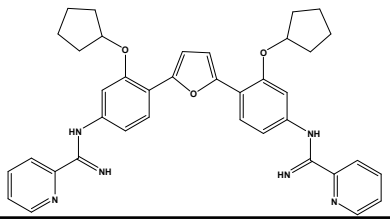
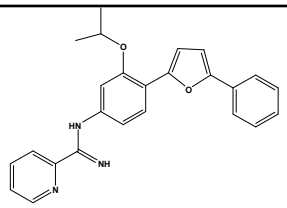
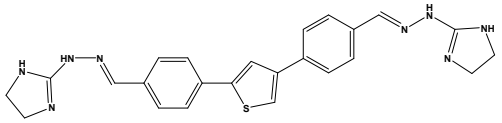
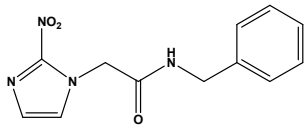
Compound class	Compound name	Chemical Structure	IC_{50} (24h/μM)	Reference
Diamidines	DB75		32	De Souza et al, 2004
	DB569		2	De Souza et al, 2004
	DB1345		0.9	Pacheco et al, 2009
	DB829		437	De Souza et al., 2010
Di-AIA	DB766		0.06	Batista et al, 2010
	DB749		1.8	SP
	DB889		0.09	Silva et al, 2007
	DB709		0.09	SP
	DB613		4	Pacheco et al, 2009
	DB1831		0.02	SP
	DB1852		0.06	SP

Table 1-continued

Compound class	Compound name	Chemical Structure	IC_{50} (24h/μM)	Reference
Mono-AIA	DB2002		20.4	SP
Guanylhydrazone	DB1080		0.24	Pacheco et al, 2009
Nitroheterocyclic	Bz		12.94	

SP = Submitted paper
Bz = Benznidazole

Table 2. Anti-*T. cruzi* activity (IC_{50}/24h) upon Y strain of epimastigotes forms and relative DNA binding affinities (ΔT_m) of amidines to minicircle sequences.

	Compound	IC_{50} (μM)	ΔT_m (°C)	
			Conserved KDNA Sequences	KDNA Entire Network
Diamidines	DB75	790 ± 296	17 ± 2	15 ± 1
	DB569	7.2 ± 2	8 ± 1	15 ± 1
	DB1345	725 ± 120	ND	14 ± 0
	DB829	938 ± 25	ND	9 ± 1
Di-AIA	DB766	0.08 ± 0.02	1 ± 1	3 ± 1
	DB749	15 ± 3	ND	8 ± 0
	DB889	0.5 ± 0.3	ND	5 ± 1
	DB709	0.8 ± 0.5	2 ± 2	8 ± 0
	DB613	22 ± 6	12 ± 0	14 ± 0
	DB1831	0.6 ± 0.09	0 ± 1	0 ± 0
	DB1852	2.3 ± 1	0 ± 0	2 ± 2
Mono AIA	DB2002	11 ± 3	0 ± 0	0 ± 0
Guanylhydrazone	DB1080	207 ± 33	ND	17 ± 1
Nitroheterocyclic	Bz	168 ±67	ND	ND

ND = not done
Bz=Benznidazole

Anti-*T. cruzi* activity (IC_{50}/24h) upon Y strain of epimastigotes forms and trypomastigotes forms.

	Compound	IC_{50} (µM) epi	IC_{50} (µM) trypo
Diamidines	DB75	790 ± 296	32
	DB569	7.2 ± 2	2
	DB1345	725 ± 120	0.9
	DB829	938 ± 25	437
Di-AIA	DB766	0.08 ± 0.02	0.06
	DB749	15 ± 3	1.8
	DB889	0.5 ± 0.3	0.09
	DB709	0.8 ± 0.5	0.09
	DB613	22 ± 6	4
	DB1831	0.6 ± 0.09	0.02
	DB1852	2.3 ± 1	0.06
Mono AIA	DB2002	11 ± 3	20.4
Guanylhydrazone	DB1080	207 ± 33	0.24
Nitroheterocyclic	Bz	168 ±67	12.94

Bz=Benznidazole

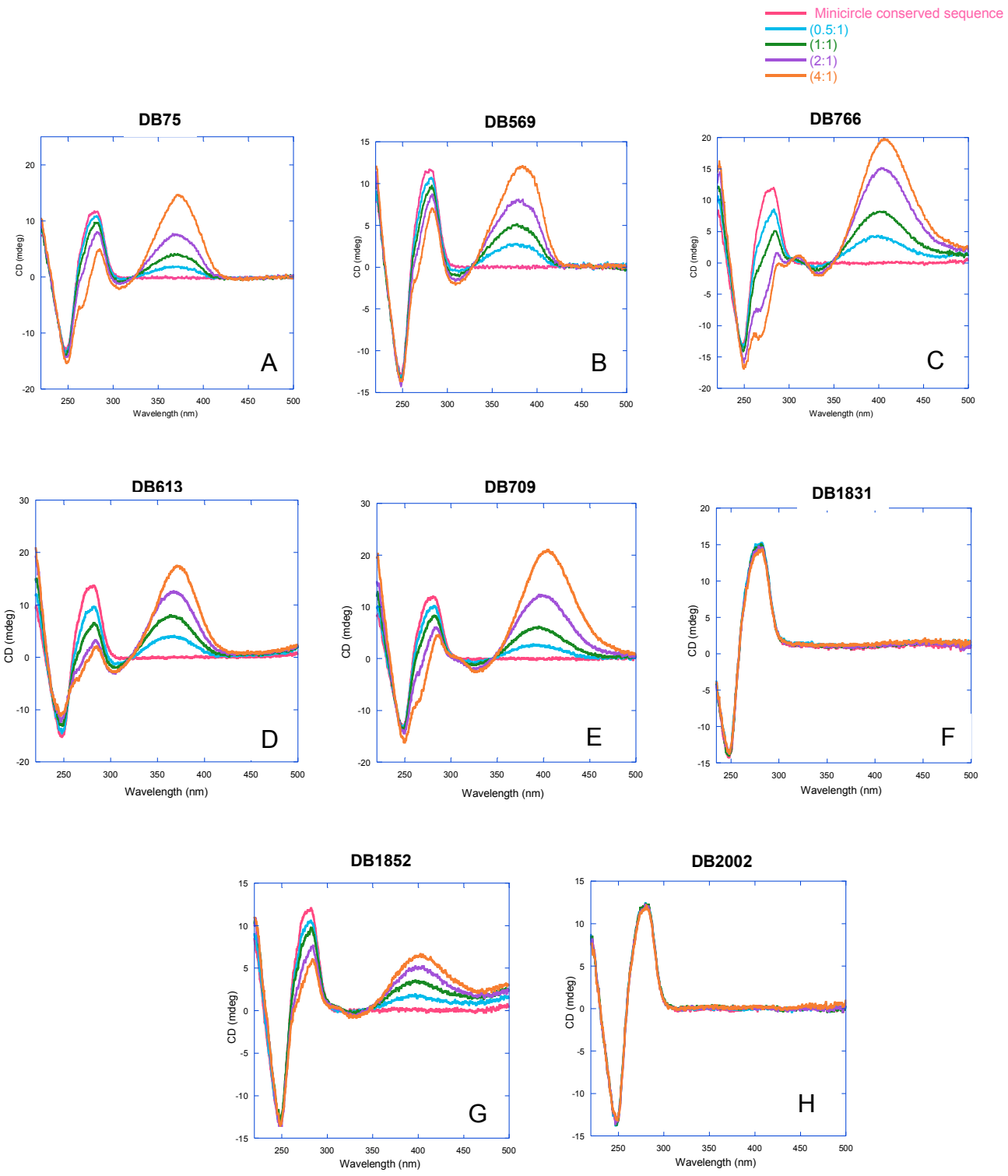


Figure 1A-H. CiD spectra titration of the indicated amidine compounds with the kDNA conserved sequence (see Material and Methods for details). A spectrum for DNA without compound is shown in red and has no induced signal.

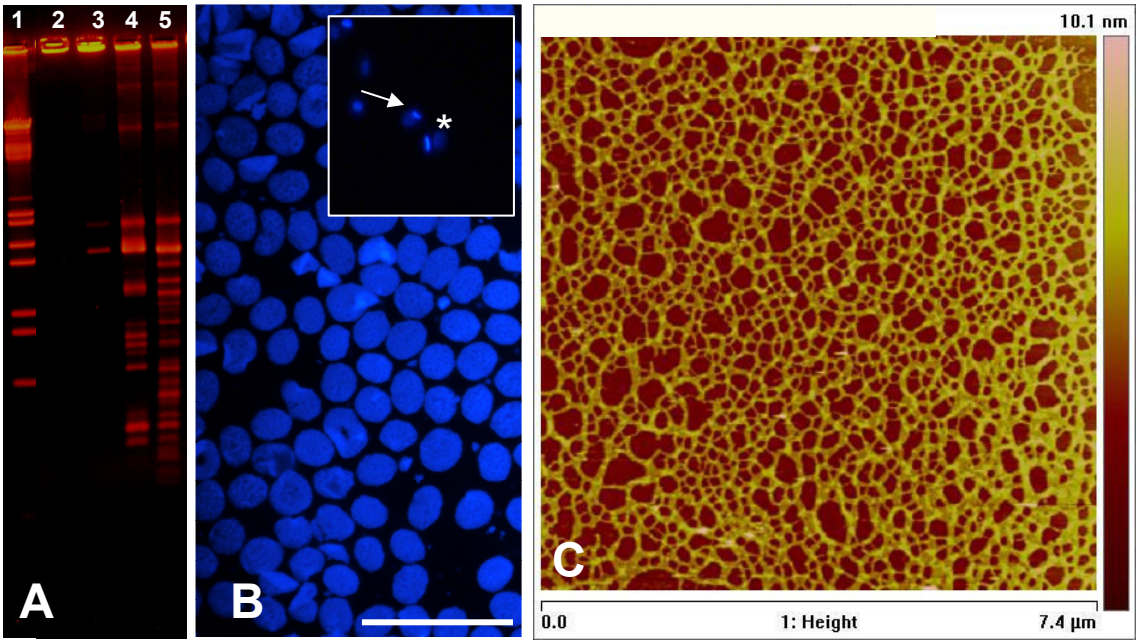


Figure 2 A-C. A: Agarose gel (0.8%) of isolated KDNA from epimastigotes forms of *T. cruzi* subjected to restriction endonucleases. 1–Lambda *EcoRI*/*HindIII* molecular marker; 2– kDNA untreated; 3–kDNA treated with *HindIII*, 4–kDNA treated with *EcorI*, 5- kDNA treated with *HinfI*. B: Fluorescente microscopy image of the purified kDNA network stained with DAPI. Inset: live epimastigotes stained with DAPI; arrow indicate the kDNA and asterisks, nuclei; Bar=20 µm. C: AFM micrography of kDNA purified network.

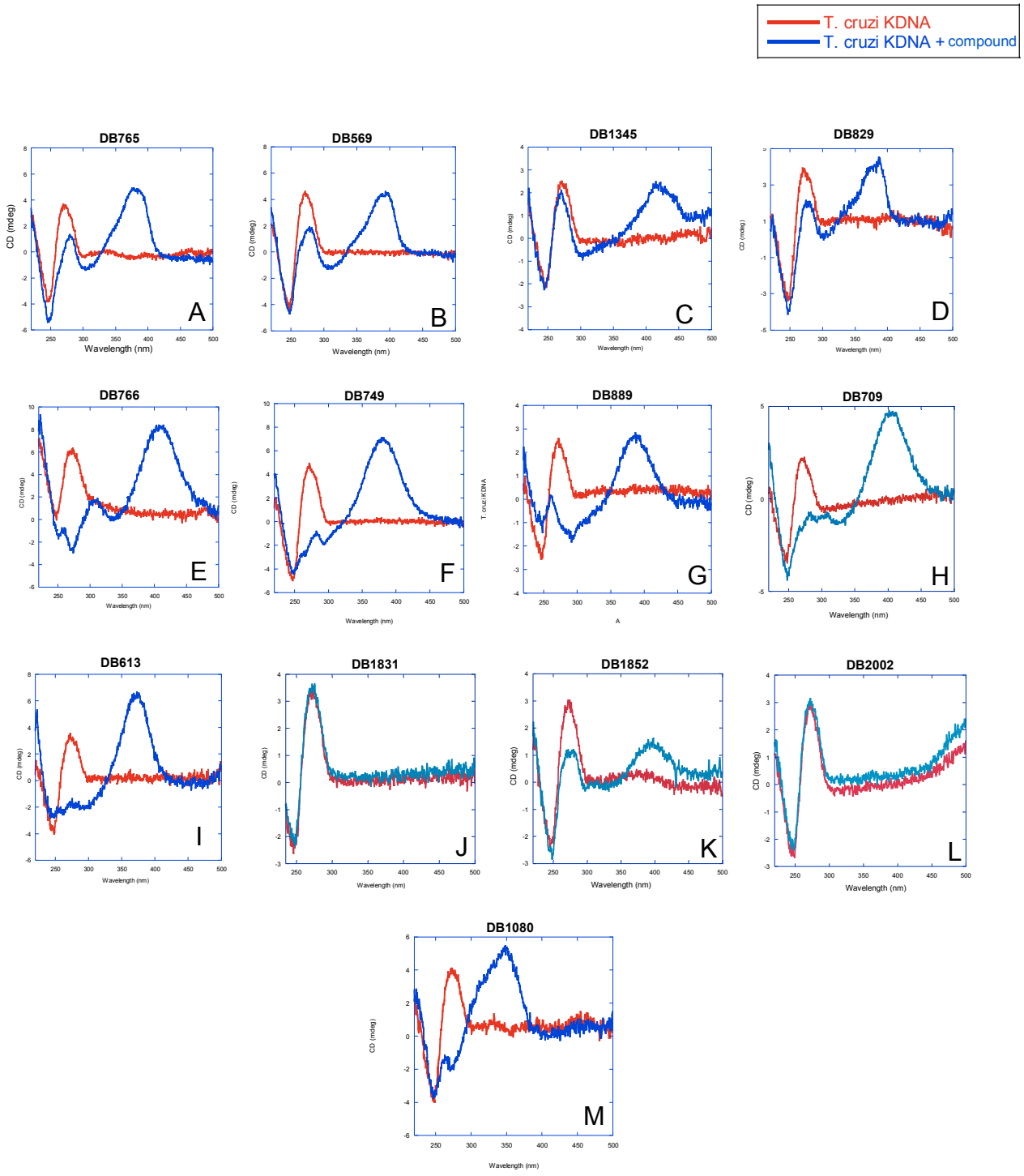


Figure 3 A-M. CiD spectrum of amidines incubated with purified kDNA network of epimastigotes of *T. cruzi.* Compound to kDNA base pair ratio was 0.2.

TRABALHO #4

Situação: à ser submetido

Referências:

Trabalho #4

Daliry A, De Castro SL, Pacheco RS, Boykin DW, Wilson WD, and Soeiro MNC. Diamidines Disrupt The Kinetoplast DNA Network of *Trypanosoma cruzi.*

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Diamidines Disrupt the Kinetoplast DNA Network of *Trypanosoma cruzi*

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Keywords: *Trypanosoma cruzi*, kDNA, aromatic diamidines.

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Abstract

Kinetoplast DNA (kDNA) is the mitochondrial DNA of the protozoan of the Trypanosomatidae family, including parasites that cause important neglected diseases that affect more than 20 million people worldwide. The kDNA is organized into networks that are composed of a few dozen maxicircles, which are similar in function to the mitochondrial DNA of other eukaryotic species, and thousands of interlocked minicircles, which specify the sites of RNA editing. DNA curvature changes such as bending or straightening can be induced by compound interactions in several sequences of the kDNA machinery. These changes may interfere with the kDNA function triggering parasite death, and thus representing an interesting approach for novel drugs development. Our present aim was test the hypothesis that diamidines and congeners such as arylimidamides (AIAs) and guanylhydrazones, which present noticeable antiparasitic activity, may induce topological changes in the kDNA of *T. cruzi*. The eletrophoretic studies conducted with endonuclease-treated *T. cruzi* kDNA fragments incubated with ten different compounds showed that many of them, especially the AIA DB889 and the diamidine DB185 induced profound mobility shifts in kDNA fragments of *T. cruzi*. Additionally, fluorescent microscopy of whole kDNA network incubated with DB75 in the presence or absence of topoisomerase II and protease inhibitors displayed an expansion of the kDNA network that was directly related to the drug concentration. Taken together our results suggest that these aromatic compounds provoke profound alterations in the normal topology of *T. cruzi* kDNA suggesting that it may represent a potential target of these compounds.

1. Introduction

Kinetoplast DNA (kDNA) is the mitochondrial DNA of parasitic protozoa of the family Trypanosomatidae, which includes *Trypanosoma brucei, Leishmania* sp. and *Trypanosoma cruzi,* the etiological agents of human African trypanosomiasis, Leishmaniasis and Chagas disease, respectively. Together these diseases affect more than 20 million people worldwide causing approximately 100,000 deaths annually (Stuart et al., 2008). The kDNA is organized into networks that are composed of a few dozen of maxicircles and thousands of interlocked minicircles (Shapiro & Englund, 1995). Maxicircles, which are similar in function to the mitochondrial DNA of other eukaryotic species codify for proteins involved in mitochondrial energy transduction, e.g. cytochrome b and subunits of cytochrome oxidase (Simpson et al., 1982; Johnson et al., 1982). The most remarkable feature of the transcripts of maxicircles is that they need to be extensively edited to generate mature transcripts, in a process called RNA editing (Benne et al., 1986; Benne, 1994; Simpson et al., 2004). The minicircles, which vary in size and sequence from species to species (0.5 to 2.9 kb), codify for small guide RNAs (gRNA) that direct the specificity of RNA editing of those transcripts (Shapiro & Englund, 1995).

In trypanosomatids, some of the minicircles sequences present intrinsic bending, which is a DNA curvature that may play a critical role in different cellular events (Marini et al., 1982, 1984; Barcelo et al., 1991). Inherent bending in kDNA was first reported in a restriction fragment of 490 bp of *Leishmania tarantolae* that presented unusual physical properties probably caused by periodicities of A-tracts in the DNA sequence (Marini et al., 1982). It is possible that the intrinsically bent kDNA could be selectively recognized by control proteins and/or complexes, acting in gene expression. In others eukaryotic cells, DNA bending play a role in packing and transcriptional regulation (Ross et al, 1999) and have been shown to be a common feature of key DNA control elements such as promoters (Garcia et al., 2007). Interestingly, despite the presence of A-tracts and AT rich sequences in *T. cruzi* minicircles, no bending fragments have been found (Ntambi et al., 1984).

DNA curvature changes such as bending or straightening can be induced by intracellular interactions with other molecules, such as RNA and/or proteins. Although there is clearly a functional role for the intrinsically bending DNA, some other exogenous factors such as natural and synthetic compounds could induce topological changes in some DNA sequences, negatively affecting its function (Ross

et al., 1999). For kDNA, such compound:DNA bindings could impair several biological functions such as transcription and RNA editing leading to parasite death, and thus represent an interesting approach for the development of new drugs.

Aromatic diamidines (ADs) and related compounds are promising chemotherapeutic agents as they present excellent *in vitro* and *in vivo* activity against a broad range of microorganisms, including trypanosomatids (Wilson, 2008; Soeiro et al., 2008). In fact, pentamidine have been extensively used against human African trypanosomiasis and leishmaniasis (Soeiro et al., 2005). In trypanosomatids, the exact mechanism of action of diamidines is still unknown and different mechanisms have been proposed. Among them, several studies suggested the mitochondrion and specifically the kDNA as potential targets (de Souza et al., 2004; Mathis et al., 2007; Daliry et al., 2009; Batista et al., 2010). In *T. cruzi*, several fluorescent ADs are localized and accumulated in high concentrations in the parasite mitochondrion, triggering profound alterations in the mitochondrial membrane potential, besides inducing noticeable damages to the kDNA structure as determined by flow cytometry and electron microscopy techniques (de Souza et al., 2004, 2006; Mathis et al., 2006). Due to the high selectivity index of some of these compounds that mainly accumulate in kDNA as compared to the parasite nuclei (Silva et al., 2010, Daliry et al., 2009), it is possible that they target specifically and more strongly the parasite kDNA due to its high content of AT sequences.

Topological changes induced by different classes of drugs or other exogenous factors on bent and non bent fragments have been investigated through thermodynamic and gel mobility analyses, which are valuable tools for accessing altered DNA organization (Fox, 1990; Cons & Fox, 1990; Ross et al., 1999). More recently, Tevis and coworkers (2009) showed that ADs such as furamidine (DB75) and other structurally related compounds are able to induce alterations on the curvature angle of A-tracts and ATATA sequences, and that such effects depend both on compound structure and DNA sequences.

Our present aim was to test the hypothesis that diamidines and congeners such as arylimidamides (AIA) and guanylhydrazones are able to induce topological changes in *T. cruzi* kDNA. These studies were conducted with ten diamidines that were shown to interact with endonuclease-treated fragments *of T. cruzi* kDNA leading to important alterations of their mobility. Additionally, fluorescent microscopy of whole kDNA network incubated with DB75 demonstrated an altered kDNA topology. Our data suggest that some of these aromatic compounds are able to

induce profound alterations in the normal topology of *T. cruzi* kDNA suggesting that at least for diamidines and some congeners, it may represent one of the potential targets of these compounds.

2. Materials and Methods

2.1. Compounds

The synthesis of the tested compounds (four ADs – DB829, DB569, DB75, DB1345, B185; four AIAs - DB749, DB766, DB613, DB889 and one guanylhydrazone – DB1080) depicted in Figure 1 has been previously reported (Wilson et al., 2008). Stock solutions were prepared at 1 mM in water or dimethyl-sulfoxide (DMSO) and kept frozen at -4°C in the dark.

2.2. Parasites

Epimastigotes of Y strain of *T. cruzi* were grown in Liver Infusion Tryptose (LIT) medium supplemented with 10% fetal calf serum with weekly passages (Camargo, 1964).

2.3. kDNA purification

The methodology used for kDNA extraction was followed as previously reported by Morel et al. (1980) with minor modification (Pacheco et al 1990). Briefly, epimastigotes harvested during the exponential phase of growth (5^{th} day of growth) were washed three times by centrifugation in cold SE buffer (0.15M NaCl, 0.1M EDTA, pH 8.0), and ressuspended at concentration of 2.5 X 10^8 cell/mL in SE. Next, the lysis was performed by addition of 0.5 mg/mL proteinase K and 3% sarkosyl (w/v) and incubation for 3 h at 60^{o}C. The lysate was then passed through a #18 needle for six times and centrifuged for 1 h at 4^{o}C using a SW55 rotor (Beckman Coulter L-100XP Optima Ultracentrifuge, USA) at 33,000 rpm. The supernatant was discarded and the pellet ressuspended in 5 mL TE buffer (10 mM Tris-HCl, 0.1 M EDTA, pH 7.4) and centrifuged as before. The supernatant fluid was discarded and the kDNA pellet was ressuspended in 200 µL TE and RNAse was added (4 µg/10^9 cells). After incubation for 1 h at 37^{o}C, the DNA solution was extracted twice with chloroform-isoamyl alcohol (24:1) and centrifuged for 7 min at 13,000 rpm at room temperature. The aqueous phase was transferred to a clean tube and 1 mL ether was added. After vigorous shaking, the upper phase was discarded and the procedure repeated twice.

Afterwards, the material was left to dry at room temperature and the DNA precipitated at -20°C, overnight with 0.2 M NaCl and 3 volumes of absolute ethanol. Then, the isolated kDNA was pelleted for 30 min at 13,000 rpm and resuspended in TE. The DNA quantification was performed reading the absorbance at 260 nm using a spectrophotometer (GeneQuant™ Pro RNA/DNA calculator, GE Healthcare Life Sciences, USA).

2.4. *EcoRI* and *CvQI* digestions

The purified kDNA was submitted to restriction endonuclease treatment with *EcoRI* and *CvQI* (Promega, Madison, WI, USA) prior to the incubation with the aromatic compounds and electrophoresis. Briefly, 1 µg of purified kDNA was digested in 30 µL total volume reaction containing: 1X respective enzyme Buffer, 2 U each enzyme, and water. The mixture was incubated for 3 h in 37ºC and then 15 µL (0.5 µg) was used for gel electrophoresis.

2.5. Gel electrophoresis

Products of *EcoRI* and *CvQI* digestions were separated on 5% native polyacrylamide gels (1.5 mM thick, 20 cm long) prepared from a 40% acrylamide solution (29:1, bisacrylamide: acrylamide, EMD, Gibbstown, NJ) in 1X TBE buffer (0.089M Tris, 0.089M boric acid, 2.0 mM EDTA, pH 8.3). A pre-running was done for gels conditioning with a 60 min, 25°C and 100V prior to sample loading. Electrophoresis was performed at 200 V (10 V/cm), 25°C for 16 h in 1X TBE buffer. Electrophoretic apparatus was connected to circulating temperature control systems to ensure constant temperature. Samples of 0.5 µg kDNA products were incubated with the compounds at a 10-100X excess of putative AT-binding sites present in the minicircles analyzed from GenBank (accession number M18814). Gels were stained with SYBR® Gold Nucleic Acid Gel Stain (Invitrogen, Carlsbad, CA) at the concentration and time recommended by the manufacturer. Stained gels were imaged using an Omega10gD Molecular Imaging System (UltraLum, Claremont, CA).

For a semi-quantitative analysis of the products shifts induced by the aromatic compounds, a mobility index was calculated (I_M) based on the distances between the slot of the gel to the bottom of selected bands of untreated (Dc) and treated samples with the different compounds (Dt). The ratio between those two determinations was determined as the mobility index:

$$I_M = D_t / D_c$$

Indexes equal to 1 indicate no mobility change. Values < 1 and >1 denote fragment bending or straightening, respectively.

2.6. kDNA treatment with DB75 and fluorescence microscopy

The purified network (1µg) were incubated at room temperature with crescent doses of DB75 (0.1- 100 µg/mL) in SSC Buffer (150 mM NaCl, 15 mM sodium citrate, pH 7.0) prior to the fluorescence microscopy analyses. The treated networks were analyzed using a methodology previously reported, with minor modifications (Simpson & Berliner, 1974). Briefly, 1 µg isolated kDNA was mixed with 1% bovine serum albumin and spread on a clean glass slide. Then, the samples were fixed for 5 min in cold methanol, dried at room temperature and stained with 5 µg/mL of DAPI. Subsequently, the samples were mounted with 2.5% DABCO and analyzed with a Zeiss photomicroscope equipped with epifluorescence (Zeiss Inc, NY, USA). The Image J software (NIH, USA) was used for the network area calculations using at least 50 parasites measurements in three different biological sample analyses.

3. Results

The first step to evaluate the possible topological changes induced in *T. cruzi* kDNA by the aromatic compounds (Fig. 1) was accessed by polyacrylamide gel electrophoresis (PAGE). The purified kDNA networks were subjected to restriction endonucleases treatment generating characteristic bands of different sizes and sequences deduced according to previous data (Degrave et al., 1988) and to the GenBank database. The typical band patterns of untreated kDNA digested by *CvQI* and by *EcoRI* are shown in Figure 2. When these *CvQI* fragments were treated with the ten structurally related amidine compounds using 100X excess of compound as related to the putative binding sites, DB1080 and DB185 induced a kDNA aggregation, impairing the access of these fragments into the gel (data not shown). These data impelled us to decrease the amount of theses compounds to a 10X excess.

The treatment of *CvQI*-digested kDNA with the aromatic compounds resulted in mobility shifts as can be seen in Figure 2A and Table 1. It is possible to see in Figure 2A that the three major upper bands (between 200 and 400 bp) displayed

similar behavior for all the tested compounds. The smaller bands (under 200 bp) were not affected by any treatment. As the most important mobility shifts of *CvQI*-digested kDNA were evidenced in the upper bands, the analysis of the indexes of mobility was performed on the major upper band of ~ 400 bp (Fig. 2A, arrow). The I_M values are shown in Table 1 and demonstrated that DB749, DB613, DB829, DB75, DB1345 and DB1080 induced no major alteration in the mobility of *CvQI* fragments. On the other hand, DB766, DB889, and DB569 reduced the mobility of the *CVQI*-digested fragments suggestive of DNA bending, inducing DB889 the higher effect. On the other hand, DB185 was the only compound of the set that increased the fragment mobility possibly due to the straightening of DNA (Fig. 2A and Table 1).

Also, shifts in the kDNA mobility were noticed with the *EcoRI* fragments (Fig. 2B and Table 1). For the quantitative measurements of *EcoRI* fragments, the reference band used for calculating the I_M was the smaller band between the four bands generated around 1.5 kb. The data showed that except for DB185, all aromatic compounds induced no major decreases in DNA mobility. On the other hand, only two compounds, the diamidines DB829 and DB75 showed an increase in the fragment motilities, possible due to the straightening of the fragments (Fig. 2B, Table 1).

In order to further evaluate the potential effect of these aromatic compounds on *T. cruzi* kDNA, fluorescence microscopy studies were performed using furamidine (DB75). Our data confirmed the presence of enriched fractions of intact networks with an area of 20-39 μm^2 (Figs 3 and Fig 4). DB75 induced a dose-dependent increase in the size of these networks (Figures 3 and 4). The incubation with 0.1 µg/mL DB75 resulted in a twofold increase in the area of 21% of the networks, whereas around 4% of the KDNA displayed 4X its normal size (Fig. 4). When the kDNA was treated with 10 µg/mL DB75 a twofold increase in 27% of the networks was noticed. Under this concentration, 24% of the networks presented an enlargement higher than 4X (Figs. 3 and 4). With the same concentration around 18% of the networks showed an increase of 7.7 X of their area. With the treatment of 100 µg/mL of DB75, 20, 59 and 18% of the networks showed and increase of 4, 8 and 11X of their total area, respectively. To rule out the possibility of any contamination during the purification steps that could be causing the noticed effect on the network, we next assayed the effect of DB75 in the presence of topoisomerase II inhibitors, namely, etoposide and novobiocin. As can be seen in Figure 5, both inhibitors were not able to avoid the

network expansion induced by DB75 (Fig. 5B and C). Also, the presence of protease inhibitors did not inhibit the effect of DB75 upon the kDNA (Fig. 5D).

4. Discussion

Due to their unique molecular characteristics, the mechanisms involved in the replication of the kDNA network as well as in the RNA editing of its transcripts represent promising and selective targets for novel anti-trypanosomatids agents. As several diamidines and their congeners are minor groove binders that induce important ultrastructural damages in the kDNA of some species (De Souza et al., 2004, Batista et al., 2010) our aim was evaluate the direct effect of these compounds on purified kDNA samples isolated from *T. cruzi*. To achieve that goal ten structurally related diamidines and congeners were assayed on endonuclease-treated kDNA fragments of *T. cruzi* using PAGE. Also, the direct effect of DB75 on intact networks was further investigated through fluorescence microscopy. As far as we know, it's the first time that native purified kDNA are used to test topological changes induced by aromatic compounds like diamidines and AIAs.

The PAGE data showed that some compounds exhibited different mobility effects depending on the type of resulting fragment. For example, although DB889 exhibited the most pronounced shift on the *CvQI* fragments, no major change on the mobility pattern was observed using *EcoRI* fragments. Interestingly the diamidine DB185 also showed different responses according to the type of the restriction enzyme: this aromatic compound increased the mobility in the *CvQI* fragments but decreased it in *EcoRI* fragments. Differences in sequence nature of each fragment could account these unlike effects resulting in different compound ability to fit into the DNA minor groove. Our findings corroborate previous studies performed by Tevis and collaborators (2009), which showed that mobility changes in ligation ladders induced by DB75 were also sequence dependent. These authors reported reduction and increase in curvature for the *cis* A5 and for *cis* ATATA tracts, respectively.

Fox (1990) demonstrated that nogalomycin, an anti-tumoral agent, was able to remove the bending of kDNA fragments. Cons and Fox (1990) found that while distamycin caused a slight increase in mobility of a restriction fragment that contained a bent kDNA fragment with primarily A-tracts, it caused a slight decrease in non-A-tract DNA. The removal of inherent curving in *Crithidia fasciculata* kDNA by

various small DNA ligands, such as distamycin and ditercalinium, has also been reported (Barcelo et al, 1991).

As DB75 is a prototype drug for the design and synthesis of novel potential chemotherapeutic agents, this diamidine was selected to be further evaluated in the topological studies performed on the purified kDNA (Fig. 1). A prodrug of this compound (DB289) that reached phase III of clinical trials against human African sleeping sickness was also used in some biophysical studies on DNA minor groove complexes before (Wenzler et al., 2009). We found that DB75 induced pronounced effects on the networks evidenced by the kDNA area expansion. This effect was dose-dependent and was not impaired by protease and topoisomerase II inhibitors confirming that DB75 triggered this molecular event. Previously, Delain & Riou (1970) demonstrated by transmission electron microscopy that live parasites treated with ethidium bromide (EtBr), a DNA intercalator that accumulates in the kDNA of parasites such as *T. cruzi*, showed strong alteration in the kDNA, with the network fractioned in numerous fibrillar spheres. These authors also demonstrated that parasites cultured with EtBr had a considerable increase in anomalous kDNA molecules, including circular dimers, trimers and pentamers (Riou & Delain, 1969). We hypothesize that DB75 could bind to specific kDNA sequences altering its topology, leading to their break down or straightening (as presently noticed using *EcoRI*-digested fragments treated with furamidine), resulting in the network expansion as observed by fluorescent microscopy. In fact, a dose-dependent increase in the number of those altered molecules was presently visualized. In fact, our work is pioneer in showing the direct effect of a diamidine on the kDNA network that was not dependent on any other protein or molecule present in live parasites. As similarly described by Riou & Delain (1969), our data suggest that one of the mechanisms of action of diamidines could include their ability to destabilize the DNA network, inducing the linearization of the maxi and/or minicircle that could rejoin leading to the formation of circular dimers, trimers as well assuming other possible configurations.

Despite the fact that a large number of compounds have been described which bind reversible to the minor groove of AT-rich DNA regions, for many years attempts have been made to synthesize new compounds with enhanced sequence selectivity that could better fit to these sequences (Abu-Daya et al., 1995). The interaction of these aromatic compounds as well as other DNA binders such as EtBr could induce important conformational changes in their structure leading to the loss

of cellular viability. Our work contribute to a better understanding of the effects induced by these interactions in order to provide additional information for the rational design of new drug that could target the kDNA network.

5. Funding

The present study was supported by grants from Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ and Pensa Rio/FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), DECIT/SCTIE/MS and MCT by CNPq, PAPES V/FIOCRUZ, Coordenação de Aperfeiçoamento Pessoal do Ensino Superior (CAPES) and Consortium for Parasitic Drug Development (CPPD).

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Legends to Figures

Figure 1. Chemical structures of the studied compounds.

Figure 2. Poliacrylamide gels showing the purified kDNA of epimastigotes of *T. cruzi* submitted to endonuclease treatment with *CvQI* (**A**) and *EcoRI* (**B**).

Figure 3. Fluorescence microscopy of the purified kDNA networks (arrow) from *T. cruzi* untreated (**A**) and treated with (**B**) 0.1 µg/mL (**C**) 10 µg/mL and (**D**) 100 µg/mL DB75.

Figure 4. Network area of purified kDNA from epimastigote forms of *T. cruzi* treated with different concentrations of DB75.

Figure 5. Fluorescence microscopy of purified kDNA networks of epimastigote forms of *T. cruzi* untreated (**A**) and treated with DB75 100 µg/mL (**B**) in the presence of 500 µg/mL etoposide (**C**) and 300 µg/mL of novobiocin (**D**) and a protease inhibitor cocktail (**E**).

Table 1. Mobility Indexes (I_M) inferred from gel mobility shifts in the presence of compounds. I_M <1 indicates bending while I_M >1 indicates straightening.

Compound	I_M *CvQI*	I_M *EcoRI*
DB749	1.009	0.993
DB613	1.007	1.002
DB766	0.995	1.004
DB889	0.987	1.004
DB829	1.004	1.021
DB75	1.000	1.021
DB569	0.995	0.995
DB1345	1.004	0.995
DB1080	1.001	1.004
DB185	1.013	0.935

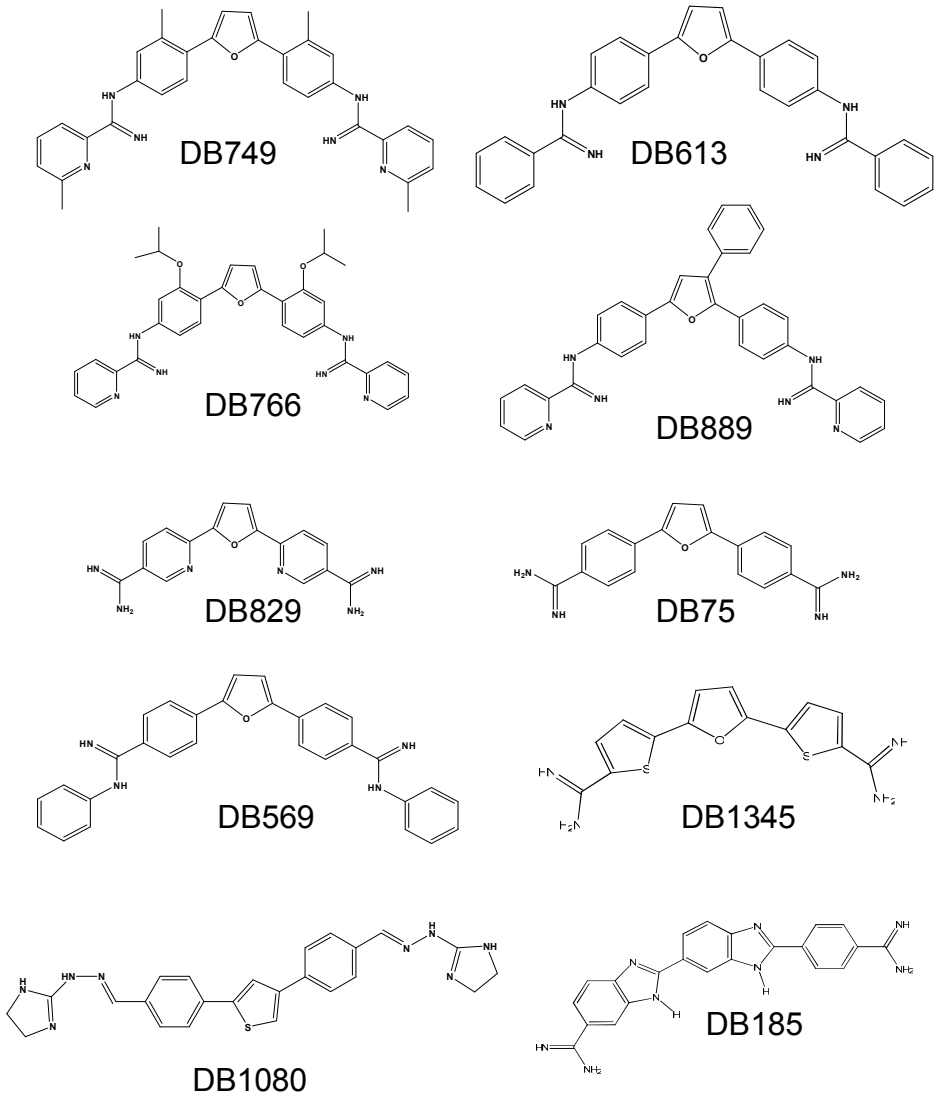

Figure 1. Chemical structures of the compounds used in the present study

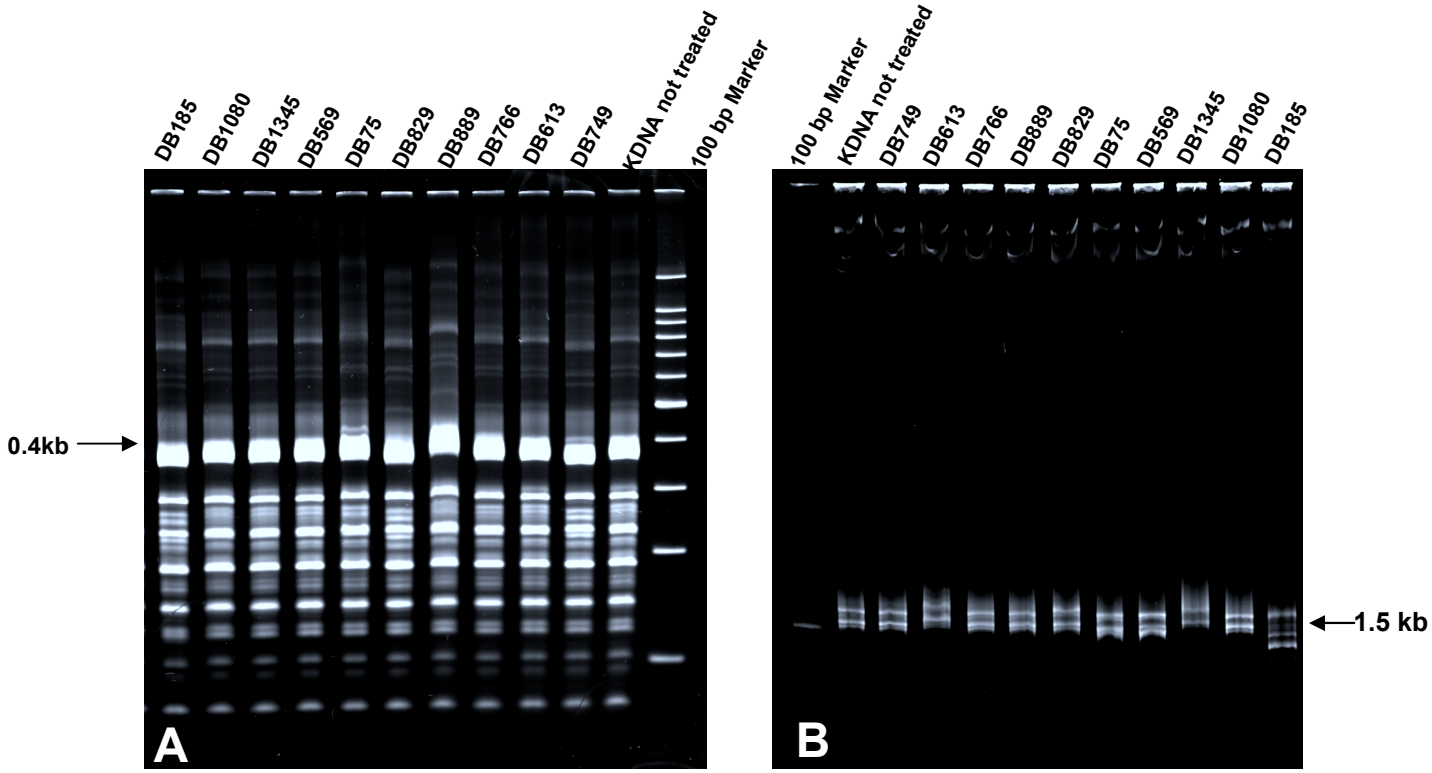

Figure 2. Poliacrylamide gels showing the purified kDNA of epimastigotes of *T. cruzi* submitted to endonuclease treatment with *CvQI* (**A**) and *EcoRI* (**B**).

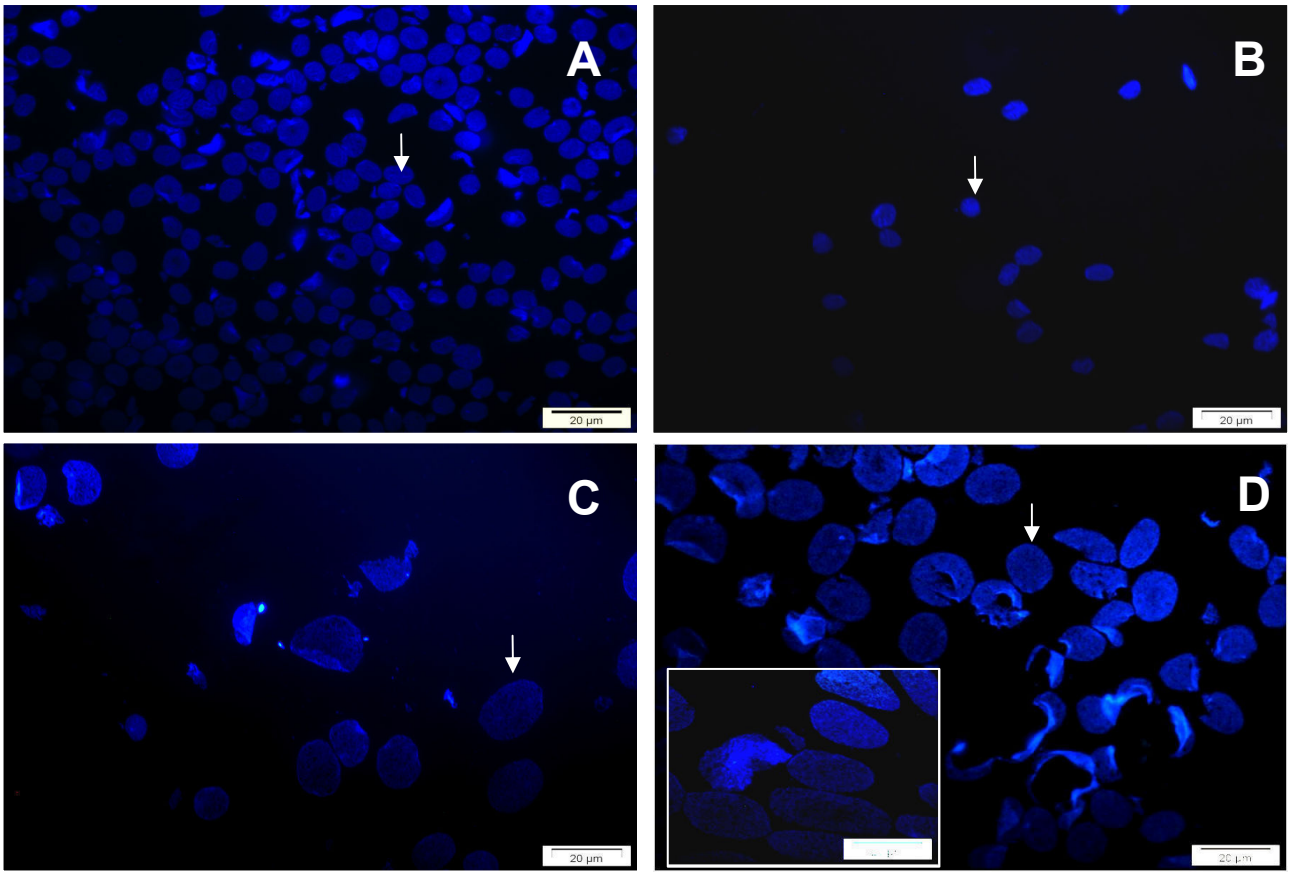

Figure 3. Fluorescence microscopy of the purified kDNA networks (arrow) from *T. cruzi* untreated (**A**) and treated with (**B**) 0.1 µg/mL (**C**) 10 µg/mL and (**D**) 100 µg/mL DB75.

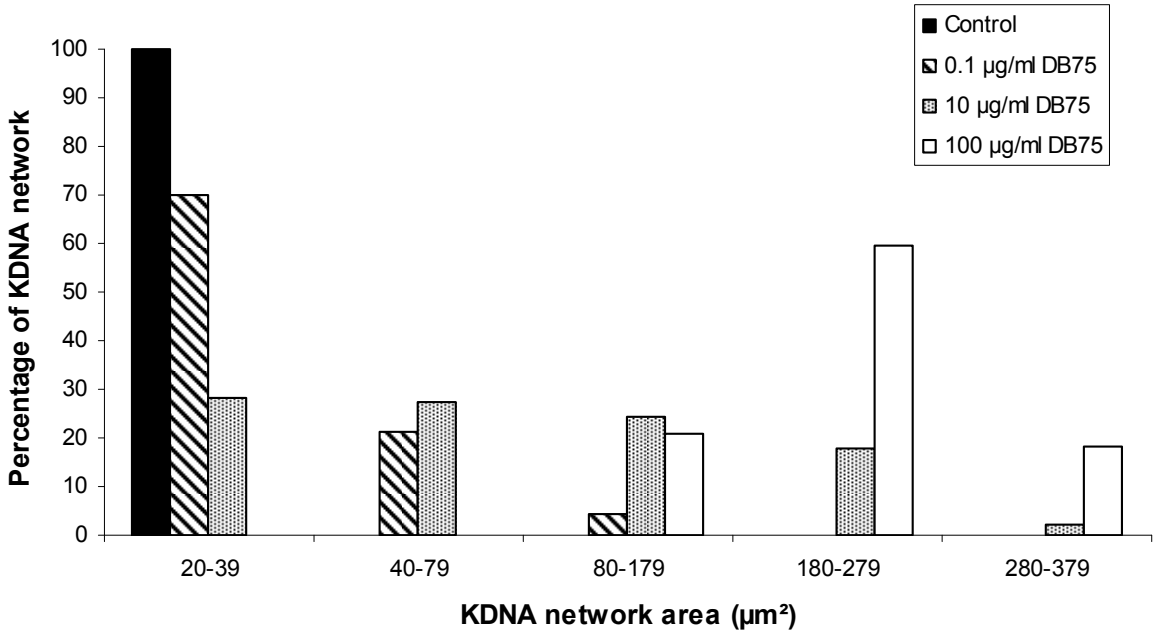

Figure 4. Network area of purified kDNA from epimastigotes forms of *T. cruzi* treated with different concentrations of DB75.

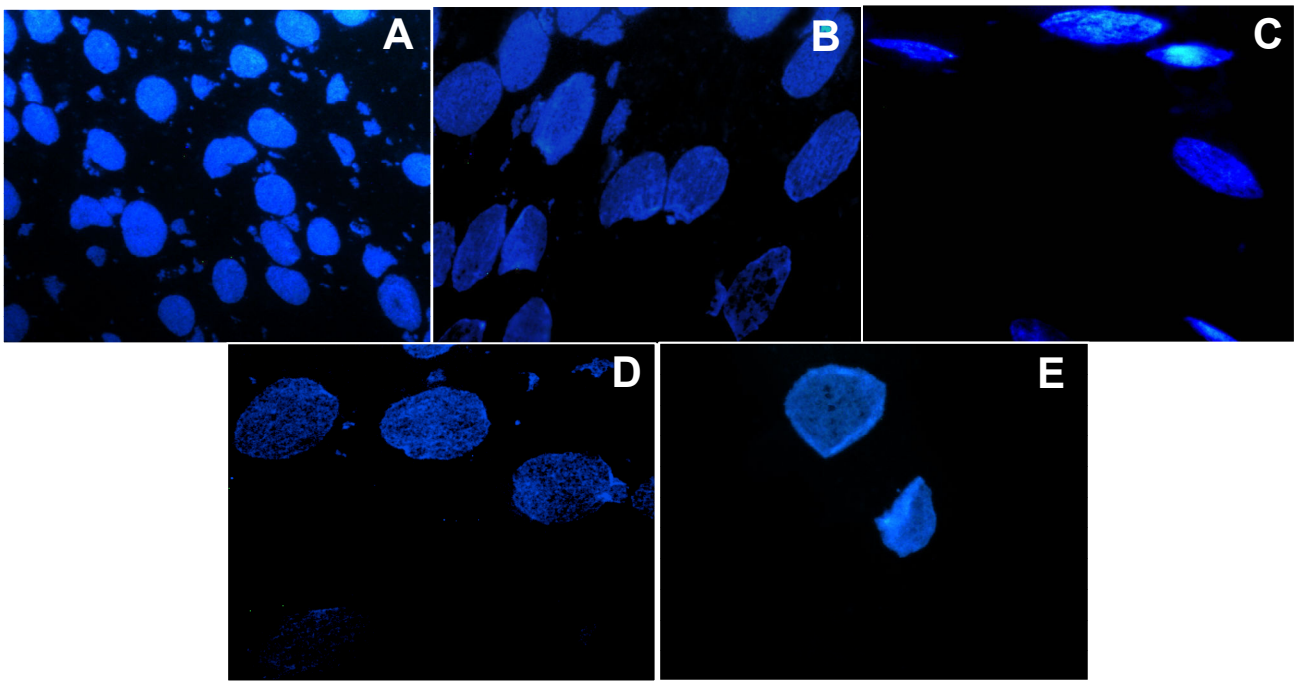

Figure 5. Fluorescence microscopy of purified kDNA networks of epimastigote forms of *T. cruzi* untreated (**A**) and treated with DB75 100 µg/mL (**B**) in the presence of 500 µg/mL etoposide (**C**) and 300 µg/mL of novobiocin (**D**) and a protease inhibitor cocktail (**E**).

IV. DISCUSSÃO

Nosso laboratório em colaboração com grupos de química medicinal do Drs. Wilson e Boykin (Departamento de Química, Georgia State University, Atlanta, GA, USA) e Dr. Tidwell (Departamento de Patologia e Medicina Laboratorial, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA) vem se empenhando na investigação da ação de compostos aromáticos como diamidinas aromáticas (DAs) e análogos como possíveis agentes tripanocidas. Dentro deste contexto resultados promissores têm sido alcançados, servindo como base para o desenho racional de novos compostos como, por exemplo, de novas AIAs, que têm se revelado potentes agentes contra *T.cruzi* (Silva et al., 2007a,b, 2010; Pacheco et al., 2009; Batista et al., 2010a,b) e espécies de *Leishmania* (Wang et al., 2010).

No primeiro trabalho (**Trabalho #1**) revisamos os principais aspectos alcançados pelo nosso grupo nos últimos 15 anos de pesquisa pré-clínica, motivados pela necessidade urgente de se avançar no desenvolvimento de novas alternativas terapêuticas para a DC. Ao longo desta seção discutiremos os resultados encontrados durante o desenvolvimento da presente tese em paralelo aos descritos nesta revisão (**Trabalho #1**) assim como de publicações da literatura.

No segundo manuscrito (**Trabalho #2**), avaliamos a atividade, seletividade e alvos celulares de onze diamidinas aromáticas (**#2**, Fig. S1) sobre formas tripomastigotas e amastigotas de *T. cruzi in vitro*. A análise por 24h a 37°C revelou quatro compostos muito ativos sobre tripomastigotas, alcançando valores de IC_{50} entre 0,7 e 2,7 µM. Observamos que três destes compostos - **2**, **5** e **7** - também apresentaram excelente atividade sobre amastigotas interiorizadas em cardiomiócitos com IC_{50} na faixa de 0,3 a 1,7 µM (**#2**, Tabela 1). Devido a baixa toxicidade sobre as células de mamíferos (culturas de células cardíacas), observamos que os compostos **5** e **7** foram os que exibiram os maiores índices de seletividade (IS), obtidos pela razão entre os valores de IC_{50} e o LC_{50}. O IS é uma importante análise que reflete o quanto o composto é seletivo para o parasito, não causando danos à célula hospedeira. O composto **5** apresentou IS >137 e >40 para tripomastigotas e amastigotas, respectivamente, enquanto que para o composto **7,** os valores de IS foram >96 e >107, respectivamente. Concluímos que o composto **7** (24SMB001) é um possível candidato a avaliação em modelos experimentais, haja visto que alcançou IS superior ao recomendado para seleção de compostos testados *in vitro* (Nwaka & Hudson, 2006; Romanha et al., 2010).

Visando avaliar a aplicabilidade destes compostos como possíveis agentes profiláticos em bancos de sangue, realizamos ensaios de atividade na presença de sangue a 4°C (Silva et al., 2007b). Os resultados mostraram que nestas condições experimentais, todos os compostos testados perderam atividade, alcançando redução de até 210X, como para o composto **9** (**#2**, Tabela 1). Silva e colaboradores (2007b) também descreveram uma parcial diminuição de atividade tripanocida na presença de sangue utilizando AIAs, porém a maior redução observada foi de 10X para a DB889. A inativação da atividade em presença de sangue pode ser explicada pela associação dos compostos a componentes plasmáticos, como por exemplo, a albumina, reduzindo assim a quantidade de composto livre. Outra possibilidade é a perda de atividade mediada pela instabilidade da droga nestas condições. A albumina é uma das mais abundantes proteínas plasmáticas, apresentando extraordinária capacidade de ligação a diferentes fatores (Fasano et al., 2005; Ascenzi et al., 2006). Esta proteína apresenta uma flexibilidade intrínseca que lhe confere a capacidade de seqüestrar diferentes ligantes extracelulares (Tajmir-Riahi, 2007). Ligantes aromáticos e heterocíclicos são capazes de se ligarem à regiões hidrofóbicas de subdomínios da albumina humana (He & Carter, 1993) demonstrando ser esta proteína um importante modulador na farmacocinética de drogas. Além disso, algumas interações com a albumina se mostraram capazes de induzir mudanças conformacionais em ligantes (Tajmir-Riahi, 2007). Sendo assim, é possível que um dos fatores responsáveis pela perda de atividade das diamidinas estudas seja a albumina do sangue de camundongo, uma vez que está apresenta as propriedades descritas e é uma proteína bastante conservada em diferentes espécies. A interação da albumina poderia induzir alterações conformacionais no composto impedindo (i) sua captação pelo parasito; (ii) sua internalização na mitocôndria e/ou (iii) o reconhecimento de seu alvo intracelular.

Neste artigo também avaliamos os possíveis alvos celulares dos compostos estudados sobre formas tripomastigotas, utilizando microscopia de fluorescência (MF) e microscopia eletrônica de transmissão (MET). Dada a fluorescência intrínsica destes compostos, foi possível localizá-los em organelas contendo DNA, como núcleo e mitocôndria (**#2**, Fig. 1), corroborando dados prévios da literatura realizados em *T. brucei* (Mathis et al., 2006; Lanteri et al., 2008) e em *T.cruzi* (De Souza et al., 2004). Além disso, os compostos foram capazes de induzir importantes alterações ultraestruturais no parasito incluindo: destacamento do envelope nuclear e da membrana plasmática além de profundos danos na mitocondrial e no kDNA (**#2**, Fig.

1). Danos mitocondriais foram também observados em *T.cruzi* em ensaios realizados com outras séries de amidinas (Silva et al., 2007b; De Souza et al., 2004; Batista et al., 2010a,b).

Outro importante achado no **Trabalho #2** foi o fato de que embora todos os compostos apresentassem acúmulo superior no kDNA em relação ao núcleo (**#2**, Fig. 1 e Tabela S1), esta característica não resultou em uma correlação direta com a atividade biológica. Resultados posteriores com outra série de diamidinas aromáticas confirmaram a falta de correlação entre acúmulo no kDNA e eficácia dos compostos (Batista et al., 2010b; Silva et al., 2010). De forma similar, Mathis e colaboradores (2007) observaram que algumas diamidinas como a DB820 e a DB75 embora sejam muito ativas sobre *T. brucei*, apresentam níveis de acúmulo intracelular semelhantes a compostos menos ativos como as DB244 e DB249. Assim, os resultados da literatura somados aos nossos achados (**Trabalho #2**) apontam para o envolvimento de outros mecanismos de ação tripanocida de diamidinas, não somente aos relacionados à interação com as organelas ricas em DNA (Singh & Dey, 2007). Uma possibilidade seria a ação sobre acidocalcisomas haja vista que algumas DAs também são capazes de se acumular nestas organelas (Batista et al., 2007; Mathis et al., 2007). Outros mecanismos possíveis para a ação de diamidinas e análogos e que já foram relatados na literatura incluem: (i) interferência direta em enzimas envolvidas na replicação do DNA, como topoisomerases, e (ii) impedimento alostérico de ligação de proteínas ou outras moléculas ao seus respectivos sítio no DNA, interferindo em funções como a replicação (Woynarowski et al., 1988; Shapiro et al., 1989; Shapiro & Englund, 1990; Soeiro et al., 2005).

Com o objetivo de melhor entender o mecanismo de ação desta classe de compostos, nosso próximo passo foi avaliar a relação entre a associação ao kDNA e atividade biológica de DAs e análogos. Para esse estudo treze compostos estruturalmente relacionados foram avaliados quanto a sua atividade tripanocida e suas respectivas afinidades ao kDNA do *T.cruzi* (**Trabalho #3**). Quatro DAs (DB75, DB569, DB1345 e DB829), oito AIAs (DB766, DB749, DB889, DB709, DB613A, DB1831, DB1852 e DB2002) e uma guanil-hidrazona (DB1080) (**#3,** Tabela 1) foram estudadas através de estudos termodinâmicos referentes a afinidades relativas ao kDNA, utilizando ensaios de desnaturação térmica (T_m) e de dicroismo circular (DiC). Ensaios biofísicos utilizando T_m e DiC têm sido amplamente utilizados para determinar a afinidade relativa de diferentes classes de ligantes, incluindo antivirais,

antibióticos e agentes anti-turmorais, a diversas sequências de DNA (Haq, 2002; Liu et al., 2007; Tanious et al., 2007; Wilson et al., 2008).

Uma vez que dados da literatura apontam para DAs e seus congêneres como excelentes ligantes da fenda menor do DNA, apresentando alta seletividade por regiões ricas em sequências AT (Abu-Daya & Fox, 1997; Wilson et al., 2008), objetivamos estudar o efeito destes 13 compostos diretamente sobre o kDNA íntegro purificado a partir de formas epimastigotas deste parasito, sendo algumas delas selecionadas para estudo com uma sequência específica de minicírculo de *T. cruzi*.

Assim, como (i) a seleção destes trezes compostos aromáticos foi baseada na atividade anteriormente reportada sobre tripomastigotas de sangue, (ii) e sabendo que a depender da forma do parasito, distintos efeitos podem ser encontrados para um mesmo agente tripanocida (Romanha et al., 2010), inicialmente estudamos a eficácia destes sobre epimastigotas. Embora todos os compostos testados tenham apresentado uma resposta dose-dependente, observamos que estas formas foram menos susceptíveis do que tripomastigotas, com exceção da mono-AIA DB2002. Dados semelhantes foram também reportados com a droga de referência (Bz) que foi cerca de 13X mais ativa sobre tripomastigotas em relação ao epimastigotas (**#3**, Tabelas 1 e 2). Testando os compostos DB766 e Bz sobre diferentes cepas selvagens de *T. cruzi,* Batista e colaboradores (2010b) também encontraram uma maior susceptibilidade de tripomastigotas quando comparadas com epimastigotas. Pentamidina e outras diamidinas como a DB75 são ativamente transportadas para dentro da célula por transportadores do tipo P2, HAPT1 e LAPT1 (Lanteri et al., 2006), sendo acumulados intracelularmente em altas concentrações (Mathis et al., 2006). Apesar de estudos conduzidos em *Leishmania mexicana* (Basselin et al., 2002), *T. brucei brucei* (De Koning, 2001) e *Plasmodium falciparum* (Barret & Fairlamb, 1999), não há qualquer evidencia sobre os mecanismos envolvidos na captação de diamidinas pelo *T. cruzi*. É possível, que os diferentes perfis de susceptibilidade observados entre epimastigotas e tripomastigotas (**Trabalho #3**) tenham relação com distintos mecanismos de captação desses compostos para cada uma das formas do parasito. Outras hipóteses incluem: (i) diferentes alvos celulares; (ii) diferentes cinéticas de acúmulo e/ou (iii) diferentes mecanismos de exclusão da droga. Recentemente a exclusão de drogas foi demonstrada como um fator de resistência de *L. mexicana* à pentamidina, (Basselin et al., 2002). Além de diferenças de atividade entre formas da mesma espécie de tripanosomatídeos, diferenças entre espécies também já foram reportadas. Um destes exemplos é a

DB829 que, em nosso trabalho, apresentou baixa atividade sobre *T. cruzi* tanto para epimastigotas (IC_{50} = 938 µM) como para tripomastigotas (IC_{50} = 437 µM), embora seja muito ativa sobre *Trypanosoma brucei rhodesiense* (18.7 nM) (Ismail et al., 2003).

Observamos ainda que dentre as classes de amidinas estudadas, as AIAs foram as que apresentaram superior atividade com valores de IC_{50} variando entre 0,02-4 µM para tripomastigotas e 0,08-22 µM para epimastigotas (**#3**, Tabelas 1 e 2). Nossos dados corroboram estudos recentes que demonstraram o superior efeito tripanocida de AIAs em relação a outras amidinas, incluindo diamidinas, monoamidinas e guanil-hidrazonas (Pacheco et al., 2009). A principal diferença estrutural entre uma diamidina e uma AIA é a natureza da ligação do grupamento imino ao anel arila, sendo no caso das AIAs mediado por um átomo de nitrogênio. É possível que esta maior eficácia tenha relação com propriedades físicas das AIAs. As diamidinas são moléculas básicas com valores de pKa próximos a 11, enquanto as AIAs tem valores próximos a 7. Assim, em pH fisiológico, as DAs são protonadas e portanto, catiônicas enquanto as AIAs são essencialmente neutras, o que facilita seu transporte passivo através de membranas biológicas, tanto da célula hospedeira como 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 (Batista et al., 2010b). A atividade das AIAs também já foi investigada sobre amastigotas e promastigotas de *Leishmania major* e *Leishmania tropica* demonstrando uma alta atividade quando comparada a pentamidina (Rosypal et al., 2008). As AIAs DB745 e DB766 também foram testadas sobre espécies de *Leishmania* causadoras da leishmaniose visceral em ensaios *in vitro* e sobre modelos experimentais, e mais uma vez AIAs apresentaram excelente atividade leishmanicida (Wang et al., 2010).

A afinidade de compostos sintéticos e naturais por sequências nucleotídicas pode ser avaliada por ensaios de T_m, através da determinação da diferença do T *melting* antes e após formação do complexo composto-DNA (ΔT_m) (Wilson et al., 2008). Quanto maior essa variação mais estável é o complexo, ou seja, maior a afinidade dos compostos ao DNA. Nossas análises iniciais foram feitas avaliando o efeito de oito compostos sobre uma sequência conservada de 22-*mer* presente em minicírculos de *T. cruzi*. Foi possível observar que os compostos apresentaram afinidade variável e que alguns deles, com alta atividade tripanocida (e.g. DB1831), não foram capazes de se ligarem à sequência estudada. Com exceção da DB613,

todas as outras AIAs, apesar de bastante ativas, não apresentaram ΔT_m condizente com uma alta afinidade pelo DNA (ΔT_m de 0 a 2°C) (**#3**, Tabela 2). A DB1831, por exemplo, um dos compostos que apresentaram maior atividade, apresentou ΔT_m de 0°C (**#3**, Tabela 2). Estes dados mostram que a afinidade à sequências do kDNA não está diretamente relacionada com a atividade tripanocida dos compostos testados.

A seguir, para análise de afinidade e da alteração topológica do DNA realizamos ensaios de DiC utilizando a mesma sequência conservada. O espectro gerado pela interação do composto com o DNA pode ser um indicativo de que tal interação esteja de fato ocorrendo, qual o tipo de ligação e o quanto esta altera a conformação do DNA (Wilson et al., 2008). Observamos que DB75, DB569, B766, DB709, DB613 e DB1852 (**#3**, Tabela 1) demonstraram capacidade de ligação a esta sequência enquanto que DB1831 e DB2002 não induziram sinais positivos no espectro, indicando que possivelmente não se ligam nesta sequência. Os compostos que induziram sinais positivos relativos à sua associação ao DNA vistos por DiC, também foram capazes de alterar a topologia de modo dose-dependente, como pode ser obervado pela alteração do espectro na região de 260 nm (**#3**, Fig. 1). Compostos que têm como alvo os minicírculos podem afetar etapas essenciais no metabolismo do parasito, como replicação, transcrição e o processo de edição de RNA. Trabalhos anteriores da literatura mostraram que o silenciamento de proteínas do editosomo, como a TbMP81 e TbMP52, leva à perda de atividade desta estrutura e consequemente à morte de *T. brucei* (Schnaufer et al., 2001; Drozdz et al., 2002; Wang et al., 2010; Salavati et al., 2006; Amaro et al., 2008). Apesar de ainda não haver evidências experimentais do papel funcional da sequência sintética utilizada no nosso estudo, ela contém uma sequência conservada (AGG GGC GTT C) localizada na região 5´ terminal de minicírculos de *C. fasciculata* recém sintetizados, podendo ter papel fundamental na origem de replicação (Ray, 1989). Portanto, compostos que se associam a essa região poderiam comprometer a síntese de novos minicírculos. Na ausência de minicírculos importantes para a geração de RNAg, os maxicírculos por sua vez seriam afetados, levando então a problemas no processo de oxidação fosforilativa e por fim a morte do parasito.

Como no **Trabalho #3**, alguns dos compostos mais ativos sobre o *T.cruzi* não apresentaram afinidade à sequência do minicírculo estudada, nosso próximo passo foi avaliar uma possível associação destas amidinas a outras seqüências presentes na rede do kDNA. Para isso realizamos análises de T_m e DiC em redes intactas

purificadas de epimastigotas de *T. cruzi*. As análises de T_m mostraram que, com exceção da DB569 e DB709, os resultados foram similares ao encontrado com a sequência sintética. DB569 e DB709 apresentaram aproximadamente 2 e 4X maior afinidade à rede intacta, sugerindo a associação destes compostos a outros sítios de ligação presentes na rede intacta. Ainda com relação ao kDNA intacto, nossos dados revelaram que embora DB75 e DB569 apresentem afinidades semelhantes (ΔT_m = 15°C), esta segunda é 100X mais ativa (**#3**, Tabela 2), confirmando que outros mecanismos de ação podem estar envolvidos no processo de morte celular. Interessantemente, o aspecto geral dos espectros de DiC utilizando a rede purificada foi bastante semelhante ao encontrado anteriormente para a sequência sintética (**#3**, Fig. 3), demonstrando um espectro típico de ligação à fenda menor do DNA e alterações de espectro na região do DNA. A identificação de potenciais alvos na rede de kDNA seria uma importante estratégia quimioterápica uma vez que é uma estrutura única de tripanosomatídeos e que não ocorre em células de mamíferos. Nossos dados mostram que apesar de AIAs não serem fortes ligantes de kDNA, elas são capazes de induzir profundas alterações topológicas no DNA mitocondrial do parasito. De fato, algumas das AIAs mais ativas, como a DB766, apesar de apresentarem baixa afinidade (visto por T_m) foram capazes de causar importantes alterações na topologia do kDNA (visto por DiC). É possível que AIAs atuem, de modo altamente específico, como "tesouras químicas" agindo em um grande número de sítios, pela sua habilidade de se desligar do DNA de forma rápida, em decorrência de sua baixa afinidade ao mesmo. Entretanto, é importante ressaltar que esta possiblidade não se extende a todas amidinas, haja vista que a baixa afinidade também não está diretamente correlacionada à alta atividade tripanocida, uma vez que compostos como a DB2002, apesar de possuírem baixa afinidade ao kDNA, também não apresentam considerável atividade tripanocida (**#3**, Tabelas1 e 2).

Assim, com relação aos parâmetros termodinâmicos estudados na presente tese, nossos dados confirmam resultados anteriores demonstrando alta afinidade da DB75 a regiões ricas em AT (Liu et al., 2007). Essa diamidina foi um excelente ligante como testado na sequência conservada (ΔT_m de 17°C e espectro induzido positivo) e sobre o kDNA purificado de *T. cruzi* (ΔT_m de 15°C e espectro induzido positivo). Porém resultados anteriores mostram maior afinidade da furamidina sobre polímeros de AATT (ΔT_m = 25°C) (Mathis et al., 2007). Essa diferença pode ser atribuída à presença de diferentes bases nucleotídicas o que também pode influenciar no tipo de associação dos compostos quando se trata de um modelo que

mimetiza uma situação *in natura*, como é o caso da rede intacta de kDNA contendo uma heterogeneidade de sítios de ligação.

Interessantemente, as AIAs DB1831 e DB2002 apresentaram baixa afinidade tanto para a sequência sintética (**#3**, Tabela 2, Fig. 1) quanto para o kDNA purificado como demonstrado pelas análises de T_m (**#3**, Tabela 2) e DiC (**#3**, Fig. 3). O inverso também é verdadeiro, alguns dos compostos que apresentaram baixa atividade tripanocida também apresentaram alta afinidade ao kDNA. Estes dados corroboram resultados anteriores que sugeriram a ausência de relação entre atividade dos compostos aromáticos e forte associação ao kDNA. Mathis e colaboradores (2007) realizaram ensaios com difenil-furanos e aza análogos da DB75 sobre *T. brucei* e também não observaram correlação direta entre atividade e afinidade a oligômeros poli(dA).poli(dT) ou oligômeros contendo um único sítio AATT. Estes achados sugerem a existência de outras etapas/eventos intermediários entre a associação dos compostos aromáticos ao kDNA e a indução da morte do parasito. Além disso, é possível que existam fatores *in vivo* necessários para que estes compostos como a DB1831 se liguem ao DNA e exibam seu efeito, como, por exemplo, proteínas mitocondriais. As metodologias utilizadas nos **Trabalhos #3** e **#**4 foram inéditas quanto ao uso de sequências nativas do parasito (kDNA íntegro) em estudos termodinâmicos, visando contribuir para um melhor entendimento do(s) mecanismo(s) de ação desses compostos sobre o *T. cruzi*.

É importante destacar que nosso estudo sugere que pequenas alterações na estrutura química podem gerar drásticos efeitos no perfil de interação com moléculas de DNA. Isto pode ser visto claramente no **Trabalho#3** através dos estudos de DiC, comparando-se a DB1831, que não causou alterações, com a DB766, que claramente gerou profundas pertubações na topologia do kDNA intacto. A única diferença entre os dois compostos é a substituição de um grupamento piridínico da DB766 por um grupamento pirimídico no caso da DB1831 (**#3**, Tabela 1). Em relação à estrutura química dos compostos, dados anteriores demonstraram que a simples adição de nitrogênio aos grupamentos aromáticos de DB75 é capaz de alterar as propriedades de lipofilicidade e polaridade deste composto, causando uma redução da habilidade de ligação ao DNA como medido por análises de T_m (Wilson et al., 2008). A redução da ΔT_m de DB75 em relação ao seu análogo DB829, no qual um átomo de nitrogênio foi adicionado (ΔT_m de 15 para 9), encontrada no presente trabalho confirma estes dados da literatura (Wilson et al., 2008).

O conjunto dos nossos dados (**Trabalho #3)** sugere que a força de associação dos compostos aromáticos ao kDNA não é o fator único e fundamental para desencadear a morte celular do *T. cruzi*. Este estudo associado com outros da literatura mostram que a ligação ao DNA pode ser apenas uma das etapas envolvidas no efeito tripanocida de diamidinas, ou mesmo que os danos encontrados no complexo mitocôndria-kDNA sejam eventos secundários.

De modo a aprofundar nosso estudo sobre o potencial efeito de amidinas sobre o kDNA do *T. cruzi*, nosso próximo passo foi avaliar a ação de 10 compostos sobre fragmentos de kDNA resultantes da digestão por endonucleases de restrição através de eletroforese em gel de poliacrilamida (PAGE) (**Trabalho #4**). Além disso, observamos por ensaios de MF o direto efeito da DB75 sobre a rede intacta purificada do parasito.

Em alguns tripanosomatídeos, excetuando-se o *T.cruzi*, as sequências de minicírculos podem apresentar uma curvatura intrínsica na molécula que pode ser monitorada por ensaios de mobilidade eletroforética, comparando géis de agarose com de poliacrilamida. Esta curvatura pode ser importante do ponto de vista funcional, atuando na regulação da expressão gênica (Marini et al., 1982; Marini et al., 1984; Barcelo et al., 1991). Por outro lado, a indução de alteração topológica no kDNA de *T.cruzi* pode ser uma importante estratégia quimioterápica. Assim, nosso próximo objetivo foi avaliar a possível alteração em fragmentos do kDNA frente a incubação com amidinas através de ensaios de PAGE. Nossos dados demonstraram que os compostos exibiram diferentes efeitos dependendo dos fragmentos utilizados. Por exemplo, a DB889 induziu um importante efeito (*bending effect*) quando incubada com fragmentos gerados pela digestão por *CvQI*, enquanto que sua interação com fragmentos obtidos pelo tratamento com *EcoRI* não resultou em alterações significativas na mobilidade destes fragmentos. A diamidina DB185 apresentou efeito oposto de acordo com a enzima testada: aumentou a mobilidade eletroforética em fragmentos gerados pela digestão com *CvQI*, enquanto diminuiu a mobilidade daqueles gerados a partir de *EcoRI*. A natureza da sequência dos fragmentos gerados por essas enzimas pode ser um dos fatores determinantes para a observação dos distintos efeitos induzidos pelo mesmo composto. Nossos dados corroboram estudos prévios de Tevis e colaboradores (2009), que mostraram mudanças topológicas induzidas no DNA pela DB75, utilizando *ligation ladders*, escadas moleculares apresentando pequenas diferenças de uma banda para outra que permitem uma precisa medição quantitativa do efeito de compostos na alteração

da mobilidade eletroforética. Os autores relataram que este efeito era dependente da sequência utilizada com redução na curvatura de sequências cis A_5 e aumento em cis ATATA.

Fox (1990) demonstrou que a nogalomicina, uma droga anti tumoral que se liga ao DNA, foi capaz de remover a curvatura de fragmentos. Cons & Fox (1990) encontraram que enquanto a distamicina, um ligante de fenda menor de DNA também usado como agente anti-turmoral, causa um aumento na mobilidade de fragmentos com curvatura intrínseca, diminui a mobilidade de fragmentos sem curvatura. A remoção de curvatura de fragmentos de kDNA de *C. fasciculata* pela presença de distamicina e por outros compostos foi também estudada através de ensaios de mobilidade em gel e por MET (Barcelo et al., 1991). Barcelos e colaboradores (1991) sugerem que a remoção de curvatura do kDNA pelas drogas é decorrente de uma alteração global da estrutura do DNA com subsequente aumento da flexibilidade levando a supressão da curvatura rígida presente em regiões AT.

A seguir, avaliamos o potencial efeito direto da DB75 na conformação da rede de kDNA de *T. cruzi*. (**Trabalho #4**, Fig. 1 e 3). Nossos dados revelaram que a furamidina é capaz de induzir importante efeito dose-dependente de expansão da área total do kDNA (**#4**, Figs. 3 e 4). Este aumento pode chegar até a 11X em aproximadamente 20% das redes quando tratadas com 100 µg/mL de DB75 (**#4**, Figs. 3 e 4). Este efeito não foi alterado na presença de inibidores de proteases e de topoisomerases do tipo II, descartando assim, possíveis efeitos proteolíticos e/ou mediados por toposoimerases presentes nas nossas amostras de kDNA. Em estudos prévios, Delain & Riou (1970) evidenciaram por MET que parasitos vivos tratados com brometo de etídio (BrEt), um intercalante de DNA que se acumula no kDNA de *T. cruzi*, induziu alterações na topologia do kDNA, resultando no fracionamento da rede em numerosas esferas fibrilares. Em outro trabalho, o mesmo grupo demonstrou também que os parasitos tratados apresentam um considerável aumento no número de minicírculos circulares anômalos, incluindo dímeros, trímeros e até pentâmeros (Riou & Delain, 1969). De acordo com os nossos achados somados aos resultados da literatura, sugerimos que a ligação da DB75 em sequências do kDNA pode induzir alterações em sua topologia, levando a quebra ou até mesmo a remoção da curvatura de alguns minicírculos, resultando em uma expansão da rede. É relevante notar que no trabalho anterior (**Trabalho #3**) observamos que a DB75 foi capaz de causar um aumento na mobilidade de

fragmentos de kDNA advindos de digestão com a enzima *EcoRI*, corroborando assim esta hipótese.

De fato, nosso trabalho é pioneiro em mostrar o efeito direto de diamidinas sobre a rede do kDNA de *T. cruzi*, sem interferências de outros fatores e moléculas como proteínas presentes no organismo intacto. De maneira semelhante à observada por Riou & Delain (1969), nossos resultados sugerem que um dos mecanismos de ação envolvidos na atividade de diamidinas pode estar relacionado com a desestabilização das redes de kDNA, presentemente observada pelos ensaios de DiC e MF. De fato, trabalhos anteriores mostram a capacidade de drogas anti-tripanosomatídeos, como pentamidina e berenil, de clivar e linearizar minicírculos de *T. equiperdum* (Shapiro & Englund, 1990). Porém este efeito foi observado em kDNA de parasitos tratados quando vivos, sendo o nosso trabalho pioneiro em demostrar o efeito direto na rede purificada.

Apesar de um grande número de compostos terem sido descritos como ligantes reversíveis da fenda menor do DNA em regiões ricas em AT, a busca por novos compostos com maior atividade e seletividade e com menor toxicidade ainda se faz necessária (Abu-Daya et al., 1995). O entendimento de mecanismos de ação é muito importante para que novos derivados sejam sintetizados. O nosso trabalho se insere nesse contexto trazendo novas contribuições a respeito da ação de diamidinas e análogos sobre o kDNA de *T. cruzi* que podem servir como base para o desenho racional de novos compostos mais ativos e menos tóxicos e que possam no futuro vir a ser utilizados como terapia alternativa para tratamento de doenças parasitárias que afligem uma grande parcela da humanidade.

V. CONCLUSÕES

- Diamidinas aromáticas (DAs) e congêneres, em especial arilimidamidas (AIAs) apresentaram um excelente efeito tripanocida *in vitro* sobre diferentes formas evolutivas do *T. cruzi* (tripomastigotas, epimastigotas e amastigotas intracelulares), confirmando dados prévios da literatura referente ao seu alto poder microbicida e potencial uso em estratégias de desenvolvimento de novos agentes quimioterápicos para tratamento de doenças causadas por tripanosomatídeos, como a doença de Chagas.

- A promissora atividade e seletividade de AIAs, como a DB766, sobre parasitos intracelulares incluindo *T. cruzi* e diferentes especies de *Leishmania*, sugerem que esta classe de compostos represente um interessante protótipo para desenho racional de novos compostos amidínicos que guardem a excelente atividade tripanocida mas que apresentem baixa toxicidade sobre células de mamíferos.

- Formas tripomastigotas revelaram-se mais suceptíveis que epimastigotas frente a ação de DAs (e análogos).

- O estudo realizado com onze DAs recém sintetizadas e que apresentam fluorescência intrínsica revelou que todos os compostos se localizam no núcleo e preferencialmente na mitocôndria do *T. cruzi*, sendo os mais ativos (compostos 5 e 7) capazes de induzir importantes danos mitocondriais, especialmente no kDNA observados por ensaios de microscopia eletrônica de transmissão (MET). No entanto, não houve correlação entre atividade tripanocida dos compostos e sua localização preferencial no kDNA, confirmando dados anteriores realizados em *T. brucei*.

- Ensaios termodinâmicos (Temperatura de *melting* – T_m - e dicroismo circular - DiC) sobre a interação de compostos aromáticos com kDNA isolado de epimastigotas de *T. cruzi* e com uma sequência conservada de 22-mer presente em minicírculos revelaram que a forte interação de algumas amidinas com a fenda menor do kDNA não é fator determinante para desencadear a atividade tripanocida, haja vista que alguns dos compostos mais efetivos (ex. DB1831) apresentaram menores índices de associação ao kDNA. Estes dados sugerem que outros mecanismos de ação

possam estar associados ao efeito direto dos compostos amidínicos sobre o kDNA do *T. cruzi*.

- Compostos da classe das AIA´s, em geral, quando testadas sobre a sequência conservada de minicírculos e sobre a rede intacta de kDNA purificada apresentaram menor afinidade porém maior capacidade de induzir alterações topológicas no DNA, como vistos por DiC, o que pode explicar sua superior atividade.

- A mobilidade eletroforética de fragmentos de kDNA, resultantes da digestão pelas endonucleases *EcoRI* e *CvQI*, incubados com 10 compostos amidínicos revelou que a natureza da seqüência dos fragmentos interfere no tipo de efeito causado pelos compostos (efeitos de *bending* ou *straightening*).

- Análises por microscopia de fluorescência (MF) revelaram que diamidinas como a DB75 são capazes de induzir consideráveis alterações na estrutura do kDNA, evidenciadas pela aumento da área da rede (até 11 vezes) mesmo na presença de inibidores de proteases e de topoisomerases do tipo II. Estes dados somados aos dados de MET, DiC e T_m sugerem que um dos mecanismos de ação envolvidos na atividade de diamidinas em *T. cruzi* possa estar relacionado com a associação destes compostos ao kDNA, induzindo alterações em sua topologia, resultando na desestabilização desta estrutura e mesmo remoção da curvatura de alguns minicírculos, culminando na morte dos parasitos.

- O conjunto de nossos dados revela que diamidinas e congêneres sugere que o kDNA representa um dos principais alvos destes compostos aromáticos. Entretanto, a falta de correlação entre a localização dos compostos, sua afinidade e alterações topológicas induzidas nesta estrutura com a atividade tripanocida também sugere fortemente que outros fatores podem estar envolvidos operando de modo primário ou secundário a interação do composto ao kDNA do parasito.

- Outros estudos serão necessários para melhor identificar os mecanismos envolvidos na ação desta classe de compostos, visando contribuir para o desenho racional de novas terapias para doenças causadas por tripanosomatídeos, como a doença de Chagas.

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ANEXO 1

Durante a realização desta tese participei também das seguintes publicações:

Capítulo de livro:

Soeiro NMC, Daliry A, Silva CF, Batista DGJ, De Souza EM, Oliveira GM, Salomão K, Menna-Barreto RFS, De Castro SL. Electron microscopy approaches for the investigation of the cellular targets of trypanocidal agents in *Trypanosoma cruzi*. *In*: Microscopy: Science, Technology, Applications and Education", Microscopy Book Series # 4, A. Méndez-Vilas A, Díaz J (Eds.), Formatex, Badajoz, Spain (2010).

Electron microscopy approaches for the investigation of the cellular targets of trypanocidal agents in *Trypanosoma cruzi*

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Chagas´ disease (CD), which is caused by the protozoan intracellular parasite *Trypanosoma cruzi*, affects at least 12 million people in the poorest areas of Latin America. Although it is the leading cause of infectious cardiomyopathy, the existing drug therapy for CD, which is based on two nitroheterocyclic compounds (Benznidazole and Nifurtimox), remains inadequate due to toxicity and low cure rates, particularly during the chronic stage. In fact, a complete lack of pharmaceutical company interest for developing new compounds against *T. cruzi* makes this tropical parasitic infection one of the major "neglected" diseases of the world. In this context, with the collaboration of different groups of medicinal chemistry, we have been performing pre-clinical experimental studies to determine the efficacy, toxicity and selectivity of different natural and synthetic compounds on *T. cruzi*. In addition, to explore the mechanism of action of the most effective compounds, scanning and transmission electron microscopy associated with biochemical analyses have been employed to identify cellular targets in the treated parasites exposed to trypanocidal agents. In this review, we summarise the most important data obtained by these electron microscopy approaches and discuss the use of Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) as valuable tools in chemotherapy studies.

Keywords chemotherapy; *Trypanosoma cruzi*; Chagas disease; electron microscopy

1. State of the Art

Chagas' disease (CD), which is caused by the intracellular parasite *Trypanosoma cruzi,* has high rates of mortality and morbidity in very poor areas of Latin America and also causes important economic losses in these developing countries (WHO, 2002). This neglected illness has two successive phases: a short acute phase characterised by patent parasitemia followed by a long, progressive chronic phase [1-3]. Due to this asymptomatic state, CD is often considered a "silent killer", impairing early specific diagnosis and treatment [4]. The main clinical manifestations of the chronic stage include cardiac and/or digestive alterations, and current evidence suggests that the pathogenesis is a consequence of a sustained inflammatory process, with anti-parasitic and/or anti-self-immune responses, that is associated with low-grade persistent parasitism [5,6]. CD is transmitted through the faeces of sucking triatominae insects (90%), congenitally, by blood transfusion, organ transplantation or oral contamination and through laboratory accidents [7]. Vectorial and transfusional transmissions have sharply declined in the past two decades mainly due to the policies of the Southern Cone countries, but several challenges still remain, particularly with respect to sustainable disease control by public policies in endemic areas [8,9].

The life cycle of *T. cruzi* involves a hematophagous triatomine insect, vertebrate hosts and different forms of the parasite. Briefly, a bloodstream trypomastigote ingested by the insect differentiates into an epimastigote, which proliferates and then differentiates into the metacyclic form in the posterior intestine. This infective form invades the vertebrate cell and undergoes differentiation into the intracellular amastigote, which proliferates and then transforms back into the trypomastigote, the main form that disseminates the infection.

Nifurtimox (5-nitrofuran-(3-methyl-4-(5'-nitrofurfurylideneamine) tetrahydro-4H-1,4-tiazine-1,1-dioxide) and benznidazole (2-nitroimidazole (N-benzyl-2-nitroimidazole acetamide - Bz), both developed empirically more than four decades ago, represent the current available therapy for CD [10,11]. Both drugs have severe limitations including long periods of treatment, side effects, variable results and low efficacy during the chronic phase, demonstrating the urgent need for new trypanocidal agents [12,13]. Although many pre-clinical studies have been performed in the last three decades and some very promising targets have been identified, only allopurinol, itraconazole and fluconazole have moved to CD clinical trials [14]. This reflects, at least in part, the lack of standardised screening protocols coupled with the absence of well-designed flow charts that allow an evaluation of the efficacy *versus* the safety of novel (natural or synthetic) anti-*T. cruzi* compounds in an optimised, safe and reproducible manner. Recently, due to an interdisciplinary meeting organised by Fiocruz and DNDi, a technical note describing the minimum criteria and decision gates related to drug screening for CD was reported, aiming to guarantee the reliability of the results while also taking into account the relevant aspects of the parasite biology and its pathogenicity to the mammalian hosts [15].

The ideal drug for CD etiological treatment should fulfil at least the following requirements: (i) orally effectiveness against the acute and chronic phases of infection, using a single or a few doses (treatment regimen under 60 days); (ii) non-toxic to the patients; (iii) availability in a paediatric formulation; (iv) activity against a broad panel of *T. cruzi*

stocks and lineages and (v) activity against the two parasitic forms found in mammalian hosts - bloodstream trypomastigotes, thus inhibiting the invasion of new host cells, and intracellularly dividing amastigotes, thus preventing the release of new infective parasites [16]. We will briefly summarise some results from our group with novel promising anti-*T. cruzi* agents obtained through pre-clinical assays that have been designed to address most of the above requirements, also considering the complexity of the parasite biology as described above. Our group has employed high stringency protocols, evaluating the toxicity on mammalian cells, the efficacy against the amastigote and trypomastigote forms and the efficacy against different parasite strains (including Bz-susceptible and -resistant stocks) as well as *in vivo* protocols using short schemes of treatment (up to 20 days) and different routes of administration.

As previously mentioned by De Souza [17], *T. cruzi* was one of the first cells to be examined by Electron Microscopy (EM) and has continued to be so by almost all EM techniques developed over the last 50 years, all of which have brought to light the main structures and organelles found in the different forms of the parasite (Fig. 1). Electron microscopy also represents a useful tool in chemotherapy studies at distinct levels, allowing the identification of subcellular targets in the parasites and corroborating with biochemical studies to elucidate the mechanism of action, as inferred by drug-induced alterations. Transmission electron microscopy (TEM) enables the analysis of thin sections usually obtained from resin-embedded samples and represents the most general approach for studying the structural organisation and sub-cellular damages in drug-treated parasites and host cells. On the other hand, scanning electron microscopy (SEM) enables the analysis of the surface and topography of the whole parasite. Any important alteration induced by a trypanocidal compound at the intracellular and/or membrane levels reveals a compromised structural organisation possibly due to a primary and/or secondary effect upon its components (lipids, carbohydrates, proteins, nucleic acid elements) and consequently upon the functional and biological properties of the parasite. Organelles as well as cell surface alterations detectable by this technique may be indicative of indirect cellular damage triggered by factors such as drug- induced oxidative stress. Additionally, both TEM and SEM approaches provide important information related to programmed cell death (PCD) and the identification of the cell death mechanisms elicited by a drug (i.e., apoptosis, autophagy or necrosis) [18,19].

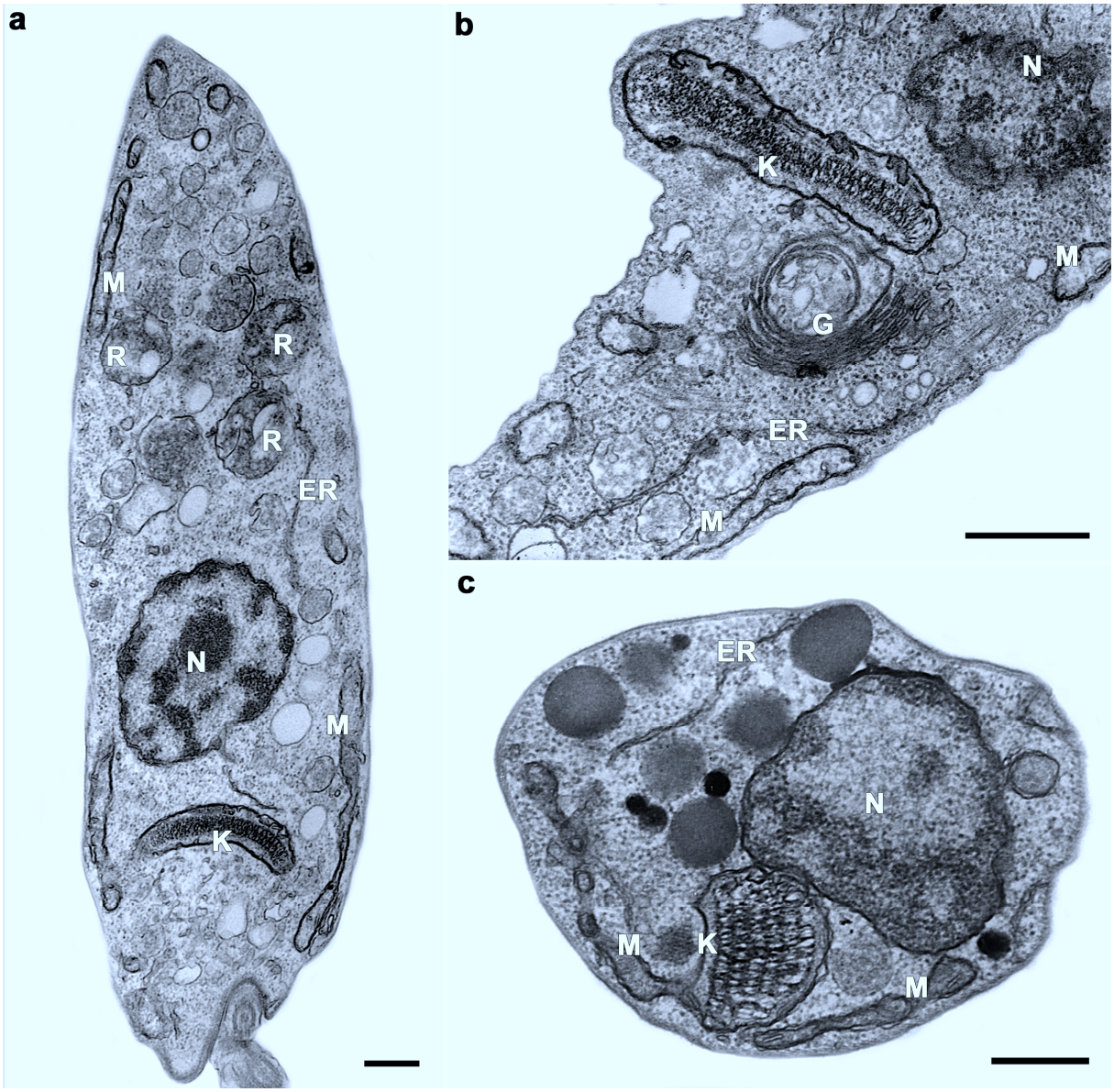

Fig. 1 Ultrastructural analysis of control *T. cruzi* epimastigotes (a,b) and trypomastigote (c). Parasites presented a typical morphology with mitochondrion (M), endoplasmic reticulum (ER), nucleus (N), reservosomes (R), kinetoplast (K) and Golgi (G). Bars = 0.5 µm.

2. Aromatic diamidines and related compounds

One class of synthetic compounds that has been investigated by our group is the aromatic diamidines (AD). ADs are highly effective against different pathogens but often present undesirable side effects and have limited oral bioavailability [20]. To overcome these limitations, different analogues and prodrugs have been developed, including arylimidamides (AIA) that display high efficacy against trypanosomatids such as *T. cruzi* [21] and *Leishmania sp* [22].

In our efforts to identify promising anti-*T. cruzi* agents from a library of ADs and their analogues, 3-bromo-4-methyl-2,5-bis(4-amidinophenyl)thiophene (DB1362) was selected due to its dose-dependent *in vitro* effect upon bloodstream trypomastigotes (IC_{50}/24 h =f 6.6 µM) and upon intracellular amastigotes (IC_{50}/48 h = 0.62 µM). This diamidine partially protected against parasitemia and reduced mortality levels in an experimental murine model of acute infection [23]. The analysis by TEM showed that the DB1362 treatment of trypomastigotes induced profound

alterations in the kinetoplast organisation and in the nucleus. Such ultrastructural findings were confirmed by flow cytometry analysis, which demonstrated an increase in the number of parasites that displayed interferences in the proton electrochemical potential gradient of the mitochondrial membrane [23].

The analysis of the effect of DB569, a phenyl substitute of furamidine (DB75), showed dose- and time-dependent activity against both the bloodstream and amastigote forms of different strains of *T. cruzi* [24]. DB569 also significantly reduced the cardiac parasitism, partially increased mouse survival rates and lowered the levels of alanine aminotransferase and creatinine, which is indicative of a protective role against renal and hepatic lesions caused by the infection [25]. DB569 also protected infected mice from electric alterations due to infection, and this protection could be correlated with a possible modulation of the immune response because this AD induced the decrease of CD8+ T cell levels within the inflamed heart [26]. Fluorescent microscopy analysis associated with TEM studies showed that treatment with DB75 and DB569 leads to the accumulation of both ADs in the nucleus and kinetoplast, inducing important alterations in both structures, where the parasite DNA is localised [24]. In treated parasites, the most striking EM findings included alterations in the nuclei morphology, mitochondrial enlargement and a final fragmentation of the kinetoplast into condensed bodies (Fig. 2). These ultrastructural alterations are directly reminiscent of those previously reported with pentamidine and its analogues for treatments *in vitro* of *L. (L) amazonensis* [27] and *L. tropica* [28] and *in vivo* of *L. donovani* and *L. major* in mouse models [29].

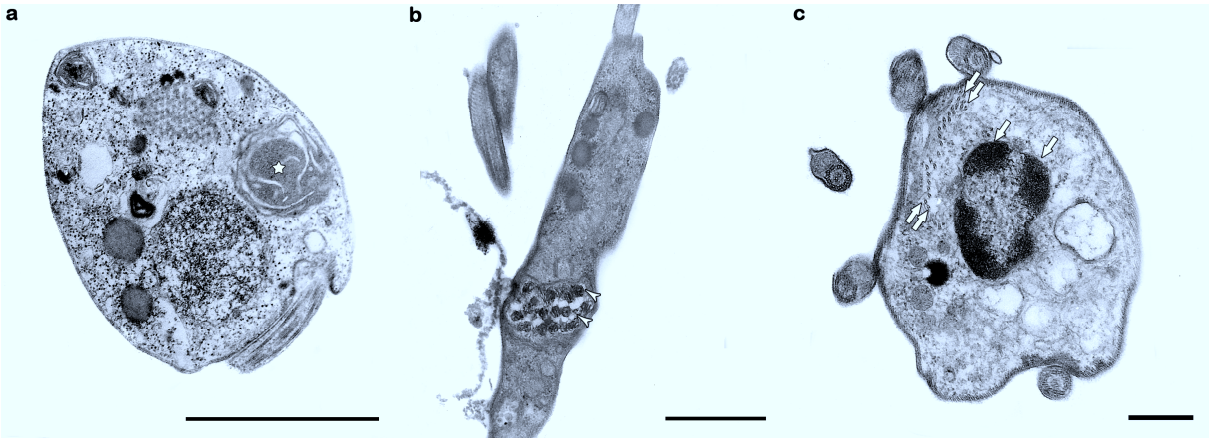

Fig. 2 Ultrastructural effects of aromatic diamidines in *T. cruzi* trypomastigotes. Treatment with 4 µM DB569 (a) and 16 µM DB75 (b,c) resulted in the appearance of concentric membranar structures in the cytosol (star), kDNA disruption (arrowheads) with abnormal chromatin condensation (arrows) and alterations of the subpellicular microtubule organization (double arrows). Bars = 1 µm.

Recently, we evaluated the *in vitro* efficacy of six dicationic compounds (DB1645, DB1582, DB1651, DB1646, DB1670 and DB1627) against trypomastigotes and intracellular amastigotes lodged within cardiac cell cultures. The most active ADs against both parasitic forms were DB1645, DB1582 and DB1651, which displayed micromolar IC_{50} values ranging from 0.15 to 13.3 µM, and all compounds displayed low toxicity towards mammalian cells, with LC_{50} values higher than 96 µM [21]. Due to their intrinsic fluorescent characteristics, the localisation of these compounds was possible, demonstrating that they also accumulated in the parasite nuclei and kDNA, with a higher concentration in the latter structure. Interestingly, DB1582 and DB1651 also localised to punctated non-DNA-containing cytoplasmic organelles - possibly acidocalcisomes - that could act as cellular targets of storage sites of these compounds as previously reported for African trypanosomes [30]. TEM revealed that these compounds caused striking alterations in the mitochondria such as swelling and the presence of many membranous structures. These diamidines provoked an intense kDNA network disruption leading to its total fragmentation and disappearance in addition to an important disorganisation in the plasma membrane with bleb formation, the appearance of concentric membranes between the cytoplasm and plasmalemma unit and its complete dissociation [21]. Striking alterations could also be noticed in sub-pellicular (Fig. 2C) and flagellar microtubules of the parasite under the action of diamidine compounds. Multiple axoneme profiles besides interference in the organisation of subpellicular microtubules were found in non-dividing bloodstream parasites incubated with the diamidines DB1582, DB1651 and DB569 without any evidence of other duplicated organelles [21,24]. Some of these uncommon cytoskeleton alterations were also noticed in *T. cruzi* treated with some AIAs such as DB889 [31]. These compounds have been shown to present superior efficacy upon *T. cruzi* as compared to ADs [32,33]. Interestingly, in trypomastigotes treated by the AIA DB889, multiple axoneme structures (flagellar microtubules) were noted. TEM analysis also showed that this drug induced alterations in nuclear

morphology, dilatations of the endoplasmic reticulum and Golgi structures and consistent damage to the mitochondria and kinetoplasts of the parasites. Flow cytometry analysis confirmed that the treated parasites presented an important loss of the mitochondrial membrane potential as revealed by a decrease in Rh123 fluorescence [31].

A novel synthesised AIA, DB766, exhibited strong trypanocidal activity (in a nanomolar range) and excellent selectivity against trypomastigotes and intracellular amastigotes lodged in the cytoplasm of cardiac cells *in vitro* [21]. This AIA also exhibited striking effects upon a large panel of *T. cruzi* strains including some of those naturally resistant to Bz and Nifurtimox (e.g., Colombiana and YuYu), showing higher efficacy *in vitro* than the reference drug (Bz). In addition, DB766 was active against diverse parasite isolates that circulate in peridomiciliar and sylvatic ecotopes from two different regions of Brazil: Ceará State and the Amazon region. DB766, similar to other AIAs, displays a high activity at 4°C in the presence of 96% mouse blood, an experimental condition that evaluates the potential use of a trypanocidal compound in blood banks, which represents another current urgent need. This AIA exhibited an activity 30.6-fold higher than that of gentian violet, a reference drug for blood therapy in CD [21], and thus, our data suggest the potential use of this class of compounds in the prophylaxis of banked blood. Currently, *in vivo* experiments are under way to confirm this hypothesis. Next, as no major acute toxicity was noted in uninfected mice treated with DB766 and due to its high selectivity index (>533), we moved to models of experimental *T. cruzi* infection. This new AIA effectively reduced the parasite load in the blood and in cardiac tissues (80-99% reduction, depending on the therapy scheme and the non-toxic doses that have been used), displaying similar efficacy to that of Bz in mouse models of infection employing Y (moderately resistant) and Colombiana (highly naturally resistant) strains, using oral and intraperitoneal doses up to 100 mg/kg/day given after the infection was established. DB766 ameliorates heart electric alterations and reduces hepatic and heart lesions induced by the infection as well as provides 90-100% protection against mortality, similar to the results seen with Bz. The effectiveness of this AIA in eradicating intracellular parasites (as noticed in cardiac cells during *in vitro* and *in vivo* infections) may be related to its pharmacokinetic properties that include a long half-life and a large volume of distribution [22]. Again, the PK characteristics of DB766 are particularly relevant given that the poor activity of the nitro-heterocyclic compounds (such as Bz and Nifurtimox) during the chronic stages of CD has been attributed, at least in part, to their unfavourable pharmacokinetic properties, such as relatively short half-lives and limited tissue penetration [14]. Although it is not a prodrug, DB766 showed good activity upon oral administration (dose of 100 mg/kg/day). Oral efficacy is an exciting finding because this represents a highly desirable route to treat CD patients, without the need for hospitalisation, which can potentially provide a convenient and economical treatment for neglected tropical diseases. This arylimidamide accumulated in the nuclei and kDNA of the parasites, with a higher concentration within the kDNA as compared with the parasite nuclei [21]. TEM studies performed with bloodstream trypomastigotes showed that parasites treated for 2 and 24 h presented consistent ultrastructural alterations of the nucleus and mitochondrion, with considerable mitochondrial swelling and cristae dilation. These results suggest that DB766 acts primarily or secondarily on the mitochondria-kinetoplast complex, although other cellular targets cannot be excluded and further biochemical studies are needed. Aromatic dicationic compounds such as pentamidine bind in a non-covalent and non-intercalative manner to the minor-groove of the DNA. The exact mode(s) of action of Ads, as well as AIAs, against trypanosomatids is still unclear, although it has been proposed that multiple modes of action are operative [34]. One of the long-hypothesised mechanisms of action of diamidines is related to their ability to bind AT-rich regions of the DNA minor groove, but other mechanisms have been proposed such as inhibition of tyrosyl-DNA phosphodiesterase, topoisomerases, protein kinase A, proteases and polymerases [35,36]. Thus, ADs possibly interfere in kinetoplast function through selective association with the unique AT rich regions of the kinetoplastid minicircle kDNA, perhaps involving DNA-processing enzymes [37]. Another interesting feature was the cytoskeleton alteration induced by ADs and AIAs. Different studies have been conducted using drugs that target microtubules such as taxol, colchicine and vinblastine, but no major alterations were noticed in both subpellicular and flagellar microtubules of *T. cruzi*. This observation is possibly due to the high content of acetylated tubulin and/or poly-glutamylation of tubulin [38,39]. Given that these structures are very resistant to microtubule disrupters in trypanosomatids as compared with mammalian cells, our findings related to the damages on the *T. cruzi* cytoskeleton induced by AIAs and ADs are very peculiar as these structures 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 [21].

In summary, our data clearly shows the trypanocidal efficacy of ADs, especially AIAs such as DB766, suggesting that these compound classes may contain promising drug candidates, validating the further exploration of others AIAs as new potential agents for CD therapy.

3. Derivatives of naphthoquinones

Naphthoquinones are present in various plant families and are considered privileged structures in medicinal chemistry due to their structural properties, participation in biological oxidative processes, biological activities and multiple roles in organisms [40,41]. Some of the bioactive quinones are lapachol, α-lapachone and β-lapachone, originally isolated from the heartwood of trees of the Bignoniaceae family (*Tabebuia* sp). These naphthoquinones presented anti-tumoural, anti-inflammatory and anti-viral biological activities as well as against pathogenic microorganisms [42,43]. Their

antiprotozoal activities have been reported, and several of them have been identified as possible leads for drug development [44-46]. The activity of β-lapachone against *T. cruzi* is related to free radical generation [47-49]. From a screening of sixty derivatives of these naphthoquinones, three naphthoimidazoles derived from β-lapachone (N1, N2 and N3) were found to be the most active against *T. cruzi* trypomastigotes [50-54]. Subsequent studies demonstrated that these three compounds were also active against epimastigotes and intracellular amastigotes. The ultrastructural effects of these three naphthoimidazoles were studied by TEM, with the mitochondrion, DNA and reservosomes being their main targets. The mitochondrion of treated parasites was remarkably swollen, with the disappearance of inner cristae and formation of concentric membranar structures inside the organelle. By flow cytometry, the collapse of the hydrogenionic potential was observed in Rh123-labelled epimastigotes and trypomastigotes [55,56]. N1, N2 and N3 also led to kDNA network disruption and abnormal chromatin condensation. Interestingly, treated trypomastigotes showed an intense DNA fragmentation as seen by electrophoresis and TUNEL techniques that was not observed in untreated trypomastigotes [56]. The remarkable blebbing formation in the trypomastigotes pointed to the plasma membrane as an important target of the naphthoimidazoles [19,57] (Fig. 3). Interestingly, plasma membrane ruffling and blebbing were also reported after treatment of different microorganisms with drugs. In *T. cruzi*, the lysophospholipid analogues edelfosine, miltefosine and ilmofosine induced blebbing and ruffling of the membrane, and such alterations were associated with interference of the phospholipid content [58,59].

In epimastigotes, two other organelles were affected by the naphthoimidazoles: the Golgi complex, especially in *Trans* Golgi Network cisternae, and the reservosome, which presented a severe disorganisation (Fig. 4). The damage on the reservosomes was reinforced by flow cytometry and fluorescence microscopy data showing decreased labelling of acridine orange, a marker of acidic compartments [60]. Reservosomes represent a crucial energy source for metacyclogenesis and are enriched in cysteine proteases [61,62]. The observed blockage of this process by the three naphthoimidazoles could be a consequence of the reservosome disruption, leading to the release of its proteases into cytosol and subsequent random proteolysis, which consequently culminates in the death of the parasite. The appearance of endoplasmic reticulum profiles surrounding organelles, including the reservosomes, was also observed in treated parasites, a morphological indicator of autophagic processes. Autophagy was reported as the most prominent phenotype associated with naphthoimidazole treatments. These ultrastructural evidences together with a strong increase in the labelling of the well-known autophagic marker monodansyl cadaverine, the complete abolishment of the activity of N1, N2 and N3 by wortmannin and 3-methyladenine and the overexpression of ATG genes in treated epimastigotes corroborates this hypothesis [57].

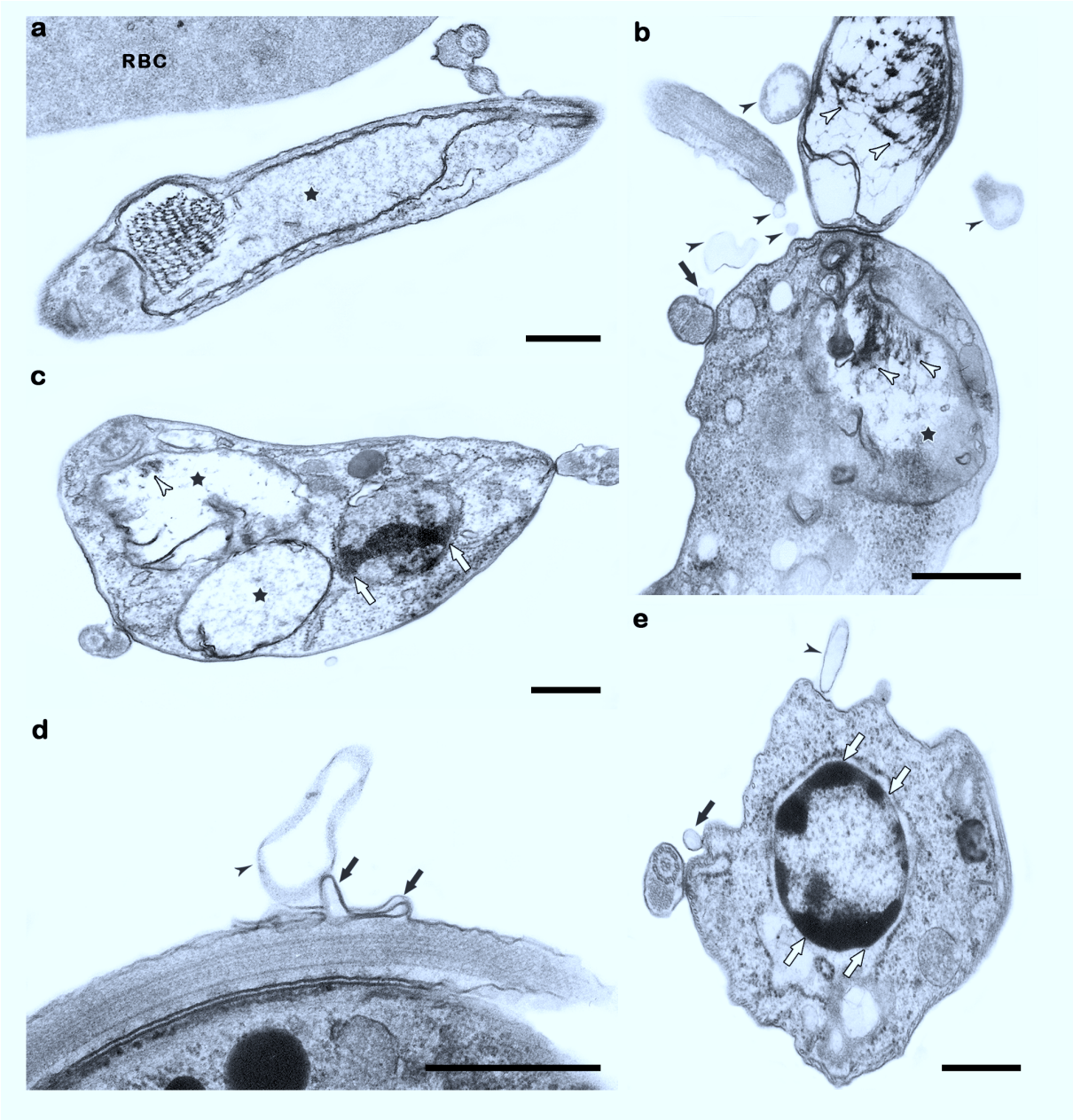

Fig. 3 Ultrastructural effects of the naphthoimidazoles in *T. cruzi* trypomastigotes. Treatment with 10 µM N2 (a-c) and 10 µM N3 (d,e) resulted in mitochondrial swelling (stars), kDNA disruption (white arrowheads), the formation of plasma membrane blebs (black arrows), abnormal chromatin condensation (white arrows) and the appearance of membranar vesicles outside the parasites (black arrowheads). Bars = 0.5 µm.

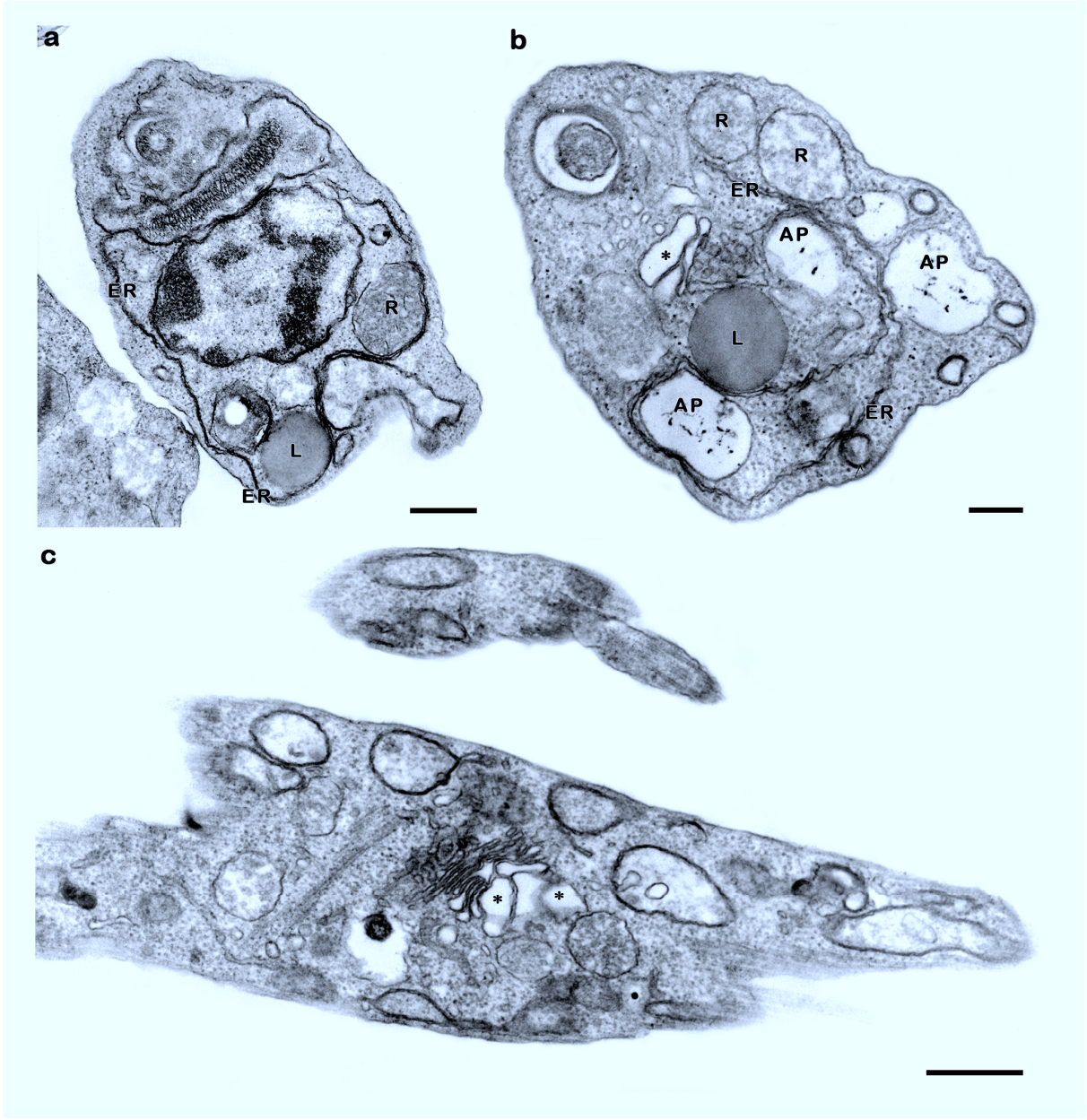

Fig. 4 Ultrastructural effects of the naphthoimidazoles in *T. cruzi* epimastigotes. Treatment with 20 µM N2 (a,b) and 20 µM N3 (c) resulted in the appearance of endoplasmic reticulum (ER) surrounding organelles and cytosolic structures such as reservosomes (R), autophagosome-like (AP) and lipid droplets (L). The treatment with the compounds also induced an extensive disruption of Golgi cisternae (asterisks) in the epimastigotes. Bars = 0.5 µm.

The mitochondrion was one of the main targets of three naphthofuranquinones synthesised from C-allyl lawsone [63]. The evaluation by TEM of treated epimastigotes and trypomastigotes demonstrated severe mitochondrial damage, with loss of the internal membrane, formation of concentric membranar structures and organelle swelling. In trypomastigotes, the three compounds also led to an increase in the number of cytoplasmic vacuoles, intense nuclear envelope dilatation and plasma membrane rupture. By flow cytometry, the high percentage of parasites labelled with propidium iodide (PI) confirmed the plasma membrane damage and the decrease in Rh123 fluorescence pointed to mitochondrial membrane depolarisation. The activities of the respiratory chain complexes I-III were partially blocked by treatment with the naphthofuranquinones, suggesting an impairment of electron transport that leads to a decrease in the respiratory rates of epimastigotes and trypomastigotes. In epimastigotes, treatment with these quinones was accompanied by an important increase in H_2O_2 production and a decrease in succinate-induced oxygen consumption

[64]. The formation of atypical membranar structures in both forms of the parasite was also observed, suggesting the involvement of autophagy in the mode of action of these naphthofuranquinones.

4. Final Comments

Even more than one century after the discovery of Chagas' disease by Carlos Chagas, this silent and neglected disease still poses critical challenges, including a more efficient and less toxic chemotherapy. This situation highlights the urgency in the search and identification of new trypanocidal compounds [15]. In this framework, interdisciplinary approaches have been performed to improve high-throughput screening of new compounds in a rational development, involving bioinformatics, proteomics, genomics and combinatorial chemistry as well as pharmacology and toxicology, all aiming to find an alternative treatment for chagasic patients.

In this regard, biochemical, molecular and morphological studies related to the identification of the mechanisms of action of anti-*T. cruzi* agents are indisputable as they can provide additional information to determine and select the compounds most active against the parasite and least toxic to the mammalian hosts as well as provide data for the synthesis and/or derivatisation of novel analogues. According to morphological studies, recent reviews focusing on the ultrastructural aspects of protozoa highlighted the interference in the ergosterol and phospholipids biosynthesis processes by new promissory anti-*T. cruzi* agents [19,65-67]. Other specific organelles and structures (see scheme 1), due to their intrinsic characteristics, are also promising targets for drug development:

DNA-enriched compartments: In the nucleus of eukaryotic cells, complexes of genomic DNA with architectural histones participate in DNA packaging to form chromosomes and to regulate gene expression. The size of the *T. cruzi* genome is 60.3 Mb, contained in 41 small chromosomes [68]. This parasite displays a particular regulation of gene expression because they do not use transcription initiation, resulting in the clustering of all protein-encoding genes in large polycistronic transcription units and the production of RNA that will be processed by trans-splicing. The exact proteins that participate in these processes are unclear [69]. Despite the conservation of some motifs and residues, trypanosomatid histones contain important modifications in their structures that provide docking sites for regulatory proteins involved in DNA replication, repair, recombination and transcription [70]. These crucial differences in the gene regulation machinery also offer another attractive point for drug intervention. In this aspect, compounds that interfere in nucleic acid composition, stabilisation and organisation are promising agents. Presently, our data indicate that AD and naphthoquinone derivatives interact with *T. cruzi* DNA, promoting a remarkable chromatin condensation (Fig. 5A) and fragmentation [25,55,56] and in some cases, leading to nuclear envelope dilation [64]. Another DNA-enriched compartment is the mitochondrion. In trypanosomatids, the single mitochondrion is a specialised organelle with unusual features such as the presence of alternative oxidases [71] and deficiency in reactive oxygen species detoxification [72]. Such peculiarities also make the mitochondrion an attractive target for therapeutic drugs to treat Chagas´ disease. Morphological alterations in the *T. cruzi* mitochondrion are well-described in parasites treated with a wide array of compounds [65-67]. Treatment with aromatic diamidines and naphthoquinone derivatives induced a severe mitochondrial swelling with the appearance of concentric membranar structures in the organelle (Fig. 5B) with a loss of functionality confirmed by flow cytometry, fluorescence microscopy and biochemical approaches [21,23,24,31,55,56,64]. It is important to keep in mind, however, that the mitochondrial alterations induced by a pharmacological stimulus may be due either to a primary effect directly on this organelle or secondary lesions caused by loss of cellular viability triggered by another cell component or metabolic pathway. A third DNA-enriched compartment is the kinetoplast of Kinetoplastida, a mitochondrial region rich in DNA that contains over 20% of the total parasite genome. kDNA comprises two circular, catenated structures called maxicircles and minicircles [75]. The maxicircles encode ribosomal RNAs and some mitochondrial proteins, similar to the mDNA of other eukaryotes, while minicircles are involved in RNA guide production, which is fundamental for successful maxicircle transcription [76]. The high percentage of AT sequences found in minicircles represents an extraordinary alternative for the drug targeting [35]. Different compounds cause *T. cruzi* kinetoplast disorganisation, such as topoisomerases II inhibitors and geranylgeraniol [77,78]. As mentioned above, trypomastigotes treated with diamidines and naphthoimidazoles showed strong kDNA disruption (Fig. 5D), suggesting a selective effect of these compounds, at doses that do not affect mammalian host cell viability.

Plasma membrane: The plasma membrane surrounds all cells and controls the entry of macromolecules. Its composition is widely variable, especially with respect to the membrane proteins responsible for essential cellular processes such as ion exchange, cell adhesion and cell signalling. Among the plasma membrane constituents, sterols have a critical role in the maintenance of its functional and structural homeostasis. In mammalian cells, cholesterol is the main sterol; however in trypanosomatids, sterol biosynthesis involves ergosterol production, pointing to this pathway as a potential drug target [67]. As noticed with fungi, *T. cruzi* synthesises ergosterol but not cholesterol [73]. In this context, the latter steps of sterol biosynthesis, which diverge from cholesterol synthesis in mammals, represent one of the most studied targets for Chagas disease chemotherapy, with many studies conducted by the group of Urbina in Venezuela [74]. Previous reports showed that different classes of compounds induce *T. cruzi* plasma membrane alterations associated with a deregulation of the lipid content [58,59,67]. Herein, it was reviewed that the

naphthoimidazoles N1, N2 and N3 induced extensive plasma membrane ruffling and blebbing (Fig. 5C) in the bloodstream forms of *T. cruzi* [19,57].

Cytoskeletal elements: The microtubules in trypanosomes are the main component of the flagellar axoneme and of the subpellicular microtubule corset, with the relative positions of each determining the morphology of each stage of the life cycle of these parasites [79]. All members of the Trypanosomatidae family display a flagellum that emerges from the flagellar pocket, showing a basic structure of 9 + 2 axonemal microtubules [80]. Alterations in the structure and organisation of microtubules are not a common event in these parasites, even after treatment with different classes of drugs. The development of strategies that interfere with these important structures also represents a potentially valuable new chemotherapeutic approach. Presently, our EM findings indicate that aromatic diamidines and some related compounds (such as the arylimidamide DB889) are able to alter the organisation of *T. cruzi* microtubules, provoking a partial loss or total disorganisation of the subpellicular microtubules besides in addition to an unusual organisation of multiple flagellum in the trypomastigote forms, which are non-proliferative [21,32]. Thus, these structures also represent interesting targets, justifying further studies on this subject.

In summary, we briefly reviewed some of the potential cellular targets of aromatic diamidines and their analogues as well as of naphthoquinone derivatives demonstrated by TEM and SEM studies. These results clearly indicate that both approaches represent valuable tools to determine the nature of the lesions triggered by these drugs and deduce their possible modes of action. These modes of action must be further explored and confirmed by biochemical and molecular analyses, some of which are currently underway in our laboratory.

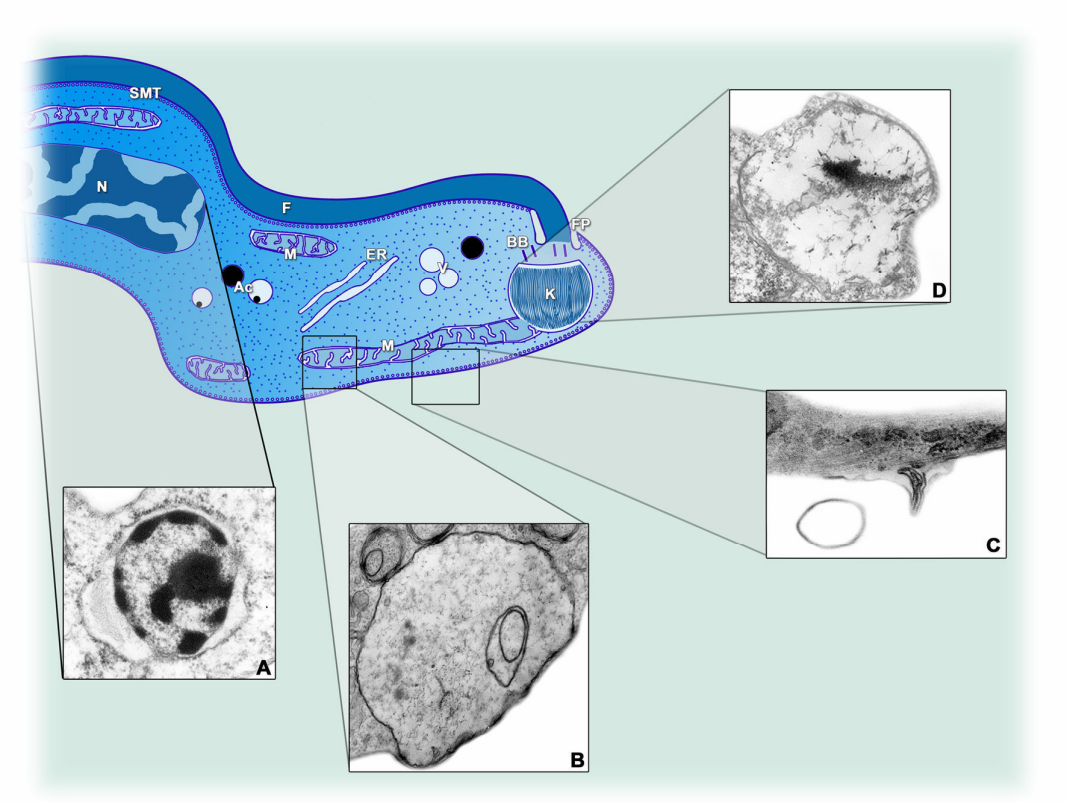

Fig. 5 Schematic view of potential targets of diamidines and naphthoquinone derivatives in the *T. cruzi* trypomastigote form. In detail, the most common organelle injuries are the following: (A) abnormal chromatin condensation and nuclear envelope dilation; (B) mitochondrial swelling, with concentric membranar structures inside the organelle; (C) bleb formation with an outside membranar vesicle, suggesting plasma membrane shedding and (D) extensive kDNA disruption. M, mitochondrion; ER, endoplasmic reticulum; N, nucleus; K, kinetoplast; V, vacuoles; Ac, acidocalcisomes; SMT, subpellicular microtubules; BB, basal bodies; F, flagellum; FP, flagellar pocket.

Acknowledgments Sponsored by CNPq, Faperj, Fiocruz, CPDD, CAPES and PAPES V.

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ANEXO 2

Artigo Publicado

Silva CF, Silva PB, Batista MM, Daliry A, Tidwell RR, Soeiro MNC. The biological *in vitro* effect and selectivity of aromatic dicationic compounds on *Trypanosoma cruzi*. Mem Inst Oswaldo Cruz, 2010; 105:239-45.

The biological in vitro effect and selectivity of aromatic dicationic compounds on *Trypanosoma cruzi*

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Trypanosoma cruzi *is a parasite that causes Chagas disease, which affects millions of individuals in endemic areas of Latin America. One hundred years after the discovery of Chagas disease, it is still considered a neglected illness because the available drugs are unsatisfactory. Aromatic compounds represent an important class of DNA minor groove-binding ligands that exhibit potent antimicrobial activity. This study focused on the in vitro activity of 10 aromatic dicationic compounds against bloodstream trypomastigotes and intracellular forms of* T. cruzi. *Our data demonstrated that these compounds display trypanocidal effects against both forms of the parasite and that seven out of the 10 compounds presented higher anti-parasitic activity against intracellular parasites compared with the bloodstream forms. Additional assays to determine the potential toxicity to mammalian cells showed that the majority of the dicationic compounds did not considerably decrease cellular viability. Fluorescent microscopy analysis demonstrated that although all compounds were localised to a greater extent within the kinetoplast than the nucleus, no correlation could be found between compound activity and kDNA accumulation. The present results stimulate further investigations of this class of compounds for the rational design of new chemotherapeutic agents for Chagas disease.*

Key words: aromatic compounds - *Trypanosoma cruzi* - chemotherapy - Chagas disease

Chagas disease is a neglected tropical illness caused by the protozoan *Trypanosoma cruzi*. Although Carlos Chagas described it 100 years ago (1909), it is still an important public health problem in Latin America (Rocha et al. 2007). The main clinical symptoms of Chagas disease are cardiac and/or digestive alterations and the overall prevalence of the disease is about 12-14 million cases, which makes it the major cause of cardiac infectious disease in endemic areas (Stewart et al. 2005, Dias 2007). In addition, despite fruitful attempts to control vectorial and blood transmission, Chagas disease still lacks prophylactic therapies and effective chemotherapeutic schemes (Rodrigues Coura & De Castro 2002, Dias 2007). Nifurtimox and benznidazole are used for the treatment of Chagas disease (Urbina 2002); although they are effective for the treatment of acute infections, they present moderate activity, exhibit undesirable side effects and require long dosing schedules for chronic infections, which frequently necessitate the cessation of treatment (Jannin & Villa 2007, Soeiro et al. 2009). In addition, the pharmaceutical industries have given little attention to the design and development of new anti-parasitic compounds aromatic dicationic compounds represent a class of DNA minor-groove binding ligands that exhibit high activity against a variety of pathogens, such as bacteria, fungi and protozoa (Werbovetz 2006, Wilson et al. 2008). Recent data showed that diamidines and related compounds, such as the reversed amidines, present considerable efficacy against *T. cruzi* both in vitro (De Souza et al. 2004, Silva et al. 2007a) and in vivo (De Souza et al. 2006a, da Silva et al. 2008) and induce striking alterations on the parasite mitochondrion-kinetoplast complex (De Souza et al. 2006b, Silva et al. 2007b). In this context, the present study investigated the activity of 10 newly synthesised aromatic dicationic compounds on trypomastigotes and intracellular amastigotes, the clinically relevant forms of *T. cruzi* and the toxicity of these compounds in cardiac cells. Due to the intrinsic fluorescent characteristics of these compounds, we also studied their sub cellular distributions to evaluate their preferred targets in *T. cruzi*.

MATERIALS AND METHODS

Compounds - The dicationic aromatic compounds 1MAA119 (Compound 1), 25DAP013 (Compound 2), 14SMB013 (Compound 3), 10SAB092 (Compound 4), 10SAB031 (Compound 5), 11SAB081 (Compound 6), 12SMB032 (Compound 7), 150XD049 (Compound 8), 18SMB092 (Compound 9) and 18SMB096 (Compound 10) (Fig. 1) were synthesised in the laboratory of R.R.T. and the previously reported protocol (Daliry et al. 2009) was used to assess the effectiveness of aromatic compounds with different shapes, cationic centres and effective motifs. Stock solutions of the drugs (5 mM) were freshly prepared in dimethyl sulfoxide and the final solvent concentration in the assays never exceeded 0.6%, which is not toxic for either parasites or mammalian cells.

Financial support: FAPERJ, Pensa Rio/FAPERJ, CNPq, PAPES V/Fiocruz, CPPD
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Received 6 August 2009
Accepted 13 April 2010

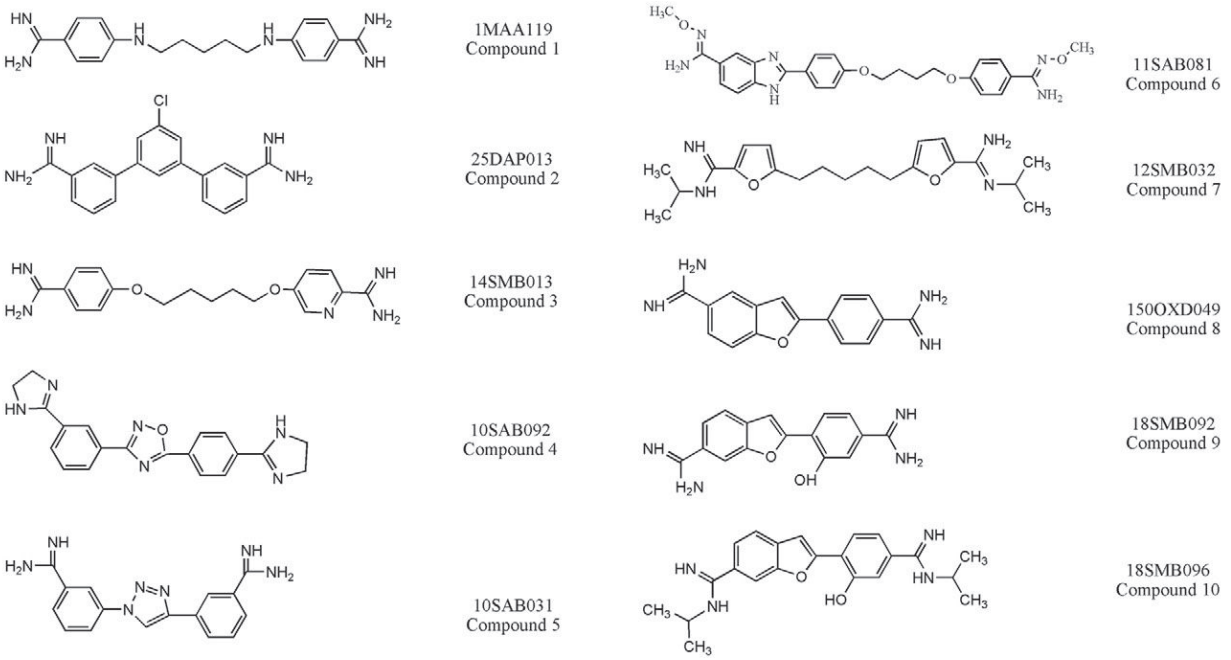

Fig. 1: chemical structure of the compounds.

Cell cultures - Primary cultures of embryonic cardiomyocytes (CM) were obtained from Swiss mice as previously described (Meirelles et al. 1986). After purification, the CM were seeded at a density of 5 × 10^4 cells/well in 96-well microplates containing gelatin-coated cover slips and sustained in Dulbecco's modified medium (DMEM) supplemented with 10% horse serum, 5% foetal bovine serum (FCS), 2.5 mM $CaCl_2$, 1 mM *L*-glutamine and 2% chicken embryo extract as described previously (Meirelles et al. 1986). The cultures were maintained at 37°C in an atmosphere of 5% CO_2 and air and the assays were performed at least three times with duplicate samples. All procedures were carried out in accordance with the guidelines established by the Fiocruz Committee of Ethical for the Use of Animals (CEUA 0099/01).

Parasites - Bloodstream trypomastigotes from the Y strain of *T. cruzi* were harvested by heart puncture from infected Swiss mice at the parasitaemia peak (Meirelles et al. 1982).

Trypanocidal assays - For the analysis of the effect of the compounds on the bloodstream trypomastigotes, 5 x 10^6 parasites/mL were incubated for 24 h at 37°C in RPMI 1640 medium supplemented with 10% FCS, in the presence or absence of serial dilutions of the compounds (0.1-32 µM). Alternatively, the treatment was performed using trypomastigotes cultured in freshly isolated mouse blood at 4°C for 24 h with the drugs at concentrations up to 32 µM. The parasite death rates were determined through direct analysis by light microscopy using a Neubauer chamber and the IC_{50} values (the compound concentration that reduces the number of parasites by 50%) were calculated (Silva et al. 2007b).

Infection assays and effect on intracellular parasites - For the analysis of the effects of the drugs on intracellular parasites, after 24 h of parasite-host cell interaction (ratio of 10:1), the infected cultures were washed to remove free parasites and then maintained at 37°C in an atmosphere of 5% CO_2 and air in the presence of the compounds (0.1 to 32 µM). The medium plus drug was replaced every 24 h. After 72 h of treatment, which corresponded to 96 h of infection, the supernatant was recovered, the number of released parasites was determined by direct quantification using light microscopy and a Neubauer chamber and the IC_{50} values were calculated.

Cytotoxicity assays - To measure the toxic effects on the host cell, uninfected CM were incubated with the compounds (up to 96 µM in DMEM) for 24 h and 72 h at 37ºC and then the cell morphology and viability were evaluated by light microscopy and the method of transcriptional and translational (MTT) colorimetric assay, respectively (Mosmann 1983). The absorbance was measured at 490 nm in a spectrophotometer (VERSAmax tunable, Molecular devices, USA) and was directly proportional to the cell viability, from which the LC_{50} values (the compound concentration that reduces cellular viability by 50%) were calculated.

Fluorescence microscopic analysis and fluorescent intensity determination - The bloodstream forms were treated for 30 min at 37ºC with 10 µg/mL of each compound, fixed with 4% paraformaldehyde and mounted with 2.5% 1.4-diazabicyclo-(2.2.2)octane (DABCO) on a slide covered with poly-L-lysine (Sigma Aldrich Corp). The fluorescence was analysed using a Zeiss photomicroscope equipped with epifluorescence (Zeiss Inc, Thornwood, NY). The fluorescence intensity of the

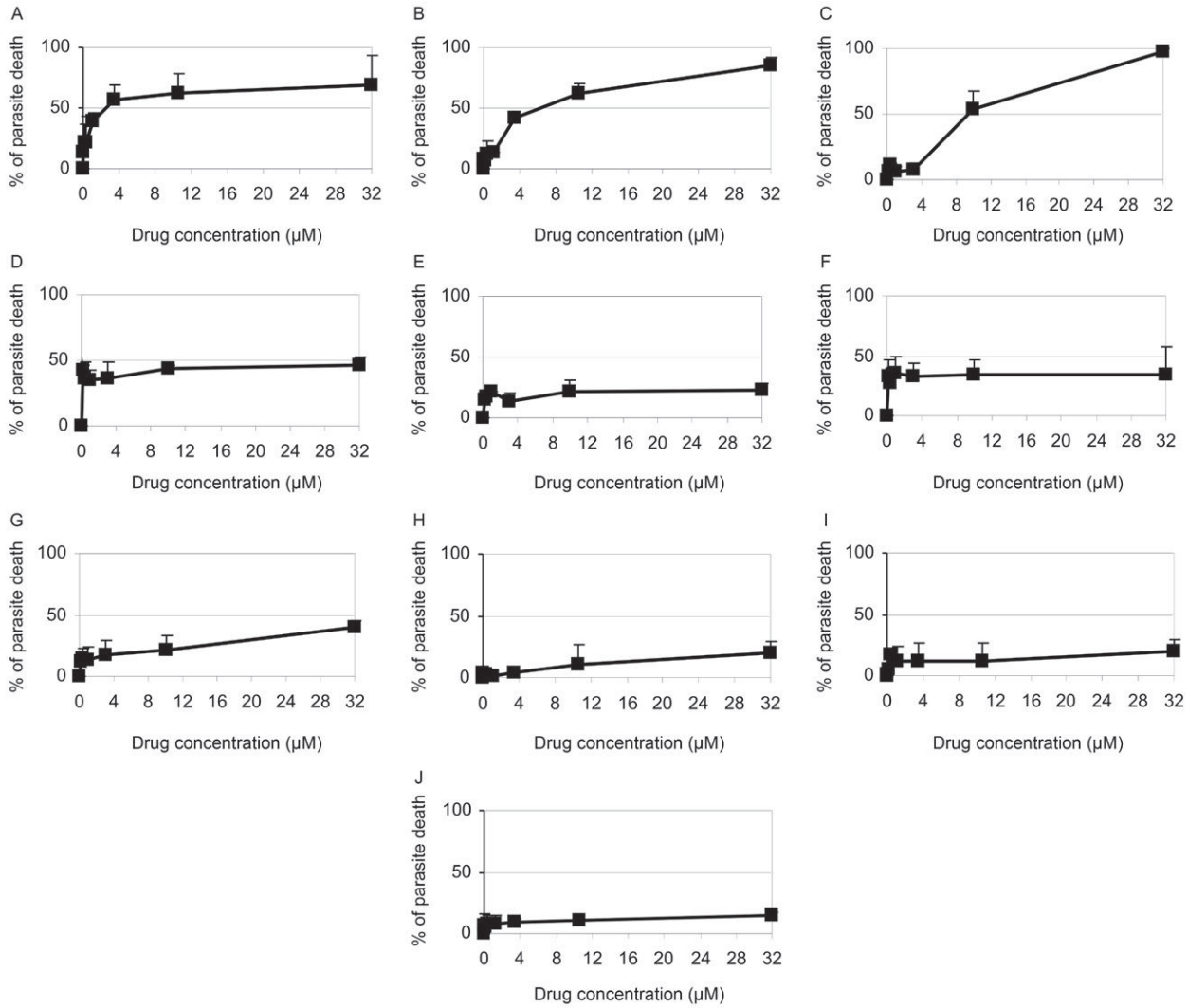

Fig. 2: effect of (A) Compound 1, (B) Compound 2, (C) Compound 3, (D) Compound 4, (E) Compound 5, (F) Compound 6, (G) Compound 7, (H) Compound 8, (I) Compound 9 and (J) Compound 10 on bloodstream trypomastigotes of *Trypanosoma cruzi* (Y strain) in vitro. The activity was evaluated during the treatment at 37ºC with the drugs diluted in culture medium. The percentage of dead parasites was measured after 24 h of treatment.

treated parasites was determined using the program Image J 1.41 (NHI, Bethesda, Maryland) as the sum of the fluorescent pixel values in the selected regions (nucleus DNA - nDNA; kinetoplast DNA - kDNA). The results were expressed as the means and standard deviations of the kDNA/nDNA ratios, which reflect the partition of the kDNA and nDNA fluorescence measurements of at least 50 individual parasites.

RESULTS

We first evaluated the direct effect of the aromatic dicationic compounds on trypomastigotes, which represent the main infective stage of *T. cruzi* (Fig. 2). The most active compounds, Compounds 1, 2 and 3, displayed dose-dependent effects, with IC_{50} values of 2.3, 6.1 and 9.3 μM, respectively (Table I) and about 70, 85 and 97% parasite death at a dose of 32 μM (Fig. 2A-C). The other seven

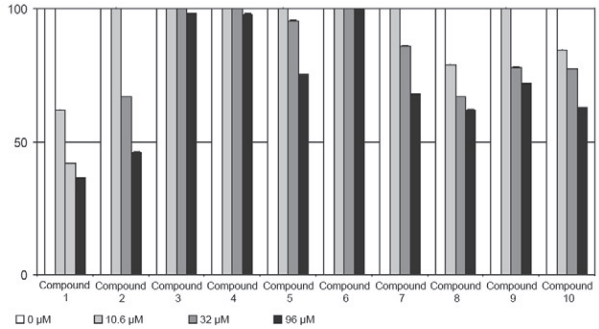

Fig. 3: effect of aromatic dicationic compounds in vitro upon primary cultures of cardiac cells assessed by method of transcriptional and translational (MTT) colorimetric assay. Cardiomyocytes were treated with 10.6, 32 and 96 μM of each compound for 72 h. Data are expressed as mean ± SD of the percentage of survival in drug-treated cells compared to untreated controls.

TABLE I

IC_{50} and selectivity index (SI) values for the effect of aromatic compounds on *Ttypanosoma cruzi*

	Trypomastigotes[a] 24 h			Intracellular parasites[b] 72 h	
	IC_{50} (μM) 4°C	IC_{50} (μM) 37°C	SI	IC_{50} (μM)	SI
Compound 1	> 32.0	2.3	> 40.0	10.6	2.3
Compound 2	> 32.0	6.1	> 15.0	> 32.0	2.7
Compound 3	> 32.0	9.3	> 10.0	0.6	> 160.0
Compound 4	> 32.0	> 32.0	3.0	0.1	> 960.0
Compound 5	> 32.0	> 32.0	3.0	0.3	> 331.0
Compound 6	> 32.0	> 32.0	3.0	2.3	> 43.0
Compound 7	> 32.0	> 32.0	3.0	0.8	> 126.0
Compound 8	> 32.0	> 32.0	3.0	20.0	> 4.7
Compound 9	> 32.0	> 32.0	3.0	20.0	> 4.9
Compound 10	> 32.0	> 32.0	3.0	> 32.0	3.0

SI corresponds to the ratio LC_{50}/IC_{50}. *a*: direct effect of the compounds on trypomastiotes performed after 24 h of incubation at 4°C in whole blood or at 37ºC, in RPMI medium; *b*: effect on intracellular parasites measured by trypomastigotes release into the supernatant culture medium (96 h of infection) performed after 72 h of treatment at 37ºC.

compounds displayed only modest activities, with IC_{50} values higher that 32 μM (Fig. 2D-J, Table I). However, when the bloodstream forms were exposed to Compounds 1, 2 and 3 in the presence of freshly isolated mouse blood, which tested the possible application of these compounds for the prophylaxis of banked blood, we observed a substantial decrease in the trypanocidal activities, with IC_{50} values higher than 32 μM (Table I).

Next, to evaluate the toxicity on mammalian host cells, uninfected cardiac cultures were incubated for 24 and 72 h with different doses of the compounds and then cellular viability was evaluated by both light microscopy and the MTT colorimetric assay. The compounds did not induce loss of cellular viability after incubation for 24 h with doses up to 96 μM (data not shown); however, most of the aromatic dicationic compounds displayed low toxicity after 72 h of incubation and Compounds 1 and 2 exhibited moderate toxicity, with LC_{50} values of 25 and 85 μM, respectively (Fig. 3).

Next, the anti-parasitic activity of the compounds against the intracellular forms of *T. cruzi* was assessed through the direct quantification of the number of parasites released in the supernatant of infected CM after 96 h of parasite interaction. Incubation for 72 h with Compounds 7, 4, 3, 6 and 5 resulted in dose-dependent effects that lead to considerable reductions in the number of parasites released into the supernatant, with micromolar and sub-micromolar IC_{50} values (Fig. 4C-G, Table I). On the other hand, Compounds 1, 8 and 9 exerted moderated activity while Compounds 2 and 10 were not active and had IC_{50} values higher that 32 μM (Fig. 4A-B, H-J, Table I). With the exceptions of Compounds 1 and 2, the other compounds displayed equal or better activity on intracellular parasites compared to the bloodstream parasites (Table I).

Based on the IC_{50} and LC_{50} values, the selectivity index (SI) of each compound was determined. This parameter reflects the quantity of compound that is active against the pathogen but is not toxic towards the host cell. For the bloodstream trypomastigotes, only one dicationic compound (Compound 1) showed a high SI value (> 40), but for the intracellular parasites, five out of 10 compounds displayed considerable selectivity: Compounds 7, 4, 3, 6 and 5 with SI ranging between > 43 and > 960. These five aromatic compounds also displayed higher anti-proliferative effects on the intracellular parasites.

Within the treated bloodstream parasites, all of the fluorescent compounds were localised in DNA-enriched structures, i.e., the kinetoplast and nucleus (Fig. 5). However, although there was consistently higher labelling within the kDNA compared to the nuclei (Fig. 5), the kDNA/nDNA ratios showed that the higher accumulation in the kDNA (ratios ≥1.28) did not correlate with compound efficacy: Compound 7, one of the less active compounds, showed the highest accumulation in the kinetoplast, with a 1.77 kDNA/nDNA ratio (Table II).

DISCUSSION

Diamidines and related dications are considered to be potential anti-parasitic agents due to their known activities against several pathogens (Soeiro et al. 2005). However, as they possess critical limitations regarding

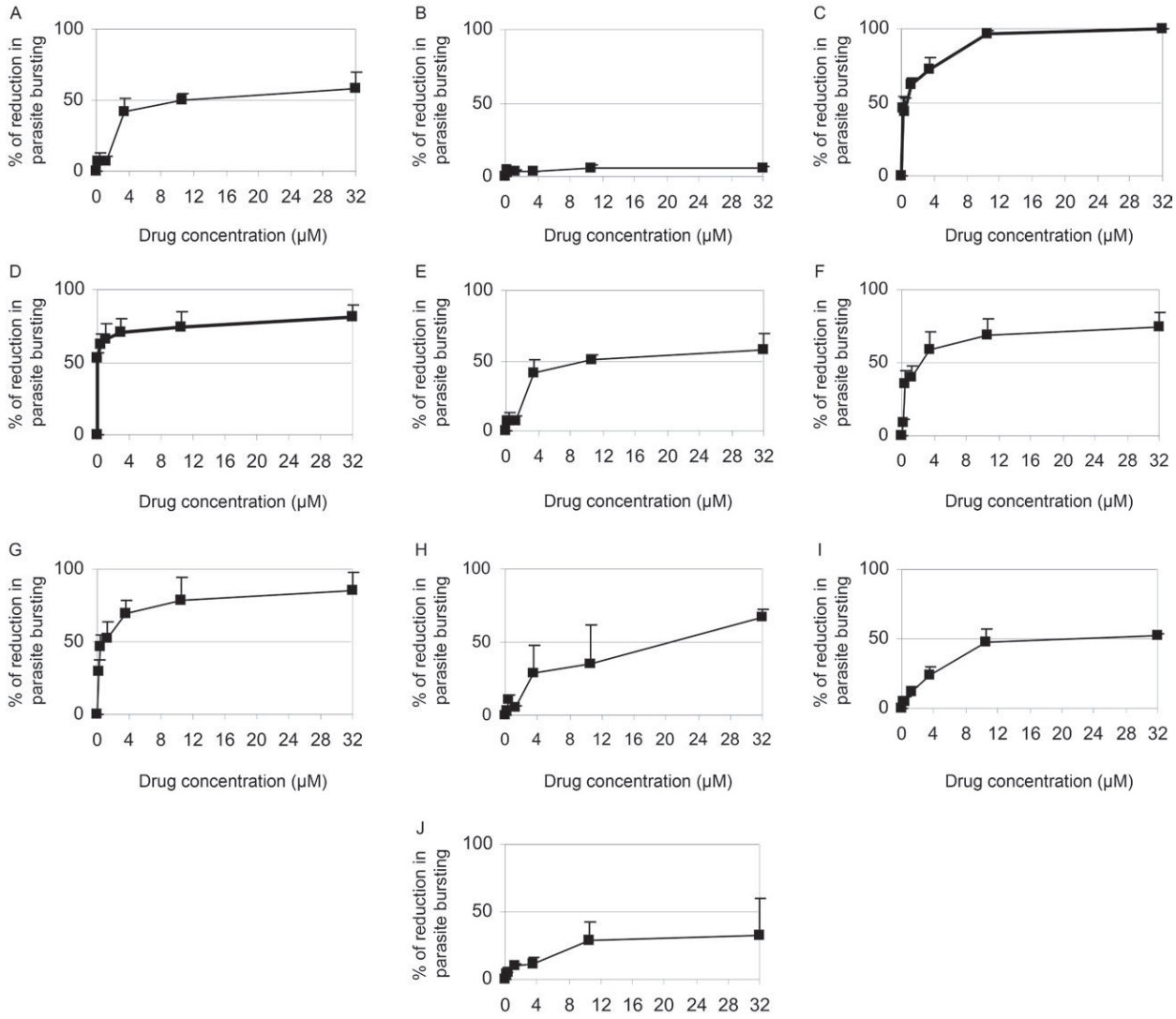

Fig. 4: activity of (A) Compound 1, (B) Compound 2, (C) Compound 3, (D) Compound 4, (E) Compound 5, (F) Compound 6, (G) Compound 7, (H) Compound 8, (I) Compound 9 and (J) Compound 10 upon intracellular parasites lodge in *Trypanosoma cruzi*-infected cardiac cells. The activity of compounds after 72 h of drug incubation is shown by the percentage of reduction in the number of released parasites into the supernatant of the infected cultures.

their poor oral bioavailability and considerable toxicity, new dicationic analogs have been synthesised to address this situation.

Our assays evaluated the effect of 10 aromatic dicationic compounds on trypomastigotes under different experimental conditions to explore their potential uses as chemotherapeutics (assays conducted at 37°C) and/or prophylactic compounds for banked blood (assays using whole blood at 4°C). Our data showed that although three compounds, Compounds 1, 2 and 3, induced high levels of parasite lysis and dose-dependent effects with low micromolar IC_{50} values when assayed at 37°C, all of them showed decreased activity in the presence of blood, possibly due to their association with and/or inactivation by serum components as reported previously (Santa-Rita et al. 2004, 2006, Silva et al. 2007a). Therefore, the decreased activity at 4°C in the presence of blood constituents demonstrated that the studied compounds are ineffective for the sterilisation of ex vivo blood batches to control Chagas disease.

In agreement with our previous studies showing that reversed amidines, also named arylimidamides, exhibited low toxicity to mammalian cells in vitro (Silva et al. 2007a), our present data showed that, except for Compounds 1 and 2, only high drug concentrations (> 96 μM) induced alterations in host cell viability.

We also found that five out of 10 Compounds (Compounds 7, 4, 3, 6 and 5) exerted considerable activity against the intracellular forms of *T. cruzi* at low micromolar and sub-micromolar doses and with high SI values (ranging between > 43 and > 960). This difference in activity on the intracellular forms compared to the

TABLE II

Mean and standard deviation values of fluorescence intensity ratios among kinetoplast and nuclei of bloodstream trypomastigotes treated for 30 min with 10 µg/mL of each compound

	Kinetoplast/nucleus
Compound 1	1.60 ± 0.44
Compound 2	1.61 ± 0.36
Compound 3	1.58 ± 0.38
Compound 4	1.50 ± 0.32
Compound 5	1.61 ± 0.30
Compound 6	1.48 ± 0.31
Compound 7	1.77 ± 0.38
Compound 8	1.28 ± 0.33
Compound 9	1.53 ± 0.30
Compound 10	1.39 ± 0.35

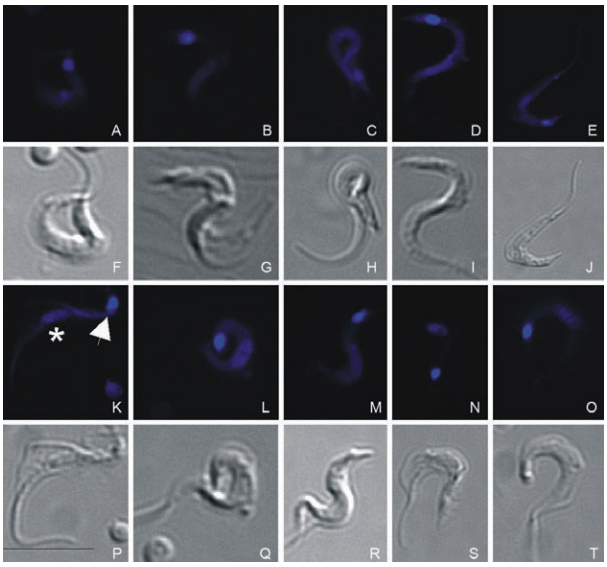

Fig. 5: fluorescent (A-E, K-O) and differential interference contrast (F-J, P-T) analysis showing intracellular localization of the aromatic dicationic compounds within bloodstream trypomastigotes of *Trypanosoma cruzi* after incubation for 30 min at the concentration of 10 µg/mL: Compound 1 (A, F), Compound 2 (B, G), Compound 3 (C, H), Compound 4 (D, I), Compound 5 (E, J), Compound 6 (K, P), Compound 7 (L, Q), Compound 8 (M, R), Compound 9 (N, S) and Compound 10 (O, T). Note that compound accumulation was higher in the kinetoplast (white arrow) than in the nucleus (asterisk). Bar = 2 µm.

bloodstream forms requires further analysis but could represent differences in drug uptake by these different parasite stages and/or different mechanisms of action upon non-dividing trypomastigotes and the highly multiplicative intracellular stages of the parasite.

Aromatic dicationic compounds, such as pentamidine, bind non-covalently and in a non-intercalative manner to the minor-groove of the DNA; however, their mechanism of action has not been fully elucidated and it has been proposed that they may possess multiple modes of action (Wilson et al. 2005). One of the long-hypothesised mechanisms of action of diamidines is related to their ability to bind to AT-rich regions of the DNA minor groove, but other mechanisms have also been proposed, such as inhibition of tyrosyl-DNA phosphodiesterase, topoisomerases, protein kinase A, proteases and polymerases (Tidwell & Boykin 2003, Soeiro et al. 2008, Soeiro & De Castro 2009).

According to our present results, we could not find any correlation between the localisation and higher accumulation of these dicationic fluorescent compounds within the *T. cruzi* kDNA and their trypanocidal activity, which we also found in another recent study of other dicationic compounds (Daliry et al. 2009). In fact, previous reports on African trypanosomes also could not correlate either intracellular accumulation or sub cellular localisation and distribution of aza analogs and diphenyl furans with their in vitro activities (Mathis et al. 2007).

Our present paper describes the potential effect of the aromatic dicationic compounds on *T. cruzi*, which supports further screening of new analogs that could be used alone or in combination with other drugs for the treatment of Chagas disease.

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