Universidade do Estado do Rio de Janeiro
Instituto de Biologia Roberto Alcantara Gomes
Centro Biomédico

Carlos Fernando Araujo Lima de Oliveira

Estudo da eficácia e segurança de compostos heterocíclicos no contexto da infecção pelo *Trypanosoma cruzi* e na prevenção de danos ao DNA

Rio de Janeiro
2018

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Tese apresentada, como requisito parcial para obtenção do título de Doutor em Ciências, ao Programa de Pós-graduação em Biociências, da Universidade do Estado do Rio de Janeiro.

Orientador: Prof. Dr. Israel Felzenszwalb

Coorientadora: Prof. Dra. Maria de Nazaré Correia Soeiro

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Obrigado!

Quem cultiva a semente do amor
Segue em frente e não se apavora
Se na vida encontrar dissabor
Vai saber esperar a sua hora

Às vezes a felicidade demora a chegar
Aí é que a gente não pode deixar de sonhar
Guerreiro não foge da luta, não pode correr
Ninguém vai poder atrasar quem nasceu pra vencer

É dia de sol, mas o tempo pode fechar
A chuva só vem quando tem que molhar
Na vida é preciso aprender
Se colhe o bem que plantar
É Deus quem aponta a estrela que tem que brilhar

Erga essa cabeça, mete o pé e vai na fé
Manda essa tristeza embora
Basta acreditar que um novo dia vai raiar
Sua hora vai chegar!

Xande de Pilares, Gilson Benini e Carlinhos Madureira

RESUMO

OLIVEIRA, Carlos Fernando Araujo Lima de. **Estudo da eficácia e segurança de compostos heterocíclicos no contexto da infecção pelo *Trypanosoma cruzi* e na prevenção de danos ao DNA**. 2018. 174 f. Tese (Doutorado em Biociências) - Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2018.

Dentre os paradigmas de saúde pública do século XXI, a busca por alternativas terapêuticas para o tratamento de doenças órfãs representa uma necessidade urgente no cenário mundial. Os compostos heterocíclicos constituem uma classe de entidades químicas de grande importância para a organização da matéria viva, pois mais da metade de todos os compostos químicos conhecidos são heterocíclicos, com destaque para os fármacos, vitaminas e princípios ativos em plantas e organismos marinhos. Uma classe amplamente investigada no contexto da infecção pelo *Trypanosoma cruzi* e que revela promissora atividade tripanocida é a das arilimidamidas (AIAs), compostos heterocíclicos oxigenados que se associam de forma não-intercalante ao DNA. Os compostos nitroimidazólicos são heterocíclicos nitrogenados, portadores do grupamento nitro (NO_2) e têm grande relevância no uso medicinal. As estatinas são inibidores competitivos da enzima HMGCoA-redutase amplamente usados no tratamento das hipercolesterolemias. Atorvastatina é considerada o último e maior dos *blockbusters* (medicamento mais rentável) da história da farmacologia sintética. Há mais de uma década vêm sendo estudados os efeitos pleiotrópicos das estatinas. Nosso objetivo foi investigar a eficácia, segurança e seletividade de compostos heterocíclicos sintéticos com potencial preventivo de lesões no DNA e no contexto de infecções parasitárias como por *T. cruzi*. Avaliamos a atividade tripanocida *in vitro* dos compostos heterociclicos sobre amastigotas e tripomastigotas de *T. cruzi* em esquemas de monoterapia e em combinação com benznidazol, além de avaliar o arranjo citoarquitetônico em cardiomiócitos infectados ou não com o *T. cruzi*. A prevenção de danos ao DNA foi avaliada em modelos bacteriano e eucariótico. Adicionalmente, o perfil mutagênico e de toxicidade sobre células de mamíferos foram avaliados através do teste de Ames, ensaio de micronúcleos e viabilidade celular. Nossos dados demonstraram que mono-AIAs são além de menos eficazes também menos seletivas que as bis-AIAs sobre o *T. cruzi*. Os resultados da análise da relação estrutura-atividade dos nitroimidazóis demonstram que o grupo nitro não é o único responsável pela atividade mutagênica ou genotóxica, tendo os substituintes próximos um papel importante na modulação de sua toxicidade. Atorvastatina foi capaz de prevenir danos no DNA, revertendo disfunções no ciclo celular de hepatócitos expostos a estresse genotóxico, exerceu atividade sequestrante de radicais $DPPH^+$ sugerindo que seu efeito quimioprotetor está associado ao sequestro de radicais potencialmente genotóxicos. A atorvastatina apresentou bom perfil de atividade tripanocida quando usada isoladamente exibindo, desse modo, interação sinérgica com o benznidazol nos ensaios de terapia combinada, levando ao aumento da eficácia e seletividade de ambos. Dois de seus derivados fenilacéticos, apresentaram moderada atividade tripanocida isoladamente contra os tripomastigotas sanguíneos da cepa Y. O derivado bromado foi considerado genotóxico e o derivado clorado demonstrou um perfil seguro para os ensaios preconizados pelas agências de toxicologia regulatória. A atorvastatina exibiu potencial de reposicionamento como medicamento tripanocida e citoprotetor, principalmente em terapias combinadas e um de seus novos derivados apresentou perfil *hit-to-lead* na quimioterapia experimental contra o *T. cruzi*. Os compostos heterocíclicos, representados neste estudo pelas classes das arilimidamidas, dos nitroimidazóis e das estatinas, são apontadas como promissoras alternativas para o tratamento da Doença de Chagas.

Palavras-chave**:** Compostos heterocíclicos. Doença de Chagas. Quimioterapia experimental. Terapia combinada. Efeito quimiopreventivo. Genotoxicidade.

ABSTRACT

OLIVEIRA, Carlos Fernando Araujo Lima de. **Study of efficacy and saafety of heterocyclic compounds on *Trypanosoma cruzi* infection contexto ando n the prevention of DNA damages.** 2018. 174 f. Tese (Doutorado em Biociências) - Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2018.

Among the 21st century's public health paradigms, the search for therapeutic alternatives for orphan diseases represents an urgent need in the world's scenario. Heterocyclic compounds constitute a class of chemical entities of great importance for the organization of living matter, since more than half of all known chemical compounds are heterocyclic, with emphasis on the drugs, vitamins and active principles in plants and marine organisms. A class widely investigated in the context of Trypanosoma cruzi infection and revealing promising trypanocidal activity is that of arylimidamides (AIAs), oxygenated heterocyclic compounds that associate non-intercalatingly with DNA. Nitroimidazole compounds are nitrogen-containing heterocyclics, which carry the nitro group (NO2) and are highly relevant in medicinal use. Statins are competitive inhibitors of the HMGCoA-reductase enzyme widely used in the treatment of hypercholesterolemias. Atorvastatin is considered the last and largest of the blockbusters (drug more profitable) in the history of synthetic pharmacology. The pleiotropic effects of statins have been studied for more than a decade. Our objective was to investigate the efficacy, safety and selectivity of synthetic heterocyclic compounds with the potential to prevent DNA damage and in the context of parasitic infections such as T. cruzi. We evaluated the in vitro trypanocidal activity of the heterocyclic compounds on amastigotes and trypomastigotes of T. cruzi in monotherapy and in combination with benznidazole schemes, in addition to evaluating the cytoarchitectonic arrangement in cardiomyocytes infected or not with T. cruzi. The prevention of DNA damage was evaluated in bacterial and eukaryotic models. In addition, the mutagenic and toxicity profile on mammalian cells were evaluated by the Ames test, micronucleus assay and cell viability. Our data demonstrated that mono-AIAs are in addition to less effective also less selective than the bis-AIAs on T. cruzi. The results of analysis of the structure-activity relationship of nitroimidazoles demonstrate that the nitro group is not alone responsible for the mutagenic or genotoxic activity, with the substituents closely playing an important role in modulating its toxicity. Atorvastatin was able to prevent DNA damage, reversing dysfunctions in the cell cycle of hepatocytes exposed to genotoxic stress, exerted sequestering activity of DPPH + radicals suggesting that its chemoprotective effect is associated with the sequestration of potentially genotoxic radicals. Atorvastatin presented a good profile of trypanocidal activity when used alone, thus exhibiting synergistic interaction with benznidazole in the combination therapy trials, leading to an increase in the efficacy and selectivity of both. Two of its phenylacetic derivatives showed moderate trypanocidal activity alone against the blood trypomastigotes of the Y strain. The brominated derivative was considered genotoxic and the chlorinated derivative demonstrated a safe profile for the tests recommended by the regulatory toxicology agencies. Atorvastatin exhibited repositioning potential as a trypanocidal and cytoprotective drug, mainly in combination therapies and one of its new derivatives showed a hit-to-lead profile in experimental chemotherapy against T. cruzi. The heterocyclic compounds, represented in this study by the classes of arylimidamides, nitroimidazoles and statins, are indicated as promising alternatives for the treatment of Chagas Disease.

Keywords: Heterocyclic compounds. Chagas disease. Experimental chemotherapy. Combined Therapy. Chemopreventive effect. Genotoxicity.

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INTRODUÇÃO

As doenças tropicais negligenciadas (DTNs) são um conjunto de 20 infecções frequentemente observadas nas regiões mais pobres de países em desenvolvimento e são causadas por diversos patógenos como vírus, bactérias, fungos, protozoários e helmintos e representam uma crescente ameaça mundial (WHO, 2015). São denominadas negligenciadas por receberem menor atenção do mercado farmacêutico para o desenvolvimento de fármacos, da mídia e das ciências médicas quando comparadas a outras doenças infecciosas, como a AIDS e as hepatites virais (BEYER et al., 2007). Esta grande incidência é justificada pela pobreza dessa grande parcela da população mundial que sofre pelas precárias condições de vida, falta de investimento em saúde e em outras áreas sócio-econômicas, além do baixo desenvolvimento de conhecimento científico relacionado (FIELD et al., 2017). A malária, as tripanossomíases africana (doença do sono) e americana (doença de Chagas - DC), as leishmanioses e a esquistossomose são responsáveis por incapacitar anualmente uma fração relevante da população de vários países em desenvolvimento, especialmente do continente africano, no qual o acesso às medidas de controle sanitário e a qualidade de vida são escassos (WHO, 2015). A maioria absoluta das mortes devido às DTNs ocorre em regiões situadas abaixo da linha do Equador e a necessidade de novas alternativas terapêuticas é clara, mas os investimentos globais em pesquisa e desenvolvimento (P&D) de fármacos e vacinas são insuficientes (WHO, 2015).

A pobreza e as condições sociais precárias, o desequilíbrio ecológico decorrente do desmatamento, a pressão econômica, a falta de investimento em saúde pública e pesquisa e, em determinados países, as guerras e outros conflitos são as principais causas pelas quais essas doenças ainda incidem nas populações mais carentes de recursos (BEYER et al., 2007; SKOLNIK; AHMED, 2010). Além disso, a ausência de tratamentos eficazes também é fator determinante para a manutenção das doenças infecciosas e negligenciadas. O resultado para a população afetada e para economia dos países é grave, pois seus indivíduos perdem a capacidade de produzir, concomitante a redução da qualidade e expectativa de vida (WHO, 2007, 2010).

Por isso, as organizações governamentais e não governamentais desenvolvem relatórios com estratégias sociais a fim de estimular países a adotarem medidas para combater a prevalência das doenças infecciosas. Dentre eles, os relatórios *Objetivos de Desenvolvimento do Milênio* e *Transformando nosso mundo: a agenda 2030 para o*

desenvolvimento sustentável, da ONU, estabelecem medidas que já beneficiaram milhares de indivíduos, protegendo-os da dor, instabilidade e pobreza, através do tratamento farmacológico e das intervenções socioeconômicas das nações afetadas. No entanto, ainda há muito a ser feito para a resolução dos problemas sociais, como a pobreza (WHO, 2007, 2010; MOLYNEUX; MALECELA, 2011; ONU, 2015; DIAS et al., 2016).

As estratégias para o desenvolvimento e inovação farmacêutica envolvem etapas sequenciais e complementares de eficácia e segurança através de diferentes plataformas incluindo: (i) a identificação de novos princípios ativos (sintéticos e biomoléculas) a partir de bibliotecas de moléculas naturais e sintéticas, (ii) o reposicionamento e investigação de fármacos já licenciados para outras patologias que compartilhem alvos celulares comuns, e (iii) desenho racional e validação de novos alvos ou vias metabólicas específicas (SOEIRO; CASTRO, 2009).

Para o desenvolvimento de inovações na área químico-farmacêutica é imprescindível avaliar o risco de efeitos adversos para saúde ou, em outras palavras, a segurança terapêutica do novo produto nas condições propostas de uso. Nenhum produto novo, quer seja ele um medicamento, aditivo alimentar, praguicida, cosmético, produto de uso doméstico, combustível, solvente industrial, e assim por diante, pode ser introduzido no mercado, ou usado em larga escala, sem ter passado previamente por uma avaliação toxicológica cuja abrangência depende do uso proposto para o produto. As exigências atuais a este respeito são muito rigorosas em nível internacional sendo, por conseguinte, a capacitação para avaliar o risco de efeitos adversos para saúde (MORTELMANS; ZEIGER, 2000).

1 **REVISÃO DA LITERATURA**

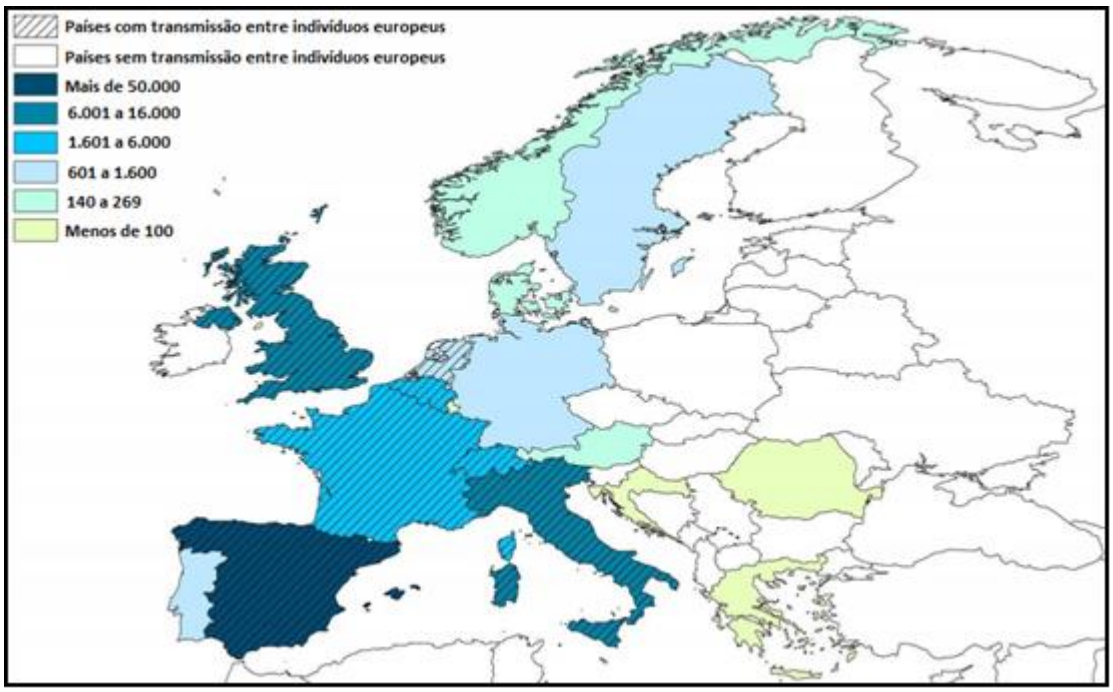
1.1. **A Doença de Chagas**

A DC é uma infecção parasitária causada pelo protozoário *Trypanosoma cruzi*. Estima-se que mais de 8 milhões de pessoas estejam infectadas na América Latina, 3 milhões delas apenas no Brasil, sendo desse modo considerada um grave problema de saúde pública mundial, devido aos fluxos migratórios e as rotas de transmissão não vetorial (Figura 1). Em média 12 mil pessoas morrem em decorrência da DC todos os anos (CROMPTON, 2013).

O principal mecanismo de transmissão da doença se dá através do vetor triatomíneo, também conhecido como barbeiro. A doença apresenta-se em duas fases: a fase aguda que ocorre logo após a infecção, com sintomatologia que varia de síndrome gripal até intensa miocardite e a fase crônica, na qual a maioria dos portadores não apresenta parasitismo patente, porém exibe sorologia positiva. Após anos ou décadas, cerca de 30-40 % pode desenvolver manifestações clínicas, em especial alterações cardíacas e/ou danos gastrointestinais (COURA et al., 2014).

A infecção pelo *T. cruzi* na natureza ocorre há milhões de anos. Evidências datam de nove mil anos atrás e foram encontradas em corpos mumificados (GUHL et al., 1999, AFDERHEIDE et al., 2004). Os vetores triatomíneos também são conhecidos desde o século XVI (LENT; WIGODZINSKY, 1979). No entanto, foi no século passado (XX), por motivos econômicos, sociais e ecológicos, que o homem se incluiu no ciclo epidemiológico da doença. A infecção evoluiu de uma zoonose entre os animais do ciclo selvagem para uma antropozoonose (COURA; DIAS, 2009).

Figura 1 - Distribuição epidemiológica da Doença de Chagas na Europa



Fonte: Adaptado de OMS, 2009.

Além disso, respondendo as pressões antrópicas no ciclo selvagem, o triatomíneo vetor se domiciliou ao encontrar um ambiente favorável nas vivendas rurais do homem interiorano (VINHAES; DIAS, 2000). Dentre as mais de 120 espécies, estima-se que de oito a dez sejam domiciliadas (PRATA, 2001; SIQUEIRA-BATISTA et al., 2011). O aumento do desmatamento e, por consequência, a diminuição da quantidade de animais silvestres que constituem fonte alimentar para os triatomíneos, foram as razões para o início da expansão da doença (COURA; DIAS, 2009). Além do desequilíbrio ecológico nas inúmeras regiões da América Latina, a questão social ligada à origem e ao desenvolvimento da doença deve ser levada em consideração. As áreas chagásicas, de endemia, demarcam os padrões sociais da população afetada. Há pobreza, má condição de vida e de educação, além da ausência do poder público ou a má organização deste (DIAS, 1985a, 1985b; DIAS et al., 2016). E mesmo depois de mais de 100 anos de descoberta da doença, ainda há muito a se fazer para diminuir a expansão e a prevalência da doença na população mundial (DIAS et al., 2016).

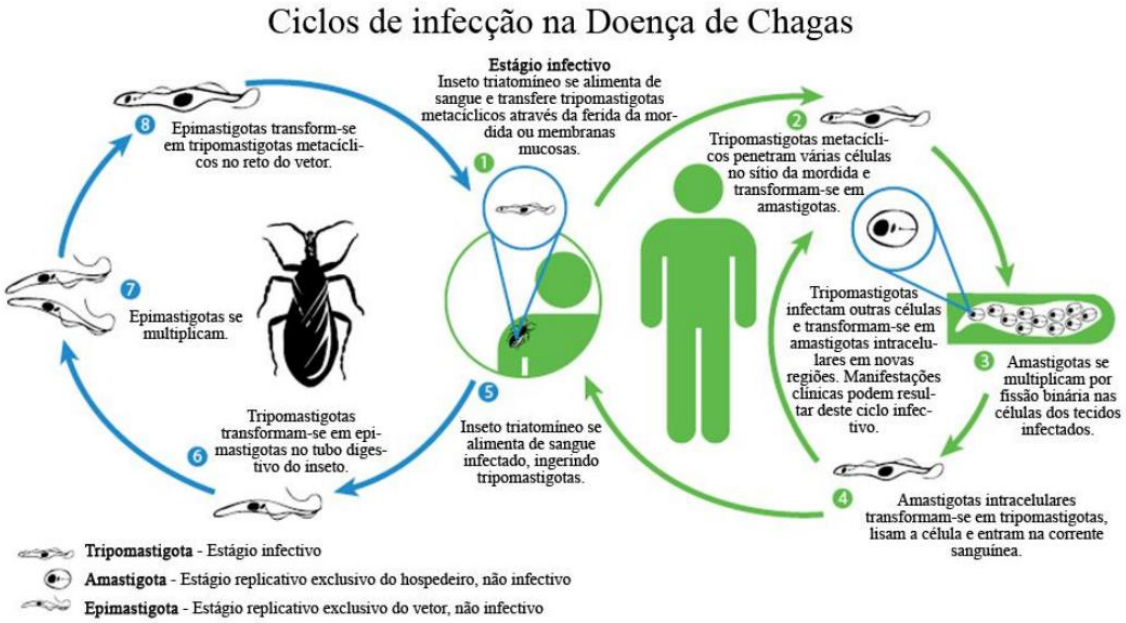
1.1.1. Ciclo biológico do *T. cruzi*

O ciclo biológico do *T. cruzi* é heteroxênico, o que significa que há presença de um hospedeiro vertebrado e um invertebrado (Figura 2). Ao se alimentar do sangue infectado do hospedeiro, o inseto ingere formas tripomastigotas que estão livres na corrente sanguínea, e os mesmos diferenciam-se em formas epimastigotas no tubo digestório do triatomíneo, forma não-infectante encontrada no vetor. Os epimastigotas se multiplicam e transformam-se, na porção final do intestino, em tripomastigotas metacíclicos, sendo eventualmente eliminados nas fezes. Na via vetorial, o parasita infecta o hospedeiro quando o conteúdo fecal entra em contato com lesões na pele induzidas pela mordida[1] do inseto ou caso o hospedeiro leve acidentalmente as excretas para mucosas. Há uma grande variedade de animais vertebrados que podem ser hospedeiros do *T. cruzi*, como gambás, ratos, cães e gatos, além do ser humano, o que confere a presença do parasita tanto no ambiente doméstico quanto no silvestre e peridomiciliar. Os tripomastigotas são capazes de invadir virtualmente qualquer tipo de célula hospedeira por endocitose, e escapam rapidamente do vacúolo parasitóforo para o citosol. No ambiente intracelular, eles transformam-se então em formas amastigotas (forma replicativa) e se multiplicam no ambiente intracelular por fissão binária simples poucas horas após a invasão celular. As formas amastigotas passam por até nove ciclos de replicação e, após aproximadamente 72 h de infecção celular as mesmas iniciam a diferenciação em tripomastigotas no citosol, lisam as células infectadas e são capazes de infectar células vizinhas ou órgãos e tecidos distantes, atingindo circulação sanguínea e linfática, podendo serem capturadas pelo inseto vetor no ato do repasto, propagando a infecção (CHAGAS, 1909; 1911).

Assim, durante seu ciclo, o *T. cruzi* apresenta formas com diferentes características morfo-funcionais específicas e compatíveis com a perspectiva biológica que se encontra. Desde a descrição de seu ciclo por Carlos Chagas, no início do século XX até os dias de hoje grandes esforços são dedicados para o entendimento da patogênese da DC e dos aspectos bioquímicos e moleculares relativos aos elementos desencadeadores desta enfermidade, assim como seu curso progressivo (GOLDENBERG E AVILA, 2011).

[1] O termo “mordida” está presente na descrição original do artigo de Carlos Chagas, 1909, apesar de nos dias atuais denominarmos repasto, em acordo com o aparelho bucal picador-sugador que dispõem os insetos triatomíneos hematófagos.

Figura 2 - Esquema ilustrativo do ciclo de vida do *T. cruzi* tendo o ser humano como hospedeiro vertebrado



Fonte: Adaptado de <http://www.cdc.gov/parasites/chagas/>. Acesso em 13 04/2018.

As transmissões transfusional e por transplante de órgãos são ainda muito frequentes e preocupantes em países ectóctones para o *T. cruzi* e seu vetor, mas que recebem imigrantes oriundos de regiões endêmicas. Ademais, essas regiões, de um modo geral não estão preparadas adequadamente para diagnosticar e lidar com essa via de transmissão, pois não implementaram estratégias na triagem de doadores de sangue, tecido e órgãos, e nem possuem estratégias de saúde pública para indivíduos portadores do *T. cruzi*, pois os serviços e profissionais da atenção básica não estão preparados para diagnosticar ou tratar pacientes que manifestem a doença (COURA, 2015; DIAS et al., 2016). No entanto, é importante ressaltar que casos de infecção provenientes da via oral, através do consumo de frutas e carne crua de animais contaminados, e que carece de controle; e a vertical, que tem diagnóstico dificultado, são recorrentes em diversos países endêmicos, e ganharam importância epidemiológica maior em relação aos meios ditos controlados, principalmente na região amazônica, além de serem responsáveis por surtos de infecção em diversos estados da federação nacional (MS, 2015; COURA, 2015; DIAS et al., 2016; ANTUNES et al., 2016). A partir do momento que ocorre a transmissão, a doença é dividida em dois estágios conhecidos como fase aguda e fase crônica, onde esta última é dividida em fase indeterminada e determinada. Os sintomas marcantes de cada fase podem se desenvolver imediatamente ou podem passar despercebidos

caso se manifestem de forma branda. Como acima relatado, em muitos casos, a sintomatologia leva anos para se manifestar nos portadores do *T. cruzi* (WHO, 2002, 2010).

1.1.2. Aspectos clínicos e fisiopatológicos

A fase aguda é o conjunto de manifestações clínicas iniciais, específicas ou não, que podem surgir após a inoculação do *T. cruzi* e após um período de incubação que varia entre diversas semanas ou meses, a depender da via de transmissão e de outras características epidemiológicas (LINDSEY et al., 2015). Quando a infecção ocorre pela via vetorial, é possível identificar uma inflamação na área pela qual o vetor se alimentou. Como o vetor costuma se alimentar pela região do rosto, é possível que o local de infecção seja perto dos olhos, e por esse motivo, há uma inflamação específica na região chamada de sinal de Romaña. Em seguida, devido à infecção generalizada (alta parasitemia), pode ocorrer o aparecimento de febre, taquicardia, arritmia cardíaca, edema, mal-estar, cefaleia, diarreia, vômito, anorexia, hepatomegalia, entre outros. Esses sintomas são comuns às demais vias de transmissão, também (PRATA, 2001; PARKER; SETHI, 2011; LINDSEY et al., 2015).

As manifestações clínicas dessa fase podem durar até oito semanas. Como elas se desenvolvem mais em crianças e adolescentes, o risco de morte nestes indivíduos é maior e ocorre em até 10 % dos pacientes (em especial crianças abaixo de 2 anos de vida). No entanto, na maioria dos casos, a fase aguda pode ser oligossintomática ou assintomática, dificultando seu diagnóstico e tratamento precoces. Além disso, a ausência dos sintomas é muito comum na transmissão congênita, tornando-se um risco maior para recém-nascidos (PRATA, 2001; WHO, 2002; PARKER; SETHI, 2011; DIAS et al., 2016).

Caso se desconfie da infecção e/ou os sintomas estejam presentes, os fatores epidemiológicos da região e da população são muito importantes para o diagnóstico correto. Como o parasita está bem disseminado no sangue, ele pode ser detectado pela análise parasitológica como a observação direta em microscopia óptica. Caso a análise direta da presença do *T. cruzi* no sangue seja negativa, mas os sintomas ou suspeitas continuem presentes, a confirmação da infecção pode ser feita por metodologias mais específicas como: análise parasitológica molecular (como PCR) além da identificação sorológica de anticorpos para o parasito, decorrente da resposta imunológica normal do paciente à alta parasitemia.

Caso os sintomas sejam mais específicos, o eletrocardiograma e o raios-X do tórax podem mostrar sinais de comprometimento cardíaco (PRATA, 2001; WHO, 2002; LINDSEY et al., 2015; DIAS et al., 2016). Após o tempo de duração da fase aguda, os sintomas, quando presentes, desaparecem, sem aparente sequela (WHO, 2002).

Frente à resposta imunológica competente ocorre o controle do parasitismo e desaparecimento dos sintomas da fase aguda que evolui para fase crônica que pode se assintomática caracterizada pela ausência de manifestações clínicas (para maioria dos indivíduos) ou sintomática, na qual até 40 % dos portadores passam a expressar importantes alterações cardiológicas associadas ou não a problemas digestivos graves (mega síndromes) (ROCHA; RIBEIRO, 1998; COURA; BORGES-PEREIRA, 2010, LINDSEY et al., 2015). Além disso, na fase crônica, o diagnóstico se dá por sorologia além de exames clínicos (análises radiológicas do coração, esôfago, sistema digestório, eletrocardiogramas, ECG, entre outros). Mas outros testes como a reação em cadeia da polimerase (PCR) e o xenodiagnóstico são ainda usados para comprovar a infecção (PRATA, 2001; COURA; BORGES-PEREIRA, 2010).

O quadro severo da forma cardíaca da doença de Chagas inclui: arritmias em consequência das lesões no miocárdio, os bloqueios átrio e intraventriculares, e a insuficiência cardíaca (PRATA 2001; WHO, 2002; PARKER; SETHI, 2011; DIAS et al., 2016).

Como acima relatado, a DC também compromete sistema digestório do portador, com alterações na motilidade, na secreção e na absorção do sistema digestório, especialmente no esófago e no cólon, que aumentam de tamanho (megaesófago e megacólon) (PRATA, 2001; DIAS et al., 2016).

O sistema nervoso periférico (SNP) e o autônomo (SNA) também podem estar comprometidos. Cerca de 10% dos pacientes que desenvolvem a doença apresentam pelo menos uma manifestação clínica relacionada a perturbações no SNP. Neurite, destruição dos neurônios motores e das fibras nervosas sensoriais são algumas das causas para as anormalidades sensoriais dos pacientes. O comprometimento do SNA se relaciona a denervação do sistema nervoso parassimpático e simpático, e justificam as manifestações clínicas relacionadas ao coração como arritmia e morte súbita, e a motilidade do sistema digestório (RIBEIRO et al., 2001; PRATA, 2001; PARKER; SETHI, 2011).

1.1.3. Aspectos eco-epidemiológicos

A maioria dos portadores chagásicos encontra-se na América Latina (

Figura **3**) (WHO, 2017; DIAS et al., 2016). Além disso, de acordo com a Organização Pan-Americana da Saúde (OPAS), 100 milhões de pessoas estejam em risco de infecção, com 56 mil novos casos e 12 mil mortes todo ano (ARMAGANIJAN; MORILLO, 2010; WHO, 2010).

Figura 3 - Estimativa global da população infectada pelo *T. cruzi*

Fonte: Adaptado de < http://revistapesquisa.fapesp.br/2009/09/01/mais-uma-tentativa/>. Acesso em 29/04/2018.

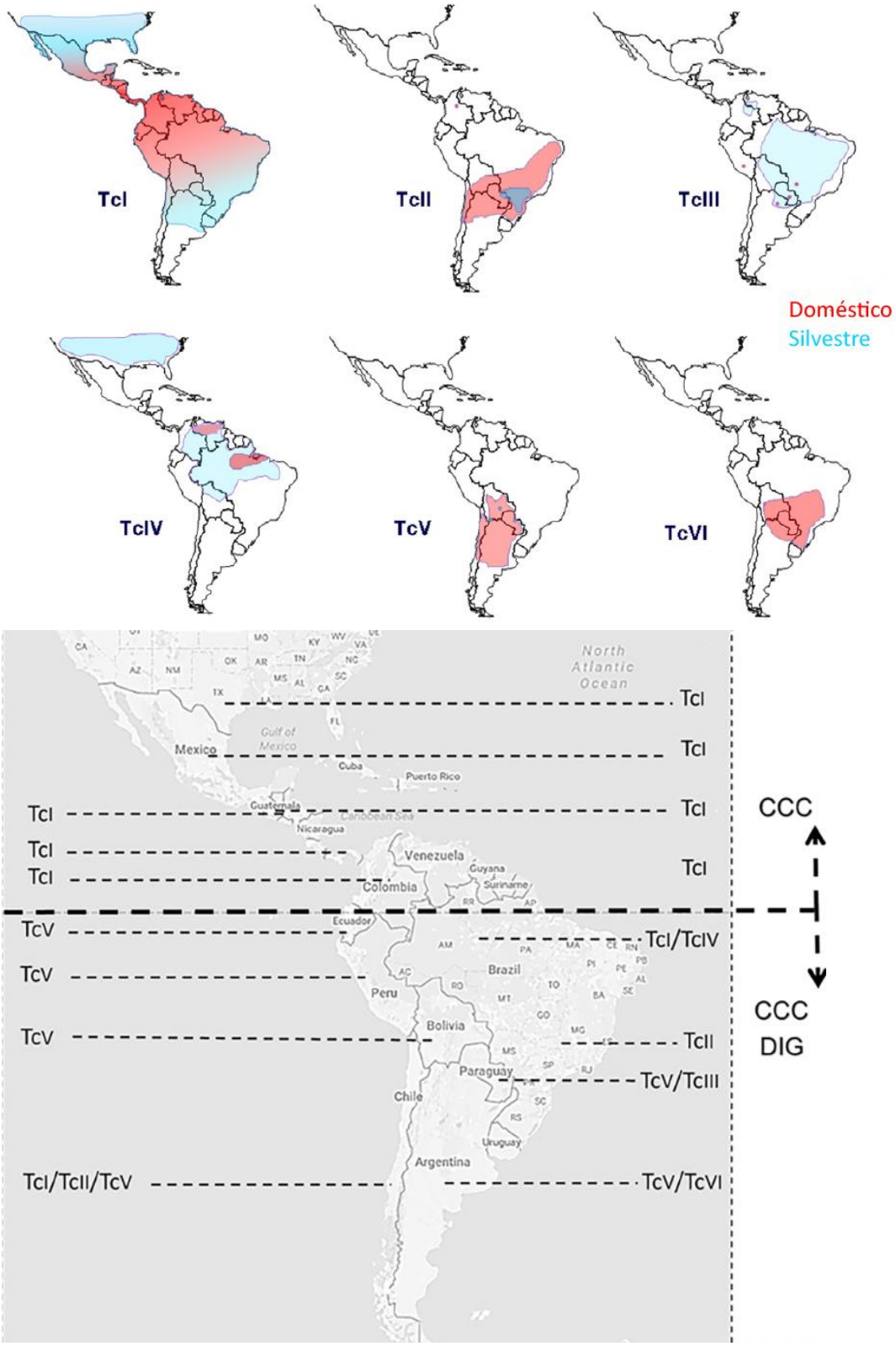
Graças à alta variabilidade genética do *T. cruzi*, existem diversas linhagens do protozoário com distintas características epidemiológicas e biológicas (PRATA, 2001; HIGUERA; GUHL; RAMÍREZ, 2013). ZINGALES et al.. (2012) apresentam um estudo filo-geográfico e ecoepidemiológico sobre a pluralidade de comportamentos das cepas de *T. cruzi*. Após consenso, as cepas de *T. cruzi* foram agrupadas de acordo com suas unidades de tipagem discreta (*discrete typing units* – DTUs), de TcI a TcVI, levando em consideração aspectos como distribuição biogeográfica, patogenicidade, prevalência de infecção humana e resistência aos medicamentos nitroaromáticos e também aspectos relativos à taxionomia molecular. Desta forma, é possível empregar melhores estratégias de combate ao parasita e ao vetor, assim como desenvolver e oferecer um tratamento mais adequado (ZINGALES et al., 2012; HIGUERA; GUHL; RAMÍREZ, 2013). O grupo *T. cruzi* I tem maior prevalência em

todo território das Américas, com exceção aos países do sul da América do Sul, e está mais associado à forma cardíaca da doença. Os demais grupos (*T. cruzi* II-VI) apresentam distribuição geográfica distinta (Figura 4). No Brasil, o grupo de maior prevalência na região central e leste DTU II, tem um ciclo de vida predominantemente doméstico e está associado a manifestações clínicas cardíacas, esofágicas e colônicas (CARRANZA et al., 2009; ZINGALES, 2011, 2012; HIGUERA; GUHL; RAMÍREZ, 2013, ZINGALES, 2017).

Apesar da América Latina ser o local de maior prevalência da doença de Chagas, existem pessoas contaminadas em diversos países não endêmicos. Em regiões ao sul dos Estados Unidos (EUA), como no Arizona e na Califórnia, existem relatos de supostos casos autóctones além da presença significativa de diversas espécies de vetores triatomíneos e mamíferos silvestres infectados. Estudos confirmaram que 41% dos insetos coletados nessa região estavam infectados pelo *T. cruzi*, evidenciando que o risco de contaminação dessa população é real (SARKAR et al., 2010; PARKER; SETHI, 2011; MONTGOMERY et al., 2016).

No entanto, a migração de portadores do *T. cruzi* para países que tradicionalmente recebem imigrantes oriundos da América Latina, como os Estados Unidos e o Canadá, na América do Norte; a Espanha e Portugal, na Europa; o Japão, na Ásia; e a Austrália, na Oceania; regiões tradicionalmente ectóctones, com exceções, é o motivo do surgimento de uma nova problemática de saúde pública. (SCHMUNIS, 2007; COURA, DIAS, 2009; SCHUMUNIS; YADON, 2010; COURA; VIÑAS; JUNQUEIRA, 2014).

Figura 4 - Distribuição ecoepidemiológica e filogeográfica das unidades subespecíficas de *T. cruzi* e as principais manifestações clínicas (CCC – forma cardíaca; DIG – forma digestória) da Doença de Chagas



Fonte: adaptado de ZINGALES, 2012; 2017.

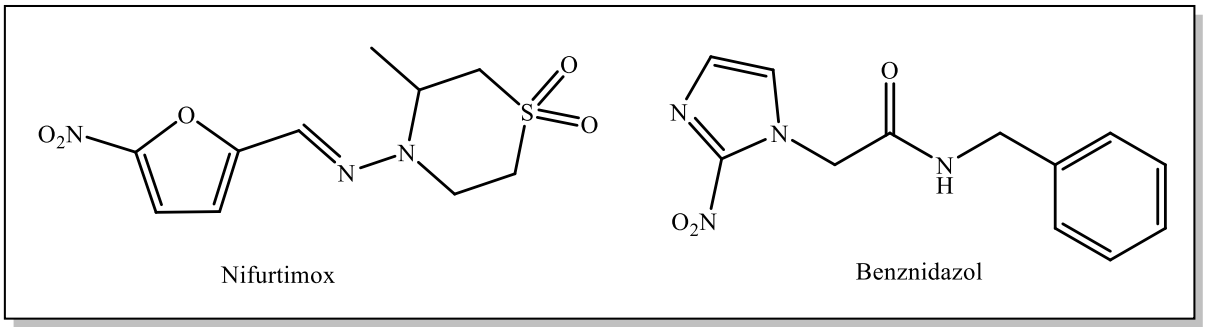
1.1.4. Tratamento

O tratamento medicamentoso para DC é limitado mesmo havendo milhões de pessoas com risco de infecção e o desenvolvimento de alternativas terapêuticas eficazes é deficiente, devido à carência de investimentos para a descoberta de novos fármacos, que afeta primariamente populações mais pobres, em virtude da falta de interesse pela indústria farmacêutica devido ao baixo retorno econômico (WHO, 2015).

As alternativas terapêuticas atuais para essa condição patológica se limitam a dois medicamentos registrados: Nifurtimox (NF) e BZ (

Figura **5**), desenvolvidos há mais de 50 anos. Ambos são nitro-heterocíclicos orais muito ativos para o tratamento da fase aguda da doença. O ciclo de tratamento pode durar 1 a 4 meses, mas seu curso habitualmente não se completa, devido aos efeitos adversos comuns apresentados e esse fator contribui para o surgimento recente da resistência aos fármacos (WILKINSON et al., 2009). Além disso, os medicamentos disponíveis para o tratamento da fase crônica da DC são inadequados e como não há perspectiva do surgimento imediato de uma vacina, novos medicamentos com melhor eficácia, segurança e tolerabilidade são urgentemente necessários (KATSUNO, 2015).

Figura 5 - Estrutura química dos medicamentos nitro-heterocíclicos utilizados na terapêutica da Doença de Chagas



Fonte: Carvalho, 2016.

O NF é um nitrofurano (3-metil-4 {(5-nitrofurfurilideno) amino} tiomorfolino-1-,1-dioxido), desenvolvido pelo Laboratório Bayer e lançado em 1967 com o nome comercial de Lampit (OLIVEIRA et al., 2008). A utilização do NF foi suspensa na década de 1980 na maioria dos países da América do Sul por conta de sua alta toxicidade (RODRIGUES

COURA; CASTRO, 2002). Em 1997, a produção foi totalmente suspensa devido à falta de demanda. No entanto, em 2000, em um acordo com a OMS, houve reativação de sua produção para suporte ao tratamento da tripanossomíase africana (JANNIN; VILLA, 2007) e atualmente pode ser administrado no Brasil, em pacientes com baixa tolerância ao BZ, através de pedido especial do Ministério da Saúde (DIAS et al., 2016). O BZ é um nitroimidazol (2-nitro-N-(fenilmetil)-1H-imidazol-1- acetamida), lançado comercialmente em 1972, que atua inibindo a síntese proteica e a cadeia respiratória, e seus metabólitos estabelecem ligações covalentes com o DNA (RODRIGUES COURA; CASTRO, 2002).

O NF e o BZ atuam por meio da formação de radicais livres e/ou metabólitos nucleofílicos. O grupo nitro de ambos os fármacos é reduzido pela ação da nitroredutase tipo I, induzindo a formação de vários radicais livres intermediários e de metabólitos nucleofílicos. Este processo começa com a reação catalisada pelo NADPH-citocromo P-450 redutase, que atua sobre o grupo nitro da molécula $R\text{-}NO_2$, levando à produção de um radical ânion nitro intermediário ($R\text{-}NO_2^{\cdot-}$). Para os nitrofuranos, o radical tem elevado potencial redox e sofre uma reação do tipo ciclo redox com o oxigênio molecular, o qual reduz e regenera parcialmente o fármaco (DIAZ et al., 1988). Paralelamente, o oxigênio é reduzido a ânion superóxido ($O_2^{\cdot-}$) pela transferência de um elétron para o oxigênio no nível do complexo da NADPH desidrogenase. A produção de peróxido de hidrogênio (H_2O_2) ocorre por dismutação do ânion superóxido. O ânion superóxido e o H_2O_2, na presença do íon férrico Fe^{3+}, formam o radical livre hidroxila (reação Haber–Weiss). Os radicais livres produzidos, principalmente o $OH^{\bullet}$, ligam-se a lipídios, proteínas e DNA, lesando as células (URBINA, 1999; MAYA et al., 2006). Em comparação ao NF, o BZ gera menos radicais livres (APT, 2010).

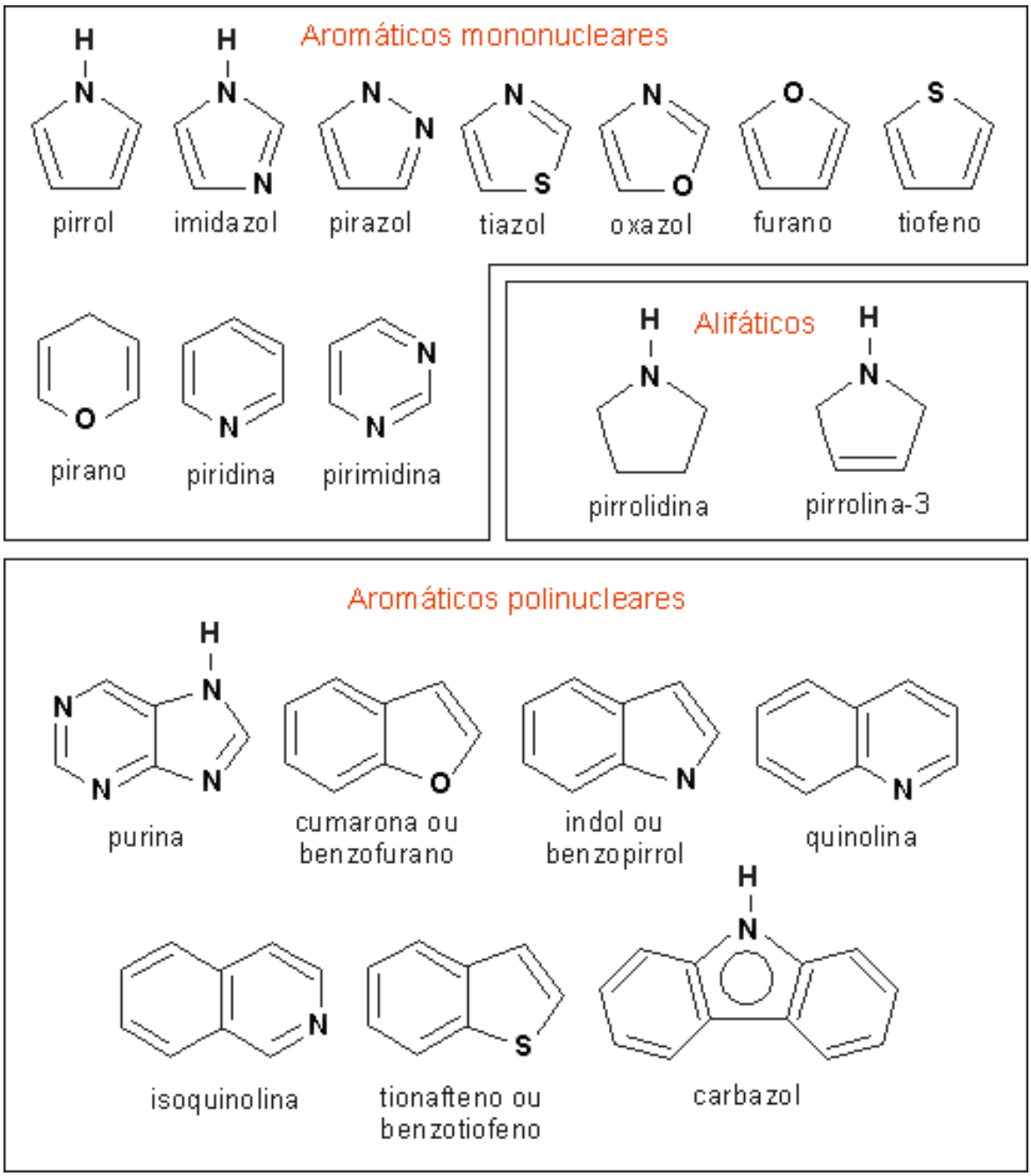
Assim como o NF, o BZ apresenta inúmeros efeitos colaterais que variam em relação à dose administrada, faixa etária dos pacientes (CARRILERO et al., 2011) e a origem geográfica destes, e por consequência, a DTU de *T. cruzi* (RODRIGUES COURA; CASTRO, 2002). Os efeitos adversos são hipersensibilidade no início do tratamento, efeito tóxico na medula óssea, dermatite, febre, dores nas articulações e músculos, e neuropatias periféricas ao final do tratamento. Esses efeitos podem levar a suspensão do tratamento (RODRIGUES COURA; CASTRO, 2002; LOUP et al., 2011). A eficácia destes dois medicamentos ainda gera muitas dúvidas devido às taxas variáveis de cura e na eliminação dos sintomas, principalmente com a baixa eficácia dos mesmos na fase crônica tardia (DIAS et al., 2016). Além disso, a tolerância dos pacientes aos efeitos colaterais deve ser considerada (MORRILO et al, 2015).

1.2. **Compostos heterocíclicos**

Compostos heterocíclicos são espécies químicas cíclicas contendo, no anel, um ou mais átomos diferentes do carbono (Figura 6).

Os heteroátomos mais frequentes são o nitrogênio, o oxigênio e o enxofre (IUPAC, 2009). Os compostos heterocíclicos constituem uma classe de entidades químicas de grande importância para a organização da matéria viva, pois mais da metade de todos os compostos químicos conhecidos são heterocíclicos, com destaque para os fármacos, vitaminas e princípios ativos em plantas e organismos marinhos (FURLANETTO, 2005). Considerando os compostos farmacologicamente ativos, pode-se admitir que a história da química medicinal se desenrole através do isolamento e desenvolvimento dos compostos heterocíclicos (SOUZA, 2012). O levantamento histórico sobre compostos heterocíclicos remonta ao século XVI onde a Quinina (ou Quinino), extraída de *Cinchona officinalis,* foi utilizada na medicina dos povos tradicionais asiáticos para a prevenção e o tratamento da malária, embora a estrutura química fosse desconhecida. Em 1887 surge a Antipirina, um composto usado como anti-térmico.

Figura 6 - Aspectos estruturais dos anéis heterocíclicos



Fonte: adaptado de IUPAC, 2009.

Verifica-se a presença de heterociclos nitrogenados na maior parte dos medicamentos disponíveis atualmente. Os azo-heterociclos aromáticos, núcleos aromáticos com um ou mais átomos de nitrogênio, têm sido objeto de estudos como potenciais antimicrobianos nos últimos anos. Estes núcleos apresentam um amplo espectro de aplicação dentro da química,

com destaque para a elucidação estrutural de produtos naturais, em química analítica e bioinorgânica (KO et al., 2001) como agentes complexantes e dendrímeros (WANG et al., 1997), em química medicinal além de alcaloides biologicamente ativos como agentes quimioterápicos (YANAGISAWA et al., 1973; MOGILAIAH et al., 2003).

Devido a isso, os compostos heterocíclicos têm um lugar especial na química medicinal. A notável capacidade de núcleos heterocíclicos de servir como unidades reativas contribuem amplamente para o seu valor como farmacóforos de diversos fármacos (BOSTRÖM et al, 2012). A maioria dos produtos farmacêuticos que se assemelham aos produtos naturais com atividade biológica são heterocíclicos e são significativos no desenvolvimento de novos potenciais fármacos contra variados tipos de doenças, incluindo a DC (MARTINS et al, 2008).

1.3. **As Arilimidamidas**

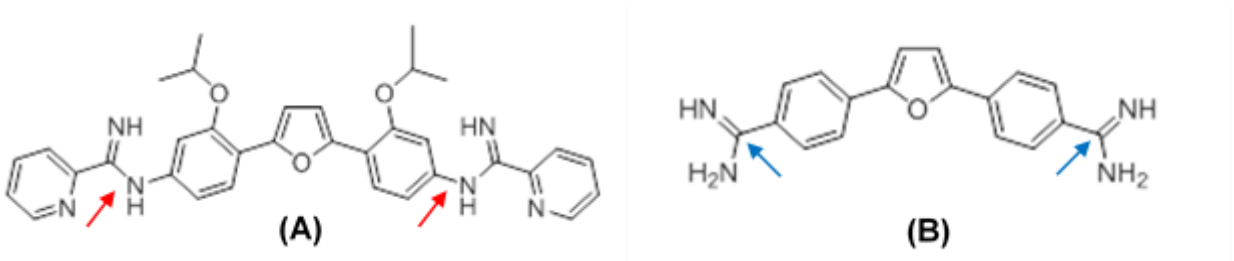
Uma classe amplamente investigada no contexto da infecção pelo *T. cruzi* e que revela promissora atividade tripanocida é a das diamidinas aromáticas (DAs), compostos heterocíclicos oxigenados que se associam de forma não-covalente e não-intercalante à fenda menor do DNA em regiões ricas em sítios AT (WILSON et al., 2008). A observação da eficácia destes compostos sobre diferentes protozoários foi feita ainda na década de 1930, com a descoberta da pentamidina e, posteriormente, de outras DAs que se mostraram efetivas para doença do sono e leishmanioses (KING et al., 1937; ASHLEY et al., 1942).

Resultados *in vitro* também demonstraram a relação entre a ação tripanocida destes compostos e algumas de suas características estruturais, tais como: i) presença dos dois grupamentos catiônicos terminais, sendo as monoamidinas menos efetivas e mais tóxicas que diamidinas (PACHECO et al., 2009); ii) linearidade ou não da molécula sendo as moléculas curvas mais ativas e seletivas sobre diferentes formas de *T. cruzi* (BATISTA et al., 2010); iii) a natureza dos componentes dicatiônicos, mostrando, por exemplo, que grupos amino levam a um melhor efeito tripanocida em relação a diguanidinas (SILVA et al., 2007a), além da presença de grupamentos piridinas e pirimidinas que resulta no incremento de potência sobre parasitos intracelulares (BATISTA et al., 2010). Com relação aos alvos celulares desta classe de compostos, estudos bioquímicos, moleculares e funcionais têm sido realizados em diferentes patógenos e os dados apontam para a ação sobre DNA, mitocôndria,

topoisomerases, citoesqueleto e síntese de poliaminas, assim como alterações na topologia do kDNA, entre outros (WILSON et al., 2014, DALIRY et al., 2011). Em *T. cruzi* foi demonstrado através de ensaios termodinâmicos a falta de correlação entre a atividade de várias moléculas amidínicas e sua força de interação com o kDNA do parasito, porém estudos de dicroísmo circular revelaram a relação entre impacto da associação de bis-AIAs e distúrbios na estrutura molecular do kDNA (DALIRY et al., 2011).

Apesar da comprovada eficácia em estudos pré-clínicos, a baixa biodisponibilidade da maioria das DAs clássicas, atribuída ao alto pK do grupo funcional amidina, dificulta seu uso clínico (SOEIRO et al., 2013). Visando transpor esta dificuldade, foram desenvolvidos derivados, anteriormente conhecidos como diamidinas reversas e hoje chamadas de arilimidamidas (AIAs), que têm revelado promissora ação em ensaios *in vitro* (SILVA et al., 2007a,b; PACHECO et al., 2009; TIMM et al., 2014) e *in vivo* (MDACHI et al., 2009; BATISTA et al., 2010; DA SILVA et al., 2012). As AIAs diferem estruturalmente dos outros análogos de DAs porque o grupo imino terminal está ligado ao anel arila através de um átomo de nitrogênio e não pelo carbono (ROSYPAL et al., 2008) (Figura 7), o que faz com que estes compostos apresentem valores de pKa mais baixos se comparados às diamidinas clássicas e, consequentemente sendo ionizados em meio biológico, o que permite a difusão passiva pelas membranas celulares e, maior biodisponibilidade (SOEIRO et al., 2013).

Figura 7 - Arranjos estruturais de arilimidamida (A) e de diamidina aromática (B)



Fonte: adaptado de De Souza et al., 2004 e Batista et al., 2010a

Ensaios *in vitro* e *in vivo* realizados com a AIA DB766 revelaram a excelente atividade desta AIA sobre formas sanguíneas e amastigotas intracelulares do *T. cruzi*. Outro ponto interessante foi a manutenção de sua excelente atividade (na faixa submicromolar) a 4°C em presença de sangue, revelando potencial uso em centros de hemoterapia. Além disso, DB766 foi igualmente ativa sobre cepas de *T. cruzi* naturalmente resistentes a nitroderivados,

de distintas áreas geográficas e que circulam em diferentes ciclos de transmissão. Ensaios *in vivo* demonstraram redução da parasitemia e do parasitismo cardíaco, apresentando atividade superior à do BZ sobre as cepas Y e Colombiana, e resultando em 90 a 100% de sobrevida (BATISTA et al., 2010). Dados semelhantes também foram encontrados para as bis-AIAs DB1831, DB1965 (DA SILVA et al., 2012), 18SAB075 e 16DAP002 (TIMM et al., 2014), que revelaram importante atividade sobre distintas cepas de *T. cruzi* e alta seletividade *in vitro*.

Estes dados estimulam a continuidade de estudos com esta classe de compostos, sobre distintas cepas representantes das diferentes linhagens de *T. cruzi*, incluindo aquelas naturalmente resistentes a nitroderivados, explorando seu perfil de toxicidade e mutagenicidade *in vitro* e *in vivo* o que representa etapas fundamentais na descoberta de compostos promissores como alternativas terapêuticas para o tratamento da DC (RIBEIRO et al., 2009).

1.4. **Os nitroimidazóis**

Os compostos nitroimidazólicos pertencem à classe dos heterocíclicos nitrogenados, portadores do grupamento nitro (NO_2) e têm grande relevância no uso medicinal, com muitos medicamentos já disponíveis no mercado (CELIK; ARAS, 2006; MITAL, 2009). O espectro de ação desses compostos é tão amplo que a atividade fenotípica dessas moléculas está sendo analisada: (i) no tratamento do câncer, como radiosensíveis (ADAMS; STRATFORD, 1986; ADAMS, 1992; ADAMS; STRATFORD, 1994; KARNTHALER-BENBAKKA et al., 2016); (ii) no controle da fertilidade (KAPOOR et al., 2003); (iii) no tratamento da tuberculose (NAGARAJAN et al., 1989; GUNAY et al., 1999; PODANY; SWINDELL, 2016); (iv) no tratamento da leishmaniose e doença do sono (POORRAJAB et al., 2009; SUNDAR; CHAKRAVARTY, 2015); (v) na inibição da enzima transcriptase reversa do vírus da imunodeficiência humana (HIV) (SILVESTRI et al., 2000; SILVESTRI et al., 2002; AL-SOUD et al., 2007); (vi) entre outros (MITAL, 2009).

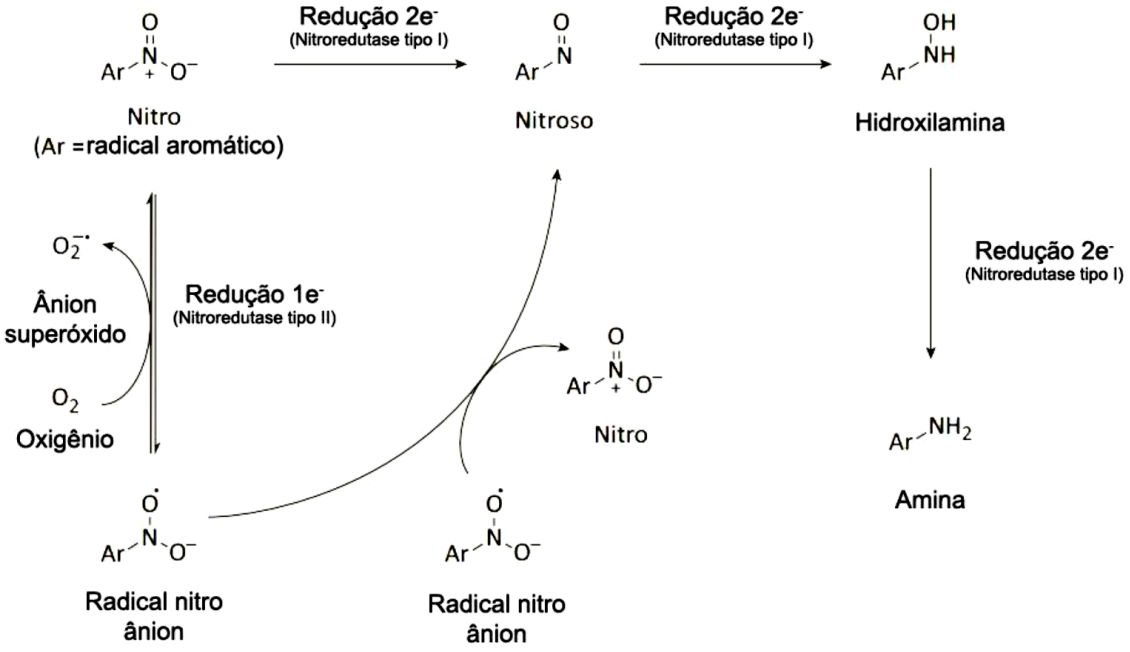
Dentre os compostos possuidores desse núcleo químico, ao qual o benznidazol e o fexinidazol fazem parte, também é relatada a atividade tripanocida, que é a razão pelo crescente interesse no desenvolvimento de novos fármacos contendo esse grupamento, como alternativa ao tratamento atual da tripanossomíase americana e africana, e outras doenças

negligenciadas (BOUTEILLE et al., 1995; ENANGA et al., 1998; BOUTEILLE; CHAUVIERE, 1999; BUSCHINI et al., 2007; MITAL, 2009; KEENAN; CHAPLIN, 2015, CHATELAIN, 2016).

No entanto, a maioria das substâncias do grupo nitroimidazol tem efeito mutagênico e tóxico em bactérias. Esse efeito pode ser explicado pela presença de nitroredutases nesses organismos (Figura **8**) (MORAIS et al., 1998). A redução de um elétron nestes compostos leva a geração de um ânion radical instável nitro que pode se decompor em ânion nitrato e radical imidazol, ou ser reduzido por um segundo elétron, formando intermediários nitrosos mais estáveis que seriam biologicamente ativos, reagindo com o DNA, lipídeos entre outras moléculas (MASON; JOSEPHY, 1985; HEIN, 1998; EDWARDS, 1993; PATTERSON; WYLLIE, 2014). Na presença de oxigênio, o elétron do ânion radical nitro poderia ser sequestrado por ele regenerando o composto-fonte e formando superóxido, gerando um ciclo fútil (MITAL, 2009; PATTERSON; WYLLIE, 2014). Mas esta via de redução enzimática não é tão eficaz nas células de mamíferos, o que diminuiria seu efeito mutagênico (EHLHARDT et al., 1988). Porém foi observada genotoxicidade em células eucariontes *in vitro* (POLI et al., 2002, NESSLANY et al., 2004; MELLO et al, 2013; BOECHAT et al., 2015).

O metronidazol (1-hidroxetil-2-metil-5-nitroimidazol) (MTZ) é um dos compostos mais estudados do grupo dos nitroimidazois (MITAL, 2009), sendo usado na clínica médica para terapia antifúngica e antiparasitária em especial para tricomoníase, candidíase, amebíase e giardíase. Ele é bem tolerado em ratos, sem apresentar toxicidade crônica em doses até 150 mg/kg, administradas até 80 semanas. Macacos ainda apresentaram menor sensibilidade em doses até 225 mg/kg, sem apresentar efeitos adversos (ROE, 1977, 1983). Em humanos, o MTZ é bem tolerado e está entre os fármacos mais utilizados por gestantes no mundo (ROE, 1985; MITAL, 2009). No entanto, O MTZ também é mutagênico nos testes bacteriológicos com *Salmonella enterica* sorovar Typhimurium e *Escherichia coli* (*E. coli*). Em ambiente anaeróbico, há interação e ligação com o DNA, além de se observar quebras. Contudo, o MTZ não apresenta efeito mutagênico em células eucariontes *in vitro*. O teste do micronúcleo é negativo e aberrações cromossômicas só são vistas em condições anaeróbias (MITAL, 2009).

Figura 8 - Reações de nitro-redução dos compostos nitroimidazólicos



Fonte: adaptado de PATTERSON; WYLLIE, 2014.

Em teoria todo nitrocomposto apresenta potencial genotóxico nos ensaios padrão de toxicologia regulatória. Essa atividade tóxica pode variar de acordo com a posição do grupo nitro na molécula, diminuindo consideravelmente este evento adverso (WALSH et al.,1987). Porém, também, é possível observar uma variável resposta tripanocida quando são realizadas alterações da estrutura, sendo este o desafio atual no desenvolvimento de novos compostos (CHAUVIÉRE et al., 2003; BOECHAT et al., 2015; PAPADOPOULOU et al., 2015).

1.5. **As estatinas**

Em 1971 o cientista Akira Endo isolou um metabólito secundário de fungos do gênero *Penicillium* capaz de bloquear a síntese do colesterol em seres humanos. Esse achado originou a primeira estatina, mevastatina, em 1976 (ENDO; KURODA; TSUJITA, 1976).

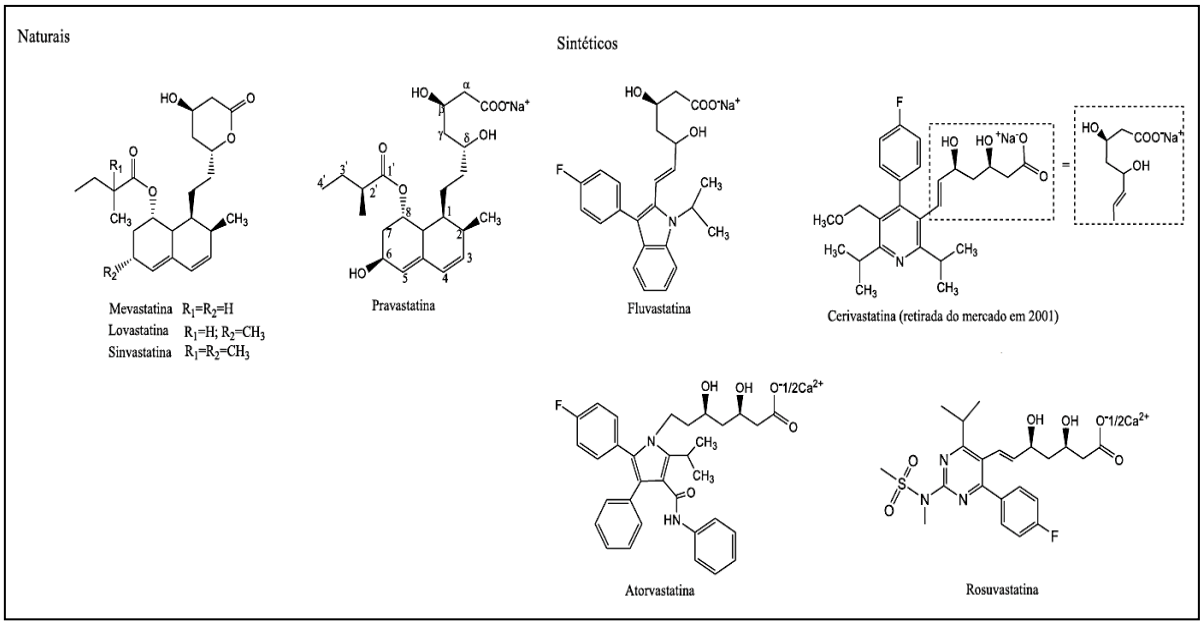
As estatinas são inibidores da biossíntese de esterois, que são fármacos inibidores competitivos da enzima hidroxi-3-metil-glutaril Coenzima A (HMGCoA) redutase, amplamente utilizados para o controle da hipercolesterolemia total e, em especial, para a redução dos níveis séricos de LDLc (ORTEGO, 2004). Estes inibidores também alteram o arranjo conformacional da enzima, limitando sua atividade funcional (FONSECA, 2005).

Mais tarde, esses inibidores da biossíntese de colesterol puderam também ser isolados de micromicetos do gênero *Aspergillus*, e *Monascus*, dando origem às estatinas de segunda geração, como a Sinvastatina (SIM) e a Lovastatina (LOVA) (CHAN et al., 1983). Cerca de 20 anos mais tarde, novos derivados sintéticos dessa estatina começaram a ser introduzidos no mercado, como a atorvastatina (AVA) em 1996, e a cerivastatina em 1997 (GÓIS *et al.*, 2011).

No ano de 2002, a AVA foi o fármaco mais vendido no mundo e rendeu para o fabricante Pfizer mais de US$ 8 bilhões (CAMPO; CARVALHO, 2007). Estudos mostram que dentre as estatinas utilizadas, a AVA é a que gera uma inibição mais prolongada sobre a HMG-CoA redutase e possui alta seletividade de atuação a nível hepático, por isso, detém a capacidade de inibição mais prolongada da enzima, promovendo maior efeito sobre os níveis de colesterol LDL e de triglicerídeos (CILLA et al., 1996 e BROWN et al., 1998).

Com a ascensão das estatinas produzidas em laboratório, as mesmas foram classificadas em dois tipos: estatinas naturais ou geradas por processos fermentativos e estatinas sintéticas (Figura **9**) (GOSWAMI et al., 2013).

Figura 9 - Estrutura química das estatinas naturais e sintéticas



Fonte: Adaptado de Campo e Carvalho, 2007.

O perfil de inibição das estatinas difere razoavelmente entre elas, fazendo que algumas possuam grande seletividade de atuação por conta, por exemplo, da lipossolubilidade, que garante o aporte do composto passando pela membrana plasmática para então exercer sua atividade inibitória no citoplasma e na mitocôndria das células-alvo (FONSECA, 2005). As características que diferem as estatinas entre si (Tabela 1), como tempo de absorção, lipossolubilidade, vias de metabolização e meia-vida plasmática, são determinadas principalmente pela estrutura química desses fármacos (MACHADO et al., 2011), responsável pela avidez para ligação ao sítio ativo da enzima HMG-CoA redutase e consequente potência do composto (LENNERNAS; FAGER, 1997 e MCTAGGART et al., 2001).

A inibição da enzima HMGCR reduz a síntese de vários isoprenóides por interromper a via do mevalonato/ácido mevalônico no fígado e em outros tecidos, impactando nos níveis de colesterol tecidual, já que essa enzima é a terceira etapa de uma cascata de mais de 20 etapas que culminam na síntese de colesterol. Isto ativa a Proteína de Ligação ao Elemento de Resposta a Esterol (SREBP), que é responsável por liberar proteínas reguladoras do retículo endoplasmático, aumentando a expressão do gene que codifica receptores de membrana celular encarregados em endocitar o colesterol LDL (LDL-R), principalmente no fígado, adrenais e adipócitos (VAUGHAN et al., 2000).

Tabela 1 - Características básicas que diferem as estatinas entre si

	Atorvastatina	Fluvastatina	Lovastatina	Pravastatina	Rosuvastatina	Sinvastatina
Química	Composto quiral (anel pirrólico)	Anel com betahidróxiácido	Anel lactônico tricíclico inativo	Anel com betahidróxiácido	Composto estruturalmente sintético	Derivado da lovastatina, semi-sintético
Ligação Protéica	CYP 450 3A4	CYP 450 2C9	CYP 450 3A4	CYP 450 3A4	CYP 450 3A4	CYP 450 3A4
Lipofilicidade	Lipofílico	Não-lipofílico	Lipofílico	Não-lipofílico	Não-lipofílico	Lipofílico
Meia-vida	15 horas	3 horas	2 horas	2 horas	20 horas	3 horas
Absorção	Rápida	Rápida	Média	Lenta	Média	Rápida
Influência de Alimentos	Sim	Não	Sim	Não	Não	Não
Dose	10 a 80mg	20 a 40mg	10 a 20mg	10 a 20mg	10 a 40mg	10 a 40mg

Fonte: Reproduzido de Machado *et al.*, 2011.

As estatinas apresentam grande importância porque, desde o início de sua administração, são considerados os mais seguros agentes redutores de colesterol dado seu perfil de segurança, eficácia e tolerância, que se, comparado a outros fármacos de mesma função, como ácido nicotínico, possuem poucos efeitos adversos (VERHULST et al., 2004). Ainda que raramente, o uso de estatinas associado a outros medicamentos específicos, principalmente os fibratos e outros inibidores de CYP3A4 e CYP2C9, pode determinar rabdomiólise, ou seja, toxicidade muscular, levando a manifestações de dores musculares, mioglobinúria e insuficiência renal (STAFFA et al., 2002).

1.6. **Efeitos Pleiotrópicos das Estatinas**

No início do século XXI, muitos estudos foram realizados com as estatinas e vários outros efeitos, não diretamente relacionados à redução da hipercolesterolemia têm sido relatados. Essas atividades múltiplas desta classe de fármacos são conhecidas como efeitos pleiotrópicos (DAVIGNON, 2004). De fato, ensaios clínicos evidenciaram que as estatinas podem impactar sobre vários órgãos, independente da redução dos níveis de colesterol, introduzindo, portanto, o conceito dos efeitos pleiotrópicos desses compostos (SOUBRIER; CHRISTIAN, 2006). Dentre os efeitos secundários mais descritos estão, por exemplo, as atividades anti-inflamatória e antioxidante desses fármacos, sugerindo que o uso de estatinas possa ser investigado para o tratamento de doenças infecciosas, degenerativas além de neoplasias, como nos casos de artrite, câncer, malária e toxoplasmose, nas quais a inflamação

tem papel central na patogênese dessas doenças (Figura 10) (CORTEZ, 2008; PARQUET, 2010; SOKALSKA, 2010).

Estatinas inibem também a síntese de intermediários isoprenoides, como o farnesilpirofosfato (FPP) e o geranilgeranilpirofosfato (GGPP), que são importantes para o aumento de proteínas intracelulares da classe das pequenas proteínas G (Ras, Rho e Rac, por exemplo), e estas são capazes de levar a sinalização intracelular relacionada a genes que codificam a síntese de citocinas, de fatores de coagulação, de óxido nítrico entre outras moléculas (MCFARLANE et al., 2002; BARRIOS-GONZÁLEZ & MIRANDA, 2010; MARZIO et al., 2010). Parte dos efeitos terapêuticos das estatinas pode ser relativo à síntese de moléculas que estimulam funções endoteliais além da redução dos níveis de mediadores inflamatórios (ROHILLA et al., 2011).

Figura 10 - Efeitos pleiotrópicos das estatinas

Fonte: O autor, 2018.

1.6.1. Efeitos na função endotelial

Segundo LIAO e LAUFS (2009), a hipercolesterolemia prejudica a função endotelial e é uma das primeiras manifestações da aterosclerose. A disfunção endotelial é caracterizada principalmente pela redução da síntese, liberação e atividade do óxido nítrico derivado do endotélio (NO). O NO promove relaxamento vascular, inibição da agregação plaquetária, proliferação do músculo liso vascular e inibição de componentes do processo aterogênico. Dessa forma, as estatinas podem melhorar a função endotelial não só pela redução do colesterol, mas através do estímulo da enzima óxido nítrico sintase endotelial (eNOS) com consequente aumento da produção de NO (LIAO; LAUFS, 2009 e MACHADO *et al.*, 2011).

Outro possível mecanismo pelo qual as estatinas podem melhorar a função endotelial é através de seus efeitos antioxidantes promovendo o relaxamento dependente do endotélio pela inibição da produção de espécies reativas de oxigênio (ROS), tais como superóxido e radicais hidroxila, através da inibição da atividade da NADH oxidase mediada pela Rac1 (LIAO; LAUFS, 2009).

1.6.2. Efeito anti-inflamatório

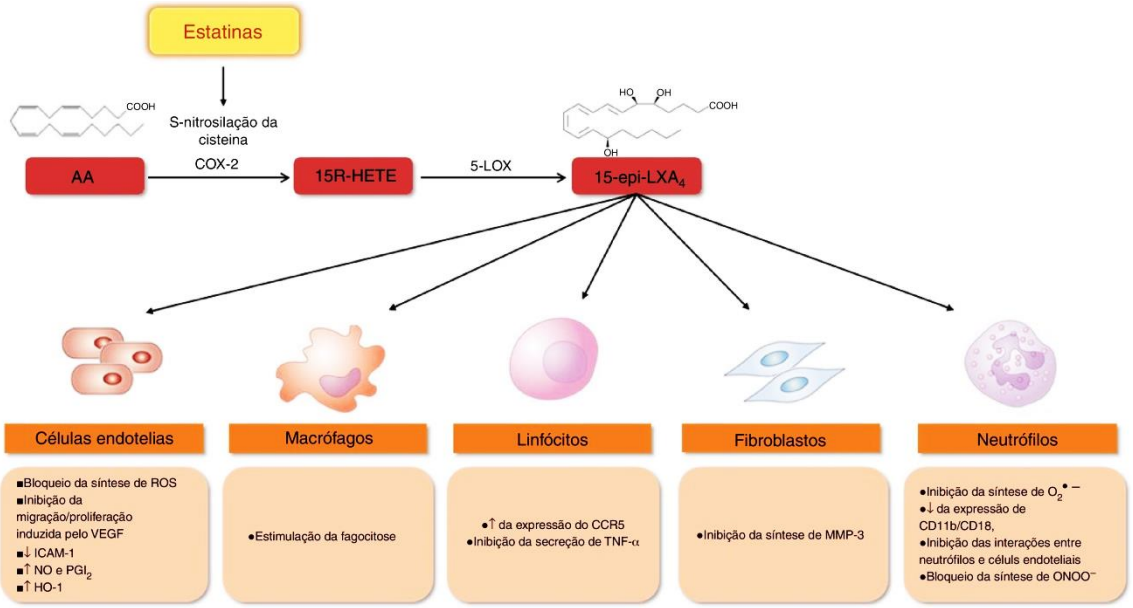
Ainda segundo LIAO e LAUFS (2009), as estatinas possuem propriedades anti-inflamatórias devido à sua capacidade de reduzir o número de células inflamatórias nas placas ateroscleróticas. Esse mecanismo ainda não foi completamente elucidado, mas sugere-se um envolvimento na inibição das moléculas de adesão, como a molécula de adesão intercelular 1 (ICAM-1). Observa-se que a diminuição da inflamação pelo uso das estatinas ocorre também através da redução dos níveis de proteína C reativa (PCR) (SOUZA, 2011). A PCR é uma molécula de fase aguda produzida pelo fígado em resposta a citocinas pró-inflamatórias, como a interleucina-6 (IL-6). Esta proteína pode contribuir para o desenvolvimento da aterosclerose pela ligação e modificação do LDL no interior das placas ateroscleróticas e, uma vez ligada, ativa o complemento, promovendo o aumento da inflamação (LIAO; LAUFS, 2009).

Estudos recentes mostram que as estatinas, como a AVA que exibem poder seletivo superior em nível hepático, possam atuar com maior eficácia no controle dos níveis de colesterol LDL e de triglicerídeos (CILLA et al., 1996 e BROWN et al., 1998). Além disso,

são moléculas capazes de suprimir a indução do complexo de histocompatibilidade principal de classe II (MHC II) induzida por interferon-gama (IFN-γ), de diminuir a secreção de citocinas pró-inflamatórias, como IFN-γ, MCP-I e IL-1β e de reduzir a proliferação e ativação dos linfócitos T, e com isso, demonstrando atividade imunomoduladora (KWAK et al., 2000 e NEURAUTER et al., 2003, SANTOS, 2017).

As estatinas induzem a s-nitrosilação de resíduos de cisteína da COX-2. Esse fenômeno gera uma reação em cadeia na via de metabolismo do ácido araquidônico, dependente de COX-2 e LOX-5, gerando a 15-epi-lipoxina A_4 (15-epi-LXA_4) (CAMPOS ESTRADA et al., 2015). Esse mediador lipídico é conhecido por suas propriedades pró-resolutivas, antioxidantes, vasodilatadoras, antiproliferativas e principalmente imunomoduladoras (Figura 11) (COSTA et al., 2015). CAMPOS-ESTRADA e seus colaboradores (2015) demonstraram que a SIM desempenha um papel importante na infectividade de *T. cruzi* por promover a desativação do endotélio e redução da resposta inflamatória sistêmica mediada por 15-epi-LXA_4. Os autores ainda sugerem que as estatinas podem ser utilizadas em combinação com benznidazol para a prevenção e tratamento de quadros agudos da doença.

Figura 11 - Efeitos anti-inflamatórios das estatinas



Fonte: Adaptado de Costa et al., 2015

Embora necessária para recuperar homeostasia tecidual e controle de infecção, uma resposta inflamatória exacerbada pode também resultar em graves injúrias, resultando em

patologia crônica inflamatória progressiva. Por esta razão, há uma grande necessidade do desenvolvimento de fármacos anti-inflamatórios que controlem suas sequelas nocivas sem interferir nos efeitos benéficos (KUMAR et al., 2010). Deste modo, as estatinas e seus derivados, têm sido desenvolvidos visando novas alternativas terapêuticas, com eficácia superior e menor indução de efeitos adversos, podem ser também alternativas para tratamento de doenças inflamatórias e autoimunes e para alguns autores, ainda em benefício para pacientes transplantados visando prevenir rejeição de aloenxertos (MACH, 2004).

1.6.3. Efeitos quimiopreventivos e citoprotetores

Um importante efeito pleiotrópico das estatinas é o efeito quimiopreventivo. De acordo com um estudo realizado por AJITH e SOJA (2006), a AVA e a LOVA são capazes de exercer efeitos quimiopreventivos contra agentes mutagênicos diretos. Em um ensaio com mutação induzida por azida sódica e 4-nitro-o-fenilenediamina em um modelo de mutação reversa bacteriana utilizando cepas de *Salmonella enterica* sorovar Typhimurium, as estatinas foram capazes de evitar danos por alteração no quadro de leitura e por substituição de pares de bases. A AVA inibiu significativamente as mutações, o que ficou evidente pela diminuição do número de colônias revertentes nas placas tratadas. Este efeito antimutagênico das estatinas pode ser explicado por uma provável proteção do genoma bacteriano contra os agentes mutagênicos diretos. Essa proteção do genoma bacteriano pode ser devido à rápida eliminação de agentes mutagênicos das bactérias antes de sua interação com o DNA. As estatinas podem facilitar ou estimular o sistema de exportação transmembrana para eliminação dos agentes mutagênicos, interferindo na sua absorção pelas bactérias. (AJITH; SOJA, 2006).

De acordo com MEKHAIL et al. (2012), em células eucariotas o efeito antineoplásico das estatinas ocorre pela supressão da biossíntese do mevalonato, um precursor de intermediários isoprenoides que são adicionados durante a modificação pós-traducional de uma variedade de proteínas, como as subunidades Ras e Rho da proteína G. Estas proteínas estão envolvidas na progressão do ciclo celular, sinalização celular e integridade da membrana. A inibição da ativação Rho reverteu o fenótipo metastático de células de melanoma humano.

A AVA é capaz de inibir o crescimento do tumor através da indução de apoptose via ativação da proteína quinase ativada por AMP, que leva à expressão de p21, resultando em estresse do retículo endoplasmático e indução de morte celular (YANG et al., 2010).

Quando da associação com quimioterápicos, as estatinas demonstram a capacidade de potencializar a atividade antitumoral através do aumento de apoptose. As estatinas detêm uma variedade de células na fase G1 do ciclo celular e estas se mostram mais sensíveis à atividade dos quimioterápicos (CAMPO; CARVALHO, 2007).

Os efeitos benéficos das estatinas na área da terapêutica do câncer vêm ganhando reconhecimento. O interesse emergente na utilização das estatinas como agentes anticâncer baseia-se em evidências pré-clínicas de suas propriedades antiproliferativas, pró-apoptóticas e anti-invasivas (CHAN et al., 2003).

A atividade antimutagênica das estatinas justifica o possível papel destas como agentes quimiopreventivos do câncer. Desta forma, o Ensaio de Ames de Mutagenicidade *Salmonella*/ Microssoma foi utilizado para avaliar a atividade antimutagênica da AVA e LOVA contra mutágenos de ação direta por AJITH E SOJA (2000).

1.6.4. Efeitos antiparasitários

Ao contrário das pesquisas envolvendo fármacos antimicrobianos, o desenvolvimento de fármacos antiparasitários esbarra em inúmeros percalços, entre eles, a maior complexidade desses organismos (BARRY et al. 2012). Os alvos terapêuticos dos parasitos apresentam maior similaridade com os presentes no hospedeiro humano, havendo, portanto, menor especificidade para esses fármacos. Uma tarefa para estabelecer medicamentos eficientes é encontrar compostos que interajam especificamente com as moléculas fundamentais ao metabolismo do parasito. Essas moléculas podem atuar, de modo seletivo, em enzimas, organelas ou rotas metabólicas do patógeno (DIAS et al., 2009).

Nesse contexto, as estatinas estão sendo empregadas tanto para a inibição da síntese de ergosterol no metabolismo de protozoários quanto na atenuação da resposta imunopatológica. MORAES et al. (2014) demonstraram que inibidores da biossíntese de ergosterol (do inglês *ergosterol biosynthesis inhibitors* – EBI) eram eficazes *in vitro* contra diversas cepas representantes dos DTUs de *T. cruzi*, apesar de terem resultados inferiores aos observados para compostos nitro-heterocíclicos (CHATELAIN; KONAR, 2015).

SILVA et al. (2006) mostraram que os EBI cetoconazol e posaconazol foram capazes de causar remodelamento citoarquitetônico em cardiomiócitos infectados com *T. cruzi*, além de exercerem atividade tripanocida desses medicamentos. O ensaio clínico de fase II "CHAGAZOL", utilizando posaconazol para o tratamento de pacientes em fase crônica da DC apresentou falha terapêutica e desempenho pior que o tratamento com benznidazol (URBINA, 2015).

Por outro lado, os trabalhos de DINIZ et al. (2013) e MARTINS et al. (2015) sugerem um efeito promissor da terapia combinada entre BZ e EBIs (inibidores da enzima 14-α demetilase - CYP51) sobre *T. cruzi.* Os azóis posaconazol e itraconazol foram ativos sobre modelos experimentais de infecção aguda por *T. cruzi*. Outro EBI promissor, inibidor de CYP51, é o imidazol VNI, que apresentou eficácia *in vitro* e em modelo animal de infecção aguda pelo *T. cruzi*, em estudo desenvolvido por nosso grupo (SOEIRO et al., 2013).

URBINA et al. (1993) observaram que a LOVA potencializa o efeito tripanocida do cetoconazol e nesse sentido PEÑA-DÍAZ et al. (1997) detectaram HMGCR funcional na via de síntese do ergosterol no *T. cruzi*. Esse achado indica que o alvo terapêutico está presente nesse protozoário e que novas estatinas, seletivas para a enzima deste parasita possam ser desenvolvidas, visando o controle antiparasitário. Foi demonstrado que a administração de SIM reduz o quadro inflamatório cardíaco e os níveis séricos e tissulares de mediadores inflamatórios durante a infecção aguda pelo *T. cruzi* em modelo experimental murino (SILVA et al. 2012), além de melhorar o remodelamento cardíaco em cães infectados com o parasito (MELO et al., 2011). Esses achados indicam que o alvo terapêutico está presente nesse protozoário e que novas estatinas, seletivas para a enzima do *T. cruzi* possam ser identificadas, visando o controle desse agente infeccioso, atuando ainda como agente anti-inflamatório no curso tardio da infecção (fase crônica).

A inibição da síntese lipídica pelas estatinas em protozoários do Filo apicomplexa é um assunto que vem sido estudado. CORTEZ et al., (2008) demonstraram que a SIM era capaz de inibir a multiplicação do *Toxoplasma gondii* em culturas de macrófagos infectadas. GRELLIER et al. (1994) detectaram a inibição do crescimento dos clones F32 e FcB1 de *Plasmodium falciparum* no interior de eritrócitos tratados com SIN e LOVA. O mesmo grupo também descreve a inibição do crescimento do hemoprotozoário *Babesia divergens,* pertencente ao filo Plasmodroma, submetidos ao mesmo tratamento.

VANDEWAA et al. (1989) demonstraram que, em condições experimentais, a HMGCR é fundamental para a fisiologia dos ovos do helminto trematódeo *Schistosoma mansoni*. Após a constatação, o mesmo grupo demonstrou a atividade antiesquistossômica da

mevastatina, concluindo que a atividade da HGMCoA-redutase no ciclo de vida do *S. mansoni* é fundamental para sua sobrevivência (CHEN et al., 1990).

Em relação à malária, a AVA se mostrou um promissor agente sinérgico para o tratamento *in vitro* de *P. falciparum* utilizando quinina (PARQUET et al., 2010). A utilização simultânea dos dois compostos foi cerca de 20% mais efetiva. TAOUFIQ et al. (2011) sugerem que a AVA é capaz de prevenir a citoaderência, penetração e dano no endotélio promovidos por *P. falciparum*. Quando células endoteliais incubadas com eritrócitos parasitados (pRBC) são tratadas com AVA, a expressão de moléculas de adesão é reduzida e, consequentemente, observa-se menor invasão de parasitos e de apoptose das células endoteliais.

REIS et al. (2012) descrevem as estatinas como redutoras da neuroinflamação em um modelo experimental de malária cerebral. Essas moléculas foram capazes de impedir o dano cognitivo em animais com lesões de neuromalária e de sepse severa. A LOVA foi capaz de proteger contra o dano causado por ROS, aumentar função microvascular cerebral, reduzir o *rolling* e a migração de leucócitos, além de aumentar a atividade da heme-oxigenase 1 (HMOX-1), importante enzima da maquinaria antioxidante. Outros efeitos foram encontrados, como menores níveis de citocinas pró-inflamatórias no cérebro de animais tratados com LOVA e, corroborando o estudo, os animais tratados com a estatina apresentaram menores taxas de peroxidação lipídica em tecidos como hipocampo e cerebelo.

AVA foi aplicada em combinação com mefloquina (SOURAUD et al., 2012) e com dihidroartemisinina (DORMOI et al., 2013) para o tratamento de malária cerebral em modelos murinos. Em ambos os casos a molécula se mostrou um eficiente adjuvante no tratamento das lesões decorrentes das respostas fisiopatológicas do hospedeiro, devido à sua característica antioxidante.

1.7. **Lesões no DNA, mutagênese, carcinogênese e quimioprevenção**

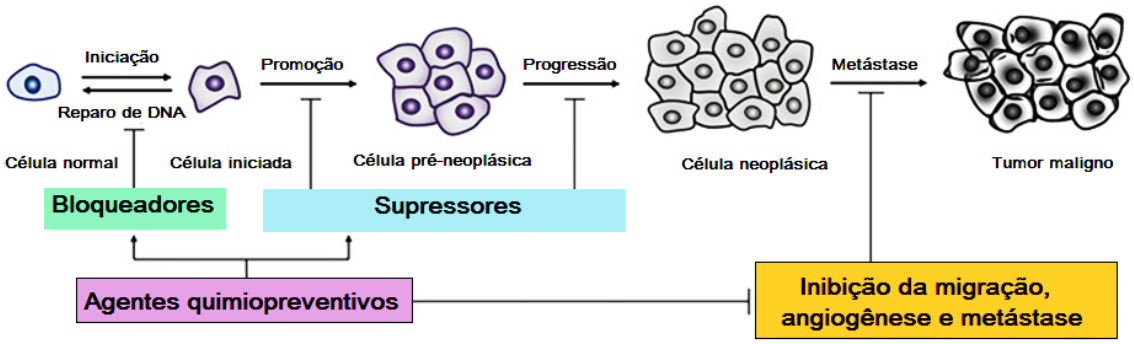
Danos ao DNA são frequentemente postulados como causadores de mutações que estão associados com a iniciação e progressão do câncer. As mutações são alterações súbitas no conjunto gênico de um organismo que são explicadas pela recombinação da variabilidade genética pré-existente (ZAHA, 1996). As mutações são criadas principalmente por fatores externos, incluindo agentes químicos e físicos, chamados mutagênicos. Além disso, mutações

podem ocorrer espontaneamente devido a erros na replicação, reparação e recombinação do DNA. Em geral, mutações podem ser agrupadas em negativas, neutras, positivas, letais e subletais.

Essas alterações podem ser divididas em mutações espontâneas, decorrentes de processos celulares normais, e mutações induzidas, devido à exposição do organismo a agentes químicos ou físicos (LEWIN, 2001). As mutações gênicas que ocorrem por alteração de um único nucleotídeo, por exemplo, podem ser subdivididas em substituição de pares de bases e alteração na matriz de leitura, ocorrendo por adição ou deleção de nucleotídeos (GRIFFITHS et al., 2006).

Os eventos celulares e moleculares regulados por esses agentes quimiopreventivos incluem indução de apoptose, alterações no ciclo celular, e na proliferação celular, reparo de DNA, diferenciação, ativação e desintoxicação de carcinógenos por enzimas metabolizadoras de xenobióticos, inativação/ativação funcional de oncogenes e genes supressores de tumor, angiogênese e metástase. Junto com estes, a mitigação da tumorigênese mediada pelo estresse oxidativo é um dos mecanismos pelos quais eles exercem seu potencial anticâncer (Figura 12) (CHIKARA et al., 2018).

Figura 12 - Mecanismo de ação dos agentes quimiopreventivos



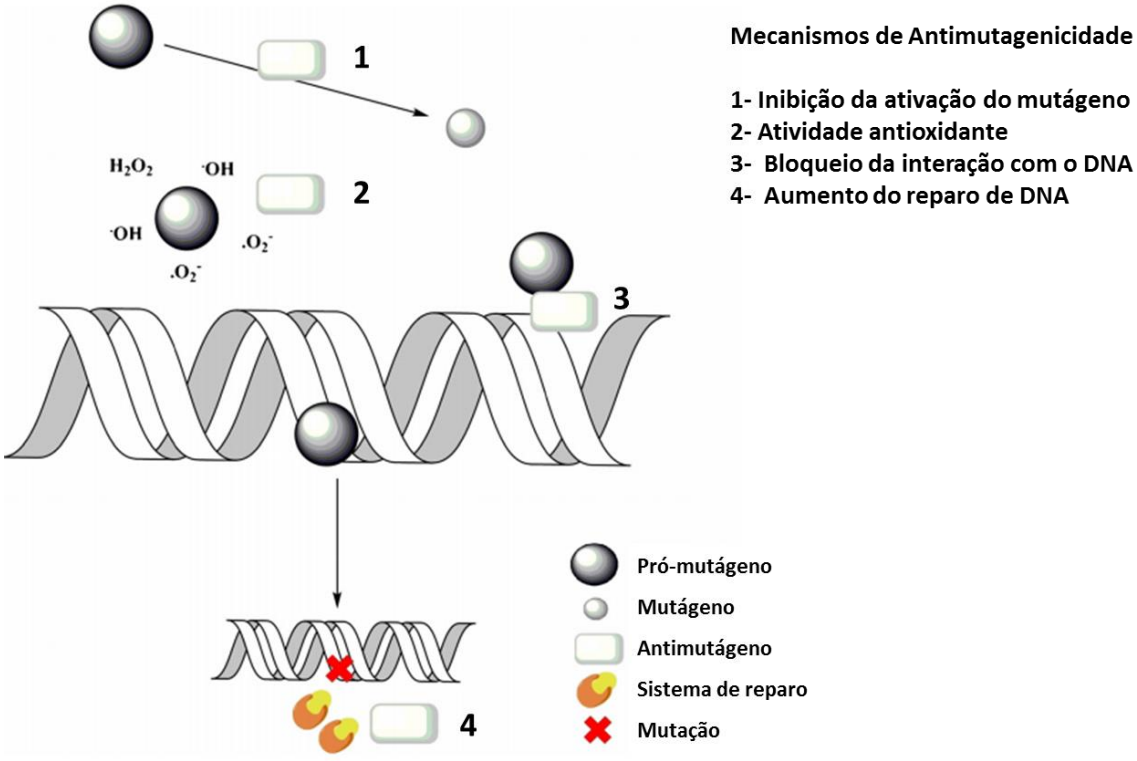
Fonte: Adaptado de RATHER; BAGHAT, 2018.

Alterações mutagênicas que ocorrem nas células germinativas podem ser passadas para as gerações futuras, enquanto as mutações somáticas contribuem para a patogênese de várias condições patológicas, incluindo câncer (MIGLIORE; COPPEDÈ, 2002; et al., 2003; IZZOTTI et al., 2003; WEAKLEY et al., 2010). O termo "genotoxicidade" é um conceito mais amplo do que a mutagenicidade e descreve a capacidade dos compostos para afetar a estrutura do DNA ou o aparelho celular e topoisomerases, responsáveis pela fidelidade do

genoma. Os efeitos genotóxicos no DNA nem sempre estão relacionados a mutações (MAURICI et al., 2005; EASTMOND et al., 2009).

Certos compostos, conhecidos como antimutagênicos, são capazes de diminuir ou mesmo remover os efeitos mutagênicos de substâncias químicas potencialmente nocivas. NOVICK e SZILARD (1952) aplicaram principalmente o termo “antimutagênico” a agentes que possuem a capacidade de diminuir a taxa ou frequência de mutações induzidas ou espontâneas. Este grupo de agentes inclui compostos naturais e sintéticos. Segundo KADA et al. (1982), podem distinguir-se dois tipos diferentes de antimutágenos, isto é, desmutágenos e bioantimutágenos. Desmutágenos que funcionam extracelularmente são capazes de inativar agentes mutagênicos antes que eles atinjam o DNA. Por outro lado, os bioantimutágenos atuam dentro da célula e participam da supressão da mutação após o dano do DNA. Estes compostos são capazes de influenciar a reparação e replicação do genoma (KADA; SHIMOI 1987; DEFLORA, 1998). Baseado em seu mecanismo de ação entre os antimutagênicos, várias classes de compostos podem ser distinguidas (Figura 13). Estes são compostos com atividade antioxidante; compostos que inibem a ativação de mutagênicos; agentes de bloqueio; bem como compostos caracterizados com vários modos de ação.

Figura 13 - Mecanismos de ação dos antimutágenos



Fonte: Adaptado de Słoczyńska et al., 2014.

Um dos principais sistemas de reparo de danos no DNA é o reparo por excisão de nucleotídeos (NER), que repara diversas lesões que resultam em distorções na hélice do DNA. Nesse mecanismo, várias proteínas podem atuar conjuntamente pela remoção da área da fita lesada e, em seguida ressintetizando um novo segmento livre de erro. Em procariotos como a *Escherichia coli* as proteínas UvrA, UvrB, UvrC e UvrD estão envolvidas neste sistema de reparo. Outro sistema de reparo muito utilizado é a via por excisão de bases (BER). Neste sistema, apenas a base nitrogenada será removida, diferente do sistema NER descrito anteriormente. Enzimas da classe da família das DNA glicosilases agem no reconhecimento de bases nitrogenadas modificadas ou inapropriadas e são responsáveis por iniciar este tipo de reparo. A remoção da base nitrogenada errônea resulta na formação de um sítio abásico, o qual é eliminado por endonucleases. Os sistemas NER e BER ocorrem de formas muito semelhantes em procariotos e eucariotos (WATSON et al., 2006).

1.8. **Eficácia e segurança experimental de novos fármacos**

A química medicinal tem evoluído significativamente nos últimos anos, sendo uma área de importância no desenvolvimento novos fármacos bioativos, correspondendo a cerca de 75% do total de fármacos disponíveis no mercado farmacêutico.

A pesquisa de produtos farmacêuticos sintéticos busca sempre uma substância mais próxima do ideal, com menor toxicidade e maior interação com os alvos biológicos, além de melhor custo efetivo (LESSA, 2001).

As estratégias para o desenvolvimento de novos agentes farmacológicos (ex. agentes antiparasitários) envolvem etapas de estudo acerca da eficácia e segurança por diferentes plataformas multidisciplinares incluindo (i) identificação de novos princípios ativos a partir de bibliotecas de compostos (sintéticos e biomoléculas), (ii) reposicionamento e investigação de drogas já licenciadas para novas aplicações quimioterápicas, e (iii) validação de alvos/vias metabólicas específicas (SOEIRO; CASTRO, 2009).

No contexto da DC, segundo recomendações da OMS, é preciso oferecer tratamento etiológico para os portadores e toda infraestrutura necessária para tal, bem como estímulo à criação de um consenso entre os países afetados para definição e padronização de ferramentas de diagnóstico e de esquemas terapêuticos visando atenção em saúde mais segura e eficaz (WHO/TDR, 2012). Além disso, a WHO (assim como o consenso acadêmico e de instituições não governamentais, como DND*i*) estabelece que o fármaco ideal para o tratamento da DC deve cumprir com vários requerimentos, incluindo: i) indução de cura parasitológica nas fases aguda e crônica; ii) eficácia em uma ou poucas doses (≤ 60 dias); iii) apresentar baixo custo; iv) não ter efeitos colaterais graves/irreversíveis ou teratogênicos; e v) não induzir resistência, entre outros (WHO, 2006). Já BUCKNER e NAVABI (2010) ressaltam que, além de ativo e seguro, é essencial a baixa interação do composto-candidato com enzimas do complexo citocromo P450, evitando, desta forma, sinergismos ou antagonismos com outros fármacos, como antiarrítmicos e anticoagulantes.

A fim de definir prioridades no tocante à pesquisa de novas opções terapêuticas para a DC e traçar as necessidades mais imediatas dos pacientes, o DND*i* apresentou um perfil de produto alvo (TPP, do inglês "Target Product Profile"). Atuando como um guia, o atual TPP contém elementos que descrevem o produto ideal para orientar o processo de desenvolvimento (Tabela 2) tendo por base os dados gerados nos ensaios clínicos recentes utilizando triazois inibidores da CYP51. Além das características de eficácia e segurança superiores aos fármacos disponíveis o TPP inclui também tempo de tratamento ≤ terapia com antifúngicos, estabilidade (3-5 anos em zona climática IV) e ação sobre todas as cepas de *T. cruzi* ou pelo menos sobre representantes das DTUs I, II, V e VI (RIBEIRO et al., 2009; DND*i*, 2015). Além do TPP para DC, há de se considerar os critérios mínimos de seleção de compostos promissores e líderes de modo a progredir no fluxograma de desenvolvimento de novos fármacos para esta patologia (ROMANHA et al., 2010; DON; IOSET, 2013) que inclui como características fundamentais a atividade ≤ 1 μM e índice de seletividade (IS) ≥ 50 (para compostos líderes).

Tabela 2 - Perfil de produto alvo para a pesquisa de compostos para a doença de Chagas

Parâmetro analisado	Aceitável	Ideal
Fase alvo	Crônica recente/Indeterminada	Aceitável + Reativações
Subespécies de *T. cruzi*	Tc I-VI	Tc I-VI
Distribuição	Todas as áreas	Todas as áreas
População alvo	Imunocompetentes	Aceitável + Imunossup.
Adultos/Crianças	Adultos	Todos

Eficácia clínica	≥ BZ em áreas endêmicas	Aceitável + portadores reativados
Resistência	Ativo contra cepas resistentes ao BZ e ao NF	Ativo contra cepas resistentes ao BZ e ao NF
Segurança	> BZ / 3 avaliações clínicas e dois testes laboratoriais padrões durante o tratamento	> BZ / Sem necessidade de monitoramento durante o tratamento
Contraindicações	Gravidez/Lactação	Nenhuma
Precauções	Sem genotoxicidade/Não prolonga o intervalo QT	Sem genotoxicidade ou efeitos indesejados/Não prolonga o intervalo QT
Interações	Ausência com antiarrítmicos, anti-hipertensivos e anticoagulantes	Nenhuma
Apresentação	Oral	Oral
Estabilidade	3 anos em zona climática IV	5 anos em zona climática IV
Regime de dosagem	≈ Antifúngicos	2x ao dia / 60 dias

Fonte: adaptado de DNDi, 2015.

O estabelecimento de parcerias público-privadas (PPP) que permitem a colaboração entre governos, setor privado, academia, organizações não-governamentais e órgãos regulamentadores tem sido uma tendência crescente (GOLDMAN, 2012). Exemplos de sucesso incluem a iniciativa DND*i* , focado no desenvolvimento de novas terapias para seis DTNs, e o programa TDR/WHO (do inglês "WHO *Special Programme for Research and Training in Tropical Diseases*"), criado para combate à filariose e cofinanciado pela UNICEF (do inglês "United Nations Children's Fund"), pelo Programa de Desenvolvimento das Nações Unidas, pelo Banco Mundial e pela OMS, em colaboração com as multinacionais farmacêuticas GlaxoSmithKline e Merck (MACKEY et al., 2014).

A maioria das grandes empresas farmacêuticas está investindo num portfolio baseado em opções de baixo risco, que incluem, entre outros, indicações secundárias e o reposicionamento fármacos para DTNs que compartilhem alvos conservados (PERSIDIS, 2012). Esta última estratégia oferece vantagens como um ciclo mais curto de desenvolvimento, custo reduzido e menor incerteza em relação à segurança e perfil farmacocinético. Diversas abordagens, como a triagem fenotípica, sistemas de imageamento e de *high throughtput screening* (HTS) além de análises *in silico* incluindo de modelagem molecular podem, de modo colaborativo, serem adotadas para identificar novos usos para medicamentos descontinuados ou pertencentes a bibliotecas comerciais (KHANNA et al., 2012).

Quando um composto com atividade biológica é sintetizado, seu perfil de toxicidade deve ser testado, em modelos preditivos e fenotípicos. É recomendada a execução de, pelo menos dois testes toxicológicos *in vitro*, antes que se prossiga, com experimentação animal. Em geral, o primeiro teste utilizado para avaliar a toxicidade de compostos químicos é o ensaio de mutação reversa bacteriana, ou ensaio de *Salmonella*/ Microssoma ou Teste de Ames, que nos permite observar padrões de mutação pontual na estrutura do DNA. Mesmo se não forem observados resultados positivos para o ensaio de Ames, é necessário que se façam testes de avaliação da clastogenicidade (danos à estrutura do DNA nuclear) em células eucariontes de, pelo menos, duas origens (FDA, 2008).

Esta identificação e avaliação de novos compostos com a possibilidade de induzir mutações são importantes etapas no desenvolvimento de novos fármacos. Testes bacterianos de avaliação da atividade mutagênica e genotóxica têm desempenhado um importante papel nesta identificação, seja pela sua simplicidade, rapidez e baixo custo quanto pela reconhecida capacidade de detectar um amplo espectro de componentes mutagênicos. Além disto, há uma associação entre um agente ser mutagênico e genotóxico para bactéria e carcinogênico para mamíferos, com uma correlação estimada em 83% (MARON; AMES, 1983). Por estes motivos, os testes bacterianos são reconhecidos na comunidade científica e institutos internacionais de regulamentação e aprovação de novos compostos, sendo utilizados largamente como avaliação inicial para determinar a mutagenicidade de agentes químicos (MARON; AMES, 1983).

O teste de Ames foi inicialmente publicado por AMES; YAMASAKI (1971) fazendo uso de mutantes de *Salmonella enterica* sorovar Typhimurium deficientes da síntese de histina para detectar o potencial carcinogênico de compostos químicos. No entanto, sabido que a maior parte dos carcinógenos conhecidos depende de metabolização para apresentar seu potencial de interação com o DNA (CLAXTON, 1997), se faz necessária a adição de um sistema de metabolização exógena (conhecido como S9), geralmente, baseado no uso de frações microssomais hepáticas de ratos (MCCANN, 1975).

A utilização de modelos bacterianos para a detecção de mutágenos é recomendada pelo *guideline* 471, de 17 de julho de 1997, da Organização para a Cooperação e Desenvolvimento Econômico (*Organization for Economic Co-operation and Development* – OECD, 1997), que é um órgão não governamental responsável pela normatização de diretrizes de desenvolvimento. Consequentemente, as demais agências reguladoras de produtos de sanidade humana e animal no mundo submetem aos testes, os produtos que porventura possam ser comercializados em seu território.

Na pesquisa por candidatos a fármacos é preciso ainda categorizar a toxicidade de moléculas novas, com possíveis aplicações quimioterapêuticas gerais. A toxicidade de um fármaco pode estar associada a elementos estruturais (sítio toxicóforo), ao alvo da atividade biológica (sítio farmacofórico primário) ou por mecanismos alvo-inespecíficos. A partir dessa caracterização, modificações planejadas nas moléculas podem ser realizadas, de modo a reduzir sua toxicidade (Tabela 3) (MAZIASZ et al., 2010).

Na era da toxicogenômica é importante avaliar não apenas o nível de interação dos intoxicantes com as biomoléculas, mas também as mudanças induzidas na estrutura dessas moléculas, a ativação de respostas de defesa e controle dos danos produzidos e a ocasional manifestação patológica da toxicidade (toxicidade observada). Deve ser verificado também o perfil característico das modificações na expressão gênica, das modificações proteicas e dos metabólitos gerados pela toxicidade observada, através de recursos de genômica, transcriptômica, proteômica e metabolômica (-omics *Research*), para a caracterização e elucidação dos mecanismos envolvidos nessas manifestações (AARDEMA; MACGREGOR, 2002).

Tabela 3 - Categorização da toxicidade de fármacos

Atividade adversa	Base molecular	Componente estrutural	Efeito da otimização estrutural	Impacto na progressão do candidato
Baseada na estrutura	Reatividade química com biomolécula ou biotransformação em metabólito reativo	Toxicóforo	Eliminação ou efeito reduzido	Rejeição
Alvo-específica	Atividade contra um alvo primário	Farmacóforo primário	Potência pode ser alterada	Aceite
Alvo-inespecífica	Atividade contra um alvo não intencional	Farmacóforo secundário OU não-seletividade contra a família de alvos relacionados ao farmacóforo primário	Eliminação ou efeito reduzido (pode haver modificação no perfil estrutural) OU Seletividade pode ser melhorada	Caso a caso

Legenda: A atividade toxicológica de fármacos pode estar baseada em três mecanismos: (a) baseada na estrutura, (b) alvo-específica e (c) alvo-inespecífica. Fonte: Adaptado de MAZIASZ et al, 2010.

O fígado desempenha um importante papel na toxicidade de drogas, pois é nele que ocorre parte da metabolização de substâncias exógenas por enzimas do complexo citocromo P450 e de fase II, como acetiltransferases, nitroredutases, entre outras (SHIMADA et al., 1994; ANTOINE; WILLIAN; PARK, 2008; ALAEJOS; PINO; AFONSO, 2008; CORSINI; BORTOLINI, 2013). As enzimas do citocromo P450 são um conjunto de genes responsáveis por mais de 70% da metabolização de xenobióticos, incluindo medicamentos. São majoritariamente expressos no fígado e desempenham papel importante na metabolização de novas substâncias bioativas (GUENGERICH, 1992; 2008). Desta forma, é crucial utilizar modelos experimentais competentes na detecção da genotoxicidade de substâncias que possivelmente necessitem destas vias metabólicas, seja para exercerem atividade ou responsáveis pela geração de metabólitos reativos intermediários.

A indústria farmacêutica e os laboratórios de síntese podem utilizar dessas informações para traçar novas estratégias na síntese de novas substâncias ou outros produtos. Em decorrência dos genes do CYP serem altamente polimórficos, algumas famílias do CYP podem ser ativadas por determinado grupo de substâncias de forma indesejável. Assim, as metodologias mais avançadas da avaliação toxicológica são descartadas para poupar tempo e recurso, pois esse grupo de substâncias podem não atender satisfatoriamente a uma grande parcela da população (GUENGERICH, 2006; EMOTO et al., 2010). Por outro lado, existe o desenvolvimento do tratamento personalizado, que pode usufruir de um mecanismo de ação particular ao indivíduo (MURRAY; PETROVIC, 2006; CHEN et al., 2011).

A interação farmacológica também deve ser avaliada através da expressão de enzimas do CYP. Caso uma nova substância interfira no metabolismo de medicamentos existentes, pela inibição da expressão de enzimas, a resposta metabólica final pode ser ineficiente ou exacerbada. Em adição, os metabólitos gerados também devem ser conhecidos, pois, caso existam e sejam responsáveis por uma resposta adversa, podem ser alvos de outras substâncias (TANAKA, 1998; BIBI, 2008).

Para reduzir a quantidade de animais utilizados na análise toxicológica de novas substâncias possivelmente biotransformadas por enzimas do CYP, cultura de células primárias de hepatócitos têm sido utilizados. No entanto, a acessibilidade e manipulação da cultura de célula primária de hepatócito humanos não é sempre ideal (WILKENING, STAHL; BADER, 2003; GERETS et al., 2012). Modelos metabolicamente ativos permitem avaliar a genotoxicidade, identificar os genes do CYP expressos e os metabólitos gerados. Cultura de

células permanentes como o hepatocarcinoma humano HepG2 tem uma capacidade de detectar hepatotoxicidade de 90% em comparação a cultura primária de hepatócito. No entanto, culturas permanentes podem não ter a mesma capacidade metabólica que a de células primárias e, principalmente, do ambiente bioquímico do organismo vivo (GERETS et al., 2012).

2 **OBJETIVOS**

2.1. **Objetivo principal** Investigar a eficácia, segurança e seletividade de compostos heterocíclicos sintéticos com potencial preventivo de lesões no DNA e no contexto de infecções parasitárias como por *Trypanosoma cruzi*.

2.2 **Objetivos específicos**

a) Investigar ação fenotípica, seletividade e perfil de mutagenicidade de arilimidamidas sobre modelos *in vitro* e *in vivo* de infecção por *T. cruzi* ;
b) Explorar o papel biológico da posição do grupamento Nitro (NO_2 -) na atividade anti-*T. cruzi* e sobre características de genotoxicidade de compostos 4- e 5-nitroimidazólicos;
c) Verificar a possibilidade de reposicionamento de atorvastatina como potencial agente tripanocida tanto isoladamente quanto em terapia combinada com benznidazol na infecção *in vitro* por *T. cruzi*;
d) Investigar ação fenotípica e seletividade de derivados híbridos de atorvastatina e fenilacetamidas sobre modelos de infecção *in vitro* por *T. cruzi*;
e) Avaliar o papel quimiopreventivo da atorvastatina na prevenção de danos ao DNA induzidos por agentes capazes de causar alquilação em bases nitrogenadas direta e/ou indiretamente.

3 **RESULTADOS**

Os resultados que compõem a presente tese de doutorado serão apresentados na forma de artigos científicos elaborados na língua inglesa e brevemente explanados em português. O item 3.1 contém o ARTIGO 1 - *In Vitro and In Vivo Studies of the Biological Activity of Novel Arylimidamides against Trypanosoma cruzi* - Publicado no periódico "Antimicrobial Agents and Chemotherapy" (IF: 4,302, Qualis A2) em 2014. Neste artigo, investigamos o potencial tripanocida de arilimidamidas em modelos *in vitro*. A DB1989 foi o composto que se mostrou mais promissor, sendo encaminhado para ensaios *in vivo* e avaliação da mutagenicidade, tendo infelizmente falhado para no modelo de infecção aguda com a cepa Y de *T. cruzi*, além de induzir mutagenicidade por *frameshift* em pares G:C.

O item 3.2 contém o ARTIGO 2 - *Studies of genotoxicity and mutagenicity of nitroimidazoles: demystifying this critical relationship with the nitro group* - Publicado no periódico "Memórias do Instituto Oswaldo Cruz" (IF: 2,605, Qualis B2) em 2015. Neste artigo foi explicitado o papel da posição do grupo nitro na genotoxicidade e eficácia tripanocida de nitroimidazois. Concluímos que, ao contrário do que se acreditava, a posição do grupamento nitro não é *per se*, um fator determinante na indução da mutagenicidade, tendo os substituintes do anel imidazólico um papel central na modulação dos danos observados.

O item 3.3 contém o ARTIGO 3 - *Repurposing strategy of atorvastatin against Trypanosoma cruzi: monotherapy and combined therapy with benznidazole using in vitro approaches* – Submetido para o periódico "Antimicrobial Agents and Chemotherapy". O estudo propõe o reposicionamento de atorvastatina como um potencial fármaco tripanocida, principalmente em terapia combinada com o benznidazol, onde houve interação farmacológica de natureza sinérgica entre os dois medicamentos. Atorvastatina, além de ser segura e causar poucos danos ao tecido cardíaco, apresentou eficácia satisfatória e perfil de *Hit*, permitindo sua otimização estrutural no contexto da quimioterapia para a Doença de Chagas.

O item 3.4 contém o ARTIGO 4 - Trypanocidal, Antiplasmodial, and genotoxicity in vitro assessment of new hybrid α,α-difluorophenylacetamide-statin derivatives – Submetido para o periódico "European Journal of Medicinal Chemistry" (IF: 4,519, Qualis A2). Neste estudo, apresentamos a síntese de oito novos compostos híbridos de atorvastatina e fenilacetamidas (diclofenaco), desenvolvidos com o objetivo de aumentar sua eficácia antiparasitária e anti-inflamatória, visto que a inflamação tem um papel central na malária e

na DC. Dos novos compostos, quatro são na forma orgânica (compostos 13a-d) e quatro na forma de sais de sódio (14a-d), tendo preservados seus substituintes. Os compostos na forma de sal de sódio não apresentaram atividade nem frente aos hemozoítos de *Plasmodium falciparum* nem aos tripomastigotas de *T. cruzi* e por conta disso não seguiram na triagem. Dois compostos restantes foram ativos contra as formas evolutivas de *T. cruzi*, mas um deles, portador de Bromo, foi mutagênico e genotóxico nos testes de Ames e Micronúcleo.

O item 3.5 contém o ARTIGO 5 – *Atorvastatin Downregulates In Vitro Methyl Methanesulfonate and Cyclophosphamide Alkylation-Mediated Cellular and DNA Injuries* – Publicado no periódico "Oxidative Medicine and Cellular Longevity" (IF: 4,593, Qualis A2). O estudo verificou o potencial quimiopreventivo de atorvastatina no contexto da indução de lesões no DNA. Observamos que atorvastatina reduziu as mutações causadas por metil-metano sulfonato e ciclofosfamida no DNA bacteriano, no modelo de pré-exposição, além de ser capaz de reduzir a morte celular, a formação de micronúcleos e as alterações no ciclo celular de hepatócitos da linhagem HepG2, evitando assim a catástrofe mitótica. Além disso, atorvastatina apresentou-se como um antioxidante direto, sendo capaz de sequestrar radicais livres e, em vista disso, sugerimos que o mecanismo de ação na proteção do DNA sob estresse genotóxico é baseado no sequestro de espécies radicalares capazes de lesar o DNA.

3.1 **Artigo 1** - ***In Vitro and In Vivo Studies of the Biological Activity of Novel Arylimidamides against Trypanosoma cruzi*** **– Publicado no periódico "Antimicrobial**

Agents and Chemotherapy”

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In Vitro and *In Vivo* Studies of the Biological Activity of Novel Arylimidamides against *Trypanosoma cruzi*

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Fifteen novel arylimidamides (AIAs) (6 bis-amidino and 9 mono-amidino analogues) were assayed against *Trypanosoma cruzi in vitro* and *in vivo*. All the bis-AIAs were more effective than the mono-AIAs, and two analogues, DB1967 and DB1989, were further evaluated *in vivo*. Although both of them reduced parasitemia, protection against mortality was not achieved. Our results show that the number of amidino-terminal units affects the efficacy of arylimidamides against *T. cruzi*.

Chagas disease (CD) is caused by *Trypanosoma cruzi* and affects more than 8 million people worldwide (1–5). Benznidazole (BZ) and nifurtimox (NF) are used for the treatment of CD, but because of their well-known toxicity and limited efficacy in the later chronic phase of the disease, new drugs are urgently needed (6–9). We have evaluated several classes of natural and synthetic compounds, including arylimidamides (AIAs), aromatic diamidine (AD) derivatives with extraordinary activity against *T. cruzi* and other trypanosomatids, both *in vitro* (10–16) and *in vivo* (17, 18). In AIAs, the imino group is linked via an anilino nitrogen, while in classical amidines, it is directly attached to an aryl ring, yielding reduced pK values (14). Here, we report the results of *in vitro* and *in vivo* activity studies and mutagenicity and selectivity assessments of new AIAs (6 bis-amidino analogues, DB1966, DB1967, DB1968, DB1979, DB1989, and DB1995, and 9 mono-amidino analogues, DB1996, DB1997, DB1980, DB2001, DB2002, DB2003, DB2004, DB2006, and DB2007), which provide insight on the relevance of one or two terminal amidino units for biological activity.

We synthesized the mono- and bis-arylimidamides (see structures in Table 1) as reported (19–21). Benznidazole (BZ) (Laboratório Farmacêutico do Estado de Pernambuco, LAFEPE, Brazil) and gentian violet (Sigma-Aldrich) were used as reference drugs (22). Primary cultures of cardiac cells (CC) were obtained as reported (18, 23). The Y strain of *T. cruzi* was used, and bloodstream trypomastigotes (BT) and intracellular trypomastigote forms were assayed as described previously (18, 23). Mammalian cell cytotoxicity of AIAs was evaluated on uninfected CC incubated up to 48 h at 37°C with each compound (0 to 32 μM); morphology, spontaneous contractibility, and cell death rates were measured for determination of the 50% effective compound concentrations (EC_{50}s) (24). For trypanocidal analysis, BT were incubated at 37°C for 24 h with nontoxic concentrations of the compounds to determine the EC_{50} (24). For analysis with intracellular amastigotes, after 24 h of parasite-host cell interaction, increasing nontoxic doses of the compounds were added for 48 h, and drug activity was estimated by calculating the infection index (II) as reported (12, 24). The data shown are the means ± standard deviations from 2 to 4 experiments run in duplicate. A bacterial reverse mutation (Ames) test and a cytotoxicity assay were performed as proposed by Maron and Ames (25) and Organization for Economic Cooperation and Development (OECD) test guideline 471 (26). Statistical analysis was performed by the analysis of variance test ($P \leq 0.05$) (22).

Male Swiss Webster mice (18 to 21 g) (Fundação Oswaldo Cruz Animal Facility [CECAL/FIOCRUZ], Brazil) were housed six per cage in a conventional room at 20 to 24°C under a 12/12-h light/dark cycle, with sterilized water and chow provided *ad libitum*. Infection was achieved by intraperitoneal (i.p.) injection of 10^4 BT (Y strain), and the mice (6 per group) were uninfected (noninfected and nontreated), untreated (infected and treated with vehicle), or treated with different doses of DB1989 and DB1967 (infected and treated with 0.2-ml i.p. daily doses up to 50 mg/kg of body weight). Infected mice were treated with 100 mg/kg/day BZ orally once a day. Treatment was given at the 5th (parasitemia onset) and 8th day postinfection (dpi) (parasitemia peak). Parasitemia levels, body weights, and percentage of cumulative mortality were checked until 30 days posttreatment, as reported (18). All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA 0028/09).

BT incubated for 24 h at 37°C showed that 12 of the 15 compounds (all but DB1996, DB1997, and DB2002) had superior trypanocidal activities ($P \leq 0.05$) compared to that of BZ (EC_{50}, 13 μM). Five of the bis-AIAs (DB1966, DB1967, DB1968, DB1979, and DB1989) yielded EC_{50}s of ≤0.1 μM. The bis-AIA DB1989, the fastest-acting trypanocidal compound, provided an EC_{50} of 2.7 μM after 2 h (Table 1). Bis-AIAs also displayed the best

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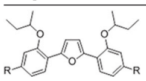
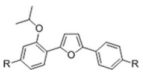
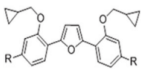
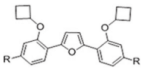
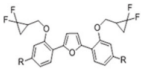
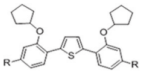
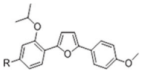
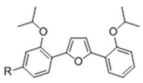
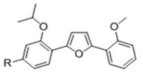
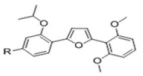
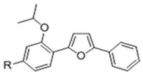
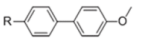
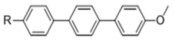
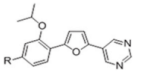
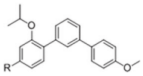
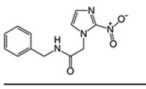
TABLE 1 AIA activity against bloodstream trypomastigotes and intracellular (amastigote) forms of *T. cruzi* (Y strain) and the corresponding selectivity indexes

Compound structure	Compound name	Bloodstream trypomastigotes					Amastigotes	
		EC_{50} (mean ± SD) (μM) at 37°C in RPMI at:		SI at 37°C at 24 h	EC_{50} (mean ± SD) (μM) at 4°C in blood at:		EC_{50} (mean ± SD) (μM) at 37°C in RPMI at 48 h	SI at 48 h
		2 h	24 h		2 h	24 h		
	DB1966[a]	>3.5	0.04 ± 0	30	>32	22 ± 3	0.09 ± 0.08	13
	DB1967[a]	>3.5	0.04 ± 0.02	88	>32	3.75 ± 0.3	0.03 ± 0.006	40
	DB1968[a]	>3.5	0.08 ± 0.02	15	>32	2.9 ± 0.4	0.1 ± 0.1	12
	DB1979[a]	3.5	0.1 ± 0.04	30	>32	10.8 ± 2.7	1 ± 1.4	3
	DB1989[a]	2.7 ± 1.6	0.05 ± 0.01	70	>32	3.9 ± 1.3	0.06 ± 0.03	20
	DB1995[a]	>32	1.0 ± 0.8	3	>32	25 ± 9	0.9 ± 0.35	1.2
	DB1980	>32	5.8 ± 4	0.2	>32	>32	>0.39	>0.39
	DB1996	>32	13 ± 12	0.09	>32	>32	>0.39	3
	DB1997	>32	15 ± 5	0.08	>32	>32	>0.39	3
	DB2001	>32	4.8 ± 1.5	0.2	>32	21 ± 6	>0.39	0.25
	DB2002	>32	20 ± 10	0.15	>32	>32	>0.39	3
	DB2003	13 ± 3	4.3 ± 1.9	0.9	>32	>32	>0.39	9
	DB2004	12 ± 3.9	2.9 ± 1.5	0.35	>32	>32	>0.39	2.5
	DB2006	12 ± 60	5.9 ± 10	0.5	>32	22 ± 0.9	>0.39	9
	DB2007	11 ± 60	1.2 ± 10	1	>32	>32	>0.39	3
	BZ	>50	13 ± 2	77	>250	>250	3.6 ± 1.7	>277

[a] Bis-AIA.

effect under blood bank conditions (in blood at 4°C); DB1967, DB1968, and DB1989 showed EC_{50}s ranging from 2.9 to 3.9 μM, while BZ was ineffective at up to 250 μM (Table 1). The most selective compounds against BT were DB1967 (selectivity index [SI], 88) and DB1989 (SI, 70) (Table 1). The mono-AIAs DB1980, DB2001, and DB2004 were the most toxic against cardiac cell cultures at 48 h. Mono-AIAs were ineffective after 48 h at 37°C on *T. cruzi*-infected cultures (Table 1). Similar to the effect against BT, four bis-AIAs (DB1966, DB1967, DB1968, and DB1989) were the most effective against intracellular parasites (EC_{50}s of ≤0.1 μM) (Table 1). Mono-AIAs displayed very low selectivities, while the bis-AIAs DB1989 and DB1967 exhibited the highest SI levels (20 and 40, respectively) against the intracellular parasites (Table 1). A bacterial reverse mutation (Ames) test indicated no major mutagenic potential (mutagenic index, <2) with DB1989 (see Table S1 in the supplemental material) or BZ (data not shown). Due to their excellent *in vitro* activities against the two parasite forms and reasonable selectivities, DB1967 and DB1989 were evaluated *in vivo*. At 8 dpi (parasitemia peak), DB1989 reduced parasitemia (by 40, 76, and 75% with 12.5, 25, and 50-mg/kg/day doses, respectively), while BZ suppressed parasitemia (Fig. 1A). BZ resulted in 100% survival of the mice, but no dose of DB1989 prevented mortality triggered by the infection (Fig. 1B); the highest dose (50 mg/kg/day) produced higher mortality rates compared to that of the untreated group, possibly due to compound toxicity (a ponderal curve shows higher weight [Fig. 1C]). DB1967 produced dose-response suppression (67 to 87%) of parasitemia but an earlier and higher mortality rate (100% for all DB1967-treated groups, likely due to toxicity; data not shown).

AIAs such as DB766 are effective *in vitro* and *in vivo* against intracellular pathogens that cause human and animal pathologies (24, 27–29) and exhibit stronger activity than those of classical diamidines (possibly due to their lower pK_a values), better bioavailability, and improved cell membrane permeability (28). Similar to their effect against *Leishmania*, bis-AIAs are highly active against *T. cruzi* (12, 15, 22). DB766 showed a selective effect against intracellular amastigotes and upon a large panel of *T. cruzi* strains, including naturally resistant strains, with a higher efficacy than those of the reference drugs (18).

This work explores the correlation between the trypanocidal activity/selectivity of AIAs with one or two terminal amidino groups. Bis-AIAs were most potent against the two parasite forms relevant to mammalian infection (the bloodstream and intracellular forms), demonstrating that two terminal amidino centers confer a higher parasiticidal effect than those bearing only one. The importance of the second amidino center is seen by comparing the results for DB1967 with those for DB2002 (500-fold activity difference; Table 1), which differ only in the absence of the second amidino group in DB1967. These results corroborate previous findings for classical diamidines, confirming the requirement of a diamidino unit for effectiveness against *T. cruzi* (15). The bis-AIAs DB1967, DB1968, and DB1989 maintained good trypanocidal activity at 4°C with 96% mouse blood, similar to the activities of other bis-AIAs, including DB766 (18), DB745 (30), and DB1831 (22).

All tested bis-AIAs have alkoxy groups of approximately the same size and with similar *in vitro* activities (EC_{50}s of ≤0.1 μM). DB1967, with only one 2-propoxy group, has essentially the same antitrypanosomal activity as that of DB766 (18), which has two such groups; yet, DB1967 is more toxic to animals than DB766,

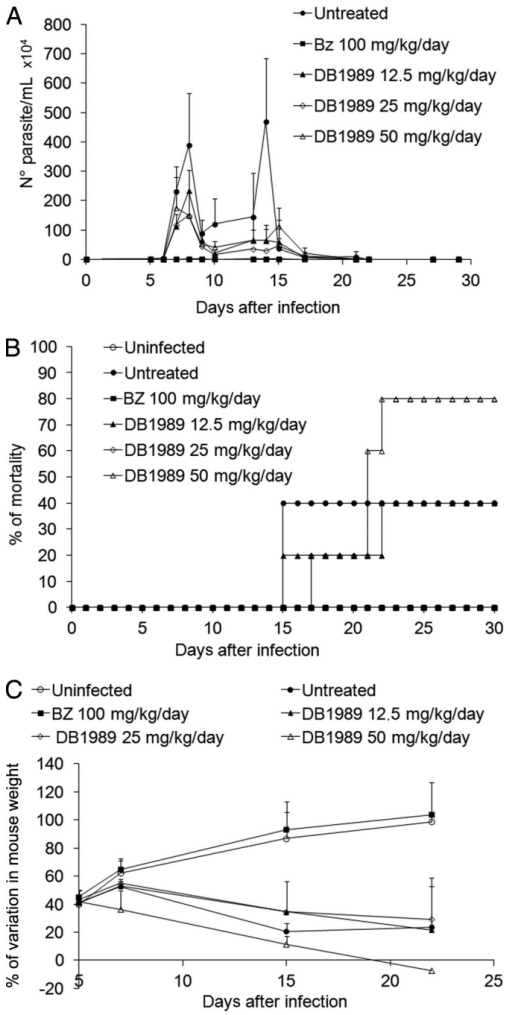


FIG 1 *In vivo* effect of DB1989 on acute mouse model of infection with the Y strain of *T. cruzi*. Parasitemia (A), mortality rates (B), and ponderal curve (C) are shown. The effects of DB1989 (i.p.) and BZ (oral) were followed using doses (up to 50 mg/kg/day for DB1989 and 100 mg/kg/day for BZ) administered at the 5th and 8th dpi.

suggesting that two moderately sized alkoxy groups reduce animal toxicity. Generally, the activities of the mono-AIAs do not vary significantly with structure (Table 1). Most bis-AIAs were also less toxic toward cardiac cells than were the mono-AIAs. Presently, up to the maximum dose tested, genotoxicity was absent, and only a mild mutagenicity profile was observed when DB1989 was assayed against the *Salmonella enterica* Typhimurium TA98 strain (see Table S1 in the supplemental material), which is suggestive of a frameshift mutation, probably during the

DNA repair or duplication process, adding GC pairs into the genome. Although OECD test guideline 471 recommends using up to 5 mg of a tested compound, the high activity of DB1989 toward the bacterial strains impaired assaying higher AIA concentrations that may mask mutagenic aspects, demanding additional toxicological studies.

DB1989 and DB1967 were moved to *T. cruzi in vivo* models due to their high *in vitro* activities and reasonable selectivities. Although parasitemia was reduced, neither DB1967 nor DB1989 protected against mortality. This is in contrast to results with DB766 (18) and DB1965, a mesylate salt form of DB1831 (22) which showed *in vivo* efficacy comparable to that of BZ. The reduction of parasitemia observed with DB1967 correlates with the *in vitro* data obtained with bloodstream and intracellular parasites (EC_{50}s of 30 to 40 nM). As low toxicity was observed *in vitro*, the higher mortality rate of the DB1967-treated mice is likely due to an organ-specific toxicity (e.g., hepatotoxicity) or arose from metabolic products of the bis-AIA.

Our data confirm the importance of two amidino centers for the trypanocidal efficacy of arylimidamides against *T. cruzi* and demonstrated that mono-AIAs are less effective and selective than bis-AIAs. Although very active *in vitro*, DB1989 and DB1967 failed to protect against *T. cruzi* infection *in vivo*, possibly due to toxicity. Since previous studies demonstrated *in vivo* efficacies comparable to that of BZ for other bis-AIAs, e.g., DB766 (18) and DB1965 (22), the synthesis of novel AIAs bearing bis-terminal pyrimidines or pyridines merits further investigation as an approach for identifying new anti-*T. cruzi* agents.

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Table S1 – Induction of His+ revertants in *Salmonella enterica (*Typhimurium strains) by DB1989 in reverse mutation test without metabolic activation

Strain	[ ] nM	*His*$^+$ ± DP[b]	MI[a]	Survival %[c]
TA97	0	120 ± 5	1.00	100
	1.11	102 ± 18	0.85	100
	2.22	114 ± 4	0.96	100
	4.45	91 ± 20	0.76	100
	8.90	69 ± 6	0.58	100
	17.75	67 ± 6	0.56	100
TA98	0	34 ± 1	1.00	100
	1.11	45 ± 6	1.32	100
	2.22	79 ± 12	**2.32**	100
	4.45	75 ± 1	**2.21**	100
	8.90	79 ± 1	**2.32**	100
	17.75	73 ± 4	**2.16**	86
TA100	0	62 ± 9	1.00	100
	1.11	57 ± 24	0.93	**35**
	2.22	83 ± 9	1.35	**15**
	4.45	81 ± 1	1.31	**27**
	8.90	76 ± 6	1.23	**52**
	17.75	91 ± 1	1.48	**50**
TA102	0	174 ± 30	1.00	100
	1.11	192 ± 37	1.11	**13**
	2.22	142 ± 10	0.82	**20**
	4.45	194 ± 2	1.11	**27**
	8.90	139 ± 18	0.80	**22**
	17.75	160 ± 10	0.92	**20**

Induction of *His*$^+$ revertants in *S. enterica (*Typhimurium strains) by DB1989 in reverse mutation test without metabolic activation. [a]Mutagenic index (M.I.): number of *His+* induced in the sample/number of spontaneous *His+* in the negative control (DMSO 5%). [b]Number of His+/plate: mean value ± SD of at least three experiments. [c]Percentage of cell survival (Surv. %) calculated in relation to the negative control. Bold numbers are statistically significant ($p \leq 0.005$).

3.2 **Artigo 2 -** ***Studies of genotoxicity and mutagenicity of nitroimidazoles: demystifying this critical relationship with the nitro group*** **– Publicado no periódico "Memórias do Instituto Oswaldo Cruz"**

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Studies of genotoxicity and mutagenicity of nitroimidazoles: demystifying this critical relationship with the nitro group

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Nitroimidazoles exhibit high microbicidal activity, but mutagenic, genotoxic and cytotoxic properties have been attributed to the presence of the nitro group. However, we synthesised nitroimidazoles with activity against the trypomastigotes of Trypanosoma cruzi*, but that were not genotoxic. Herein, nitroimidazoles (11-19) bearing different substituent groups were investigated for their potential induction of genotoxicity (comet assay) and mutagenicity (*Salmonella*/Microsome assay) and the correlations of these effects with their trypanocidal effect and with megazol were investigated. The compounds were designed to analyse the role played by the position of the nitro group in the imidazole nucleus* (C-4 or C-5) *and the presence of oxidisable groups at N-1 as an anion receptor group and the role of a methyl group at C-2. Nitroimidazoles bearing NO_2 at C-4 and CH_3 at C-2 were not genotoxic compared to those bearing NO_2 at C-5. However, when there was a CH_3 at C-2, the position of the NO_2 group had no influence on the genotoxic activity. Fluorinated compounds exhibited higher genotoxicity regardless of the presence of CH_3 at C-2 or NO_2 at C-4 or C-5. However, in compounds 11 (2-CH_3; 4-NO_2; N-CH_2OHCH_2Cl) and 12 (2-CH_3; 4-NO_2; N-CH_2OHCH_2F), the fluorine atom had no influence on genotoxicity. This study contributes to the future search for new and safer prototypes and provide.*

Key words: nitroimidazoles - genotoxicity - mutagenicity - trypanocidal activity

The class of nitroimidazoles includes compounds that are important antiparasitic agents, which have a broad spectrum of action and high biological activity. For instance, metronidazole (1), secnidazole (2), tinidazole (3), ornidazole (4), dimetridazole (5), carnidazole (6) and panidazole (7) (Fig. 1) are some examples of drugs currently used to treat infections of anaerobic *Bacteroides* sp. and protozoans, such as *Trichomonas* sp., *Entamoeba* sp., *Giardia* sp. and *Histomonas* sp. (Yakugaku 1971, William et al. 1975, Buschini et al. 2007, Mital 2009, Valdez et al. 2009). In addition, nitroimidazoles have other interesting properties, including antitubercular and antifungal activities, in the control of fertility (Bone et al. 1997, Cooper et al. 1997), as radiosensitisers (Paul & Abdel-Nabi 2007, Khabnadideh et al. 2009, Lee et al. 2011) and against the recombinant reverse transcriptase of human immunodeficiency virus (HIV)-1 (Silvestri et al. 2002, Al-Soud et al. 2007).

The 2-nitroimidazole benznidazole (BZ) (8) (Fig. 2) and nifurtimox (NFX) are the only available drugs for the treatment of Chagas disease. However, these nitro derivatives exhibit poor activity in the late chronic phase, with severe collateral effects and limited efficacy against different parasitic isolates, justifying the urgent need to identify alternatives to treat chagasic patients (Soeiro & de Castro 2011, Urbina 2014). This disease is caused by *Trypanosoma cruzi* and affects approximately eight million individuals in Latin America. Furthermore, it is emerging in nonendemic areas, associated with the immigration of infected individuals (Gascon et al. 2010, Schmunis & Yadon 2010, França et al. 2014). Megazol (9) (Fig. 2) is a nitroimidazole-thiadiazole with high in vitro and in vivo activity against *Trypanosoma cruzi,* including against strains resistant to 8 (Filardi & Brener 1982, de Castro & de Meirelles 1986, Lages-Silva et al. 1990, Salomão et al. 2010) and *Trypanosoma brucei*, the causative agent of human African trypanosomiasis (HAT) (Enanga et al. 1998, 2000, Boda et al. 2004). The mode of action of 9 is associated with the interference with the parasite's oxygen metabolism, as well as acting as a trypanothione scavenger (Viodé et al. 1999, Maya et al. 2003). Despite its notable trypanocidal activity, 9 was not approved for clinical use due to reports of in vitro mutagenic and genotoxic effects associated with the reduction of the nitro group (Ferreira & Ferreira 1986, Poli et al. 2002, Nesslany et al. 2004), but the nature of the mutagenic metabolite was not yet characterised.

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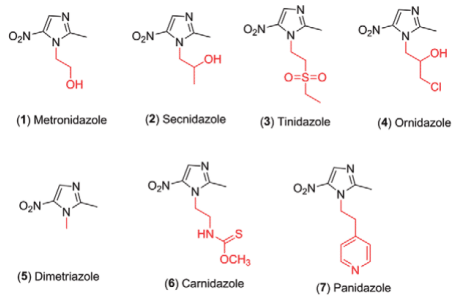

Fig. 1: structures of the nitroimidazole drugs (1-7).

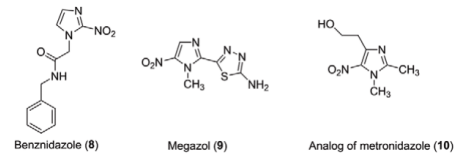

Fig. 2: chemical structures of the nitroimidazoles: benznidazole (8), megazol (9) and a metronidazole analog (10).

Several nitroimidazoles possess good oral therapeutic activity against protozoal parasites; however, concerns over toxicity, mutagenicity and genotoxicity have made drug development problematic. These adverse properties appear to be related to DNA damage by the products of the bio-reduction of the nitro group. In fact, positive Ames tests were observed for 1 and 8 using *Salmonella typhimurium* (Rosenkranz Jr et al. 1976). Despite these results, nitroimidazoles are employed for the clinical treatment of bacterial and protozoal infections and 1 was included the World Health Organization list of Essential Medicines, a list of the arsenal of key antimicrobial drugs (WHO 2011).

Studies of the mutagenicity have shown that there are differences in the ability of mammalian cells, bacteria and protozoa to reduce nitroimidazoles (Moreth et al. 2010). However, to date, there are no conclusive results from analogous studies of mutagenicity performed in vitro in animals or in humans (Paula et al. 2009). Voogd et al. (1979) described the influences of different substituent groups in the nitroimidazole ring on the redox system, while Walsh et al. (1987) group demonstrated that the mutagenic action of 1 is affected by different substituents at position 1 of the imidazole nucleus. For example, compound 10 (Fig. 2), which is a position structural analogue of 1, maintained its biological activity without toxic effects. Although some hypotheses exist, active research in this area has yet to produce a comprehensive mechanism to explain the toxic and therapeutic activities of these compounds. Thus, further studies to elucidate the relationship between the biological activity, genotoxicity and mutagenicity of nitroimidazoles are needed.

Our group has investigated new nitroimidazoles as trypanocidal agents and their mutagenic/genotoxic activities. We have observed that these activities do not only depend on the nitro group (Boechat et al. 2001, Carvalho et al. 2004, 2006, 2007, 2008, 2014, Mello et al. 2013). Encouraged by this observation, in the present work we evaluated the genotoxic, mutagenic and antitrypanocidal activities of nine nitroimidazoles (11-19) (Fig. 3). The following key structural aspects of the nitroimidazoles were examined: the importance of the position of the nitro group in the imidazole nucleus (C-4 or C-5), the incorporation of an oxidisable group at N-1 of the nitroimidazole ring, as an anion receptor group and the importance of the methyl group at C-2. This study may serve as a guide to the search for new lead compounds for the chemotherapy of human trypanosomiasis and may also identify safer compounds that may serve as the basis for investigation into the mutagenic activity of nitroimidazoles.

Although the focus of this work is studying the genotoxic and mutagenic effects of different nitroimidazole moieties, the synthesised compounds (11-19) were also assayed against *T. cruzi,* even though they do not possess the structural requirements required for trypanocidal activity, i.e., two aromatic and/or heteroaromatic rings.

MATERIALS AND METHODS

Chemistry - 4 or 5-nitroimidazoles with different substituents at the 2 position (11-19) were synthesised following the method described by Skupin et al. (1997). Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO) and in all subsequent assays control sets with the highest required solvent concentrations (5%) were included.

In vitro trypanocidal assay - Bloodstream trypomastigotes *of T. cruzi* were obtained from mice intraperitoneally inoculated with the Y strain of *T. cruzi* and were resuspended in Dulbecco's modified Eagle's medium plus 10% foetal calf serum at a concentration of 10^7 parasites/mL. This suspension (100 µL) was added to an equal volume of 11 to 19 and incubated for 24 h at 37ºC under a 5% CO_2 atmosphere. Cell counts were then performed using a Neubauer chamber and the concentration that produced lysis of 50% of the parasites (IC_{50}) was determined. Untreated and BZ-treated parasites were used as controls (Carvalho et al. 2014). The experiments were conducted in accordance with the guidelines established by the Oswaldo Cruz Foundation Committee of Ethics for the Use of Animals (LW 16-13).

In vitro treatment and cytotoxicity assay in whole blood - Heparinised human blood was obtained by venipuncture immediately before the assays. Whole blood was treated for 2 h at 37ºC with different concentrations of 9 (380-4,000 µM) and of 11 to 19 (148-6,400 µM). The cell viability was determined using the fluorescein diacetate (FDA)/ethidium bromide (EtBr)-assay, in which viable cells are labelled in green, while dead ones display orange-stained nuclei (Hartmann & Speit 1997). Whole blood (50 µL) was mixed with an equal volume of a freshly prepared staining solution consisting of 30 µg/mL FDA plus 8 µg/mL EtBr in phosphate-buffered saline (PBS). Samples (50 µL) were spread on a micro-

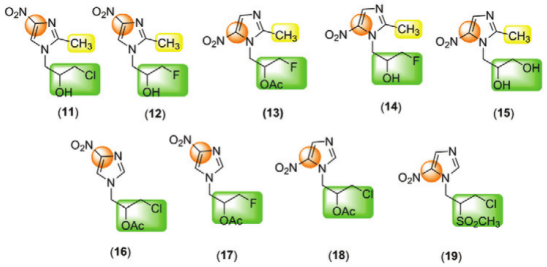

Fig. 3: chemical structures of the studied nitroimidazoles 11-19.

scope slide, covered with a coverslip and observed using a fluorescence microscope. Two hundred cells were analysed for each treatment. This toxicity test was performed to determine the range of drug concentrations in the subsequent approaches: concentrations that led to more than 30% loss of viability were excluded (Anderson et al. 1998, Henderson et al. 1998).

In vitro alkaline single cell gel electrophoresis (SCGE) or the comet assay - At the end of a 2 h-treatment, DNA damage in the whole blood was evaluated in duplicate using an alkaline comet assay (Speit & Hartmann 2006). Methyl methane-sulfonate (MMS) (160 µM) (Aldrich, USA) was used as a positive control. Aliquots of 5 µL of whole blood were mixed with 120 µL of 0.5% low melting-point agarose (LMPA) (Sigma-Aldrich, USA) in PBS at 37ºC and were applied to microscope slides previously covered with 1.5% normal melting-point agarose (Sigma-Aldrich). Then, the slides were covered with coverslips and after LMPA solidification (3 min/4-5ºC), the coverslips were removed and the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% N-lauroylsarcosine sodium salt, 1% Triton X-100, 10% DMSO, pH 10) for at least 1 h at 4-5ºC, protected from light. Afterwards, the slides were placed in a horizontal gel electrophoresis unit (Bio-Rad) filled with a freshly prepared alkaline buffer at a pH > 13 (300 mM NaOH, 1 mM EDTA). The times for the alkali treatment and the electrophoresis of the DNA (0.86 V/cm and 300 mA) were 20 min each in an ice bath. The slides were neutralised by washing three times (5 min) in Tris buffer (0.4 M Tris, pH 7.5), dried, fixed in absolute ethanol (99.8%) for approximately 10 min and left at room temperature overnight. Then, each slide was covered with 30 µL of EtBr (20 µg/mL) and a coverslip and analysed using a fluorescence microscope at 400X magnification.

Fifty random cells per slide (200 cells per treatment) were visually analysed in a blinded manner according to tail size into four classes of damage: 0 (undamaged, i.e., no visible tail), 1 (slightly damaged), 2 (moderately damaged) and 3 (maximally damaged, i.e., the head of comet was very small and most of the DNA was in the tail) (Kobayashi et al. 1995). DNA damage was expressed as arbitrary units (AU) as percentage of cells into each of the four classes according to the formula AU = (0 x M0) + (1 x M1) + (2 x M2) + (3 x M3).

The codes M0, M1, M2 and M3 correspond to the number of cells within damage classes 0, 1, 2 and 3, respectively. Thus, the total DNA damage score [total arbitrary units (TAU)] for 200 comets range from zero (undamaged) to 600 (maximally damaged). The experiments were performed in triplicate and the statistical significance ($p < 0.05$) between the treated and control groups was evaluated using Student's one-tailed *t* test (Kobayashi et al. 1995, OECD iLibrary 1997)

Salmonella/Microsome mutagenicity test - Four samples with nitro group at positions 4 (16 and 17) and 5 (13 and 18) and different substituent groups at position N-1 were selected for this analysis (Fig. 3). The *Salmonella*/Microsome mutagenicity test evaluates mutations in the DNA of genetically modified *Salmonella enterica* serovar *Typhimurium* strains that lack the ability to synthesise histidine (*His*$^-$). The strains TA97, TA98, TA100, TA102 and TA1535 were utilised following the guidelines for the testing of chemicals (Maron & Ames 1983). At least five strains of bacteria were used, including four strains of *S. enterica* serovar *typhimurium* (TA1535; TA1537 or TA97a or TA97; TA98 and TA100).

A volume of 100 µL of the nitroimidazole (25-2,500 µM), 500 µL of PBS and 100 µL of the bacterial suspension (2 x 10^9 cells/mL) were combined in a test tube. Next, 2 mL of top agar (7 g/L agar; 5 g/L NaCl; 0.0105 g/L L-histidine; 0.0122 g/L biotin; pH 7.4, 45ºC) was added and the mixture was poured into a Petri dish containing minimal agar {15 g/L agar, Vogel-Bonner E medium 10X [10 g/L $MgSO_4.7H_2O$; 100 g/L $C_6H_8O_7.H_2O$; 500 g/L K_2HPO_4; 175 g/L $Na(NH_4)HPO_4.4H_2O$]} with 20 g/L glucose. The plates were incubated at 37ºC for 72 h and the *His*$^+$ revertant colonies were counted. The positive controls included 1.0 µg/plate 4-nitroquinoline-N-oxide (4-NQO) for TA97 and TA98, 0.5 µg/plate sodium azide for TA100 and TA1535 and 0.5 µg/plate mitomycin C for TA102. All of the chemicals were obtained from Sigma Co (USA). DMSO was used as the negative control. The sample was considered positive for mutagenicity when the following conditions were observed: the number of revertant colonies in the assay was at least twice the number of spontaneous revertants [mutagenicity index (MI) ≥ 2], a significant response to the analysis of variance ($p \leq 0.05$) was observed and a reproducible, positive dose-response curve ($p \leq 0.01$) was obtained.

The MI was calculated by dividing the number of *His*$^+$ colonies induced in the sample by the number of *His*$^+$ colonies in the negative control. All the experiments were performed in triplicate and were repeated twice. Statistical significance was evaluated using analysis of variance, including a one-way ANOVA and Tukey's honestly significant difference HSD *post hoc* analysis ($p \leq 0.01$). The mutagenic potency slope was obtained using the Bernstein model (Bernstein et al. 1982).

Survival experiments - Quantitative evaluations were performed to determine the cytotoxic effects of the nitroimidazoles by the Ames test. Ten microlitres of each treated bacterial suspension (10^8 cells/mL) was diluted in a saline solution (NaCl, 9 g/L-0.9%). Then, 100 µL of the solution was placed on a Petri dish containing nutrient agar [0.8% bacto nutrient broth (Difco), 0.5% NaCl and 1.5% agar] and incubated at 37ºC for 24 h. The colonies were counted and the survival percentage was determined with respect to the negative control. All the experiments were performed in triplicate and were repeated twice (Aiub et al. 2004).

RESULTS

The present study focused on the use of SCGE to evaluate DNA damage induced by the nitroimidazoles 11-19 with the objective of mapping genotoxic activity, as displayed in Table I. The treatment of blood cells with 9 over the range of 380-4,000 µM for 2 h at 37°C did not reduce the cell viability. However, a highly significant ($p < 0.01$) genotoxic effect was observed at concentrations higher than of 1,562 µM. The positive control, MMS, was highly active at 160 µM ($p < 0.0001$), causing a clear genotoxic effect with a TAU value of 454.3 ± 9.2. In the range of 148-6,400 µM, the nitroimidazoles 11, 12, 16 and 18 did not alter cellular viability and caused no DNA damage compared to the control group ($p > 0.05$). On the other hand, at the same concentrations, 13, 15 and 19 caused moderate alterations to the DNA, whereas 14, 17 and 9 caused strong alterations of the DNA structure.

The nitroimidazoles were also assayed against *T. cruzi*. Compounds 11-17 and 19 exhibited an IC_{50} in the range of 902-1,749 µM, whereas for 18, this parameter was > 2000 µM. Under the same experimental conditions, the values for 8 and 9 were 9.7 ± 2.4 and 9.9 ± 0.8 µM, respectively (Table I). As expected, the compounds were not active trypanocides because they do not possess the structural requirements required for trypanocidal activity, i.e., two aromatic and/or heteroaromatic rings.

Fig. 4 illustrates the mutagenicity and cytotoxicity induced by nitroimidazoles 13, 16, 17 and 18 using *S. typhimurium* strains TA97, TA98, TA100, TA102 and TA1535. At concentrations between 25-2,500 µM, these compounds did not induce frameshift mutations in TA97. However, compounds 17 and 18 were capable of inducing frameshift mutations in TA98, whereas 18 also induced transversions and transitions in TA102 and base pair substitutions in TA1535. Furthermore, all of the nitroimidazoles were capable of inducing dose-dependent base pair substitutions for at least two tested doses in TA100, the most mutagen-responsive strain (Fig. 5). In addition, cytotoxic activity was present only in the higher concentration (2,500 µM) for at least one sample and strain. Using the Bernstein correlation, the number of revertant colonies per µg (rev/µg) was deduced. In addition, higher mutagenic potency correlated with an increased risk of being a mutagen.

TABLE I

Anti-*Trypanosoma cruzi* and genotoxic in vitro activities of nitroimidazoles 11-19

Compound	IC_{50}/24 h (µM)	Genotoxic
9	9.9 ± 0.8	3
11	1,690.9 ± 371.8	0
12	1,147.3 ± 111.6	0
13	1,749.7 ± 184.6	1
14	1,170.7 ± 100.5	3
15	1,585.8 ± 218.3	1
16	1,132.4 ± 86.3	0
17	1,049.4 ± 63.7	3
18	> 2,000	0
19	902.6 ± 83.4	1

0: undamaged; 1: slightly damaged; 2: moderately damaged; 3: maximally damaged (see Materials and Methods for more details).

DISCUSSION

Although nitroimidazoles have been clinically used for chemotherapy against several parasites, the mechanisms underlying their genotoxic and biological activities are not fully understood. On the basis of the high activity of 9 against trypanosomatids (Filardi & Brener 1982, Enanga et al. 1998, Darsaud et al. 2004) together with the success of NFX-eflornithine for the treatment of HAT (Yun et al. 2010), the Drugs for Neglected Diseases initiative triggered a systematic review of more than 700 nitroheterocycles (mostly nitroimidazoles) (Torreele et al. 2010). In this context, fexinidazole (5-nitroimidazole), first described in 1978 by Winkelmann and Raether (1978), emerged as a potential candidate. The development of this compound was previously abandoned largely due to the prejudice against nitroaromatic compounds (Patterson & Wyllie 2014). More recently, analysis of pharmacological and toxicological profiles suggested that fexinidazole is a promising candidate for both HAT (Torreele et al. 2010) and Chagas disease (Bahia et al. 2012). These facts and our previous results prompted us to re-evaluate the role of the nitro group in the toxic effects of nitroimidazoles. The presence of a nitro group in a compound can result in several toxicity issues, including genotoxicity and mutagenicity (Walsh & Miwa 2011). However, at the same time, this functional group is necessary for the desired biological activity. Consequently, nitrocompounds are not included in screening libraries due to this unwanted functionality (Brenk et al. 2008) and are not synthesised in medicinal chemistry programs [reviewed in Patterson and Wyllie (2014)].

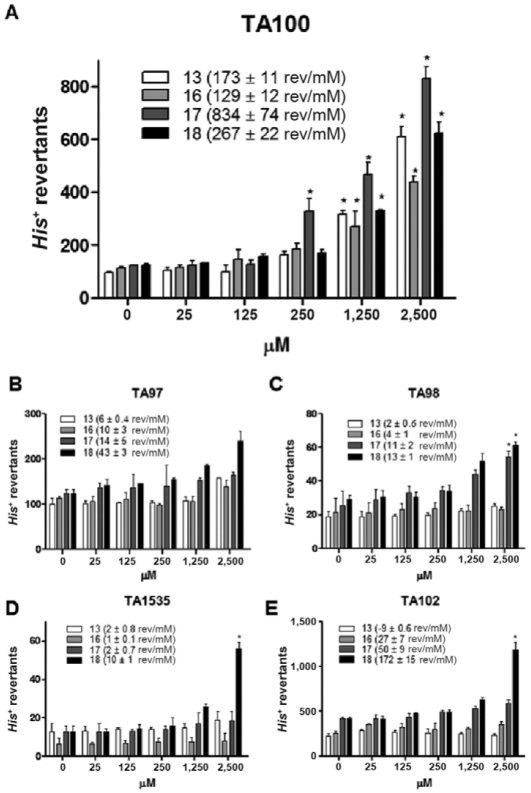

Fig. 4: *Salmonella*/Microsome assay of the nitroimidazoles 13, 16, 17 and 18. rev/mM: revertant colonies per nM.

This work investigated the importance of different functional groups of C-4 or C-5 nitroimidazoles having oxidisable groups bonded at N-1 on the biological and mutagenic activities of the compounds.

The results of the genotoxicity assays are described in Table I. Nitroimidazoles 11 and 12, which contain a NO_2 group at C-4 and a CH_3 group at C-2, were not genotoxic compared to 13, 14 and 15, which possess a NO_2 group at C-5 and exhibited moderate to high genotoxicity. Nitroimidazole 13 had moderate mutagenic effects. We also observed a comparable result between 11 (2-CH_3 and 4-NO_2), which was not genotoxic and its analogue 4 (2-CH_3 and 5-NO_2), which is known to be a mutagenic agent (Ferreiro et al. 2002). However, when the nitroimidazole had no CH_3 group at C-2, the position of the NO_2 group had no influence on genotoxic activity. This is the case for 16 (4-NO_2) and 18 (5-NO_2), which exhibited behaviours similar to that of 11 and 12 (no genotoxicity). When comparing pairs of similar compounds, for instance, 16 (4-NO_2 and N-CH_2OAcCH_2Cl) with 17 (4-NO_2 and N-CH_2OAcCH_2F) and 14 (2-CH_3; 5-NO_2; N-CH_2OHCH_2F) with 15 (2-CH_3; 5-NO_2; N-CH_2OHCH_2OH), we observed that the presence of fluorine induced genotoxicity. The fluorinated compounds 14 and 17 showed higher genotoxicity regardless of the presence of CH_3 at C-2 or NO_2 at C-4 or C-5. However, when comparing compounds 11 (2-CH_3; 4-NO_2; N-CH_2OHCH_2Cl) and 12 (2-CH_3; 4-NO_2; N-CH_2OHCH_2F), the fluorine atom had no influence on genotoxicity.

Four compounds were selected for the Ames assays [nitro group at positions 4 (16 and 17) and 5 (13 and 18) and different substituent groups at position N-1] with the aim of clarifying whether the increase in mutagenicity of nitroimidazoles was dependent only on the position of the nitro group or was related to the presence of more or less reactive halogens.

The results of the cytotoxicity and mutagenicity analyses are described in Fig. 4 and Table II. The four *S. typhimurium* strains have GC base pairs at the primary reversion site and as a result, it is known that these

TABLE II

Genotoxicity, mutagenicity and mutagenic potency slope of nitroimidazoles 13, 16, 17 and 18

	Genotoxicity	Ames test (MI > 2) - Mutagenicity			Mutagenic potency slope (revertants/µg)				
Compound	1,562 µM	250 µM	1,250 µM	2,500 µM	TA97	TA98	TA100	TA102	TA1535
13	1 (300 TAU)	-	TA100	TA100	0.30 ± 0.22	NA	8.98 ± 0.71	NA	NA
16	0 (10 TAU)	-	TA100	TA100	NA	NA	6.60 ± 0.63	NA	NA
17	3 (411 TAU)	TA100	TA100	TA98, TA100	NA	0.55 ± 0.08	41.70 ± 3.71	2.51 ± 0.44	NA
18	0 (32 TAU)	-	TA100	TA98, TA100, TA102, TA1535	2.13 ± 0.14	0.69 ± 0.05	8.18 ± 0.15	NA	0.51 ± 0.07

MI: mutagenicity inde; NA: not applicable; TAU: total arbitrary units for the positive control, methyl methane-sulfonate value was 454.3 ± 9.2.

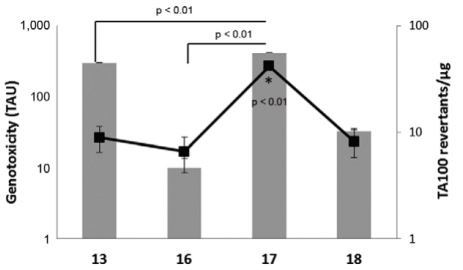

Fig. 5: genotoxic (comet assay) and mutagenic (*Salmonella*/Microsome assay) response of the nitroimidazoles 13, 16, 17 and 18.

strains may not detect certain oxidising mutagens, cross-linking agents and hydrazines. Such substances may be detected by *S. typhimurium* TA102, which has an AT base pair at the primary reversion site (OECD iLibrary 1997). The data showed that 17 is a highly potent mutagen (41.7 rev/µg to TA100), whereas 13, 16 and 18 are low-potency mutagens, causing approximately 8-9 rev/µg. The data in Fig. 5 further corroborate this finding, on the basis that the number of TAU in the comet assay is high for 17, similar to the number of rev/µg.

In conclusion, we observed that the type and position of different substituents bonded to the imidazole ring have a significant influence on the toxicological activity. Whereas nitroimidazoles 11 and 12 were not genotoxic, nitroimidazoles 13, 15 and 19 were moderately genotoxic and mutagenic. Nitroimidazole 16 was neither genotoxic nor mutagenic and 18 was moderately mutagenic but not genotoxic. These results demonstrate that the nitro group is not solely responsible for the mutagenic or genotoxic activity.

Furthermore, the data suggest that the nitroimidazole may be the moiety most likely to be responsible for the genotoxic and mutagenic effects, but in the analogues tested, this moiety was unable to provide anti-*T. cruzi* activity.

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3.3 Artigo 3 - *Repurposing strategy of atorvastatin against Trypanosoma cruzi: in vitro monotherapy and combined therapy with benznidazole exhibits synergistic trypanocidal activity* – Submetido para o periódico "Antimicrobial Agents and Chemotherapy"

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Dear Mr. Araujo-Lima,

On May 10, 2018, we received the manuscript "Repurposing strategy of atorvastatin against *Trypanosoma cruzi*: *in vitro* monotherapy and combined therapy with benznidazole exhibits synergistic trypanocidal activity" by Carlos Araujo-Lima, Raiza Peres, Patricia Bernardino da Silva, Marcos Batista, Claudia Alessandra Aiub, Israel Felzenszwalb, and Maria de Nazaré Soeiro. The submission form indicates that this paper should be processed as a(n) Full-Length Text intended for publication in the section Experimental Therapeutics.

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Repurposing strategy of atorvastatin against *Trypanosoma cruzi*: *in vitro* monotherapy and combined therapy with benznidazole exhibits synergistic trypanocidal activity

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Running title:

Repurposing of atorvastatin against *Trypanosoma cruzi*

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Summary

Statins are inhibitors of cholesterol synthesis but other biological properties, like antimicrobial effects, have been assigned leading to the designation of their pleiotropic aspect. Our goal was investigate the activity and selectivity of atorvastatin (AVA) against *T. cruzi* using *in vitro* models aiming more effective and safer therapeutic options through drug repurposing proposals under monotherapy and in combination with benznidazole (BZ). Phenotypic screening used different strains (Tulahuen (DTU VI) and Y (DTU II)) and forms (intracellular forms, bloodstream and tissue-derived trypomastigotes) of the parasite. While assaying Tulahuen strain, AVA is more active against intracellular amastigotes (SI=3). Also, against another parasite DTU (Y strain), this statin was more active (2.1-fold) and selective (2.4-fold) against bloodstream trypomastigotes (SI=51) than upon the intracellular forms (SI=20). The cytomorphological approach using phalloidin–TRITC permitted to verify that AVA did not induced cell density reduction and CC maintained their typical cytoarchitecture. Combinatory approaches using fixed-ratio methods showed that AVA and BZ gave synergistic interactions against both trypomastigotes and intracellular forms (mean ΣFICIs = 0.46 ± 0.12 and 0.48 ± 0.03 respectively). In this sense, the repurposing strategy of AVA, especially in combination with BZ leading to a synergic effect, encourages future studies in order to identify novel therapeutic protocols for Chagas disease treatment.

Keywords: Trypanocidal, Chagas Disease, Experimental Chemotherapy, Selectivity, Statin, Atorvastatin, HMGCR inhibitor

Key findings:

- Statin repurposing represents a promising approach for drug discovery of novel anti-*T.cruzi* candidates
- Atorvastatin (AVA) is active against trypomastigotes and intracellular forms of *T.cruzi* from different DTUs
- AVA was more potent and selective against trypomastigotes as compared to intracellular forms
- Combined therapy between AVA and BZ demonstrated synergic drug interaction against both BT and intracellular forms of *T. cruzi*

1. Introduction:

Statins are competitive hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors of cholesterol synthesis from the mevalonate pathway. These molecules also enhance the expression of low-density lipoprotein (LDL) receptors and increase the incorporation of LDL, leading to reduced serum levels of cholesterol (1). Several new roles have been assigned to statins, some of them related to their hypocholesterolemic activity, but others not directly involved into this primary activity (2). These multitasking statin properties known as pleiotropic effects, include anti-oxidative, anti-inflammatory, anti-atherogenic and chemotherapeutic activity (3), suggesting also their potential use for prevention and treatment of infectious diseases such as Toxoplasmosis and Malaria (4-8). Chagas disease (CD), caused by the protozoan intracellular parasite *Trypanosoma cruzi*, is an important public health problem, affecting more than 06 million people, with more than 14,000 annual deaths (9). Current therapeutic options for CD are limited to benznidazole (BZ) (N-benzyl-2-nitroimidazole acetamide) and nifurtimox (NF) (3-methyl-4-(5'-nitrofurfurylideneamine tetrahydro-4H-1, 4-tiazine-1, 1-dioxide), and both display severe limitations including side effects, lack of efficacy in later chronic phase and the occurrence of naturally resistant strains (10).

Different therapeutic strategies have been used to identify new treatments more effective and safer and several classes of compounds have been studied (9, 10). One of this are the inhibitors of the lipid biosynthetic route (11,12) as sterols are essential components in biological membranes, exhibiting key roles in controlling the fluidity and permeability, ion channel functionality, as well as upon the cycles of cell division (13). Differently to mammalian cells that have cholesterol as major sterol, like fungi, protozoan agents such as *T. cruzi* synthesize, in endogenous character, ergosterol and; therefore the inhibition of this biosynthetic pathway has been considered an important drug target for CD (11,14).

Unfortunately, clinical trials demonstrated a therapeutic failure of azoles inhibitors of the CYP51 enzyme although experimental models of *T. cruzi* infection (*in vitro* and *in vivo*) have previously demonstrated promising pre-clinical findings (9).

Interestingly, Urbina et al. (15) reported that the anti-parasitic effect of ketoconazole is improved by lovastatin (LOV) when murine experimental models of Chagas disease were used. Simvastatin (SIM) reduces heart inflammation and decreases the levels of serum and tecidual inflammatory mediators in mouse models of acute *T. cruzi* infection (17). Also, SIM improved cardiac remodeling in *T. cruzi* infected dogs (18) although was not effective in reducing the parasitaemia peak levels. In this sense, as Peña-Diaz et al. (16) found a functional HMGCoA-reductase on *T. cruzi*, our present goal was evaluated the impact of drug repurposing schemes using a third generation statins, the Atorvastatin (AVA) alone and in combination with the clinical drug for CD, the BZ. Then, the activity, mammalian toxicity and cardiac cell viability of AVA was assessed against different strains and forms of *T. cruzi*, considering its selectivity among the distinct *T. cruzi* DTUs, through well standardized *in vitro* approaches, besides exploring its efficacy profile in combination with BZ.

2. Results

We started our analysis by evaluating the effect of AVA against trypomastigotes of *T. cruzi* (BT from Y strain, representative of DTU II). Our findings demonstrate that AVA displayed a high trypanocidal effect *in vitro* exhibiting an EC_{50} of 7.1 ± 1.8 µM when BT forms were assayed, being slightly more active than the reference drug (EC_{50} = 13± 2 µM) (Table 1). Regarding *in vitro* cardiac toxicity, AVA demonstrated a low toxicity profile, presenting LC_{50} = 360.7 ± 18.2 µM after 24 h of compound exposure and thus this low cardiotoxicity behavior led to high selectivity: 51 for BT. Following our screening flowchart, we next evaluated the effect of AVA upon intracellular forms of the parasite, also exploring two different *T. cruzi*

strains belonging to the DTUs VI and II. As described in Table 2, after 48 h of incubation, AVA reached an EC_{50} of 15 ± 2.8 µM when CC were infected with the Y strain. Due to the high LC_{50} values (320.0 ± 14.1 µM) of AVA after 48 h of exposure, the SI was also encouraging, reaching 21-fold (Table 2).

At figure 1, CC incubated with the corresponding EC_{50} values reached against intracellular forms, did not exhibit toxic alterations at their morphology and density (Figure 1B) neither on their cytoskeleton organization (Figure 1D), displaying similar aspects in comparison to uninfected cells (Figure 1E). AVA largely reduced the percentage of infected cells and the number of parasite per host cell as compared to infected and untreated cultures (Figure 1A and 1C). Next, as AVA was less potent against intracellular forms as compared to BT forms, we next assessed if AVA could have a similar behavior (less activity on intracellular forms) testing it against other host cells infected with another parasite strain and DTU. Then, infected L929 cell cultures infected with Tulahuen and exposed to non-toxic concentrations of AVA confirmed that this statin was less active against intracellular forms (EC_{50} = 25 µM), regardless the parasite strain (Table 3). There were no statistical differences ($p > 0.05$) between EC_{50}s values against intracellular forms of neither Tulahuen nor Y strains.

Next step was investigate if the association of AVA with BZ could improve the trypanocidal effect upon intracellular and trypomastigote forms of *T. cruzi*. Then *in vitro* interaction of AVA and BZ was evaluated and mean FICIs and representative isobolograms are presented in Fig. 2. The findings showed an important leftward shift of the combined therapy curves in both BZ (Fig 2a) and AVA (Fig 2b) when compared to monotherapy. A similar behavior in the treatment was also noticed when BT for assayed (Fig. 2d) with leftward shift in AVA combination therapy (Fig. 2e) *versus* monotherapy curve. The interaction of AVA and BZ was classified as synergic, in both BT (Fig. 2c) and intracellular forms (Fig 2f), presenting mean ΣFICIs equal to 0.46 ± 0.12 and 0.48 ± 0.03 respectively.

3. Discussion

According to Don and Ioset (19), the characteristic of a hit compound for anti- *T. cruzi* drug screening includes an EC_{50} value up to 10 µM against intracellular forms and SI of at least 10 besides the absence of structural alerts of safety concerns (such as genotoxicity) in addition to druggability properties, at least evaluated using *in silico* platforms. Also, the Target Product Profile (TPP) for CD recommends the analysis upon the both relevant parasite forms for mammalian hosts (trypomastigotes and intracellular forms), also looking the effects towards different representatives of *T.cruzi* DTUs (20).

Although de-Souza and Rodrigues (21) had point AVA as a promising Ergosterol Biosynthesis Inhibitor (EBI) in their 2009's review about sterol pathways as target for anti-trypanosomatid drugs, few studies have been performed testing the effect of statins upon animal *T.cruzi* infection, displaying controversial data (18, 22). In this sense, due to the well-known pleiotropic profile of AVA acting as anti-parasitic agent, presently a repurposing study using this molecule was conducted, taking into consideration phenotypic steps preconized for a novel anti-*T.cruzi* entity (23). The repurposing approach is largely encouraged for the drug discovery of neglected pathologies like CD since it may reduce costs and time due its already approval and use for other clinical conditions (10, 23). Concerning the safety aspects, as demonstrated through pre-clinical and clinical toxicological screenings performed by Ciaravino et al., (24), AVA is not mutagenic neither genotoxic, being considered a safe drug currently in the market. Regarding its potential application as anti-parasitic drug, Carvalho et al. (25) pointed that AVA pyrrole region hybridized with quinolines were effective against *P. falciparum* in parasitized red blood cells, suggesting that the pyrrole ring of this molecule has a central role in its microbicidal effect, possibly acting as a secondary pharmacophoric region.

As suggested previously by Kaiser et al. (26), due to the large databank of anti-malarial agents, the repurposing of the hit-to-lead compounds merits further screening upon other kinetoplastid diseases, including CD. In this sense, our present study evaluated the potential activity of AVA as anti-*T.cruzi* agent, using a well-standardized pipeline for CD (23).

Our finding demonstrated a promising effect of AVA when *T.cruzi* was assessed, especially towards trypomastigotes were assayed, giving considerable selectivity indexes (>50), which is a desirable characteristic for a novel hit drug for CD (20). Another aspect is that AVA displayed low cardiotoxicity profile that is favorable characteristic since heart is one of the main targets of *T.cruzi* infection and inflammation in CD (12). Our data corroborates in part the literature data that demonstrated the beneficial cardiovascular pleiotropic effect of statins such as AVA (2, 3, 22).

Florin-Christensen et al. (27) and Melo et. al. (18) demonstrated the activity of lovastatin and SIM, respectively, against epimastigotes of *T. cruzi* (Y strain). However, this form is not the relevant one for assaying the effect of novel drugs for CD since epimastigotes are the proliferative forms found only in the vector and thus not adequate to explore the activity and selectivity of novel anti-*T.cruzi* agents (23).

It is important to highlight that trypomastigotes were consistently more vulnerable (2-3- fold) to AVA than intracellular forms. This differential susceptibility according to the parasite forms of *T. cruzi* may be due to the intracellular cytoplasmatic microenvironment localization of amastigotes. It is possible that the plasma membrane of the parasites are more permeable to AVA as compared to the membranes of the mammalian hosts, reducing the access of free AVA directly towards the intracellular parasites (e.g. as result of the host plasma membrane barrier). Thus, as alternative to increase AVA potency against intracellular forms, the use of

drug carrier and novel formulation could allow higher permeability across the host cell and/or reduce the drug efflux.

Other hypothesis is regarding differences related to the parasite stage, including (i) localization into distinct microenvironment (blood *vs* intracellular *milleu*) with thus differential lipid levels and access; and (ii) distinct metabolism, uptake or efflux levels among the non-proliferative *vs* highly multiplicative parasite forms, among others, which merit be further explored.

A remarkable present result was achieved using combinatory schemes, resulting ΣFICIs values lower than 0.5, indicating synergism with the reference drug for CD, benznidazole. Our data corroborates previous studies using combinatory repurposing approaches (AVA and reference drug such as quinine) on several strains of *Plasmodium falciparum*, reporting a synergistic profile (28).

In fact, *de novo* drug discovery and development are very long and costly processes, so the repurposing strategy has been largely stimulated as an alternative to treat several orphan pathologies, since the steps related to drug pharmacokinetics and safety profile are approved by the regulatory authorities (25). Drug repurposing comes side by side with the possibility of combining drugs, as it is already the established treatment protocol for infectious diseases as tuberculosis and malaria (29). Recently, the repurposing of NF and its combination to eflornithine to treat African Trypanosomiasis has been made available and demonstrated positive outcomes (30-31). The combination of licensed drugs enables cost reduction and reduces side effects, as well as provides a wider arsenal against neglected diseases, improved efficacy and finally possible changes in posology and administration, which encourages patient adherence to treatment (32). Besides, this strategy allows as much as the attack of

different targets in the parasite, as the bypass or even avoidance of drug resistance, and has been largely claimed as an alternative for CD therapy (32-34).

Novel alternatives for Chagas disease therapy still represent an urgent issue (35). The therapeutic failure of EBIs, such as posaconazole and ravuconazole, in clinical trials for CD, along with the withdrawn of nitroderivative fexinidazole from recent clinical trials due to its low safety aspects for the CD patients justify the search for alternative anti-*T.cruzi* molecules as well as novel therapeutic approaches (20). By the way, a hybrid molecule derived from the EBI ravuconazole (E1224) presented satisfactory results in a recent clinical trial in with chronic CD patients in Colombia (36).

The bulk of our data suggests the promising therapeutic approach of statins in combination with BZ to treat parasitic infections, also justifying the synthesis of novel statin molecules more selective for parasite's enzyme aiming to contribute for the discovery of novel alternative therapeutic options for parasitic pathologies. Our combined therapy results are thus very encouraging, reaching synergic effect between AVA and BZ, ameliorating the efficacy and potency of both drugs in combination, against both trypomastigotes and intracellular forms of *T. cruzi,* belonging to different parasite DTUs, which are relevant for human infection.

4. Methods

4.1 Compounds

For the analysis of the compounds against *T. cruzi* and upon mammalian viability, stock solutions of AVA (FarManguinhos, Fiocruz, Brazil) were prepared in dimethyl sulfoxide (DMSO) with the final concentration of the solvent never exceeding 0.6 %, which do not exert any toxicity (data not shown). BZ (Laboratório Farmacêutico do Estado de Pernambuco

[LAFEPE], Brazil) was used as trypanocidal reference drug control and aliquots were stored at -20°C.

4.2 Cell cultures

Mouse L929 fibroblasts obtained from the American Type Culture Collection (Manassas, VA) were cultured at 37°C in RPMI-1640 medium (Gibco BRL) supplemented with 10 % fetal bovine serum (FBS) and 2 mM glutamine, as reported in Timm et al., 2014 (37). For both drug cytotoxicity and infection assays, primary cultures of cardiac cells (CC) were obtained as reported in Batista et al. (38). The cultures were sustained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6 % FBS, 2.5 mM $CaCl_2$, 1 mM L-glutamine, and 2% chicken embryo extract maintained at 37°C in an atmosphere of 5 % CO_2 and air.

4.3 Parasites

Tissue culture derived trypomastigotes (CT) from Tulahuen strain (expressing the *E. coli* β-galactosidase gene) were maintained in L929 cell lines. After 96 h of parasite infection, the supernatant enriched with released trypomastigotes was collected and the parasite purified following previously established protocol (37). Bloodstream trypomastigote forms (BT) of the Y strain were obtained from the blood samples of infected albino Swiss mice at the peak of parasitaemia and the purified parasites resuspended RPMI as reported (40). Intracellular amastigotes from Y and Tulahuen strains were also evaluated. For the analysis using Y strain, CC were infected with BT for 24 h (parasite/CC ratio of 10:1), then rinsed to remove non-internalized parasites and further incubated for another 48 h with increasing but nontoxic doses of the test compound (38). For the Tulahuen strain, L929 cell cultures were infected for 2 h with CT (10:1 ratio) and then the cultures rinsed to get rid of the non-internalized parasites. Next, the cell cultures were further incubated for another 24 h at 37°C and then non-toxic compound concentrations added and followed for 96 h (37).

4.4 Mammalian cytotoxicity in vitro tests

CC were incubated for 24-48 h at 37°C with different concentrations of AVA (up to 500 µM) diluted in DMEM (without phenol red), then the morphology and spontaneous contractibility evaluated by light microscopy and their cellular viability determined by the PrestoBlue® assay. For this colorimetric bioassay, 10 µL PrestoBlue® (Invitrogen) was added to each well and the plate further incubated for 5 h. A similar protocol was used to evaluate L929 cell viability after 96 h of exposure to the compounds, using 10 µL AlamarBlue® (Invitrogen). As negative controls, AlamarBlue® assay was also performed in the lack of mammalian cells, running only DMEM and DMEM containing AVA and BZ (at higher concentration). The absorbance was determined (at 570 and 600 nm) and the results expressed as percentage of reduction between compound- and vehicle-treated cell cultures following the manufacturer instructions. Then, the values of LC_{50}, which corresponds to the concentration that reduces in 50 % the cellular viability calculated as reported (37).

4.5 Trypanocidal analysis

BT forms of the Y (5 X 10^6 per mL) were incubated for up to 24 hours at 37°C in RPMI in the presence or absence of serial dilutions of AVA (0 to 32 µM) and BZ (up to 100 µM). After compound incubation, the parasite death rates were determined by light microscopy through the direct quantification of the number of live parasites using a Neubauer chamber, and the EC_{50} (compound concentration that reduces 50 % of the number of parasites) calculated (38). For the assay on intracellular forms, Y and Tulahuen strains were employed using CC and L929 cells, respectively, as above mentioned. Briefly, Tulahuen-infected-L929 cultures were exposed to serially diluted non-toxic concentrations of AVA diluted in RPMI to determine the EC_{50} value (39). After 96 h of compound incubation at 37°C, chlorophenol red glycoside (500

μM) in 0.5% Nonidet P40 was added to each well and the plate incubated for 18 h at 37°C. Next the absorbance was measured at 570 nm. Uninfected and *T.cruzi*-infected cultures submitted to only vehicle and BZ exposure were run in parallel. The results are expressed as the percentage of parasite growth inhibition in compound-tested cells as compared to the infected cells and untreated cells (40). Triplicate samples were run in the same plate and at least two assays performed in each analysis. For the analysis of the effect against intracellular amastigotes from Y strain, after 24 h of parasite-host cell interaction, the infected CC were washed to remove free parasites and then incubated for another 48 h with increasing concentrations of the test compounds. CC were maintained at 37°C in an atmosphere of 5 % CO_2 and air and the medium replaced every 24 h. Then, untreated and treated infected CC were fixed and stained with Giemsa solution and the mean number of infected host cells and of parasites per infected cells scored as reported (39). Only characteristic *T. cruzi* nuclei and kinetoplasts were counted as surviving parasites since irregular structures could mean parasites undergoing death. The compound activity was estimated by calculating the infection index (II - percentage of infected cells times the average number of intracellular amastigotes per infected host cell) (38). At least two assays were performed in duplicates in each analysis. Statistical analysis was performed by analysis of variance (ANOVA) as reported in De Araujo et al. (39). All *in vitro* assays were run at least twice in triplicate. Statistical Analysis were performed by two-way ANOVA and Bonferroni's post-hoc test, considering $p < 0.01$.

4.6 Determination of drug interactions against BT and Intracellular forms of T. cruzi

In vitro drug interactions were assessed using a fixed-ratio method (29) by combining AVA and BZ. In these assays, predetermined EC_{50}s were used to determine the top concentrations of the individual drugs to ensure that the EC_{50} fell near the midpoint of a six-point 2-fold dilution series. The top concentrations used were 100 μM for BZ and 50 μM for AVA in a 24

h assay (against Y strain; BT) and 15 μM for BZ and 50 μM for AVA in a 96 h assay (against Tulahuen strain; intracellular forms). The top concentrations were used to prepare AVA and BZ solutions at fixed ratios of 5:0, 4:1, 3:2, 2:3, 1:4, 0:5, as reported previously (41-42).

4.7 Determination of FICI index, isobologram construction, and classification of the nature of the interaction

Fractional inhibitory concentrations (FICIs) and the sum of the FICIs (ΣFICIs) were calculated as follows: FICI of AVA = EC_{50} of AVA in combination/EC_{50} of AVA alone. The same equation was applied to the partner drug (BZ). ΣFICIs were calculated as the FICI of AVA plus the FICI of BZ. An overall mean ΣFICI was calculated for each combination and used to classify the nature of the interaction. Isobolograms were constructed by plotting the AVA's EC_{50} against the BZ's EC_{50}. Statistical analysis was performed individually for each assay using an analysis of variance program, and the level of significance was set at a *P* value of ≤ 0.05. Interactions were categorized as described by Simões-Silva et al. (40), in which synergism was classified as a mean ΣFICI $\leq$ of 0.5, antagonism was classified as a mean ΣFICI > of 4.0, and indifference (or an additive effect) was classified as a mean ΣFICI of between > 0.5 and ≤ 4.0. All the assays were conducted in triplicate, and results are averages from at least three independent experiments (40).

4.8 Fluorescence cytoarchitectonic arrangement assay

In an attempt to assess cardiac cells viability through contractibility apparatus, sub-cellular cytoskeleton configuration was checked. The cultures were treated during 48 h with AVA at EC_{50} obtained against intracellular forms (15 μM, Table 2) and after, washed and stained with 800 μg/ml phalloidin-rhodamine and 1 μg/mL 4,6-diamidino-2-phenylindole (DAPI) for actin filaments and DNA visualization, respectively (43). Doxorubicin (10 μM) was used as

cytoarchitectonic derangement induction control and 2.5 µM BZ was used as *T. cruzi* infection positive control (44). The samples were observed at a Zeiss microscope equipped with epifluorescence.

4.9 Ethics

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

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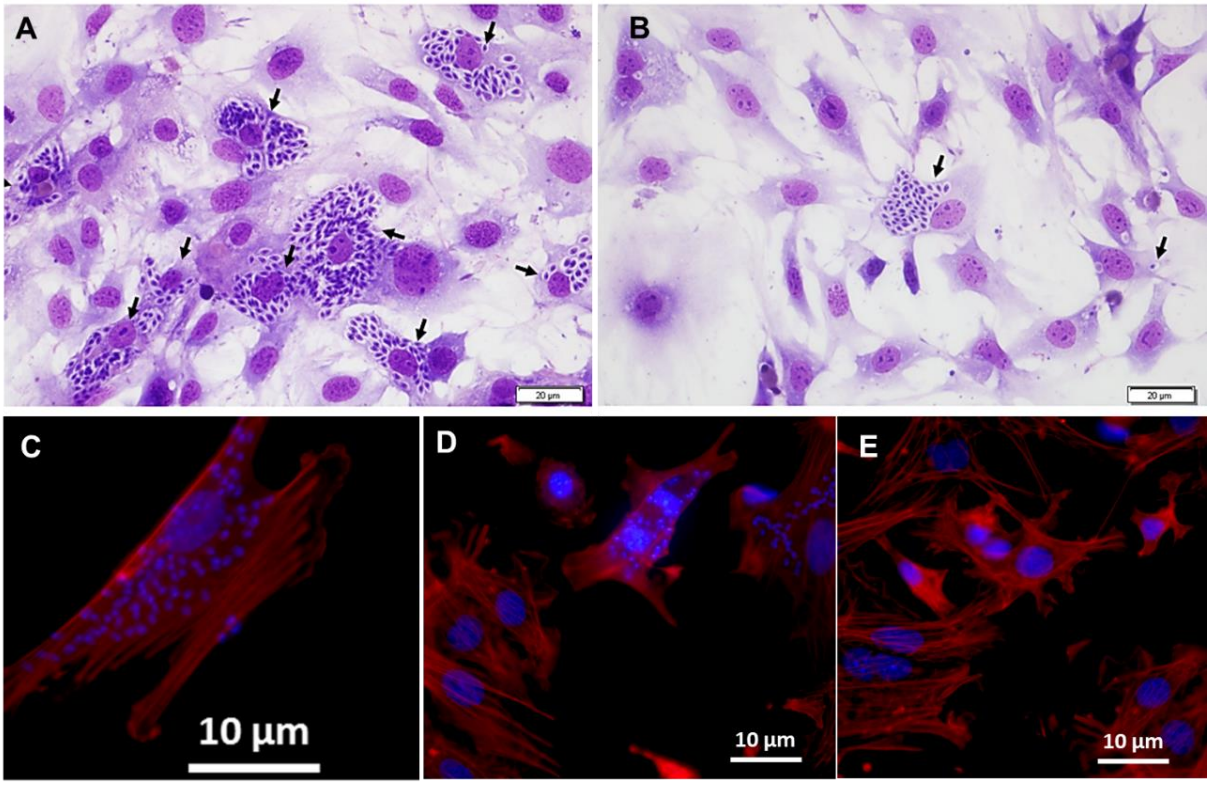

Figure 1. Activity of AVA against intracellular forms of *T. cruzi* (Y strain) after treatment for 48 h at 37°C. (A) *T. cruzi*-infected cardiac cells incubated with vehicle and (B) *T. cruzi*-infected cardiac cells incubated with AVA at EC_{50} (15.0 µM). The arrows point to intracellular parasites. Magnification of 40x, Giemsa's stained cells. (C) *T. cruzi*-infected cardiac cells, (D) AVA *T. cruzi*-infected cardiac cells incubated with AVA at EC_{50} (15.0 µM) and (E) uninfected and untreated cardiac cells. Epifluorescence micrographs ant magnification of 63x, labelled with Phalloidin-rhodamine (red colored) and DAPI (blue colored).

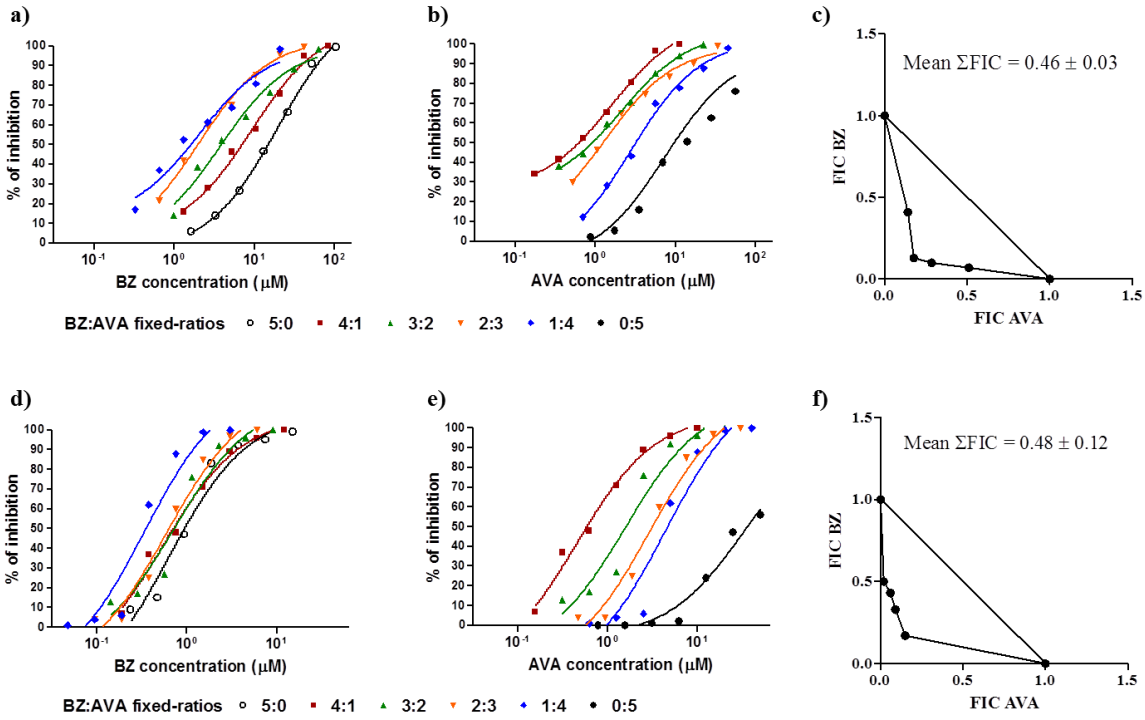

Figure 2. ***In vitro*** **trypanocidal combinatory analysis of atorvastatin (AVA) and benznidazole (BZ) against bloodstream trypomastigotes (Y strain - a, b and c) or intracellular forms (Tulahuen strain – d, e and f).** Dose-response curves of benznidazole (BZ) (a and d) and atorvastatin (AVA) (b and e), and the respective fixed-ratio combinations. Representative isobolograms of *in vitro* interaction of AVA and BZ combined against *T. cruzi* (c and f). The EC_{50} of AVA is plotted on the abscissa, and the EC_{50} of BZ is plotted on the ordinate. The plotted points are the EC50 of each fixed ratio of 5:0, 4:1, 3:2, 1:4, and 0:5 of AVA and BZ. ΣFICs of < 0.5 indicate synergism. ΣFICs of >0.5 and ≤4.0 indicate additivity, and ΣFICs of >4.0 indicate antagonism.

Table 1. *In vitro* effect (EC_{50}) of AVA and BZ against trypomastigote forms of *T. cruzi* (Y strain; BT) and cardiac muscle cell cultures (LC_{50}) after 24h of treatment, and their corresponding Selectivity Index (SI)

	LC_{50} (µM)	EC_{50} (µM)	SI
AVA	360.7 ± 18.2 *	7.1 ± 1.8	51
BZ	> 1000	13.0 ± 2.0	>77

AVA: atorvastatin; BZ: benznidazole; EC_{50}: efficacy concentration of 50%; LC_{50}: lethal concentration of 50%; SI: Selectivity Index; * $p < 0.01$ *vs.*Benznidazole at two-way ANOVA and Bonferroni's post-hoc test.

Table 2. *In vitro* different effects (EC_{50}) of AVA and BZ against intracellular amastigote forms of *T. cruzi* (Y strain) and cardiac muscle cell cultures (LC_{50}) after treatment for 48 h, and their corresponding Selectivity Index (SI)

	LC_{50} (µM)	EC_{50} Infected cells (µM)	EC_{50} Parasites per cell (µM)	EC_{50} Infection Index (µM)	SI
AVA	320.0 ± 14.1	12.3 ± 2.1	7.3 ± 1.7	15.0 ± 2.8	21
BZ[#]	>1000	n.d.	n.d.	2.8 ± 1.9	>357

AVA: atorvastatin; BZ: benznidazole; EC_{50}: efficacy concentration of 50%; LC_{50}: lethal concentration of 50%; SI: Selectivity Index; n.d.: not determined. [#] Data from De Araujo et al. 2014.

Table 3. *In vitro* effect (EC_{50}) of AVA and BZ against intracellular forms of *T. cruzi* (Tulahuen strain transfected with β-galactosidase) and L929 cell cultures (LC_{50}) after 96 h of treatment, and their corresponding Selectivity Index (SI)

	LC_{50} (µM)	10 µM % of parasite kill	EC_{50} (µM)	SI
AVA	76.1 ± 2.2	31.7 ± 5.8	25.3 ± 3.1	3
BZ	169.1 ± 27.2	87.8 ± 7.1	1.8 ± 0.7	92

AVA: atorvastatin; BZ: benznidazole; EC_{50}: efficacy concentration of 50%; LC_{50}: lethal concentration of 50%; SI: Selectivity Index;

3.4 **Artigo 4 -** ***Trypanocidal, Antiplasmodial, and genotoxicity in vitro assessment of new hybrid α,α-difluorophenylacetamide-statin derivatives*** **– Submetido para o periódico "European Journal of M edicinal Chemistry"**

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Co-Authors: Carlos F Araujo Lima, PhD; Rita de Cássia C Carvalho, PhD; Kelly Salomão, PhD; Sandra L Rosário; Debora I Leite, MSc; Mariana M Costa Lima; Julianna S De Araujo, MSc; Anna Carolina C Aguiar, PhD; Antoniana U Kretlli, PhD; Carlos R Kaiser, PhD; Monica M Bastos, PhD; Claudia Alessandra F Aiub, PhD; Israel Felzenszwalb, PhD; Nubia Boechat, PhD;

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European Journal of Medicinal Chemistry

Trypanocidal, antiplasmodial and genotoxicity *in vitro* assessment of new hybrid α,α-difluorophenylacetamide-statin derivatives

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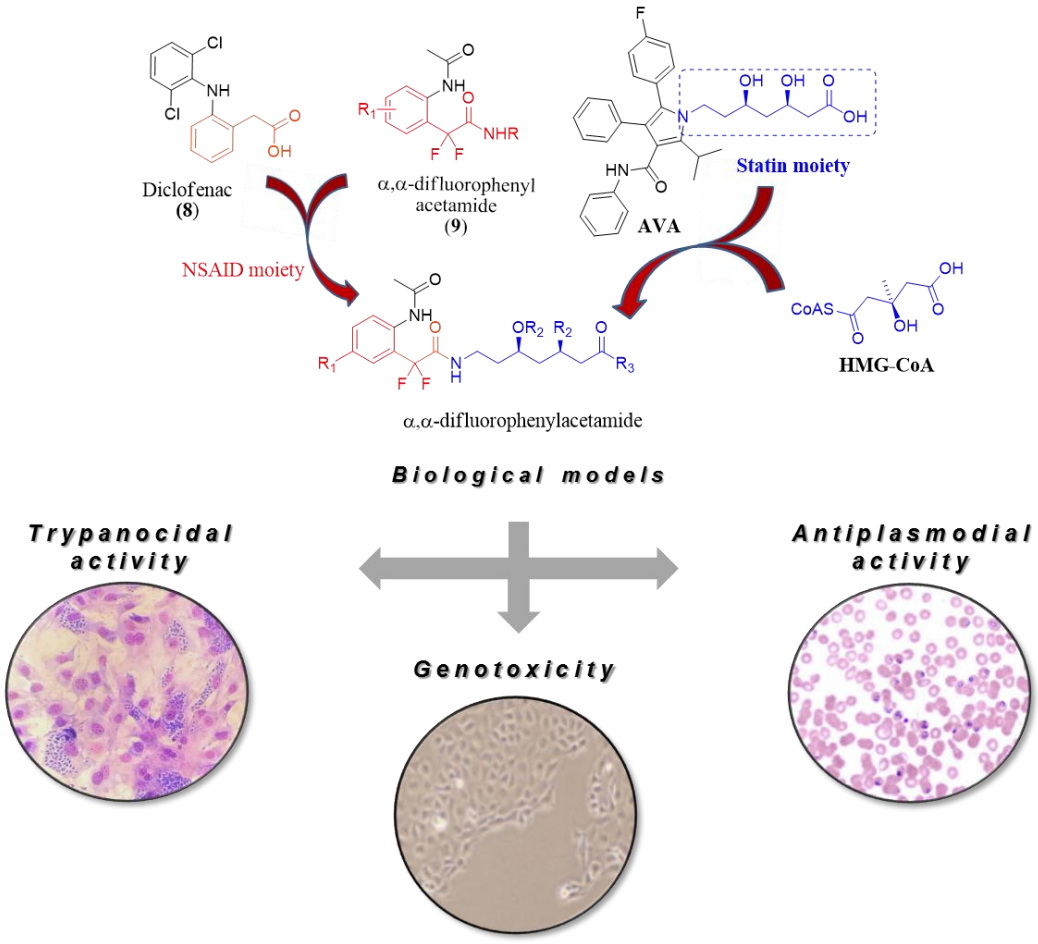
Running title: antiparasitic effect of α,α-difluorophenylacetamide-statin hybrids

Highlights

- Eight novel α,α-difluorophenylacetamide-statin hybrid derivatives were synthetized;
- Acetate derivatives were more active than sodium salt derivatives against *P. falciparum*
- Acetate derivatives were more active than sodium salt derivatives against *T. cruzi*
- Halogen-substituted compounds presented mild antiparasitic activity
- Brominated-substituted acetate form compound induced genotoxic responses in our models

Graphical Abstract



Abstract

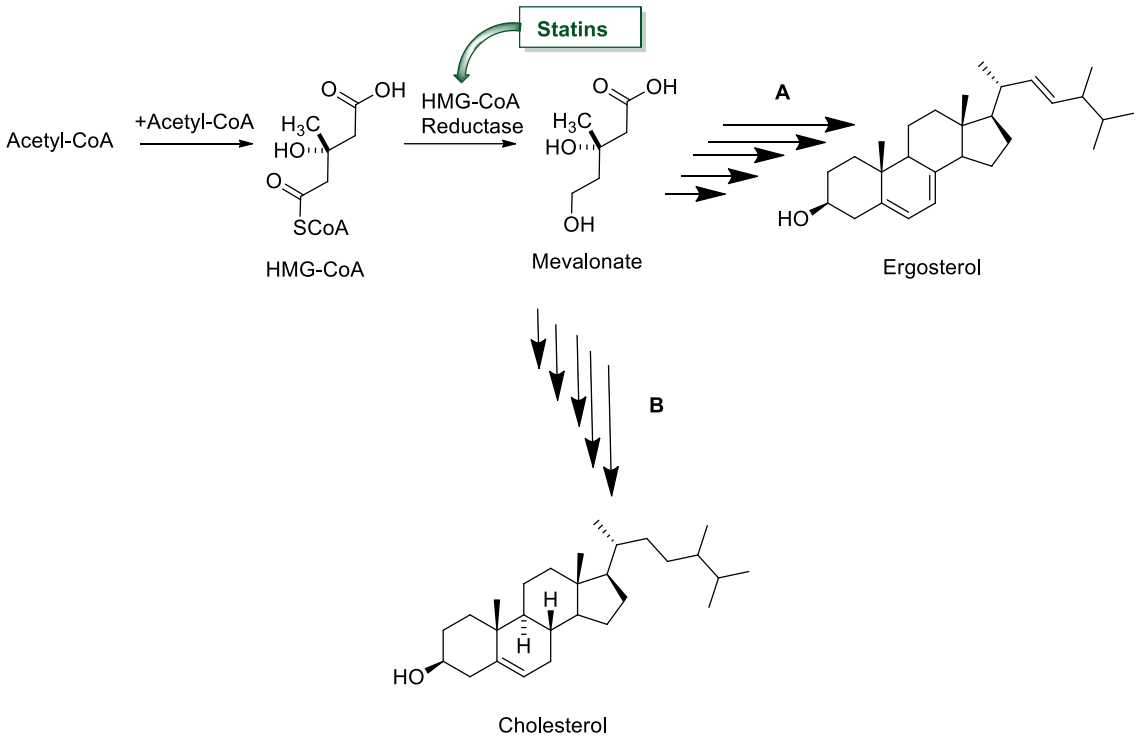
Statins present a plethora of pleiotropic effects including anti-inflammatory and antimicrobial responses. A,α-difluorophenylacetamides are anti-inflammatory non-steroidal drugs, analogs of diclofenac which have been used as adjuvant treatment in for malaria. The molecular hybridization based on the combination of pharmacophoric moieties has emerged as an strategy for the development of new drugs. Considering the anti-inflammatory and microbicidal potential of phenylacetamides and statins against *P.falciparum* and *T.cruzi*, the objective of this work was synthesize eight new hybrid compounds of α,α-difluorophenylacetamides with the moiety of statins and assay the phenotypic screening against *in vitro* models of these parasitic infections and their genotoxicity profile. The hybrid compounds were presently synthetized and their *in vitro* biological aspects evaluated. None of sodium salt compounds presented antiparasitic activity and two acetate form compounds presented mild anti-*P. falciparum* activity. Against *T. cruzi*, the acetate halogenated hybrids presented moderate effect against both parasite forms relevant for human infection. However, despite the considerable trypanocidal activity, the brominated compound displayed a genotoxic profile that turns it unfeasible for moving it to *in vivo* testing. The chlorinated derivative presented the most promising chemical and biological characteristics as it did not also present genotoxicity *in vitro* and its eligible to *in vivo* experiments.

Key- words: Bioisosteric hybridization; Antiplasmodial; Trypanocidal; Anti-inflammatory; Genotoxicity; Statin; α,α-difluorophenylacetamides

1.Introduction

Chagas disease (CD), caused by the protozoan *Trypanosoma cruzi*, is an important public health problem, affecting more than 6 million people, about 7,000 annual deaths and expected 528,000 DALYs [1,2]. Current therapeutic options for CD are limited to benznidazole (Bz) (**1**) and nifurtimox (**2**) (Figure 1), and both display limitations including severe side effects and lack of efficacy in the later chronic phase; also naturally resistant strains were identified. Different strategies have been used to identify new drugs more effective and safer and different parasite molecules/metabolic pathways have been studied [3,4]. Among these strategies, the repurposing approach of drugs used for other clinical conditions and that share common targets has been largely recommended to reduce costs and time on pipeline [5].

Statins are competitive inhibitors of hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, enzyme that catalyzes the NADPH-dependent reduction of HMG-CoA to mevalonate in the cholesterol biosynthesis (Scheme 1A). Besides their role in cholesterol control, statins present pleiotropic effects including acting on the inflammatory responses [6,7] and antimicrobial effect [8]. Some of them, like atorvastatin (AVA, **3**, Figure 1), which is a lipid-lowering agent acting on HMG-CoA reductase present promising antiparasitic activity against intracellular protozoan parasites such as *Toxoplasma gondii* [9] and *Plasmodium falciparum* [10,11]. Similarly as fungi, trypanosomatids require specific sterols for cell viability and proliferation and do not use the abundant supply of cholesterol from the mammalian host. Thus, since *T. cruzi* has this strict requirement for specific endogenous ergosterol, enzymes involved in this biosynthetic pathway have been proposed as potential targets for drug development as showed in Scheme 1B [12-14]. The statin lovastatin (**4**) (Figure 1), able to competitively inhibit the HMG-CoA of *T. cruzi* epimastigotes [15], is primarily localized in the parasite mitochondrion [16]. Lovastatin in combination with ketoconazole or terbinafine was active *in vivo* against *T.cruzi* infection [17]. However, studies conducted with simvastatin (**5**) (Figure 1) in a mouse model of this parasite infection revealed reduction of total cholesterol and of inflammatory mediators in the serum and in the heart tissue but did not demonstrate reduction on parasitemia levels and cardiac parasite load [18]. Also, simvastatin improved cardiac remodeling in *T. cruzi* infected dogs [19]. These findings indicate that besides acting as antiparasitic candidates, some statins may dually play a role in controlling the inflammatory profile on chronic conditions such as the later phase of Chagas disease.



Scheme 1. The simplified ergosterol and cholesterol biosynthesis pathway, highlighting the inhibition of hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) by statins.

Malaria, another parasitic disease, is considered a public health problem in more than 95 countries with 214 million cases in 2015, 438,000 deaths, 3.2 billion people at risk and about 55,111,095 DALYs [20,21]. The disease is caused by protozoa of the genus *Plasmodium* and the life cycle in mammalians occur under different stages infecting liver and erythrocytes. In endemic areas such as on the Africa continent where one child die each day due to this infection, the deaths are mainly related to cerebral malaria, the most severe manifestation caused by *Plasmodium falciparum*. The socio-economic development of the affected regions is highly impaired, corroborating the importance of prevention and treatment of this pathology [22]. Thus, due to the well-known pleiotropic effects such as neuroprotective and anti-inflammatory activity, AVA has been tested as adjuvant in the treatment of experimental malaria [23,24] and its cerebral models [8]. Owing to the increased ability of *Plasmodium* to acquire resistance to chemotherapeutic agents associated to well-known toxicity aspects of the current antimalarial drugs [20,25], the discovery and development of alternatives for this severe pathology is an urgent need. Up to now, studies fail to demonstrated the cross-resistance *in vitro* between AVA and antimalarials, suggesting the occurrence of different modes of action, that is a potential prediction that AVA may be an

promising alternative for malaria therapy [10,11, 26-29]. In this context, our group synthesized and assayed the biological effect of some pyrrolic hybrids of AVA with aminoquinolines against *P. falciparum*, and found an improved activity than chloroquine (**6**) and also being less toxic than primaquine (**7**) [30].

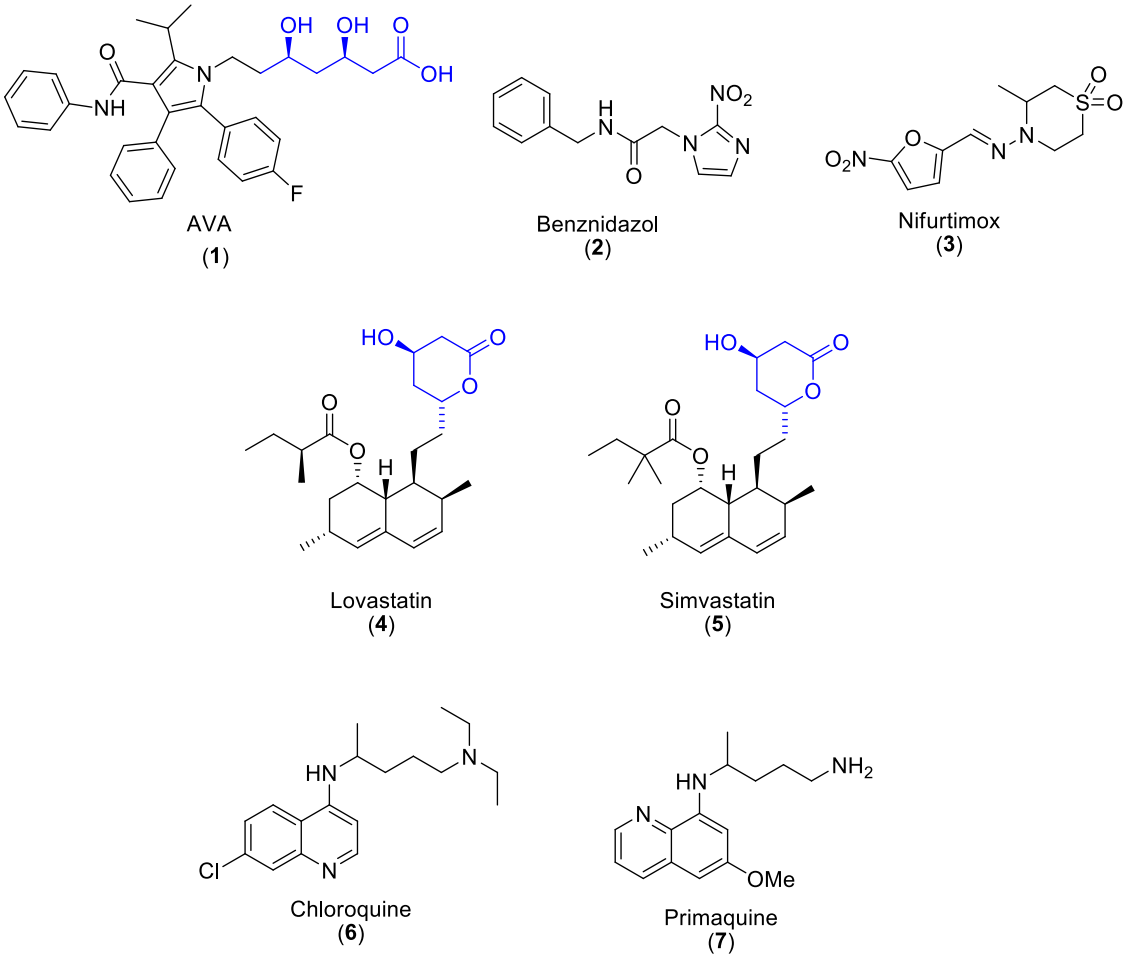


Figure 1. Structures of compounds that reduces inflammatory condition during *T. cruzi* infection.

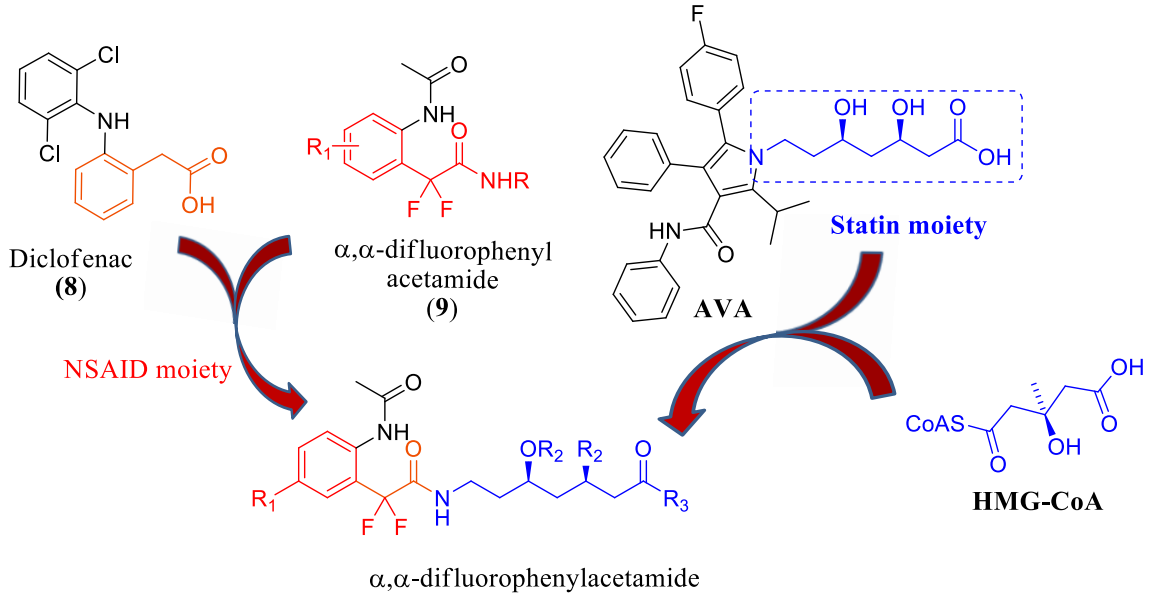
Additionally, as found for *T. cruzi*, although not reaching *in vivo* potency to reach parasitological cure on malaria mouse models, statins acts on the animal inflammation [8, 24, 26] The association of statins with several antimalarial drugs improved the clinical profile of experimental malaria due to a milder progress of this inflammatory process [11, 26, 31-34].

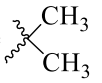
Hybrid molecules represent a current approach for anti-parasitic drug development. The molecular hybridization based on the combination of pharmacophoric moieties has emerged as an important strategy for the development of new drugs that are able to act as multitarget ligands [30]. In this sense, anti-inflammatory non-steroidal drugs (NSAIDs like diclofenac (**8**) Figure 2) have been used as adjuvant treatment in patients with malaria [22]

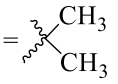
and α,α-difluorophenylacetamides **9** (Figure 2) are potent pre-clinical NSAID analogs of diclofenac [35,36]. Both **8** and **9** have chemical structures based in phenylacetic acid and phenylacetamide, respectively. Phenylacetamides also display a large spectrum of biological activity [37,38].

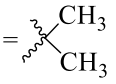
Finally, on the drug pipeline, whenever a new hit compound is identified, its toxicological activity must be explored and as recommended by OECD at least two *in vitro* toxicological assays are needed before proceeding *in vivo* experimentation [39]. Usually, the first set of analysis regarding toxicological evaluation is related to the *Salmonella*/Microssome reverse mutation assay that allows to observe point mutations in DNA sequence, following to evaluation of clastogenicity potential, through micronucleus assay [40].

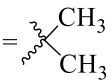
Having in account the anti-inflammatory activity of phenylacetamides and also the potential microbicidal action of statins against *P. falciparum* and *T. cruzi*, the objective of this work was synthesize new hybrid compounds of α,α-difluorophenylacetamides with the moiety of statins (3*S*,5*S*)-3,5-dihydroxyheptanoic **13** (**a-d**) and **14** (**a-d**) as represented in the planning in Figure 2 and assay the phenotypic screening against *in vitro* models of these parasitic infection and their genotoxicity safety profiles.



13a R_1= H; R_3= $C(CH_3)_3$; R_2 = 

13b R_1= CH_3; R_3= $C(CH_3)_3$; R_2 = 

13c R_1= Cl; R_3= $C(CH_3)_3$; R_2 = 

13d R_1= Br; R_3= $C(CH_3)_3$; R_2 = 

14a R_1= H; R_2= OH; R_3= O^-Na^+

14b R_1= CH_3; R_2= OH; R_3= O^-Na^+

14c R_1= Cl; R_2= OH; R_3= O^-Na^+

14d R_1= Br; R_2= OH; R_3= O^-Na^+

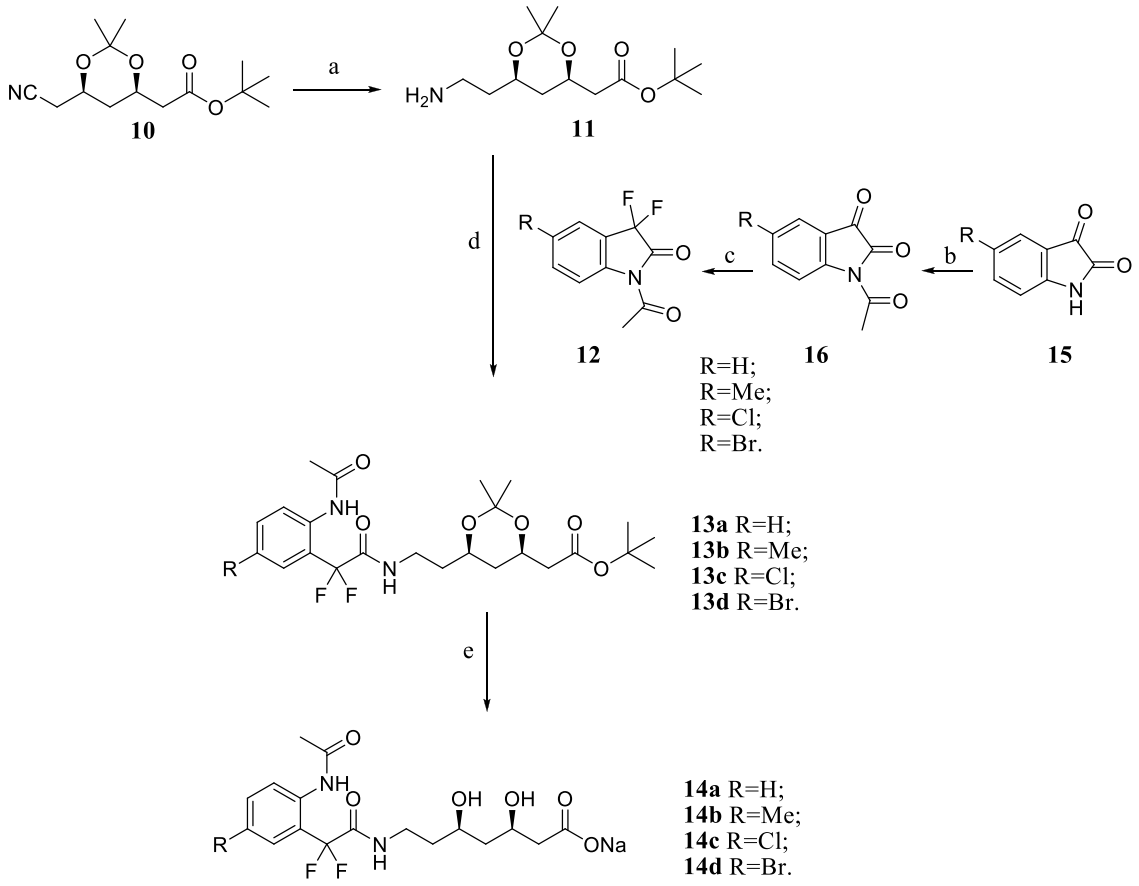
Figure 2. Design of novel hybrids α,α-difluorophenylacetamide-statin derivatives **13** (**a-d**) and **14** (**a-d**) as antiplasmodium and anti-*T. cruzi.*

2. Results and discussion

2.1. Chemistry

The synthetic route (Scheme 2) starts with hydrogenation of the commercially available nitrile compound **10**, which is an intermediate of synthesis of AVA, in Parr reactor, using Raney nickel or palladium on carbon 10% as catalyst to produce the amino intermediate **11** [41,42]. This amino intermediate **11** is used in an nucleophilic addition on *N*-acetyl-3,3-difluoro-2-oxoindoles **12** (**a-d**) to furnish the protected α,α-difluorophenylacetamides **13** (**a-d**) [36]. *N*-acetyl-3,3-difluoro-2-oxindoles **12** (**a-d**) were obtained from fluorination with DAST (diethylamino sulphortrifluoride) of the corresponding *N*-acethylisatins **16** (**a-d**), which were obtained by acetylation of isatins **15** (**a-d**).

The products **14** (**a-d**) were obtained by hydrolysis under acidic conditions and transformed into salt with aqueous NaOH [43]. All compounds **13** (**a-d**) and **14** (**a-d**) were full characterized by, FTIR, NMR ^1H and ^{13}C, proving the formation of the desired products.



Experimental conditions: a) H_2 (50 psi), Ni-Ra or Pd(C), MeOH, NH_4OH, rt, 48 h, 90%; b) Acetic anhydride, 140 °C, 2-6 h, 82-89%; c) DAST, CH_2Cl_2, rt, 18 h, 88-96%; d) DMF, 70 °C, 24 h, 25-70%; e) *i.* MeOH, HCl_{aq} 4% (w/v), rt; ii. NaOH*aq.*70% (w/v), rt, 48 h, 13-84%

Scheme 2. Synthesis of the novel atorvastatin analogues **13a-d** and **14a-d**.

2.2. Biological evaluation

The results of the antiplasmodial activity and cytotoxicity on HepG2 of the new compounds are summarized in Table 1. Compounds **13b**, **13c** and **AVA** were active against *P. falciparum*, with EC_{50} values of 14.26 μM, 11.78 μM and 10.30 μM, respectively. The preliminary cytotoxicity results demonstrated lack of toxicity up to 400 μM (according to the compound solubility) being insufficient to define the accurately the LC50 values. Among the tested compound, **13b** and **13c** were the best exhibiting similar potency as AVA and can be considered as future prototypes for the development of new antimalarial drugs.

Table 1. Evaluation of activity against *P. falciparum* parasites (W2 clone) chloroquine-resistant, cytotoxicity against a human hepatoma cell line (HepG2) and drug selectivity index (SI) of AVA, compounds **13** (**a-d**), **14** (**a-d**) and chloroquine as reference drug, after treatment for 24 h at 37 °C.

Compounds	EC_{50} (µM)	LC_{50} (µM)	SI
AVA	10.30 ± 1.2	>1000	>98
13a	>62	>1000	Inactive
13b	14.26 ± 0.2	>1000	>70
13c	11.78 ± 2.0	>1000	>85
13d	>89	>1000	Inactive
14a	>122	>488	Inactive
14b	>118	>472	Inactive
14c	>112	>450	Inactive
14d	>102	>409	Inactive
Chloroquine	0.59 ± 0.03	1,219	2,066

EC_{50}: Efficacy concentration for 50% of parasite kill, evaluated in two to four different experiments for each test; LC_{50}: lethal concentration for 50% of HepG2 cells; IS: selectivity index (LC_{50}/EC_{50}).

Table 2 shows the effects of **13** (**a-d**) and **14** (**a-d**) against bloodstream trypomastigotes of *T. cruzi* Y strain (moderately resistant to nitroderivatives as Bz) and mouse cardiac cell cultures (CC). It is possible to observe that **13c** and **13d** presented mild activity on the parasite, with EC_{50} corresponding to 28.20 µM and 23.18 µM, respectively, while AVA was slightly more potent than Bz (EC50 7.07 µM and 13 µM, respectively) (Table 2). All the tested compounds **13** (**a-d**) and **14** (**a-d**) had LC_{50} higher than 500 µM after 24 h of exposure of the CC.

Table 2. Evaluation of the trypanocidal activity against trypomastigote forms of T. cruzi (Y strain) partially benznidazole-resistant, cytotoxicity against murine cardiac muscle cell cultures and drug selectivity index (SI) of AVA, compounds 13 (a-d), 14 (a-d) and benznidazole as reference drug, after treatment for 24 h at 37 °C. ND= not determined

Compounds	EC_{50} (µM)	LC_{50} (µM)	SI
AVA	7.07 ± 1.78	360.7 ± 18.2	51
13a	>500	>500	ND
13b	>500	>500	ND
13c	28.20 ± 0.75	>500	>17.7
13d	23.18 ± 3.13	>500	>21.6
14a	>500	>500	ND
14b	>500	>500	ND
14c	>500	>500	ND
14d	>500	>500	ND
Benznidazole	13.00 ± 2.0	>1,000	>77

EC_{50}: Efficacy concentration for 50% of parasite kill, evaluated in two to four different experiments for each test; LC_{50}: lethal concentration for 50% of murine cardiac cells; IS: selectivity index (LC_{50}/EC_{50}).

The inactivity of the salts **14** (**a-d**) could be attributed to their instability and thus only the **13** (**a-d**) were moved to further in vitro analysis to investigate their efficacy against intracellular forms of *T.cruzi* (Table 3) and genetic toxicological *in vitro* screening (Table 4, Figures 3 and 4).

Using a first filter with a fixed concentration of each compound (10 µM), our findings revealed that only **13c** and **13d** displayed more than 50% of reduction on the parasite intracellular infection, being more active than AVA (Table 3). Next, the further analysis using increasing concentrations (up to 50 µM) confirmed the promising effect of both derivatives presenting EC_{50} values of 9.24 µM and 11.42 µM for **13c** and **13d**, leading to SIs of 16.7 and 10.0 respectively. On the other hand, **13a** and **13b** were less effective against intracellular forms of *T. cruzi*, exhibiting EC50 higher than 50 µM (Table 3).

Table 3. *In vitro* effect of the compounds against intracellular forms of *T. cruzi* (Tulahuen strain transfected with β-galactosidase) using fixed concentration of 10 µM (trypanocide rates as % of parasite lysis) and corresponding EC50 values of the studied compounds besides viability assays using L929 cell cultures treated with the compounds (LC50 values) for 96 h at 37 °C, and their corresponding selectivity indexes (SI). ND= not determined

Compounds	10µM (% of parasite lysis)	EC50 (µM)	LC50 (µM)	SI
AVA	31.73 ± 5.80	45.33 ± 3.06	76.13 ± 2.16	1.7
13[a]	17.92 ± 11.02	>50	112.33 ±28.29	ND
13b	25.41 ± 10.90	>50	113.67 ± 35.44	ND
13c	57.83 ± 2.84	9.24 ± 1.06	153.33 ± 25.17	16.7
13d	54.92 ± 16.94	11.42 ± 3.29	114.00 ± 5.29	10.0
Benznidazole	87.84 ± 7.08	1.83 ± 0.73	169.12 ± 27.2	92.4

EC_{50}: Efficacy concentration for 50% of parasite kill, evaluated in two to four different experiments for each test; LC_{50}: lethal concentration for 50% of L929 cells; IS: selectivity index (LC_{50}/EC_{50}).

Next, as the risk assessment by Ames test is mandatory for potential chemical entities for the pharmaceutical clinical arsenal, this analysis was conducted with all the 13 series (Table 4). In this bacterial model, the compound **13a** presented cytotoxicity to TA102 and TA104 exhibiting LC50 in the absence and in the presence of metabolic activation corresponding to 1478.7 ± 56.1 µM 1280.0 ± 21.1 µM, respectively. Derivative **13c** presented toxic concentrations to TA104 in the presence of metabolic conditions (LC_{50}= 1390.0 ± 14.8 µM). **13b** showed LC_{50} >1500 µM for all tested strains both in absence or presence of S9 mix. **13d** was the most active against the tested bacteria, being toxic to TA97 (LC_{50} = 1490.0 ± 36.5 µM), TA98 (LC_{50}= 1260.0 ± 46.8 µM), TA100 (LC_{50}= 120.5 ± 14.1 µM) and TA102 (LC_{50}= 14.7 ± 1.8 µM) in absence of S9 mix and toxic to TA100 (LC_{50}= 1450.0 ± 18.2 µM)

and TA102 (LC_{50}= 442.8 ± 32.1 µM) after exogenous metabolic activation. Besides the cytotoxicity, **13a** and **13d** were capable to induce significant mutagenicity to TA98 (-S9) and TA97 (-S9/+S9), respectively, at concentrations above 300 µM. Despite cytotoxicity, both **13b** and **13c** were not mutagenic at all of the described experimental conditions (Table 4).

Finally, the mutagenic profile was checked using micronuclei approach through mammalian cell cultures employing both CHO-K1 and HepG2 cell lineages since Westerink and coworkers [44] validated those protocols to detect *in vitro* genotoxicity using *in vitro* micronucleus assays. These authors classified the performance scores of these cell lineages for *in vivo* genotoxicity and found a high translation of both models in the beginning of drug discovery steps, representing a useful tool to assess genotoxic potential at the earlier stages. Our present data conducted in parallel by WST-1 colorimetric assay showed that all the derivatives (**13a-d**) presented LC_{50} >1000 µM after exposures of 3 h and 24 h with CHO-K1 or HepG2 cell lineages (data not shown). In micronuclei assay using CHO-K1 (Figure 3), after 3 h of exposure, **13a** induced a significant increase in micronuclei formation at 200 µM and presented a significant concentration-response decrease in survival rate, being considered cytotoxic (Figure 3A). **13b** and **13c** (Figure 3B and 3C) were non-cytotoxic, non-clastogenic and did not affect the mitotic index of the ovarian cells. **13d** (Figure 3D) was significant cytotoxic to ovary cell culture in all tested concentrations and genotoxic upon 1000 µM. *In vitro* micronuclei assay in HepG2 showed that the compounds **13a** (Figure 4A) and **13c** (Figure 4C) did not induce micronuclei formation in HepG2 cells, after 3 h of exposure, but just **13a** and AVA presented significant cytotoxic effects upon 1000 µM. The compound **13b** (Figure 4B) had a statistical enhance, in comparison to the control, in genotoxicity and cytotoxicity parameters to hepatic cells at 2000 µM and **13d** (Figure 4D), besides it was non-cytotoxic, induced a significant micronuclei formation in HepG2 cells after the exposure to the three tested concentrations.

Table 4. Mean values (±SD) of revertant *His*$^+$ colonies of *Salmonella enterica* serovar Typhimurium strains used in *Salmonella*/microssome assay after co-incubation with **13** (**a-d**).

		µM	TA97	TA98	TA100	TA102	TA104
13a	-S9	0	76 ± 6	25 ± 4	117 ± 5	386 ± 32	213 ± 3
	-S9	3	78 ± 6	24 ± 3	117 ± 43	458 ± 19	264 ± 27
	-S9	15	82 ± 8	29 ± 6	136 ± 13	435 ± 22	307 ± 18
	-S9	30	79 ± 8	31 ± 8	139 ± 13	433 ± 7	314 ± 26
	-S9	150	69 ± 10	32 ± 3	142 ± 5	380 ± 12	315 ± 36
	-S9	300	80 ± 11	**52 ± 4 ***	162 ± 37	345 ± 48	316 ± 33
	-S9	1500	90 ± 9	**61 ± 5 ***	179 ± 34	Cytotoxic	350 ± 21
	+S9	0	225 ± 9	29 ± 8	213 ± 20	344 ± 38	369 ± 44
	+S9	3	214 ± 21	25 ± 8	211 ± 37	384 ± 32	390 ± 49
	+S9	15	217 ± 9	25 ± 4	223 ± 15	399 ± 32	417 ± 15
	+S9	30	235 ± 23	26 ± 5	241 ± 36	407 ± 21	467 ± 19
	+S9	150	237 ± 26	27 ± 1	244 ± 14	411 ± 46	330 ± 9
	+S9	300	237 ± 4	33 ± 1	270 ± 45	448 ± 52	268 ± 37
	+S9	1500	290 ± 28	36 ± 3	290 ± 31	501 ± 20	Cytotoxic
13b	-S9	0	76 ± 6	15 ± 3	111 ± 13	370 ± 20	210 ± 3
	-S9	3	69 ± 12	18 ± 2	145 ± 11	388 ± 20	210 ± 11
	-S9	15	76 ± 7	19 ± 3	146 ± 1	393 ± 18	267 ± 15
	-S9	30	75 ± 12	21 ± 5	145 ± 17	423 ± 11	27 ± 13
	-S9	150	74 ± 12	22 ± 2	143 ± 40	503 ± 23	326 ± 13
	-S9	300	84 ± 7	22 ± 2	144 ± 8	543 ± 39	350 ± 22
	-S9	1500	89 ± 27	26 ± 4	178 ± 10	560 ± 41	400 ± 11
	+S9	0	185 ± 8	27 ± 8	183 ± 20	354 ± 38	314 ± 5,7
	+S9	3	189 ± 43	27 ± 5	179 ± 38	365 ± 37	355 ± 12
	+S9	15	183 ± 43	27 ± 1	188 ± 23	386 ± 42	356 ± 14
	+S9	30	216 ± 47	27 ± 5	228 ± 46	403 ± 19	421 ± 67
	+S9	150	231 ± 5	27 ± 5	260 ± 32	396 ± 31	447 ± 45
	+S9	300	244 ± 23	39 ± 1	306 ± 60	372 ± 40	449 ± 18
	+S9	1500	288 ± 33	45 ± 4	333 ± 45	399 ± 18	510 ± 13
13c	-S9	0	68 ± 5	21 ± 4	127 ± 5	385 ± 31	180 ± 9
	-S9	3	81 ± 6	21 ± 2	135 ± 38	373 ± 50	195 ± 9
	-S9	15	84 ± 8	21 ± 5	135 ± 21	386 ± 35	199 ± 7
	-S9	30	84 ± 6	21 ± 3	135 ± 30	387 ± 33	192 ± 3
	-S9	150	70 ± 6	21 ± 2	140 ± 31	386 ± 5	201 ± 17
	-S9	300	71 ± 11	21 ± 1	164 ± 17	400 ± 24	206 ± 8
	-S9	1500	62 ± 9	23 ± 4	168 ± 15	450 ± 32	241 ± 10
	+S9	0	225 ± 8	30 ± 4	213 ± 19	344 ± 19	369 ± 44
	+S9	3	219 ± 16	34 ± 4	223 ± 39	392 ± 8	384 ± 34
	+S9	15	245 ± 22	35 ± 3	223 ± 47	390 ± 12	401 ± 31
	+S9	30	247 ± 14	39 ± 3	228 ± 17	402 ± 24	445 ± 49
	+S9	150	249 ± 17	37 ± 7	278 ± 5	401 ± 66	305 ± 7
	+S9	300	259 ± 1	47 ± 4	286 ± 18	403 ± 12	297 ± 4
	+S9	1500	261 ± 13	49 ± 5	300 ± 15	430 ± 14	Cytotoxic
13d	-S9	0	71 ± 4	20 ± 3	103 ± 15	325 ± 2	403 ± 1
	-S9	3	119 ± 8	22 ± 7	99 ± 4	356 ± 7	469 ± 65
	-S9	15	137 ± 1	23 ± 5	96 ± 12	Cytotoxic	515 ± 61
	-S9	30	135 ± 4	25 ± 1	93 ± 1	-	570 ± 53
	-S9	150	134 ± 4	25 ± 1	Cytotoxic	-	605 ± 61
	-S9	300	**146 ± 6 ***	27 ± 5	-	-	615 ± 15
	-S9	1500	Cytotoxic	Cytotoxic	-	-	620 ± 31
	+S9	0	119 ± 3	24 ± 3	139 ± 17	124 ± 1	382 ± 30
	+S9	3	134 ± 3	28 ± 1	156 ± 18	153 ± 13	490 ± 52
	+S9	15	135 ± 1	28 ± 5	166 ± 11	155 ± 20	513 ± 19
	+S9	30	201 ± 4	31 ± 7	173 ± 1	160 ± 11	530 ± 7
	+S9	150	203 ± 1	34 ± 2	174 ± 21	162 ± 31	541 ± 29
	+S9	300	**288 ± 6 ***	34 ± 4	181 ± 15	176 ± 11	589 ± 5
	+S9	1500	**410 ± 10 ***	35 ± 3	Cytotoxic	Cytotoxic	591 ± 11

SD: standard deviation; -S9: absence of metabolic activation; +S9: presence of metabolic activation;
Positive controls without S9: 4NQO (1.0 µg/pl.) for TA97, 286±17 revertants and TA98 120±10 revertants; AS (1.0 µg/pl.) for TA100, 607±56 revertants; MMC (0.5 µg/pl.) for TA102, 2968±34 revertants; MMS (50 µg/pl.) para TA104 746±58 revertants. With S9: 2AA (1.0 µg/pl.) for TA97, 587±11 revertants, for TA98, 305±1 revertants and for TA100, 1436±40 revertants; B[a]P (50 µg/pl.) for TA102, 1448±79 revertants and for TA104 763±11 revertants.

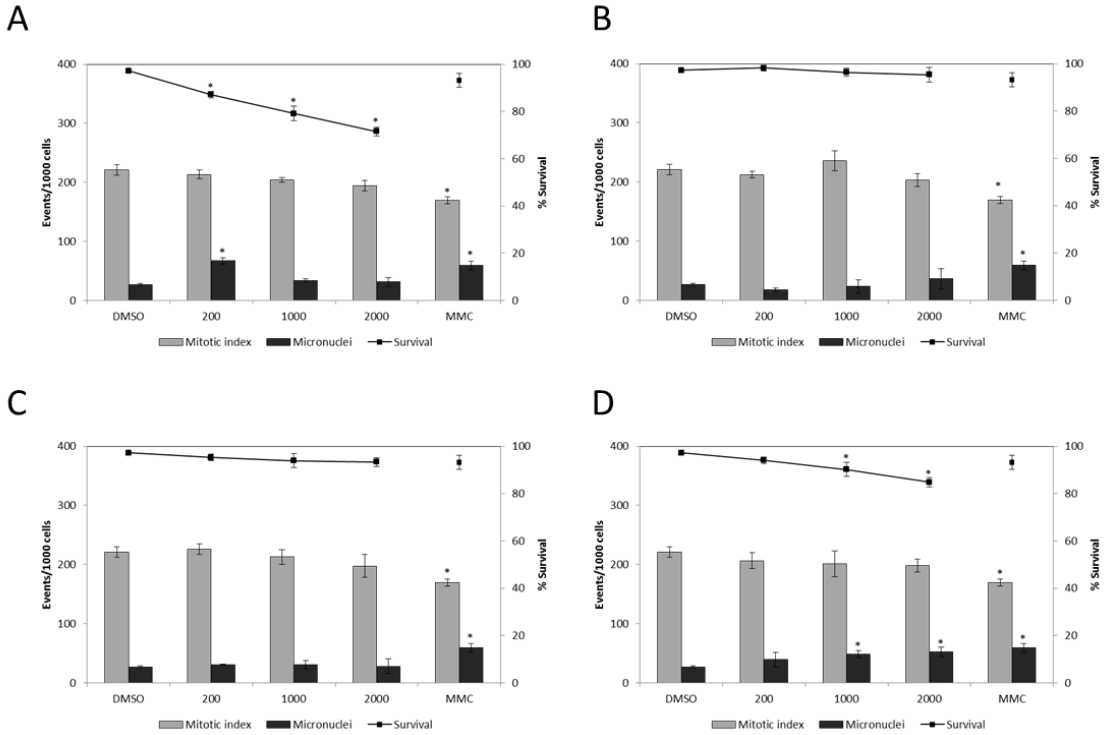


Figure 3. Mitotic index, micronuclei and survival rate of CHO-K1 cells. After 3 h of exposure, it is possible to observe that **13a** (A) was cytotoxic and induced micronuclei in CHO-K1 cells at 200 µM. There were no cytotoxic or genotoxic response of **13b** (B) and **13c** (C). **13d** (D) reduced the survival and induced micronuclei at 1000 and 2000 µM concentrations.

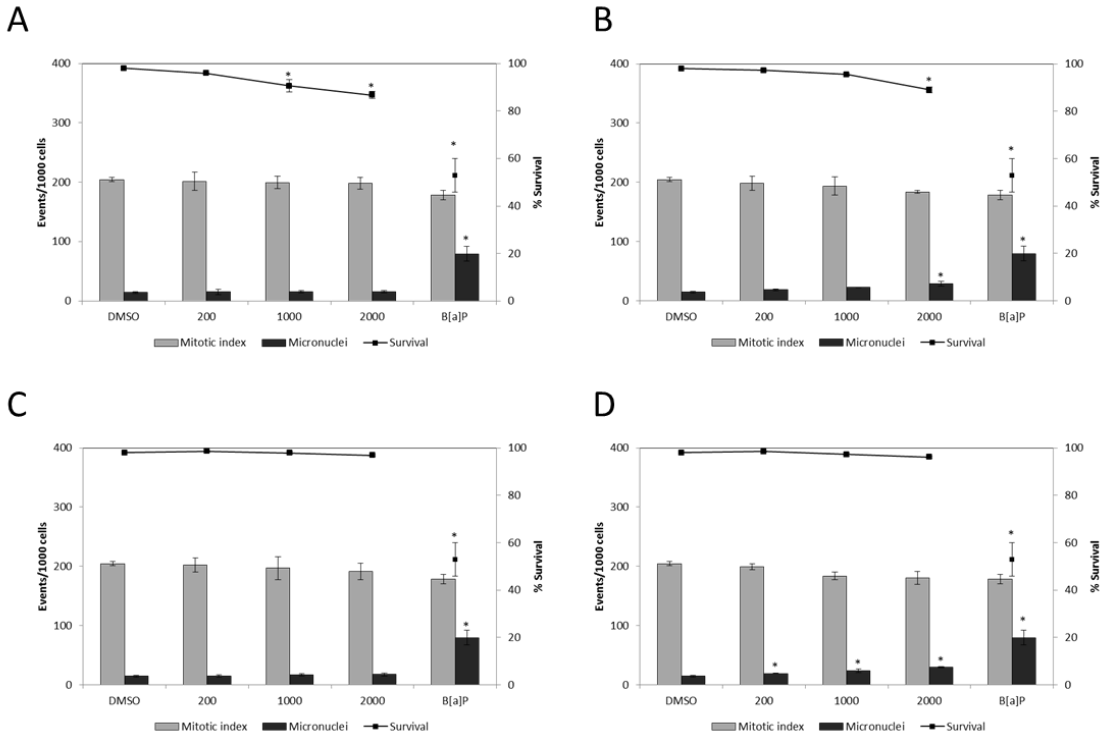


Figure 4. Mitotic index, micronuclei and survival rate of HepG2 cells. After 3 h of exposure, it is possible to observe that **13a** (A) and **13c** (C) did not induce micronuclei formation in HepG2, but just **13a** presented

cytotoxic effects upon 1000 µM. The compound **13b** (B) was genotoxic and cytotoxic to hepatic cells at 2000 µM and **13d** (D) it was non-cytotoxic and induced a significant micronuclei formation in HepG2 cells.

Regarding the trypanocidal activity of the present statins, just the halogenated derivatives presented potential chemotherapeutic effects. **13c** and **13d** presented the same range of activity, toxicity and selectivity against the two different DTU of *T. cruzi* used in this phenotypic screening. Against the intracellular forms of a DTU VI (Tulahuen strain), the chlorinated compound **13c** was more selective than the brominated **13d** one while no major differences could be noticed when bloodstream trypomastigotes were assayed using Y strain (DTU II). Our data corroborate previous in vitro studies that demonstrated the efficacy of lovastatin and simvastatin against epimastigote forms of Y strain [19,45]

Our finding using the statin derivatives demonstrated a similar toxicity profile of AVA that has been previously reported as non-mutagenic neither genotoxic after *in vitro* and *in vivo* toxicological screening, being pointed as a safe drug [46]. In our evaluation, the derivative **13c**, that did not presented genotoxic response and was cytotoxic just to TA104 in the presence of metabolic activation, presenting a LC_{50} > 1200 µM (Figure 3 and Figure 4). Besides **13b** had no mutagenic potential in our bacterial model, and only increased micronucleated cells were found in HepG2 cells exposed to higher concentrations such as 2000 µM that was followed by cytotoxicity at the same concentrations. This **13b** genotoxic effect can be related to the aromatic ring metabolism in its terminal region. The presence of a toluyl radical, after oxidative process by CYP enzymes can produce epoxide radicals that are capable to produce DNA adducts, inducing its genotoxicity [47].

The compound **13a** was capable to enhance the number of revertants for TA98 in absence of exogenous metabolic condition, which suggest that this derivative can act as a direct mutagen promoting frameshift mutations by insertion or deletion of G:C base pairs in *hisD3052* hotspot genic locus [48]. On the other hand, after metabolic activation, its mutagenic response was not observed and, probably, CYP enzymes can inactivate **13a** mutagenic effect. The evidence that **13a** causes DNA damage directly and loses its genotoxic potential after metabolization are reinforced by the data on eukaryotic cells since it was genotoxic and cytotoxic to CHO-K1 cells and non-genotoxic to HepG2 cells even though its cytotoxicity remains present.

The presence of bromine on the aromatic ring of **13d** can explain its cytotoxic and genotoxic effects [49]. This compound was mutagenic to TA97 both in absence or presence of exogenous metabolization, acting as a frameshift mutagen, causing addition of G:C base pairs

in *hisD6610* locus. Notwithstanding, **13d** exposure was induced genotoxicity in ovarian and hepatic cell eukaryotic cultures (Figure 3 and Figure 4). The presence of bromine in organic molecules is related to its genotoxic potential. Similarly, Tsuboy and coworkers [50] detected the genotoxic, mutagenic and cytotoxic effects of a commercial dye whose presents a bromide in a phenyl-amine ring.

The cytotoxic effects in bacterial model and the trypanocidal activity presented by **13c** and **13d** can be related to the presence of halogens in their structures. In studies of another group was synthetized some new halogen-containing 1,2,4-triazolo-1,3,4-thiadiazines and observed that the chlorinated and brominated compounds were effective as antimicrobials against gram positive and negative bacteria [51].

According to Don and Ioset [52] a hit compound in an anti- *T. cruzi* screening needs to reach $EC_{50} \leq 10$ μM against intracellular forms using representative strains relevant for mammalian infection (such as Y and Tulahuen strains belonging TcII or TcIV discrete typing units (DTU) besides demonstrating lack of structural alerts of genotoxicity at least performed by *in silico* platforms. According to the literature, to move a compound from hit to lead classification it is necessary to reach at least a 10-20 fold increase in selectivity without presenting signals of *in vitro* mutagenicity or genotoxicity evidences. According to these, the most promising derivative presently evaluated against *T.cruzi* are **13c** and **13d** that displayed considerable activity against both parasite forms and upon different strains belonging to distinct DTUs. However, due to the genotoxicity profile of **13d**, the prototype **13c** merits chemical optimization aiming to identify a novel lead for CD therapy. In this sense, the optimization of the statin hybrid derivatives is largely recommended in order to identify novel alternatives for neglected diseases and justify further studies *in vitro* and *in vivo.*

3. Conclusion

Eight new hybrid compounds of α,α-difluorophenylacetamides with the moiety of statins (3S,5S)-3,5-dihydroxyheptanoic **13** (**a-d**) and **14** (**a-d**) were presently synthetized and their biological aspects evaluated. None of the sodium salt form compounds (**14a-d**) presented antiparasitic activity and two acetate form compounds (**13b** and **13c)** presented mild anti-*P. falciparum* activity when compared with AVA and the reference drug (chloroquine). Against *T. cruzi*, just **13c** and **13d** presented moderate effect against both parasite forms relevant for human infection although less potent than Bz. However, despite the considerable trypanocidal activity, compound **13d** displayed a genotoxic profile that turns it unfeasible for moving it to *in vivo* testing. The derivative **13c** was the only which presents the promising

chemical and biological characteristics as it does not also present genotoxicity *in vitro* and has a quite considerable selectivity (>17) and thus this compound is ongoing to *in vivo* experiments as the next step of the CD experimental chemotherapy flowchart.

4. Material and Methods

4.1. Chemistry

All reagents and solvents used were analytical grade. Thin layer chromatography (TLC) was performed using a Merck TLC Silica gel 60 F254 aluminium sheets 20 x 20 cm (eluent chloroform/methanol 9:1). The melting points (m.p.) were determined using a Büchi model B-545 apparatus. The ^{1}H, ^{13}C and ^{19}F nuclear magnetic resonance (NMR) spectra were generated at 400.00, 100.00 and 376.00 MHz, respectively, in a BRUKER Avance instrument equipped with a 5 mm probe. Tetramethylsilane was used as an internal standard. The chemical shifts (δ) are reported in ppm, and the coupling constants (J) are reported in Hertz. The Fourier transform infrared (FT-IR) absorption spectra were recorded on a Shimadzu mode IR Prestige-21 spectrophotometer through KBr reflectance to **13** (**a-d**) samples and attenuated total reflection technique (ATR) for **14** (**a-d**) samples. Mass spectrometry with electrospray ionization in positive mode [ESI-MS (+)] was carried out in Waters ® Micromass ZQ4000 equipment. Values are expressed as mass/charge ratio (*m/z*) and are equivalent to the molecular mass of the substance plus a proton. The HRMS data were obtained using LC–MS Bruker Daltonics MicroTOF (analyzer time of flight). The analysis by high performance liquid chromatography (HPLC) was performed on Shimadzu liquid chromatograph using Supelcosil LC-8 column (250 mm x 4.6 mm x 3 μm) and as mobile phase acetonitrile: potassium phosphate buffer 0.01 mol/L, pH 5.8, flow 1 mL/min.

4.2. Synthesis

4.2.1. Preparation of tert-butyl 2-((4R,6R)-6-(2-aminoethyl)-2,2-dimethyl-1,3-dioxan-4-yl)acetate (**11**)

In a Parr reactor vessel was added intermediate 1,3-dioxane-4-acetic acid, 6-(cyanomethyl)-2,2-dimethyl-1,1-dimethylethyl ester **10** (10.3882 g, 0.0386 mol), methanol (110 mL), Raney-Ni catalyst (8.07 g, 0.1375 mol) or Pd (C) 10% (1 g) and 15-18% NH_4OH solution (8 mL). The reaction was kept for 48 h under stirring and hydrogen atmosphere (50 psi/3.4 atm). Then the reaction was filtered, concentrated, washed with methanol and concentrated again, giving an oil product. Yield: 90%. ESI-MS: *m/z* 274.0 $[M+H]^+$. MSGC

(*m/z*): 200 (70%); 158 (54%); 142 (100%); 100 (81%); 72 (74%). ^{1}H NMR (400 MHz, MeOD) δ: 1.17-1.26 (1H, m, CH$\underline{CH_2}CH_2$); 1.33 (3H, s, C($\underline{CH_3}$)$_2$); 1.45 (9H, s, C($\underline{CH_3}$)$_3$); 1.48 (3H, s, C($\underline{CH_3}$)$_2$); 1.61; 1.64 (1H, dt, J = 2; 12 Hz; CH$\underline{CH_2}CH_2$); 1.74-1.93 (2H, m, CH$\underline{CH_2}$CH); 2.27-2.42 (2H, m, CH$\underline{CH_2}$Ac); 3.04-3.20 (2H, m, $\underline{CH_2}$NH); 4.06-4.13 (1H, m, CH); 4.30-4.36 (1H, m, CH). ^{13}C NMR (100 MHz, MeOD) δ: 20.22; 28.46; 30.46; 33.68; 37.10; 43.63; 46.13; 67.50; 68.56; 81.99; 100.39; 172.16. IR (ATR, cm^{-1}): 3408; 2979; 2944; 2732; 1719; 1366; 1258; 1154; 943.

4.2.2. General preparation of tert-butyl 2-((4R,6R)-6-(2-(2-(2-acetamido-5-subtitutedphenyl)-2,2-difluoroacetamido)ethyl)-2,2-dimethyl-1,3-dioxan-4-yl)acetate **13** (**a-d**)

In a flask was added amine **11** (0.2753 g, 0.001 mol), 5-substituted-1-acetyl-3,3-difluoroindolin-2-ones **12** (**a-d**) (0.001 mol), prepared according to the methodology previously described [36], and anhydrous DMF (5 mL) under magnetic stirring. The reaction mixture was stirred at 80 °C for 24 h. DMF solvent was removed in a rotary evaporator and pure products **13** (**a-d**) were precipitated in pure form with 25-70% yields with ice cold water.

tert-Butyl 2-((4*R*,6*R*)-6-(2-(2-(2-acetamidophenyl)-2,2-difluoroacetamido)ethyl)-2,2-dimethyl-1,3-dioxan-4-yl)acetate (**13a**)

Yellow solid. Yield: 70%. m. p. 93-96 °C. HRMS (ESI) calc. for $C_{24}H_{34}F_2N_2O_6$ 484.2385, found $[M+1]^+$ 484.2385. HPLC grade: 96%. ^{1}H NMR (400 MHz, CD_3OD) δ: 1.12 (1H, q, *J*= 12 Hz, CH$\underline{CH_2}$CH); 1.28 (3H, s, C($\underline{CH_3}$)$_2$); 1.34 (3H, s, C($\underline{CH_3}$)$_2$); 1.44 (9H, s, C($\underline{CH_3}$)$_3$); 1.51 (1H, dt, *J* = 2; 12 Hz, CH$\underline{CH_2}$CH); 1.59-1.70 (2H, m, CH$\underline{CH_2}CH_2$); 2.16 (3H, s, NHCO$\underline{CH_3}$); 2.24-2.35 (2H, m, CH$\underline{CH_2}$Ac); 3.38-3.44 (2H, m, $\underline{CH_2}$NH); 3.86-3.90 (1H, m, CH); 4.19-4.24 (1H, m, CH); 7.30 (1H, t, *J* = 6 Hz, H5); 7.51 (1H, t, *J* = 6 Hz, H4); 7.61 (1H, d, *J* = 6 Hz, H6); 7.86 (1H, d, *J* = 6 Hz, H3). ^{13}C NMR (100 MHz, CD_3OD) δ: 19.85; 23.95; 28.35; 30.36; 36.11; 37.26; 37.40; 43.59; 67.55; 68.37; 81.77; 100.02; 115.76 (t, *J* = 253 Hz); 126.37; 126.65-127.04 (m); 127.19; 132.71; 137.03 (t, *J* = 3 Hz); 166.62 (t, *J* = 31 Hz); 171.60; 172.03. ^{19}F (376 MHz, CD_3OD) δ: -104.50. IR (KBr, cm^{-1}): 3307; 3240; 2983; 2941; 2862; 1728; 1699; 1674; 1554; 1369; 1257; 1147; 1087.

tert-Butyl 2-((4*R*,6*R*)-6-(2-(2-(2-acetamido-5-methylphenyl)-2,2-difluoroacetamido)ethyl)-2,2-dimethyl-1,3-dioxan-4-yl)acetate (**13b**)

Ivory-white solid. Yield: 44%. m. p. 116-119 °C. HRMS (ESI) calc. for $C_{25}H_{36}F_2N_2O_6$ 498.2541, found $[M+1]^+$ 498.2541. HPLC grade: 94%. ^{1}H NMR (400 MHz, CD_3OD) δ: 1.12

(1H, q, *J*= 12 Hz, CHC<u>H</u>$_2$CH); 1.26 (3H, s, C(C<u>H</u>3)$_2$); 1.33 (3H, s, C(C<u>H</u>3)$_2$); 1.44 (9H, s, C(C<u>H</u>3)$_3$); 1.51 (1H, dt, *J* = 2; 12 Hz, CHC<u>H</u>$_2$CH); 1.59-1.70 (2H, m, CHC<u>H</u>$_2$CH$_2$); 2.14 (3H, s, NHCOC<u>H</u>$_3$); 2.23-2.35 (2H, m, CHC<u>H</u>$_2$Ac); 2.36 (3H, s, Ar-<u>Me</u>); 3.28-3.43 (2H, m, <u>CH$_2$</u>NH); 3.85-3.89 (1H, m, CH); 4.18-4.23 (1H, m, CH); 7.32 (1H, d, *J* = 8 Hz, H4); 7.42 (1H, s, H6); 7.68 (1H, d, *J* = 8 Hz, H3). ^{13}C NMR (100 MHz, CD$_3$OD) δ: 19.88; 21.02; 23.87; 28.40; 30.40; 36.13; 37.32; 37.47; 43.65; 67.61; 68.49; 81.82; 100.08; 115.79 (t, *J* = 252 Hz); 126.95 (t, *J* = 24 Hz); 127.33 (t, *J* = 9 Hz); 127.58; 133.23; 136.75 (t, *J* = 4 Hz); 166.65 (t, *J* = 30 Hz); 171.73; 172.07. ^{19}F (376 MHz, CD$_3$OD) δ: -104.58. IR (KBr, cm^{-1}): 3348; 3246; 2985; 2935; 2864; 1728; 1683; 1666; 1519; 1367; 1267; 1151; 1083.

tert-Butyl 2-((4*R*,6*R*)-6-(2-(2-(2-acetamido-5-chlorophenyl)-2,2-difluoroacetamido)ethyl)-2,2-dimethyl-1,3-dioxan-4-yl)acetate (**13c**)

White solid. Yield: 58%. m. p. 128-130 °C. HRMS (ESI) calc. for $C_{24}H_{33}ClF_2N_2O_6$ 518.1995, found $[M+1]^+$ 518.1999. HPLC grade: 96%. ^{1}H NMR (400 MHz, CD$_3$OD) δ: 1.13 (1H, q, *J*= 12 Hz, CHC<u>H</u>$_2$CH); 1.26 (3H, s, C(C<u>H</u>3)$_2$); 1.35 (3H, s, C(C<u>H</u>3)$_2$); 1.44 (9H, s, C(C<u>H</u>3)$_3$); 1.52 (1H, dt, *J* = 2; 12 Hz, CHC<u>H</u>$_2$CH); 1.60-1.70 (2H, m, CHC<u>H</u>$_2$CH$_2$); 2.15 (3H, s, NHCOC<u>H</u>$_3$); 2.23-2.38 (2H, m, CHC<u>H</u>$_2$Ac); 3.28-3.42 (2H, m, <u>CH$_2$</u>NH); 3.86-3.90 (1H, m, CH); 4.19-4.23 (1H, m, CH); 7.52 (1H, dd, *J* = 2; 8 Hz, H4); 7.61 (1H, d, *J* = 2 Hz, H6); 7.89 (1H, d, *J* = 8 Hz, H3). ^{13}C NMR (100 MHz, CD$_3$OD) δ: 20.00; 24.07; 28.51; 30.52; 36.24; 37.45; 37.64; 43.78; 67.74; 68.53; 81.96; 100.20; 115.11 (t, *J* = 254 Hz); 127.16 (t, *J* = 9 Hz); 128.55 (d, *J* = 25 Hz); 128.84; 131.75; 132.81; 136.10 (t, *J* = 3 Hz); 166.16 (t, *J* = 32 Hz); 171.75; 172.20. ^{19}F (376 MHz, CD$_3$OD) δ: -104.84. IR (KBr, cm^{-1}): 3344; 3240; 2980; 2937; 2868; 1735; 1683; 1666; 1516; 1367; 1263; 1153; 1089; 829.

tert-Butyl 2-((4*R*,6*R*)-6-(2-(2-(2-acetamido-5-bromophenyl)-2,2-difluoroacetamido)ethyl)-2,2-dimethyl-1,3-dioxan-4-yl)acetate (**13d**)

Pale brown solid. Yield: 25%. m. p. 126-128 °C. HRMS (ESI) calc. for $C_{24}H_{33}BrF_2N_2O_6$ 562.1490, found $[M+1]^+$ 562.1472. HPLC grade: 83%. ^{1}H NMR (400 MHz, acetone-d$_6$) δ: 1.13 (1H, q, *J*= 12 Hz, CHC<u>H</u>$_2$CH); 1.27 (3H, s, C(C<u>H</u>3)$_2$); 1.37 (3H, s, C(C<u>H</u>3)$_2$); 1.43 (9H, s, C(C<u>H</u>3)$_3$); 1.56 (1H, dt, *J* = 2; 12 Hz, CHC<u>H</u>$_2$CH); 1.62-1.78 (2H, m, CHC<u>H</u>$_2$CH$_2$); 2.13 (3H, s, NHCOC<u>H</u>$_3$); 2.22-2.41 (2H, m, CHC<u>H</u>$_2$Ac); 3.34-3.53 (2H, m, <u>CH$_2$</u>NH); 3.94-4.01 (1H, m, CH); 4.20-4.28 (1H, m, CH); 7.67 (1H, s, H4); 7.68 (1H, s, H6); 8.20 (1H, d, *J* = 8 Hz, H3). ^{13}C NMR (100 MHz, acetone-d$_6$) δ: 19.86; 24.50; 28.28; 30.42; 35.68; 36.97; 37.56; 43.33; 67.05; 68.10; 80.47; 99.28; 114.66 (t, *J* = 255 Hz); 118.01; 125.79

(t, *J* = 25 Hz); 126.53; 129.08 (t, *J* = 9 Hz); 135.33; 137.40 (t, *J* = 3 Hz); 165.36 (t, *J* = 30 Hz); 168.70; 170.35. ^{19}F (376 MHz, acetone-d_6) δ: -104.82. IR (KBr, cm^{-1}): 3356; 2989; 2930; 1735; 1685; 1668; 1517; 1367; 1263; 1155; 1091.

4.2.3. General preparation of sodium (3R,5R)-7-(2-(2-acetamido-5-substituted-phenyl)-2,2-difluoroacetamide)-3,5-dihydroxyheptanoate salts **14** (**a-d**)

Product **13** (**a-d**) (1-3 mmol) and methanol (5 mL) were added under magnetic stirring at room temperature. After 30 min, was added 0.5 mL of 4% HCl solution (w/v) after some hours, the pH was measured (pH 1-2), aqueous NaOH solution 7% (w/v) were added until pH 11-12 again. The stirring was switched off when the pH was measured pH 7 and was added 5 mL of distilled MeOH/H_2O (3:7) and activated carbon, which was under magnetic stirring for more 5 min. The solution was evaporated and the products was precipitated in acetone giving the salts **14** (**a-d**). Overall reaction time were 48 hours.

Sodium (3*R*,5*R*)-7-(2-(2-acetamido-phenyl)-2,2-difluoroacetamide)-3,5-dihydroxyheptanoate salt (**14a**)

White solid. Yield: 84%. m. p. >240 °C. HRMS (ESI) calc. for $C_{17}H_{21}F_2N_2NaO_6$ 410.1265, found $[M+1]^+$ 410.1273. HPLC grade: 86%. ^{1}H NMR (400 MHz, D_2O) δ: 1.61-1.84 (4H, m, CH<u>CH_2</u>CH; CH<u>CH_2</u>CH_2); 2.17 (3H, s, CO<u>Me</u>); 2.31-2.41 (2H, m, CH<u>CH_2</u>Ac); 3.39 (2H, t, *J* = 7 Hz, <u>CH_2</u>NH); 3.78-3.84 (1H, m, CH); 4.07-4.13 (1H, m, CH); 7.40 (1H, d, *J* = 8 Hz, H6); 7.54 (1H, t, *J* = 8 Hz, H4); 7.65 (1H, t, *J* = 8 Hz, H5); 7.79 (1H, d, *J* = 8 Hz, H3). ^{13}C NMR (100 MHz, D_2O) δ: 22.41; 35.21; 36.87; 43.12; 44.98; 67.45; 67.55; 114.29 (t, *J* = 251 Hz); 127.05 (t, *J* = 8 Hz); 128.44; 129.17; 130.07; 132.68; 133.67; 165.37 (t, *J* = 30 Hz); 174.32; 180.42. ^{19}F (376 MHz, D_2O) δ: -101.07. IR (in MeOH, cm^{-1}): 3420; 2950; 2854; 1710; 1490; 1415; 1190; 1065.

Sodium (3*R*,5*R*)-7-(2-(2-acetamido-5-methylphenyl)-2,2-difluoroacetamide)-3,5-dihydroxyheptanoate salt (**14b**)

White solid. Yield: 33%. m. p. >240 °C. HRMS (ESI) calc. for $C_{18}H_{23}F_2N_2NaO_6$ 424.1422, found $[M+1]^+$ 424.1415. HPLC grade: 91%. ^{1}H NMR (400 MHz, MeOD) δ: 1.55-1.78 (4H, m, CH<u>CH_2</u>CH; CH<u>CH_2</u>CH_2); 2.14 (3H, s, CO<u>Me</u>); 2.27-2.37 (2H, m, CH<u>CH_2</u>Ac); 2.38 (3H, s, Ar<u>Me</u>); 3.37 (2H, t, *J* = 7 Hz, <u>CH_2</u>NH); 3.67-3.82 (1H, m, CH); 4.05-4.12 (1H, m, CH); 7.32 (1H, d, *J* = 8 Hz, H4); 7.43 (1H, s, H6); 7.62 (1H, d, *J* = 8 Hz, H3). ^{13}C NMR (100 MHz, MeOD) δ: 21.02; 23.79; 37.00; 37.17; 44.81; 45.50; 69.00; 69.15; 115.78 (t, *J* =

251 Hz); 119.3; 127.40 (t, *J* = 8 Hz); 127.89; 133.19; 134.18; 137.00; 166.75 (t, *J* = 7 Hz); 172.02; 180.34. ^{19}F (376 MHz, MeOD) δ: -101.17. IR (KBr, cm^{-1}): 3322 (broad); 2925; 1663; 1556; 1514; 1424; 1273; 1184; 1151; 1077.

Sodium (3*R*,5*R*)-7-(2-(2-acetamido-5-chlorophenyl)-2,2-difluoroacetamide)-3,5-dihydroxyheptanoate salt (**14c**)

White solid. Yield: 13%. m. p. >240 °C. HRMS (ESI) calc. for $C_{17}H_{20}ClF_2N_2NaO_6$ 444.0876, found $[M+1]^+$ 444.0867. HPLC grade: 75%. ^{1}H NMR (400 MHz, MeOD) δ: 1.58-1.76 (4H, m, CH<u>CH$_2$</u>CH; CH<u>CH$_2$</u>CH$_2$); 2.15 (3H, s, CO<u>Me</u>); 2.30-2.34 (2H, m, CH<u>CH$_2$</u>Ac); 3.38 (2H, t, *J* = 7 Hz, <u>CH$_2$</u>NH); 3.80 (1H, s, CH); 4.07 (1H, s, CH); 7.52 (1H, dd, *J* = 0,5; 2 Hz, H4); 7.62 (1H, d, *J* = 0,5 Hz, H6); 7.84 (1H, d, *J* = 2 Hz, H3). ^{13}C NMR (100 MHz, MeOD) δ: 23.89; 37.04; 38.16; 44.83; 45.54; 69.20; 69.21; 115.00 (t, *J* = 253 Hz); 127.17 (t, *J* = 8 Hz); 128.50; 129.00; 131.81; 132.66; 135.90; 166.10; 171.87. ^{19}F (376 MHz, MeOD) δ: -101.88. IR (KBr, cm^{-1}): 3734-3649; 3325-3064; 2972; 2941; 2929; 1670; 1597; 1558; 1423; 1261; 1153; 1085.

Sodium (3*R*,5*R*)-7-(2-(2-acetamido-5-bromophenyl)-2,2-difluoroacetamide)-3,5-dihydroxyheptanoate salt (**14d**)

Yellow solid. Yield: 24%. m. p. >240 °C. HRMS (ESI) calc. for $C_{17}H_{20}BrF_2N_2NaO_6$ 488.0371, found $[M+1]^+$ 488.0369. HPLC grade: 97%. ^{1}H NMR (400 MHz, MeOD) δ: 1.56-1.78 (4H, m, CH<u>CH$_2$</u>CH; CH<u>CH$_2$</u>CH$_2$); 2.15 (3H, s, CO<u>Me</u>); 2.23-2.35 (2H, m, CH<u>CH$_2$</u>Ac); 3.38 (2H, t, *J* = 7 Hz, <u>CH$_2$</u>NH); 3.79-3.82 (1H, m, CH); 4.04-4.08 (1H, m, CH); 7.66 (1H, dd, *J* = 2; 9 Hz, H4); 7.75 (1H, d, *J* = 2 Hz, H6); 7.80 (1H, d, *J* = 9 Hz, H3). ^{13}C NMR (100 MHz, MeOD) δ: 23.94; 37.00; 38.17; 44.85; 45.56; 69.05; 69.19; 114.91 (t, *J* = 253 Hz); 119.08; 128.89; 129.06; 130.06 (t, *J* = 9 Hz); 135.71; 136.41; 166.13 (t, *J* = 31 Hz); 171.79; 180.41. ^{19}F (376 MHz, MeOD) δ: -101.58. IR (KBr, cm^{-1}): 3326 (broad); 2940; 1663; 1556; 1507; 1404; 1261; 1153; 1076.

4.3. Biological evaluation

4.3.1 Bacteria

Salmonella enterica serovar *Typhimurium* (*S. typhimurium)* strains TA97, TA98, TA100, TA102 and TA104from the authors' stock were used as described by Maron and Ames [53] in the mutagenicity assay.

4.3.2 Cell cultures

Human hepatocellular carcinoma (HepG2) and Chinese Hamster Ovary (CHO-K1) cells obtained from the American Type Culture Collection (Manassas, VA) were cultured in Eagle's medium (MEM, GIBCO®, USA) containing 10% fetal bovine serum (FBS) plus 100 µg/mL streptomycin and 100 µg/mL penicillin at 37 ºC in a 5% CO_2 atmosphere. Logarithmic-phase cells were used in all the experiments [54].

Mouse fibroblasts (L929) obtained from the American Type Culture Collection (Manassas, VA) were cultured at 37° C in RPMI-1640 medium (Gibco BRL) supplemented with 10% FBS and 2 mM glutamine, as reported in Timm and coworkers [55].

Primary cultures of cardiac cells (CC) were obtained as reported in Meirelles and coworkers [56]. The cultures were sustained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2.5 mM $CaCl_2$, 1 mM L-glutamine, and 2% chicken embryo extract. Cell cultures were maintained at 37 °C in an atmosphere of 5% CO_2, and assays were run at least three times in triplicate.

4.3.3 Cellular viability in cell cultures

Fresh HepG2 and CHO-K1 cells were seeded at a density of 1×10^4/well. The water-soluble tetrazolium salt assay (WST-1) (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (Roche Co., South San Francisco, CA) was used to determine the number of viable cells after 3 and 24 h of exposure to the compounds (0 to 1000 µM). Briefly, after treatment the culture medium was replaced by 90 µL fresh culture medium and 10 µL WST-1 reagent and incubated at 37 °C and 5% CO_2 for 3 h. The absorbance was then measured at 440 nm according to the kit protocol [57]. The intensity of the yellow color in the negative control (DMSO 1%) wells was designated as 100% viability and all further comparisons were based upon this reference level to determine the lethal concentration (LC_{50}) to 50% of cultured cells.

Cardiac cell cultures were incubated for 24 h at 37 °C with different concentrations of the compounds (up to 200 µM) diluted in DMEM (without phenol red). Their morphology and spontaneous contractibility were evaluated by light microscopy and then the cellular viability determined by the PrestoBlue® assay. For this colorimetric bioassay, PrestoBlue® (Invitrogen) was added to each well (using a 10:1 medium/dye), the plate further incubated for 24 h followed by DO measurement performed at 570 and 600 nm, as recommended by the manufacturer. A similar protocol was used to evaluate L929 cell viability after 96 h of exposure to the compounds, using 10 µL AlamarBlue® (Invitrogen). The results were

expressed as the difference in the percentage of reduction between treated and untreated cells and the LC_{50} value, corresponds to the concentration that reduces in 50% the cellular viability [14].

4.3.4 Cultures of P. falciparum-infected erythrocytes and in vitro assays

The chloroquine-resistant and mefloquine-sensitive [58] *P. falciparum* W2 clone was maintained in continuous culture as previously described [59]. Briefly, parasites were cultivated in human erythrocytes (A+) at 37 °C in Petri dishes using complete medium (RPMI 1640 supplemented with 10% human sera blood group A+, 2% glutamine and 7.5% $NaHCO_3$) and kept either in a candle jar, or in an environment containing a gas mixture atmosphere (3% O_2, 5% CO_2 and 91% N_2). Prior to testing, the ring-stage parasites were synchronized using sorbitol [60]. The parasite suspension was adjusted for 0.05% parasitemia and 1.5% hematocrit and then distributed in 96-well microtiter plates (Corning, Santa Clara, CA, USA), 180 μL per well, to which 20 μL of different concentrations of the test drugs and controls had previously been added. The maximum concentration of 50 μg/mL (~157 μM) was tested at least three times for each compound against *P. falciparum*; the drug activity was evaluated using the anti-HRPII assay [61] with commercially available monoclonal antibodies (MPFM ICLLAB-55A®, MPFG55P ICLLAB®, USA) raised against a *P. falciparum* histidine and alanine-rich protein (HRP2).

The test quantification was read at 450 nm on a spectrophotometer (SpectraMax340PC384, Molecular Devices Sunnyvale, CA, USA), and the drug activity was expressed as the half-maximal inhibitory concentration (EC_{50}) compared to the drug-free controls using the curve-fitting software Origin 8.0 (OriginLab Corporation, Northampton, MA, USA) [62].

4.3.5 Trypanocidal in vitro phenotypic screening

Bloodstream trypomastigote forms (BT) of the Y strain were obtained from infected albino Swiss mice at the peak of parasitaemia, isolated by differential centrifugation and resuspended in DMEM to a parasite concentration of 10^7 cells/mL in the presence of 10% of mouse blood. This suspension (100 μL) was added in the same volume of **13** (**a-d**) and **14** (**a-d**) previously prepared at twice of the desired final concentrations. Cell counts were performed in Neubauer chamber and the trypanocidal activity was expressed as EC50, corresponding to the concentration that leads to the lysis of 50% of the parasites [63].

Tissue culture-derived trypomastigotes (Tulahuen strain expressing the *E. coli* β-galactosidase gene) were maintained in L929 cell lines and collected from the supernatant after 96 h of parasite infection, following previously established protocols [64]. Briefly, after 24 h of L929 platting ($4x10^3$cels/well), the cultures were incubated with trypomastigotes (10:1 parasite/host cell ratio) for 2 h at 37 °C. Then, the cell cultures were rinsed to remove non-internalized parasites and then further incubated for 24 h at 37 °C. Then the infected cultures were followed using two sequential protocols. In the first one, the infected cultures were exposed to each tested compound diluted at a fixed concentration of 10 µM (that corresponds to the EC90 value of Bz) in RPMI and incubated for 96 h at 37 °C. After this period, 50 µL chlorophenol red glycoside (500 µM) in 0.5% Nonidet P40 was added to each well and the plate incubated for more 18 h at 37 °C, after which the absorbance was measured at 570 nm. Then, the compounds were next evaluated using dose response assays which Tulahuen-infected-L929 cells were exposed to different non-toxic concentrations of each compound (0-50 µM) and then after 96 h of incubation the same procedure was conducted as above reported. Controls with untreated and Bz-treated cells are run in parallel. The results were expressed as the percent difference in the reduction between treated and untreated cells [55, 64].

4.3.6 Salmonella/Microsome assay

The mutagenicity reverse mutation test was carried out to investigate the potential of **13** (**a-d**) to induce genetic mutation in *S. typhimurium* TA97, TA98, TA100, TA102 and TA104 strains. The test was carried out according to the pre-incubation method, both in the absence and presence of a metabolic activation system (4% S9 mix, Aroclor-pre-induced, from Moltox Inc., USA). DMSO 10% served as the negative control while known mutagens were used as the positive control substances. The positive controls without the S9 mix were: 4-nitroquinoline 1-oxide (4-NQO, CAS Number 56-57-5) (5 µg per plate) for TA97 and TA98; sodium azide (SA, CAS Number 26628-22-8) (10 mg per plate) for TA100; mitomycin C (MMC, CAS Number 50-07-7) (1 µg per plate) for TA102 and methyl methanesulfonate (MMS, CAS Number 66-27-3) (200 µg per plate) for TA104. The positive controls with the S9 mix were: 2-amineanthracene (2-AA, CAS Number 613-13-8) (10.0 µg per plate) for TA97 and TA98; and benz(a)pyrene (BaP, CAS Number 50-32-8) (50.0 mg per plate) for TA100, TA102 and TA104. A dose-finding test was carried out with and without the

metabolic activation system (S9 mix) for each tester strain. A total of six concentrations were set, from 0 to 1500.0 μM.

For the assays without metabolic activation, 0.5 mL of 0.1 mol/L sodium-phosphate buffer (pH 7.4) was added, and for the assays in the presence of metabolic activation, 0.5 mL S9 mix was mixed with 0.1 mL culture medium (2×10^8 cells/mL) plus 0.1 mL of each compound solutions. The mixtures were incubated in a shaker at 37 °C (pre-incubation). After 30 min pre-incubation under light protection, the mixture was added to and mixed with 2 mL top agar containing 0.05 mmol/L L-histidine and D-biotin for the *S. typhimurium* strains. Each of these was then spread on a minimum glucose agar plate. After the top agar solidified, the plates were incubated at 37 °C for 60-72 h. Each tester strain was assayed in triplicate, and the number of revertant colonies counted for each tester strain and treatment group [65]. The results were judged to be positive when the average number of revertant colonies in each treated group increased with increase in the compound concentration, reaching at least twice the number in the negative control group.

To determine the cytotoxic effects, after 30 min pre-incubation the assay mixtures were diluted in 0.9% NaCl (w/v) to obtain a suspension containing 2×10^2 cells/mL. A suitable aliquot (100 μL) of this suspension was plated on nutrient agar (0.8% bacto nutrient broth (Difco), 0.5% NaCl and 1.5% agar). The plates were then incubated at 37 °C for 24 h and the colonies counted. All the experiments were done in triplicate and were repeated at least twice. Statistical differences between the groups were analyzed by a two-way ANOVA ($p < 0.05$) and Tukey's post-hoc test [66].

4.3.7 In vitro micronuclei assay in the cell culture (MNvit)

Fresh HepG2 and CHO-K1 cells were seeded at the density of 1×10^5/mL into 24-well plates (1 mL/per well). The compounds **13** (**a-d**) were then added to the medium to final concentrations of 500, 1000 and 2000 μM and incubation continued for 3 h or 24 h. DMSO 10% was used as the negative control, and, BaP (80 μM) for HepG2 and MMC (5 μM) for CHO-K1 were the positive controls. After exposure to the compounds, the cells were incubated for more 24 h under growth conditions before quantification of the micronuclei and cytotoxicity. The cytogenetic studies were carried out in triplicate as described previously [54]. To determine the mitotic index and the number of cells with micronuclei, the medium was replaced by a cold methanol-glacial acetic acid (3:1) fixative for 30 min, and the cells then rinsed with distilled water for 2 min and air dried. The fixed cells were stained with 4,6-diamidino-2-phenylindole (DAPI) (0.2 pg/mL) dissolved in McIlvaine buffer (0.1 M citric

acid plus 0.2 M Na_2HPO_4, pH 7.0) for 60 min, washed with McIlvaine buffer for 5 min, briefly rinsed with distilled water and mounted in glycerol. To determine the mitotic index and the number of cells with micronuclei, 1000 cells per well (3000 cells per concentration) were analyzed under a fluorescence microscope. The results for micronuclei were presented as the percentage of cells containing micronuclei in 3000 cells/concentration group analyzed. Cells that glowed brightly and had homogenous nuclei were considered as having normal phenotypic morphology. Apoptotic nuclei were identified by the condensed chromatin at the periphery of the nuclear membrane or by fragmented nuclear body morphology. Necrotic cells presented chromatin forms with irregularly-shaped aggregates, a pyknotic nucleus (shrunken and darkly stained) and cell membrane disruption, with cellular debris spilled into the extracellular milieu. Once again, 3000 cells were counted and the percentage of viable cells evaluated, discounting apoptotic and necrotic cells. Statistical differences between the groups were analyzed by two-way ANOVA ($p < 0.01$) and Tukey's post-hoc test.

5. Author contributions

RCCC, SLR and DIL synthetised the compounds. RCCC, MMB CRK and NB analysed the chemistry data. CFAL, JSA and SK carried out the trypanocidal activity evaluation. ACCA and AUK performed the antiplasmodial activity evaluation. CFAL, MMCL and CAFA performed the mutagenicity assay (Ames test). CFAL, JSA and IF performed the eukaryotic cytotocity and genotoxicity evaluation. CFAL, SK, IF and MNCS analysed the biological data. CFAL and RCCC firstly wrote the manuscript and NB, IF and MNCS reviewed and presented the final version of manuscript. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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3.5 **Artigo 5 -** ***Atorvastatin Downregulates In Vitro Methyl Methanesulfonate and Cyclophosphamide Alkylation-Mediated Cellular and DNA Injuries*** **– Publicado no periódico "Oxidative Medicine and Cellular Longevity"**

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Research Article

Atorvastatin Downregulates *In Vitro* Methyl Methanesulfonate and Cyclophosphamide Alkylation-Mediated Cellular and DNA Injuries

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Statins are 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, and this class of drugs has been studied as protective agents against DNA damages. Alkylating agents (AAs) are able to induce alkylation in macromolecules, causing DNA damage, as DNA methylation. Our objective was to evaluate atorvastatin (AVA) antimutagenic, cytoprotective, and antigenotoxic potentials against DNA lesions caused by AA. AVA chemopreventive ability was evaluated using antimutagenicity assays (*Salmonella*/microsome assay), cytotoxicity, cell cycle, and genotoxicity assays in HepG2 cells. The cells were cotreated with AVA and the AA methyl methanesulfonate (MMS) or cyclophosphamide (CPA). Our datum showed that AVA reduces the alkylation-mediated DNA damage in different *in vitro* experimental models. Cytoprotection of AVA at low doses (0.1–1.0 μM) was observed after 24 h of cotreatment with MMS or CPA at their LC_{50}, causing an increase in HepG2 survival rates. After all, AVA at 10 μM and 25 μM had decreased effect in micronucleus formation in HepG2 cells and restored cell cycle alterations induced by MMS and CPA. This study supports the hypothesis that statins can be chemopreventive agents, acting as antimutagenic, antigenotoxic, and cytoprotective components, specifically against alkylating agents of DNA.

1. Introduction

Alkylating agents (AAs), at the widest sense, are compounds able to substitute a hydrogen atom in other molecules by an alkyl radical, involving electrophilic attack by the AA. The definition is extended to the reactions involving addition of the radical to a molecule containing an atom in a lower valence state, as the sulfonates [1]. These agents that induce DNA methylation can act through covalent modification of DNA to generate mismatching base derivatives and lesions that interrupts genetic replication [2].

Statins are drugs largely used to inhibit cholesterol synthesis by blockage of HMG-CoA reductase [3]. Statin pleiotropic effects are the nonhypocholesterolemic-related new roles that this class of drugs presents [4]. In eukaryotic cells, the antineoplastic effect of statins occurs by suppression of mevalonate biosynthesis, a precursor of important isoprenoid intermediates which are added during posttranslational modification of a variety of proteins such as subunits Ras and Rho of small G protein [5]. These modifications in Rho GTPases can induce actin cytoarchitectonic rearrangement by reducing the focal adhesion regions, stress fiber formation, and cell pseudopod emission, disfavoring cellular migration and phagocytosis [6]. In this sense, our intent was to observe possible chemopreventive effects of the compounds on different

biological models exposed to chemical injury induced by AA.

2. Materials and Methods

2.1. Compounds. For antimutagenesis and cytoprotection assays, AVA (CAS #134523-00-5) and the AA (methyl methanesulfonate (MMS; CAS #66-27-3), cyclophosphamide (CPA; CAS #50-18-0)) stock solutions were prepared in dimethyl sulfoxide (DMSO) with the final concentrations of the solvent never exceeding 1.0%, which did not exert any toxicity (data not shown), and aliquots were stored at −20°C.

2.2. Scavenging of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay. The free radical scavenging activity was measured by following microplate procedures as previously described [7]. One hundred microliters of the sample dilutions with five concentration levels (varying from 0 to 2000 μM in DMSO) was added to two identical groups of wells in a 96-well microplate. The same volume of 0.1 mM DPPH-methanol solution was added to each well of one group (samples), and methanol (100 mL) was added to the other group (blanks). The control was prepared by mixing the DPPH-methanol solution with the sample solvent or butylated hydroxytoluene (BHT). The solutions were mixed thoroughly, covered, and allowed to react in the dark at room temperature for 40 min. The absorbance was measured at 517 nm using a microplate reader (Quant, BioTek Instruments Inc.), and the scavenging activity was calculated from the absorbance values according to the following equation: % scavenging = (control sample)/(control blank) × 100%. The antioxidant properties of the samples were expressed as half the maximal effective concentrations (EC_{50}) obtained by interpolation from the linear regression analysis. BHT was used as the positive control.

2.3. Biological Models

2.3.1. Bacteria. Salmonella enterica serovar *typhimurium (S. typhimurium)* strains TA100, TA1535, TA104, and TA102 from the authors' laboratory stock were used as described by Maron and Ames [8] in the antimutagenicity assay.

2.3.2. Cell Culture. Human hepatocellular carcinoma cells (HepG2) obtained from the American Type Culture Collection (Manassas, VA) were cultured in a minimum Eagle's medium (MEM, Gibco®, USA) containing 10% fetal bovine serum (FBS) plus 100 μg/mL streptomycin and 100 μg/mL penicillin at 37°C in a 5% CO_2 atmosphere. Logarithmic-phase cells were used in all the experiments [9].

2.4. Antimutagenicity in a Bacterial Model. We carried out the coexposure protocol of the antimutagenicity assay to investigate the potential of the compound to protect against alkylation-mediated genetic mutation in *S. typhimurium* TA100, TA102, TA104, and TA1535 strains according to Ajith and Soja [10]. The test proceeded both in the absence and presence of a metabolic activation system (4% S9 mix, Aroclor preinduced, from MOLTOX Inc., USA). DMSO 1% served as the negative control. For the assays without metabolic activation, 0.5 mL of a 0.1 mol/L sodium phosphate buffer (pH 7.4) was added, and for the assays in the presence of metabolic activation, 0.5 mL of S9 mix was mixed with a 0.1 mL culture medium (2×10^8 cells/mL) plus 0.1 mL of AVA solutions (0–1000 μM) and 0.1 mL MMS (100 μg/plate) in the absence of metabolic activation and CPA (100 μg/plate) in metabolic active conditions. The mixtures were incubated in a shaker at 37°C (preincubation) under light protection. After a total of 60 min of cotreatment, the mixtures were added to and mixed with 2 mL top agar containing 0.05 mmol/L L-histidine and D-biotin for the *S. typhimurium* strains. Each of these was then spread on a minimal glucose agar (1.5% agar, Vogel-Bonner medium E, containing 2% glucose) plate. After the top agar solidified, the plates were incubated at 37°C for 60–72 h. Each tester strain was assayed in triplicate and repeated at least twice, and the number of revertant colonies was counted for each tester strain and treatment group [11]. The counts of revertant colonies were obtained to build a dose-response curve and calculate the percentage of reduction. Statistical differences between the groups were analyzed by a one-way ANOVA ($p < 0.05$) and Tukey's post hoc test.

When we did not detect a significant reduction in cotreatment, we carried out the pretreatment and posttreatment protocols, according to our previous study [12]. In the pretreatment protocol, the bacterial suspensions were incubated in a buffer or S9 mix with AVA for 30 minutes. After this period, the mutagen (MMS in −S9 condition and CPA in +S9 condition) was added and the mixtures were incubated for 30 minutes. The posttreatment protocol consisted in the incubation of the bacterial suspension with the mutagen for 30 minutes, and after the addition of AVA, the mixtures were incubated for 30 minutes more. The % of reduction was determined by linear regression considering 0% the background count and 100% the group exposed only to MMS or CPA.

To determine the cytotoxic effect, after 60 min incubation, the assay mixtures were diluted in 0.9% NaCl (*w*/*v*) to obtain a suspension containing 2×10^2 cells/mL. A suitable aliquot (100 μL) of this suspension was plated on nutrient agar (0.8% bacto nutrient broth (Difco), 0.5% NaCl, and 1.5% agar). The plates were then incubated at 37°C for 24 h, and the colony-forming units (CFU) were counted to obtain the percentage of survival. All the experiments were done in triplicate and were repeated at least twice. Statistical differences between the groups were analyzed by a one-way ANOVA ($p < 0.05$) and Tukey's post hoc test [12].

2.5. Cytoprotective Assay of HepG2 Cells. Fresh HepG2 cells were seeded at a density of 1×10^5/well. The water-soluble tetrazolium salt assay (WST-1) (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) (Roche Co., South San Francisco, CA) was used to determine the number of viable cells after 24 h of exposure to AVA and the AAs (0 to 1000 μM. Briefly, after treatment, the culture medium was replaced by a 90 μL fresh culture medium and a 10 μL WST-1 reagent and incubated at 37°C and 5% CO_2 for 2 h. The absorbance was then measured at 440 nm according to the kit protocol and according to Ferraz et al. [13]. The intensity of the

yellow color in the negative control (DMSO 1%) wells was designated as 100% viability, and all further comparisons were based upon this reference level to determine the lethal concentration (LC_{50}) to 50% of cultured cells.

After the determination of LC_{50} of AVA, MMS, and CPA, fresh HepG2 cells were seeded at a density of 1×10^5/well and were coincubated with each AA at its LC_{50} and AVA (from 0 to 100 μM) for its cytoprotective capacity evaluation. After 24 h of coexposure, the culture medium was replaced by a 90 μL fresh culture medium and 10 μL WST-1 and incubated at 37°C and 5% CO_2 for 2 h. The absorbance was then measured following the protocol as described before. The survival rates were determined in comparison to the negative control. Statistical differences between the groups were analyzed by a one-way ANOVA ($p < 0.05$ to <0.001) and Tukey's post hoc test.

2.6. Micronuclei in HepG2 Cells. The HepG2 cells were seeded at a density of 1×10^5/mL into 24-well plates (1 mL/well). The CPA at 60 μM or MMS at 0.5 μM was coincubated with AVA at 10 μM and 25 μM or incubated alone for 6 h or 24 h. DMSO 1% was used as the negative control. After exposure to the compounds, the cells were incubated for 24 h more and the cytogenetic studies were carried out in triplicate and $N = 3$ [14]. In order to determine the mitotic index and the number of cells with micronuclei, the medium was replaced by a cold methanol-glacial acetic acid (3 : 1) fixative for 30 min and the cells were then rinsed with distilled water for 2 min and air dried. The fixed cells were stained with 4,6-diamidino-2-phenylindole (DAPI) (0.2 pg/mL), dissolved in a McIlvaine buffer (0.1 M citric acid plus 0.2 M Na_2HPO_4, pH 7.0) for 60 min, washed with a McIlvaine buffer for 5 min, briefly rinsed with $_{dd}H_2O$, and mounted in glycerol. To determine the mitotic index and the number of cells with micronuclei, 2000 cells per well (6000 cells per concentration) were analyzed using fluorescence microscopy (Reichert Univar) with an excitation wavelength of 350 nm. The results for micronuclei were presented as the percentage of cells containing micronuclei in 6000 cells/concentration. Statistical differences between the groups were analyzed by a one-way ANOVA ($p < 0.01$) and Tukey's post hoc test.

2.7. Flow Cytometry Cell Cycle Analysis. The cells (1×10^5) were washed in PBS solution and centrifuged at 400 ×g for 5 min, and after, the cells were suspended in DNA staining solution (0.3% Triton X-100 and 50 μg/mL propidium iodide (PI) in a 43 mM citrate buffer), as previously described. After 45 minutes of treatment with 50 μg/mL ribonuclease A (Sigma, EUA), the PI fluorescence was determined (10,000 events per sample) in a Gallios flow cytometer (Beckman Coulter, USA). Data were analyzed by the Summit v4.3 software. The experiments were done at least three times, and statistical analysis was performed by one-way ANOVA followed by a Tukey's post hoc test [15].

3. Results

3.1. DPPH Assay. After incubation with $DPPH^+$, AVA was capable to exert DPPH free radicals scavenging dose-dependently (Figure 1). AVA obtained an $EC_{50} = 274 \pm 3\ \mu$M and showed itself to be a good direct antioxidant, with similar results to the positive control, even though BHT's EC_{50} was lower ($83 \pm 2\ \mu$M).

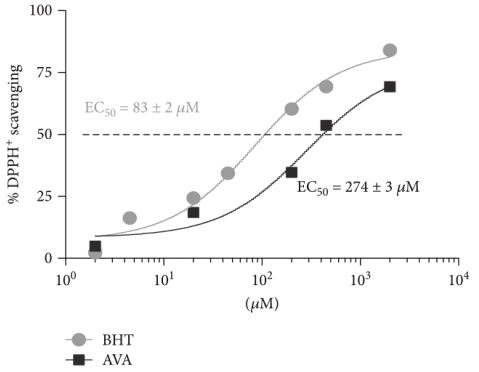

Figure 1: Atorvastatin (AVA) direct antioxidant activity by a $DPPH^+$ scavenging model. After 50 minutes of incubation with $DPPH^+$ free radicals, AVA scavenging potential was measured by spectrophotometry at 517 nm. A clear Q-curve ($R^2 = 0.9733$) can be evidenced, representing a dose-response phenomenon, and AVA's EC_{50} was $274 \pm 3\ \mu$M. Butylated hydroxytoluene (BHT) was used as an antioxidant (positive control) and presented as $R^2 = 0.9712$ and $EC_{50} = 83 \pm 2\ \mu$M. The experiments were done in triplicate and repeated 3 times ($n = 3$).

3.2. Antimutagenicity in a Bacterial Model. In the antimutagenicity evaluation using a bacterial model, AVA presented no cytotoxic effect to *S. enterica* serovar *typhimurium*-tested strains, both in the presence and absence of exogenous metabolic activation (data not shown).

After the cotreatment with alkylating agents, AVA presented dose-dependent antimutagenic effects against MMS-directed DNA damage for TA104 (32.3% and 55.7% of reduction at 200 μM and 1000 μM, resp.), TA102 (29% and 41.2% of reduction at 200 μM and 1000 μM) (Table 1), and TA1535 (from 45.7% to 91.3% of reduction in all the tested concentrations) (Table 2). AVA also presented antimutagenicity against CYP metabolism-dependent DNA injury caused by CPA for TA104 (25%, 29%, and 41% of reduction, respectively, at 100 μM, 200 μM, and 1000 μM), TA102 (30% of reduction at 1000 μM) (Table 1), and TA1535 (30.6% and 42.2% of reduction at 200 μM and 1000 μM, resp.) (Table 2). At last, AVA did not protect TA100 (Table 2) against MMS nor CPA mutagenicity.

Due to this lack of chemopreventive effects just on TA100, we carried out pretreatment and posttreatment protocols using this strain (Table 3). AVA exerted antimutagenic activity to TA100 on all of the pretreated concentrations both in the presence and absence of metabolic conditions, presenting the highest percentages of reduction of revertants. Notwithstanding, posttreatments with AVA concentrations

Table 1: Effects of atorvastatin after cotreatment with alkylating agents on *Salmonella enterica typhimurium* strains TA104 and TA102.

Atorvastatin (μM)		Coincubation	TA104			TA102		
			His$^+$	MI	% reduction	*His*$^+$	MI	% reduction
—	−S9	DMSO 1%	400 ± 31	1.00	—	250 ± 31	1.00	—
0	−S9	MMS (100 μM)	897 ± 55	2.24	0.00	578 ± 55	2.31	0.00
20	−S9		801 ± 32	2.00	19.36	525 ± 32	2.10	16.03
100	−S9		776 ± 64	1.94	24.19	515 ± 64	2.06	19.08
200	−S9		736 ± 91	1.84	32.26*	483 ± 91	1.93	29.01*
1000	−S9		620 ± 28	1.55	55.65*	443 ± 28	1.77	41.22*
—	+S9	DMSO 1%	455 ± 41	1.00	—	280 ± 41	1.00	—
0	+S9	CPA (150 μM)	1092 ± 85	2.40	0.00	588 ± 85	2.1	0.00
20	+S9		969 ± 54	2.13	19.29	566 ± 54	2.02	7.27
100	+S9		933 ± 38	2.05	25.00*	560 ± 38	2.00	9.09
200	+S9		905 ± 42	1.99	29.29*	543 ± 42	1.94	14.55
1000	+S9		829 ± 11	1.82	41.43*	496 ± 11	1.77	30.00*

MMS: methyl methanesulfonate; CPA: cyclophosphamide; *His*$^+$: revertant colonies; MI: mutagenicity index. *$p < 0.01$ versus only MMS or only CPA (one-way ANOVA followed by a Dunnett's post hoc test).

Table 2: Effects of atorvastatin after cotreatment with alkylating agents on *Salmonella enterica typhimurium* strains TA1535 and TA100.

Atorvastatin (μM)		Cotreatment	TA1535			TA100		
			His$^+$	MI	% reduction	*His*$^+$	MI	% reduction
—	−S9	DMSO 1%	25 ± 2	1.00	—	100 ± 5	1.00	—
0	−S9	MMS (100 μM)	71 ± 5	2.84	0.00	212 ± 11	2.14	0.00
20	−S9		50 ± 4	2.00	45.65*	204 ± 18	2.04	7.14
100	−S9		40 ± 5	1.60	67.39*	198 ± 22	1.98	12.5
200	−S9		31 ± 2	1.24	86.95*	190 ± 13	1.90	19.64
1000	−S9		29 ± 3	1.16	91.30*	186 ± 14	1.86	23.21
—	+S9	DMSO 1%	20 ± 3	1.00	—	112 ± 9	1.00	—
0	+S9	CPA (150 μM)	56 ± 6	2.80	0.00	239 ± 30	2.13	0.00
20	+S9		53 ± 3	2.63	9.44	235 ± 22	2.10	2.65
100	+S9		52 ± 7	2.60	11.11	230 ± 31	2.06	6.19
200	+S9		45 ± 4	2.25	30.56*	224 ± 15	2.00	11.50
1000	+S9		41 ± 8	2.04	42.22*	216 ± 25	1.93	17.70

MMS: methyl methanesulfonate; CPA: cyclophosphamide; *His*$^+$: revertant colonies; MI: mutagenicity index. *$p < 0.01$ versus only MMS or only CPA (one-way ANOVA followed by a Dunnett's post hoc test).

were not able to reduce the DNA injuries caused directly by MMS or those related to the metabolism of CPA.

3.3. Cytoprotection of HepG2 Cells. The hepatotoxicity of the compounds using HepG2 cells at 24 h of exposure is presented in Table 4. AVA showed $LC_{50} > 1000\,\mu$M. The AA presented different grades of hepatotoxicity. CPA's LC_{50} was $98.71 \pm 11.50\,\mu$M. MMS was more hepatotoxic, presenting $LC_{50} = 18.67 \pm 6.67\,\mu$M. Using the alkylating agent concentrations around the LC_{50} to evaluate the AVA cytoprotective effects, which means that there is the potential to reduce cell death induced by the DNA AA in our specific case, it is possible to observe that AVA induced a significant protection in hepatic cells coexposed to MMS at 1.0 and 10.0 μM (Figure 2(a)). The same effect was observed against CPA (Figure 2(b)) from 0.1 to 10.0 μM.

3.4. Micronuclei in HepG2 Cells. Figure 3 shows the micronucleated HepG2 cell counts of coexposure to AVA and 10.0 μM MMS after 6 h (Figure 3(a)) and 24 h (Figure 3(b)). After exposure to MMS, it is possible to observe a significant decrease in micronucleus formation in coincubated cells to AVA at 6 and 24 h, from 6-7 fold (in only MMS-exposed cells) to 3-4 fold and 1-2 fold in comparison to the negative control at 10.0 μM or 25.0 μM, respectively. After 6 h (Figure 3(c)) and 24 h (Figure 3(d)) of coexposure to 60.0 μM CPA, AVA showed the same behavior, decreasing

Table 3: Effects of atorvastatin after pretreatment and posttreatment with alkylating agents on *Salmonella enterica typhimurium* strain TA100.

Atorvastatin (μM)			TA100					
			Pretreatment			Posttreatment		
			His$^+$	MI	% reduction	*His*$^+$	MI	% reduction
—	−S9	DMSO 1%	102 ± 17	1.00		127 ± 4	1.00	—
0	−S9		230 ± 23	2.26	0	264 ± 31	2.09	0
20	−S9		171 ± 14	1.68	45.97*	257 ± 26	2.03	5.69
100	−S9	MMS (100 μM)	117 ± 2	1.14	88.57*	248 ± 23	1.96	11.86
200	−S9		113 ± 6	1.11	91.43*	237 ± 22	1.87	20.10
1000	−S9		103 ± 4	1.02	98.7*	233 ± 26	1.84	23.00
—	+S9	DMSO 1%	100 ± 16	1.00	—	105 ± 1	1.00	—
0	+S9		244 ± 8	2.44	0	278 ± 33	2.66	0.00
20	+S9		130 ± 30	1.30	79.4*	257 ± 5	2.46	12.04
100	+S9	CPA (150 μM)	127 ± 19	1.27	81.6*	227 ± 3	2.37	17.44
200	+S9		111 ± 9	1.11	92.59*	216 ± 8	2.34	18.49
1000	+S9		103 ± 2	1.03	97.92*	207 ± 23	2.30	21.48

MMS: methyl methanesulfonate; CPA: cyclophosphamide; *His*$^+$: revertant colonies; MI: mutagenicity index. *$p < 0.01$ versus only MMS or only CPA (one-way ANOVA followed by a Dunnett's post hoc test).

Table 4: HepG2 cytotoxicity of compounds after 24 h of exposure.

Compound	LC_{50} (μM)
AVA	>1000
MMS	18.67 ± 6.67
CPA	98.71 ± 11.50

LC_{50}: lethal concentration of 50%; MMS: methyl methanesulfonate; CPA: cyclophosphamide; AVA: atorvastatin.

the fold from 5-6 fold to 3-4 fold and 1-2 fold in comparison to the negative control at 10.0 μM or 25.0 μM.

3.5. Cell Cycle Analysis. We observed that after exposure to MMS, HepG2 cell subsets at different stages of the cell cycle were significantly different from what was observed in the unexposed control (Figure 4). AVA reduced the sub-G1 percentage of cells (Figure 4(a)) in a dose-dependent manner, from 19% in untreated cells to 12%, 4%, and 2% in its cotreatment at 1 μM, 10 μM, and 25 μM, respectively. AVA also reduced the polyploid subpopulation (Figure 4(b)), from 15% after exposure just to MMS to the background counts (3-4%) in cotreatment. AVA and MMS cotreatment did not affect G1 (Figure 4(c)) and S (Figure 4(d)) phases and restored the number of cells in the G2 phase (Figure 4(e)) that was reduced in only MMS-exposed cells. The representative histograms demonstrated that, in comparison to the control (Figure 4(f)), 25 μM AVA (Figure 4(g)) did not induce alterations on the cell cycle pattern. On the other hand, 20 μM MMS (Figure 4(h)) induced several modifications on the cell cycle pattern, but the cotreatment with 25 μM AVA (Figure 4(i)) in MMS-exposed cells restored the cell cycle pattern.

The same behavior was observed after exposure to CPA with HepG2 cell subsets at different stages of the cell cycle presenting significantly different counts from what was observed in the unexposed control (Figure 5). AVA also reduced the sub-G1 percentage of cells (Figure 5(a)), from 17% in untreated cells to the background counts (3-4%) that did not exert dose dependence. AVA also reduced the polyploid subpopulation (Figure 5(b)) from 13% after exposure just to CPA to the background counts (3–5%) in cotreatment; besides, the incubations with different AVA treatments increased the number of polyploidy cells, even though there is no significance. AVA and CPA cotreatment did not affect G1 (Figure 4(c)), S (Figure 4(d)), and G2 phases (Figure 4(e)). The representative histograms demonstrated that, in comparison to the control (Figure 5(f)), 25 μM AVA (Figure 5(g)) did not induce alterations on the cell cycle pattern. On the other hand, 20 μM MMS (Figure 5(h)) induced several modifications on the cell cycle pattern, but the cotreatment with 25 μM AVA (Figure 5(i)) in MMS-exposed cells restored the cell cycle pattern.

4. Discussion

According to the study of Ajith and Soja [10], atorvastatin (AVA) and lovastatin (LOVA) were able to exert chemopreventive effects against direct mutagens in a bacterial reverse mutation model using *Salmonella enterica* serovar *typhimurium* TA98 and TA100 strains in the absence of metabolic activation. The antimutagenic effects of AVA and LOVA against the direct mutagens sodium azide or 4-nitro-*o*-phenylenediamine in a bacterial reverse mutation model using *Salmonella enterica* serovar *typhimurium* TA98 and TA100 strains were described previously. AVA significantly inhibited the mutagenic response, which was evident by the decrease in revertant colony counts in cotreated plates [10].

In our study, we used four *Salmonella enterica typhimurium* strains to be able to detect DNA damage caused by base-pair substitution/transition. Our results corroborate the Ajith

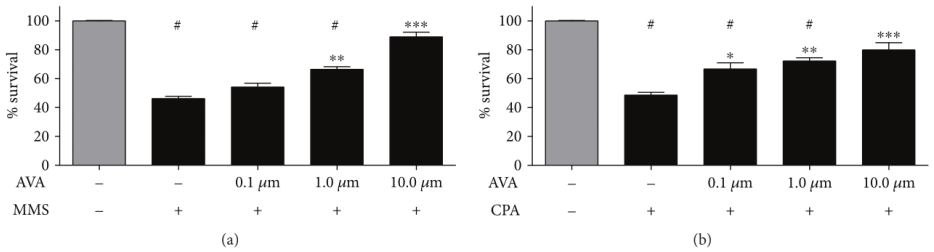

Figure 2: Effect of cotreatment with atorvastatin (AVA) after 24 h of coexposure with alkylating agents. HepG2 cells were coexposed to AVA from 0.1 to 100 μM. It is possible to observe that AVA induced a significant cytoprotective effect in hepatic cells coexposed to (a) 20 μM MMS at 1.0 and 10.0 μM. The same effect was observed against (b) 100 μM CPA from 0.1 to 10.0 μM ($^{\#}p > 0.001$ versus the negative control and $^{*}p > 0.05$; $^{**}p > 0.01$; $^{***}p > 0.001$ versus CPA or MMS only; $n = 4$ in triplicate; one-way ANOVA followed by a Tukey's post hoc test).

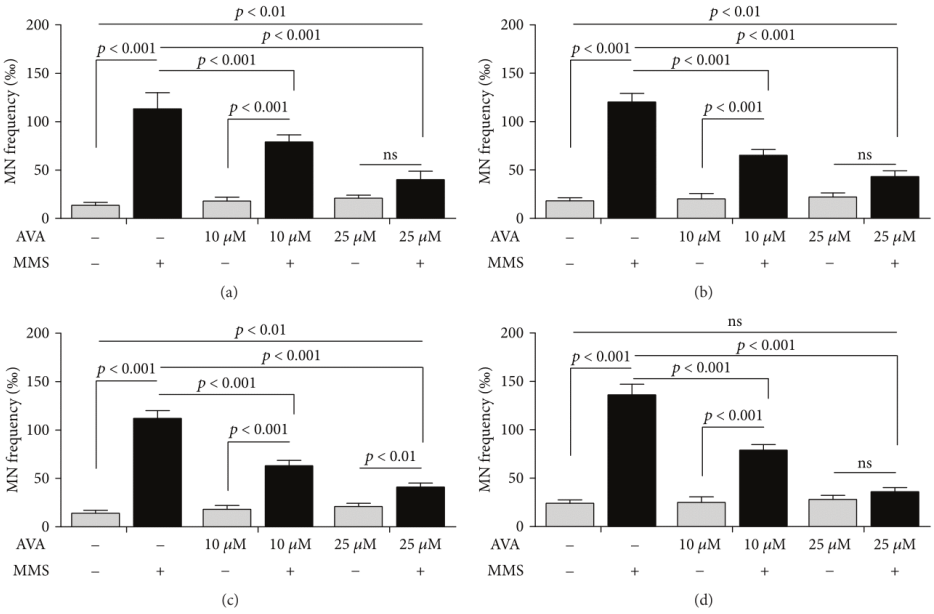

Figure 3: Effect of cotreatment with atorvastatin (AVA) on methyl methanesulfonate- (MMS-) or cyclophosphamide- (CPA-) induced micronuclei in HepG2 cells. HepG2 cells were coincubated with AVA at 10 and 25 μM with10 μM MMS after (a) 6 h or (b) 24 h of exposure. The coincubation with 60 μM CPA during (c) 6 h or (d) 24 h followed the same protocol. 2000 cells were scored per treatment for each experiment ($n = 3$ in triplicate; one-way ANOVA followed by a Tukey's post hoc test).

and Soja study [16], once AVA showed itself being more protective against direct than indirect induction in a bacterial model. Mutagenesis is not a passive process, and the modifications in DNA sequence can be mediated by mechanisms of repair [16]. This active and multifactorial process of DNA modifications based on DNA impairment and repair is named genomic instability [17]. TA1535 and TA104, strains that are deficient in error-prone recombination repair (REC), were more effective than the REC-proficient correspondent strains (TA100 and TA102, resp.) in exerting chemoprevention against AA damage. These REC-proficient variants can produce an endonuclease mediated by *RecA* SOS response, which could play a role in "nick and gap" formation in the mutagenized DNA [18]. Besides this, TA100 and TA102 can activate DNA repair mediated by an error-prone polymerase [19].

In relation to TA1535/TA100 (TA1535, *pKM101*$^{+}$), these strains are capable to detect mutations by substitution of G:C to A:T pairs in GGG sites of hotspot locus *hisG46*. They can detect primary DNA modifications, after a replication cycle,

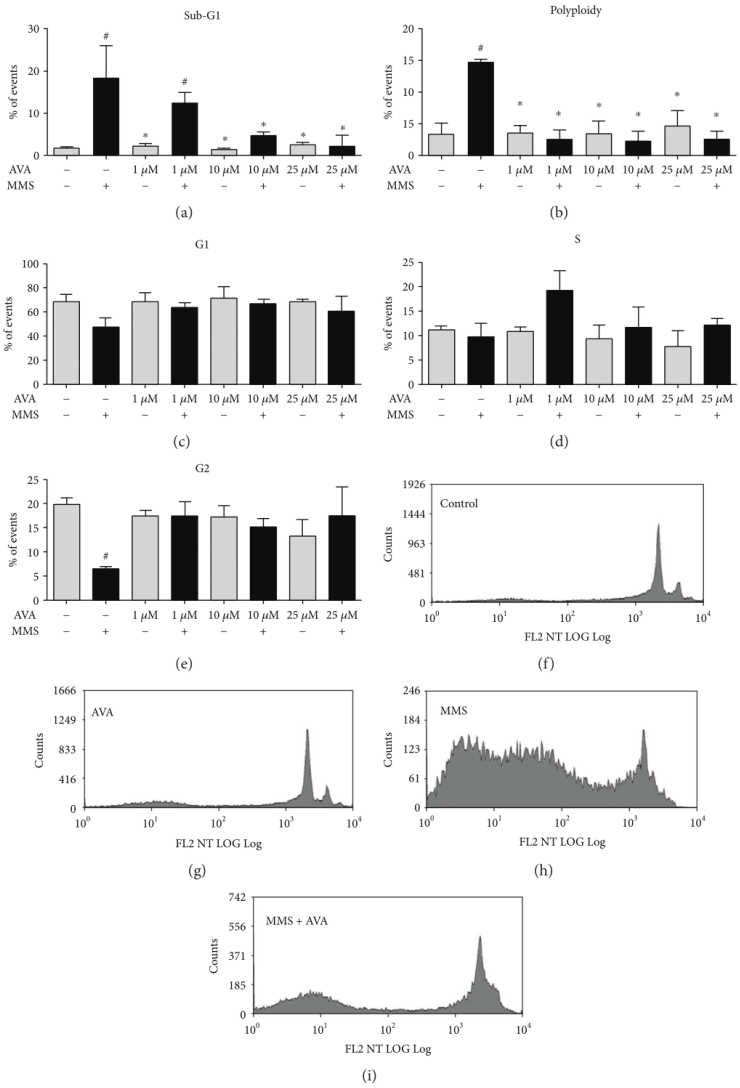

Figure 4: Cell cycle analysis of HepG2 cells after treatment with atorvastatin (AVA) and also cotreatments with AVA and methyl methanesulfonate (MMS). HepG2 cells were incubated with 1, 10, and 25 μM AVA or 20 μM MMS and also coincubated with 1, 10, and 25 μM AVA plus 20 μM MMS during 24 h. The negative control was DMSO 1%. The histograms represent the percentages of cell cycle phases in each condition by flow cytometry. Data of 104 cells were analyzed using the Summit v4.3 software (Dako Colorado Inc., USA). Cotreatment with AVA reduced the sub-G1 percentage of cells in a dose-dependent manner (a) and polyploid cells (b), in comparison to only MMS-exposed cells, without affecting G1 (c) and S (d) phases and restored the number of G2 cells (e). The representative histograms demonstrated that in comparison to the control (f), 25 μM AVA (g) did not induce alterations on the cell cycle pattern. On the other hand, 20 μM MMS (h) induced several modifications on the cell cycle pattern, but the cotreatment with 25 μM AVA (i) in MMS-exposed cells restored the cell cycle pattern ($n = 3$; $^{\#}p > 0.001$ versus the control and $^{*}p > 0.001$ versus MMS only; one-way ANOVA followed by a Tukey's post hoc test).

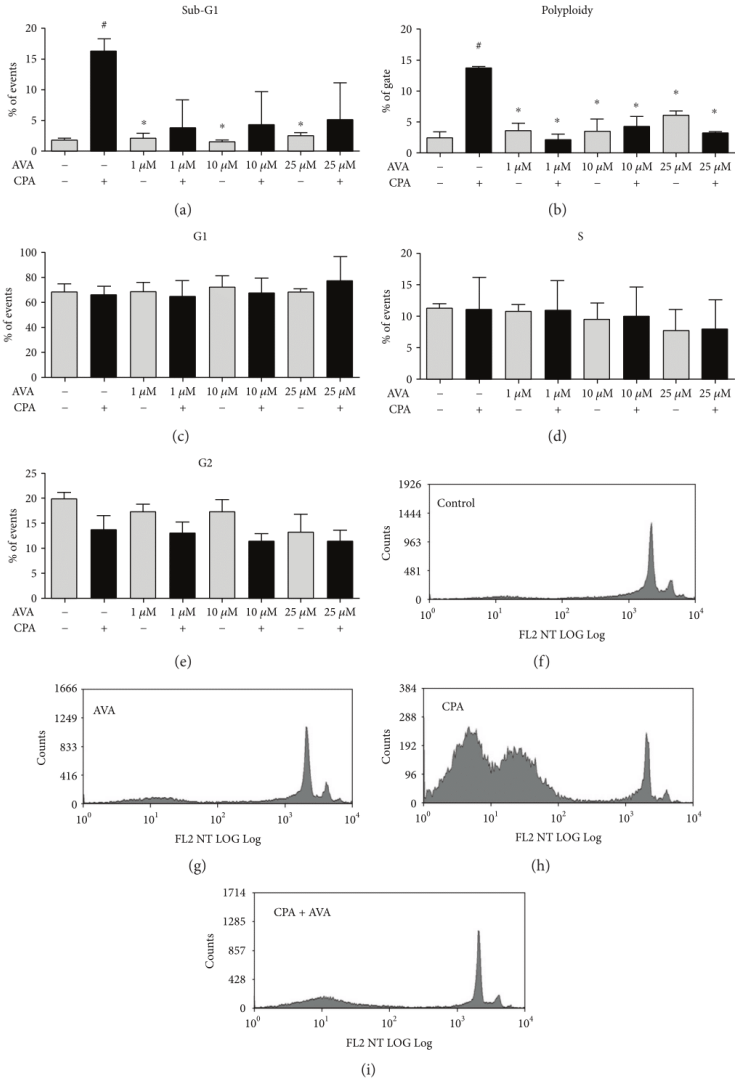

Figure 5: Cell cycle analysis of HepG2 cells after treatment with atorvastatin (AVA) and also cotreatments with AVA and cyclophosphamide (CPA). HepG2 cells were incubated with 1, 10, and 25 μM AVA or 60 μM CPA or coincubated with 1, 10, and 25 μM AVA plus 20 μM CPA during 24 h. The negative control was DMSO 1%. The histograms represent the percentages of cell cycle phases in each condition by flow cytometry. Data of 104 cells were analyzed using the Summit v4.3 software (Dako Colorado Inc., USA). Cotreatment with AVA reduced the sub-G1 percentage of cells (a) and polyploid cells (b), in comparison to only CPA-exposed cells, without affecting G1 (c), S (d), and G2 (e) phases. The representative histograms demonstrated that in comparison to the control (f), 25 μM AVA (g) did not induce alterations on the cell cycle pattern. On the other hand, 60 μM CPA (h) increased sub-G1 percentage of cells, but after the cotreatment with 25 μM AVA (i) in CPA-exposed cells, it was restored ($n = 3$; $^{\#}p > 0.001$ versus the control and $^{*}p > 0.001$ versus CPA only; one-way ANOVA followed by a Tukey's post hoc test).

as alkylation in purines, mainly in guanine, as N-(2-chloroethyl)-N-[2-(7-guaninyl)ethyl]amine or an hydroxylated mustard arm (N-(2-hydroxyethyl)-N-[2-(7-guaninyl)ethyl]amine) [20], the kind of damage induced by CPA and O^6-alkyl-G formation and induced by MMS [21]. The protective effect was more evident against MMS because this mutagen acts predominantly by alkylating guanines and favoring adduct formation [22]. In relation to TA104 and TA102 (TA 104, *pKM101*$^+$), both strains are capable to detect thymine alkylation by formation of O^4-alkyl-T due to A:T to G:C transition and mismatch recognizing [20–22], and AVA was more antimutagenic to TA104 than to TA102. Specifically in this case, AVA was protective to TA1535 and was not to TA100 in coincubation, which means that probably REC has an important role in AVA antimutagenesis, and also, base excision repair (BER) can play a primordial role in this process.

According to De Flora et al. [11], the implementation of protocols that include pre- and posttreatments are scientifically relevant because it allows predicting some aspects about the mechanism of action (MoA) in antimutagenesis assays. In general, the literature recommends to perform cotreatment protocol as a trial model, once the most part of antimutagens can demonstrate some protection in combined exposure, and then perform pre/posttreatments after, to obtain more mechanistic information. Antimutagenicity's MoA in cotreatment is related to general antimutagenic activity and also can be related to membrane responses. If a compound just exerts antimutagenic effect on pretreatment, the MoA is related to extracellular events as an interruption of promutagen shift, free radical scavenging capacity or other antioxidative property. Withal, if a compound is antimutagenic just on posttreatment, it means that this MoA is related to this compound ability to reduce the DNA attachment of the mutagen or activation of repair mechanisms and/or induction of DNA dismutation [23]. In this sense, the antimutagenic activity observed for TA100 just in pretreatment suggests that AVA can exert directly free radical scavenging, which is in accord with our DPPH model results.

Rossini et al. [24] demonstrated that the most frequent *TP53* mutations in esophageal cancer varies according to the injury that the tissue was exposed. The frequency of G:C to A:T CpG or non-CpG mutations in TP53 was higher in patients exposed to inflammatory injuries. In our model, the antimutagenic effect of AVA was more relevant on *Salmonella* strains that detect G:C to A:T substitution which corroborate the hypothesis that the chemopreventive effects of AVA are mediated by downregulation of the redox status, reducing the genomic instability.

In eukaryotic cells, statins can contribute to oxidative stress modulation in different tissues. AVA was able to enhance glutamate via glutamate synthase activity in hippocampal neural cells after hypoxia and starvation conditions [25]. Comparatively, cells treated with AVA produced less ROS than the untreated cells. In the same sense, LOVA were capable to prevent genotoxic and cytotoxic effects caused by doxorubicin, etoposide, and MMS in human umbilical vein endothelial cells (HUVEC) by reduction of FASr, procaspase 2, and phosphorylated JNK-1 [26].

On the other hand, Gajski et al. [27] observed AVA-mediated genotoxic damage in human lymphocyte chromosome aberrations, sister chromatid exchange and increasing in tail length and intensity in lymphocyte comet assay even at nM concentrations. According to the authors, this DNA damage was caused by oxidative stress, observed in Fpg-modified comet assay. These evidences go against the original study about the AVA's safety profile that demonstrated in a complete toxicological screening that AVA is a safe drug [28]. Reis et al. also showed LOVA's capacity to enhance heme oxygenase 1 and reduction of lipid peroxidation in cerebral tissues [29]. AVA also induced antioxidative effect and reduced pathophysiological impairments mediated by host immunity in malaria infection [30].

The preantineoplastic effect of statins occurs by suppression of mevalonate biosynthesis, a precursor of important isoprenoid intermediates which are added during posttranslational modification of a variety of proteins such as subunits Ras and Rho of small G protein. These proteins are involved in cell cycle progression, cell signaling, and membrane integrity. The inhibition of Rho activation reversed the metastatic phenotype of human melanoma cells [5].

Jialal et al. [31] demonstrated a reduction in reactive protein C and hepatic acute phase proteins after treatment with statins in a follow-up clinical trial, suggesting that possibly these drugs can act in hepatic oxidative damage chemoprevention. Our results go in the same way of this evidence, showing an AVA capacity to reduce HepG2 cell death in coexposure to different AAs. On the micronucleus assay, we choose the AA concentration based on using noncytotoxic doses (a concentration lower than LC_{50}) and it was possible to observe that AVA presented a dose-response antigenotoxic effect against the AAs. In addition, against the nonmetabolism-dependent AA (MMS), AVA reduced the frequency in damaged cells earlier at the lower concentration, reaching the level of micronucleated cells to the same range of the negative control at 6 h. Against the metabolism-dependent AA (CPA), AVA just reached the level of micronucleated cells to the range of the negative control after 24 h of coexposure, displaying a late response.

At last, the cell cycle analysis by the flow cytometry approach allowed us to confirm the cytoprotective aspects that were observed by the other methodologies. Exposing HepG2 cells to the same AA concentration that we used on micronucleus assay and co-incubating the cells with AA and AVA treatments, we observed a reduction on Sub-G1 subpopulations, in comparison to only MMS or CPA groups, which represents a diminishment of cell death, as on cell viability assay. We also observed a reduction on the subpopulation with polyploidy after treatment with AVA, a fact that can be related to its antigenotoxic effect, which was the outcome observed on micronucleus assay. It is important to emphasize that there were no important changes on G1, S, and G2 phases, even after severe cell damage, and the maintenance of the cell cycle is a fundamental aspect to the reliability of micronucleus assay [32].

Iwashita et al. [33] demonstrated that pravastatin and fluvastatin reduced micronucleus formation in CHO-K1 cells after exposure to the antineoplastic bleomycin. The statins,

at concentrations from 10 μM to 100 μM, were capable to reduce the micronucleated cell rate in pretreatment, in minor responses, and in cotreatment and posttreatment schemes being high effectives. This preventive effect was not observed in exposure to X-radiation. This corroborates with our results that demonstrated a reduction in MMS- or CPA-induced micronuclei in HepG2 cells after 6 h and 24 h of cotreatment. The earlier response of AVA against MMS is related to nitrogen heterocyclic compound capacity to reduce the reactivity of sulfonates [34] and probably the later response against CPA was due to AVA's neutralization of epoxide radicals, from CPA metabolism by CYP coenzymes [35]. So, AVA was able to act as a scavenger, protecting DNA from direct and indirect alkylation-mediated point mutations, genotoxicity, and cellular death, reducing the redox status and the genomic instability. These protective effects can avoid mitotic catastrophe [36] and are expected for a good antimutagen.

In summary, our data showed that AVA reduces the alkylation-mediated DNA damage in different *in vitro* experimental models. In a bacterial model, AVA was more effective to prevent direct than indirect damage in TA1535 (cotreatment) and TA100 (pretreatment). Cytoprotection of AVA at low doses (0.1–10.0 μM) was observed after 24 h of cotreatment with MMS or CPA at their LC_{50}, causing an increase in HepG2 survival rates. AVA had decrease effect in AA-induced micronucleus formation and cell cycle alterations in HepG2 cells.

5. Conclusion

This study supports the hypothesis that atorvastatin can be considered a chemopreventive agent, acting as antimutagenic, antigenotoxic, and cytoprotective compound, and permits to clarify about its mechanism of action, reducing the oxidative microenvironment, scavenging alkylating agents directly, or neutralizing their metabolites, and thus protecting specifically against DNA damages.

Conflicts of Interest

The authors declare that there are no conflict of interest during the execution of this study.

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4 **DISCUSSÃO**

Apesar de passados mais de 100 anos após a descrição canônica da DC, as alternativas terapêuticas para o tratamento desta patologia ainda são escassas e ineficazes para muitos dos casos, principalmente aqueles com diagnóstico tardio. Além disso, por conta do cenário epidemiológico atual, denotando grande aumento da incidência da infecção por vias de transmissão não vetoriais, o aumento da ocorrência de triatomíneos em ambientes urbanos e periurbanos e a globalização da DC, com o aumento significativo do número de casos em áreas não endêmicas renova os interesses das iniciativas públicas e privadas, com ou sem fins lucrativos, para o desenvolvimento de novas abordagens terapêuticas e de diagnóstico. Um olhar crítico sobre a problemática de saúde pública que envolve uma doença, cujo diagnóstico é difícil e impreciso e o tratamento, quando não iniciado precocemente, tende à falha terapêutica, faz com que o diagnóstico e tratamento da DC sejam ainda nos dias de hoje uns dos maiores paradigmas a serem solucionados pelos gestores dos sistemas de saúde no mundo todo.

Nesse sentido, o presente estudo, através de abordagens multidisciplinares se baseando primariamente em estratégias de prospecção tecnológica, teve a intenção de gerar conteúdos de inovação farmacêutica na área de quimioterapia experimental para o tratamento da doença de Chagas, levando em conta aspectos das respostas biológicas de eficácia, segurança e seletividade de diferentes famílias de compostos heterocíclicos sintéticos.

Segundo DON e IOSET (2014), as características de um composto *hit* para triagem de fármacos anti-*T. cruzi* inclui um valor de EC_{50} até 10 µM contra formas intracelulares e índice de seletividade de pelo menos 10, além da ausência de alertas estruturais de segurança (como a genotoxicidade estrutural – toxicóforo primário), presença de propriedades de "drogabilidade", pelo menos avaliadas usando plataformas *in silico*. Além disso, o TPP de CD recomenda a análise de ambas as formas relevantes para hospedeiros mamíferos (formas tripomastigotas e intracelulares), observando também os efeitos para diferentes representantes de DTUs de *T. cruzi* (CHATELAIN E KONAR, 2015).

De fato, a descoberta e o desenvolvimento de novos fármacos são processos muito longos e onerosos, de modo que a estratégia de reposicionamento tem sido amplamente estimulada como uma alternativa para tratar várias patologias órfãs, já que as etapas relacionadas à farmacocinética e perfil de segurança já estão aprovadas pelas autoridades reguladoras (CHATELAIN; KONAR, 2015). O reposicionamento de fármacos vem lado a

lado com a possibilidade de terapia combinada, já que este esquema já é protocolo de tratamento para diversas patologias incluindo as doenças infecciosas como a tuberculose e a malária (NZILA; MA; CHIBALE, 2011). Recentemente, o reposicionamento de NF e sua combinação com eflornitina para tratar a tripanossomíase africana foi disponibilizado e demonstrou resultados positivos (SCHMID et al., 2012; ALIROL et al., 2013). A combinação de fármacos licenciados possibilita a redução de custos e reduz os efeitos colaterais, além de proporcionar um arsenal mais amplo contra doenças negligenciadas, maior eficácia e, finalmente, possíveis mudanças na posologia e administração, o que encoraja a adesão do paciente ao tratamento (HOTEZ et al., 2016). Além disso, essa estratégia permite tanto o ataque a diferentes alvos no parasita, como redução e prevenção da resistência a medicamentos, tendo sido amplamente reivindicada como uma alternativa para a terapia da DC (COURA, 2009; CHATELAIN, 2016; HOTEZ et al., 2016).

Novas alternativas para a terapia da DC ainda representam uma questão urgente (MOLINA et al., 2018). A falha terapêutica dos EBIs, como posaconazol e ravuconazol, em ensaios clínicos para DC, juntamente com a retirada do nitro-derivativo fexinidazol de ensaios clínicos recentes devido a seus baixos aspectos de segurança para os portadores chagásicos justificam a busca por moléculas alternativas anti-*T. cruzi* bem como novas abordagens terapêuticas (CHATELAIN; KONAR, 2015). A propósito, uma molécula híbrida derivada do EBI ravuconazol (E1224) a semelhança do posaconazol, resultou em altas taxas de falha terapêutica em ensaio clínico com portadores com DC crônica, na Bolívia (TORRICO et al., 2018).

O primeiro trabalho aqui apresentado explora a correlação entre a atividade tripanocida (e a seletividade) de AIAs e a presença de um (mono-AIAs) ou dois (bis-AIAs) grupos amidinos terminais. Foram sintetizadas 15 novas AIAs, submetidas a um *screening* fenotípico envolvendo diferentes formas e condições de incubação do *T. cruzi*. A maioria das bis-AIAs foi mais potente contra as duas formas de parasitas relevantes para a infecção de mamíferos (formas sanguínea e intracelular), demonstrando que dois centros amidinos terminais conferem um efeito antiparasitário superior que aqueles que possuem apenas um. A importância do segundo centro amidino pode ser observada claramente comparando os resultados da DB1967 com os da DB2002 que diferem apenas na ausência do segundo grupo na DB1967 que resulta em perda de atividade em cerca de 500 vezes. Esses resultados corroboram achados prévios para diamidinas clássicas, confirmando a relevância da presença de dois grupos catiônicos terminais efetividade sobre o *T. cruzi* (PACHECO et al., 2009). As bis-AIAs DB1967, DB1968 e DB1989 apresentaram ainda boa atividade tripanocida a 4°C na

presença de 96% de sangue de camundongo, protocolo para identificação de compostos ativos para bancos de sangue, corroborando dados prévios com outras bis-AIAs, incluindo DB766 (BATISTA et al., 2010), DB745 e DB1831 (DA SILVA et al., 2011; 2012).

A maioria das bis-AIAs também foi menos tóxica para as células cardíacas que as mono-AIAs. Em nossa investigação toxicológica, até a dose máxima testada, a genotoxicidade estava ausente, e somente um perfil de mutagenicidade leve foi observado quando DB1989 foi testada frente à cepa TA98 de *Salmonella enterica* sorovar Typhimurium, o que é sugestivo de uma mutação tipo *frameshift*, provavelmente durante o processo de reparo ou duplicação de DNA, adicionando pares de G:C ao genoma bacteriano. Embora a diretriz de teste da OCDE 471 recomende usar até 5 mg de um composto-teste, a alta atividade bactericida da DB1989 em relação às cepas bacterianas impossibilitou ensaios com concentrações mais altas de AIA que poderiam mascarar aspectos mutagênicos, exigindo estudos toxicológicos adicionais.

DB1989 e DB1967 foram transferidas para modelos *in vivo* de infecção aguda pelo *T. cruzi* devido às suas altas atividades *in vitro* e seletividades razoáveis. Embora a parasitemia tenha sido reduzida, ambas moléculas foram incapazes de proteger contra mortalidade. Os resultados contrastam com o que foi observado para DB766 (BATISTA et al., 2010) e DB1965, uma forma de sal mesilato de DB1831 (DA SILVA et al., 2011) que mostraram eficácia *in vivo* comparável ao BZ. Nossos dados confirmam a importância de dois centros amidínicos para a eficácia de AIAS contra o *T. cruzi* e demonstraram que mono-AIAs são além de menos eficazes também menos seletivas que as bis-AIAs. Embora ativos *in vitro*, DB1989 e DB1967 falharam em proteger contra a infecção por *T. cruzi in vivo*, possivelmente devido às suas toxicidades. Como estudos prévios demonstraram eficácias *in vivo* comparáveis àquelas de BZ para outras bisAIAs, a síntese de novos compostos da mesma família contendo pirimidinas bis-terminais ou piridinas merece atenção em futuros estudos de triagem de compostos tripanocidas.

Outra família de moléculas alvo de nossos estudos foram os nitroimidazois, que embora venham sendo usados clinicamente no tratamento de diversas doenças infecciosas há mais cinco décadas, seus perfis de respostas genotóxicas e biológicas não são ainda totalmente compreendidos. Sabe-se que o grupo nitroimidazol é responsável tanto pelas atividades biológicas, quanto pela indução de respostas mutagênicas, agindo, até então, como um farmacóforo toxicóforo. Em nosso segundo artigo componente desta tese, investigamos a importância de diferentes grupos funcionais nas atividades biológicas e mutagênicas dos nitroimidazois de posição C-4 ou C-5 com grupos oxidáveis ligados em N-1. No presente

estudo foram testados 9 nitroimidazois, intermediários da síntese de megazol. Este último também foi testado em paralelo. Todos os compostos foram analisados quanto a sua atividade frente às formas tripomastigotas sanguíneas de *T. cruzi*, e pelo seu potencial genotóxico, avaliado pelo ensaio de cometa em sangue total.

Os nitroimidazois 11 e 12, que têm um grupo NO_2 em C-4 e um grupo CH3 em C-2, não foram genotóxicos em comparação com os compostos 13, 14 e 15, que têm um grupo NO_2 em C-5 e que exibiram genotoxicidade moderada a alta. O nitroimidazol 13 teve efeitos mutagênicos moderados. Também observamos um resultado comparável entre 11 (2-CH_3 e 4-NO_2), que não foi genotóxico, e seu análogo ornidazol (4) (2-CH_3 e 5-NO_2), que é conhecido por ser um agente mutagênico (GISELL et al., 2002). Os compostos fluorados 14 e 17 mostraram maior genotoxicidade, independentemente da presença de CH_3 em C-2 ou NO_2 em C-4 ou C-5. No entanto, quando se comparam os compostos 11 (2-CH_3; 4-NO_2; Cl) e 12 (2-CH_3; 4-NO_2; F), o átomo de flúor não teve influência na genotoxicidade.

Quatro amostras foram selecionadas (grupo nitro nas posições 4- e 5- e diferentes grupos substituintes na posição N-1) visando determinar se o aumento da mutagenicidade dos nitroimidazois dependia apenas da posição do grupo nitro ou estaria relacionado à presença de halogênios mais ou menos reativos. Os resultados da citotoxicidade e mutagenicidade mostraram que dentre os quatro, apenas um (4-nitroimidazol, portador de flúor) era um mutágeno altamente potente (41,7 rev/µg para TA100), enquanto os outros são mutágenos de baixa potência, apresentando aproximadamente 8-9 rev/µg para linhagem TA100. Estes dados corroboram a observação do ensaio cometa, onde a genotoxicidade deste composto mutagênico foi comparável aos danos ao DNA causados por megazol. Em conclusão, observamos que a natureza e a posição de diferentes substituintes ligados ao anel imidazol têm influência significativa na atividade toxicológica. Enquanto os nitroimidazois 11 e 12 não eram genotóxicos, os nitroimidazois 13, 15 e 19 eram moderadamente genotóxicos ou mutagênicos. O nitroimidazol 16 não foi nem genotóxico nem mutagênico, e o 18 revelou-se moderadamente mutagênico, mas não genotóxico. Estes resultados demonstram que o grupo nitro não é o único responsável pela atividade mutagênica ou genotóxica, tendo os substituintes próximos um papel importante na modulação de sua toxicidade.

Uma terceira classe de compostos que foi estudada em três abordagens distintas no presente relato é a família das estatinas. No contexto da infecção pelo *T. cruzi*, embora DE SOUZA e RODRIGUES (2009) tivessem apontado AVA como um promissor EBI em sua revisão sobre as vias do esterol como alvo para agentes antitripanossomatídeos, poucos estudos foram realizados testando o efeito das estatinas sobre a infecção por *T. cruzi*,

mostrando dados controversos (MELO et al., 2012; ZHAO et al., 2016). Nesse sentido, devido ao conhecido perfil pleiotrópico de AVA como agente antiparasitário em especial sobre *Plasmodium* e *Toxoplasma* (CORTEZ et al., 2008; PARQUET et al., 2012), realizamos um estudo de reposicionamento dessa molécula, levando-se em consideração as etapas do *screening* fenotípico preconizadas para uma nova entidade química anti-*T. cruzi* (ROMANHA et al., 2010). A abordagem de reposicionamento é amplamente encorajada para a descoberta de drogas para o tratamento de doenças tropicais negligenciadas como a DC, uma vez que pode reduzir custos e tempo devido à sua aprovação e uso para outras condições clínicas (SOEIRO; DE-CASTRO, 2009; ROMANHA et al., 2010).

Como sugerido anteriormente por KAISER et al. (2015), devido ao grande banco de dados de agentes antimaláricos, o reposicionamento dos compostos promissores e líderes (*Hit* e *Lead*) merece uma triagem adicional sobre outras doenças causadas por cinetoplastídeos, incluindo a DC. Nesse sentido, nosso presente estudo avaliou a atividade potencial do AVA como agente anti-*T. cruzi*, utilizando um *pipeline* bem padronizado para DC (ROMANHA et al., 2010). Nosso achado demonstrou um efeito promissor do AVA quando o *T. cruzi* foi avaliado, especialmente em relação aos tripomastigotas, fornecendo índices consideráveis de seletividade (>50), que é uma característica desejável para um novo medicamento para DC (CHATELAIN; KONAR, 2015).

Outro aspecto é que o AVA apresentou baixo perfil de cardiotoxicidade, característica favorável, uma vez que o coração é um dos principais alvos da infecção por *T. cruzi* e da inflamação na DC (12). Nossos dados corroboram, em parte, os dados da literatura que demonstraram o efeito pleiotrópico cardiovascular benéfico das estatinas, como o AVA (DAVIGNON, 2004; LIAO; LAUFS, 2005; ZHAO et al., 2016). É importante ressaltar que os tripomastigotas foram consistentemente mais vulneráveis (2-3 vezes) à AVA que as formas intracelulares. Esta susceptibilidade diferencial de acordo com as formas parasitas do *T. cruzi* pode ser devido à localização intracelular do microambiente citoplasmático de amastigotas. É possível que a membrana plasmática dos parasitas seja mais permeável ao AVA em comparação com as membranas dos hospedeiros mamíferos, reduzindo o acesso do AVA livre diretamente aos parasitas intracelulares (por exemplo, como resultado da barreira da membrana plasmática do hospedeiro). Assim, como alternativa para aumentar a potência de AVA contra as formas intracelulares, o uso de veículo de fármaco e nova formulação poderia permitir uma maior permeabilidade através da célula hospedeira e / ou reduzir o efluxo de fármaco.

Um resultado presente notável foi alcançado usando esquemas combinatórios, resultando em valores ΣFICI inferiores a 0,5, indicando sinergismo com o medicamento de referência para DC, benzonidazol. Nossos dados corroboram estudos prévios utilizando abordagens de reaproveitamento combinatório (AVA e drogas de referência, como a quinina) em várias cepas de *Plasmodium falciparum*, relatando um perfil sinérgico (PARQUET et al., 2010). Nossos dados sugerem uma promissora abordagem terapêutica das estatinas em combinação com BZ no tratamento de infecções parasitárias, justificando também a síntese de novas moléculas da família das estatinas mais seletivas para a enzima do parasito, visando contribuir para a descoberta de novas alternativas terapêuticas para patologias parasitárias. Nossos resultados de terapia combinada são muito encorajadores, alcançando efeito sinérgico entre AVA e BZ, melhorando a eficácia e potência de ambas as drogas em combinação, sobre tripomastigotas e formas intracelulares de *T. cruzi*, pertencentes a diferentes DTUs, relevantes para a infecção em seres humanos.

Por conta disso, foram sintetizados novos compostos híbridos portando o motivo químico do sítio farmacofórico das estatinas (região de lactona), conjugados ao sítio ativo de fenilacetamidas, que são potentes anti-inflamatórios não esteroidais (AINES) derivados do diclofenaco. Em relação à atividade tripanocida destas novas oito estatinas, apenas os derivados halogenados apresentaram potenciais efeitos antimicrobianos, na mesma faixa de atividade, toxicidade para células cardíacas e seletividade contra duas diferentes DTUs de *T. cruzi* utilizadas na triagem fenotípica desenvolvida em nosso estudo. Frente às formas intracelulares da DTU VI (cepa de Tulahuen), o composto clorado foi mais seletivo que o bromado, enquanto nenhuma diferença importante pode ser notada quando os tripomastigotas sanguíneos da cepa Y (DTU II) foram tratados. Nossos dados corroboram estudos anteriores *in vitro* que demonstraram a eficácia da lovastatina e da sinvastatina contra formas epimastigotas da cepa Y (FLORIN-CHRISTENSEN et al., 1990; MELO et al., 2011), assim como os nossos dados acima relatados de AVA sobre tripomastigotas (ARAÚJO-LIMA et al., 2018). Em nossa avaliação, o derivado clorado não apresentou resposta genotóxica.

A presença de bromo no anel aromático pode explicar os efeitos citotóxicos e genotóxicos deste halógeno (PÉREZ-GARRIDO et al., 2014). Este composto foi mutagênico para TA97 tanto na ausência quanto na presença de metabolização exógena (S9), atuando como um mutágeno de deslocamento de estrutura, causando adição de pares de bases G: C no loco hisD6610, além de induzir genotoxicidade em células ovarianas e hepáticas. A presença de bromo em moléculas orgânicas está relacionada ao seu potencial genotóxico. TSUBOY et

al. (2007) detectaram os efeitos genotóxico, mutagênico e citotóxico de um corante comercial que apresenta um brometo em um anel fenil-amina.

Os efeitos citotóxicos no modelo bacteriano e a atividade tripanocida podem estar relacionados à presença de halogênios em suas estruturas. Em estudos de outro grupo foram sintetizados novas 1,2,4-triazolo-1,3,4-tiadiazinas contendo halogênio e observou-se que os compostos clorados e bromados foram eficazes como antimicrobianos contra bactérias gram positivas e negativas (HOLLA et al., 2001).

Os dados alcançados sugerem que o protótipo clorado merece otimização química visando identificar uma nova entidade química para terapia antichagásica. A otimização dos derivados de híbridos de estatina é de fato, largamente recomendada, a fim de identificar melhores alternativas para doenças negligenciadas, justificando novos estudos fenotípicos *in vitro* e *in vivo*. Nenhum dos compostos de sal sódico apresentou atividade antiparasitária e dois compostos na forma de acetato mostraram ação anti-*P. falciparum* e anti-*T. cruzi* superior ou semelhante quando comparados com AVA e aos medicamentos de referência. No entanto, apesar da considerável atividade tripanocida, o composto bromado exibiu um perfil genotóxico que inviabiliza sua movimentação para testes *in vivo*. O derivado clorado foi o único que apresentou características químicas e biológicas promissoras, uma vez que não exibiu genotoxicidade *in vitro*, com seletividade bastante considerável (> 17). Portanto, estudos sobre a eficácia e segurança em modelos animais de infecção para ambas as doenças são recomendados.

Por fim, em relação aos possíveis efeitos quimiopreventivos das estatinas, desenvolvemos um estudo que teve a intenção de entender mais claramente os mecanismos envolvidos na proteção exercida por AVA frente aos danos no DNA e ao estresse genotóxico promovido por alguns agentes alquilantes. De acordo com o estudo de AJITH e SOJA (2006), AVA e LOVA foram capazes de exercer efeitos quimiopreventivos contra mutágenos diretos em um modelo de mutação reversa bacteriana usando cepas de *Salmonella enterica* sorovar Typhimurium TA98 e TA100 na ausência de ativação metabólica. AVA inibiu significativamente a resposta mutagênica, que foi evidente pela diminuição na contagem de colônias revertentes no cotratamento. Os efeitos antimutagênicos de AVA e LOVA contra os mutágenos diretos azida sódica e 4-nitro-o-fenilenodiamina em um modelo de mutação reversa bacteriana.

Em nosso presente estudo (ARAÚJO-LIMA et al., 2018b) foram utilizadas quatro cepas de *Salmonella enterica* Typhymurium capazes de detectar danos no DNA causados por substituição/transição de pares de bases. Nossos resultados corroboram o estudo de AJITH e

SOJA (2006), uma vez que a AVA induziu superior proteção contra mutações diretas quando comparadas aquelas indiretas geradas no modelo bacteriano. A mutagênese não é um processo passivo e as modificações na sequência do DNA podem ser mediadas por mecanismos de reparo (RADMAN, 1975). Esse processo ativo e multifatorial de modificações do DNA com base no comprometimento e reparo do DNA é denominado instabilidade genômica (SHEN, 2001). As cepas TA1535 e TA104, que são deficientes nas vias de reparo propenso ao erro e recombinacional (REC), foram mais eficazes que suas cepas correspondentes que são competentes para o reparo REC (TA100 e TA102, respectivamente) em exercer quimioprevenção contra danos pelos alquilantes. Estas variantes proficientes de REC podem produzir uma endonuclease mediada pela resposta SOS de RecA, que poderia desempenhar um papel na formação de "nick and gap" no DNA danificado (MCCANN et al., 1975). Além disso, TA100 e TA102 podem ativar o reparo propenso ao erro mediado por uma DNA polimerase (MORTELMANS; ZEIGER, 2000).

Segundo DE-FLORA (1992), a implementação de protocolos que incluem pré e pós-tratamento são cientificamente relevantes, pois permitem prever alguns aspectos sobre o mecanismo de ação (MA) em ensaios de antimutagênese. Em geral, a literatura recomenda a realização do protocolo de cotratamento como primeira abordagem, pois a maior parte dos antimutágenos pode demonstrar alguma proteção na exposição combinada e, em seguida, realizar pré/pós-tratamentos para obter informações mais mecanísticas. O MA de antimutagenicidade no cotratamento está relacionado à atividade antimutagênica geral e também pode estar relacionado às respostas da membrana. Se um composto exerce efeito antimutagênico apenas no pré-tratamento, o MA está relacionado a eventos extracelulares como interrupção da conversão de promutágenos, capacidade de eliminação de radicais livres ou outra propriedade antioxidante. Além disso, se um composto é antimutagênico apenas no pós-tratamento, isso significa que este MA está relacionado à capacidade desse composto de reduzir a ligação do DNA ao mutágeno, ou a ativação de mecanismos de reparo e / ou indução de dismutação do DNA (BARENEK, 1990). Nesse sentido, a atividade antimutagênica observada no TA100 apenas no pré-tratamento sugere que o AVA pode exercer diretamente a eliminação de radicais livres, o que está de acordo com os resultados do modelo colorimétrico de sequestro do DPPH.

Os estudos com células de mamífero permitiram analisar danos na estrutura do DNA, através do ensaio de micronúcleo e a análise do ciclo celular por citometria de fluxo permitiu confirmar os aspectos citoprotetores induzidos por AVA. Expondo as células HepG2 às concentrações de 10 μM de MMS e 60 μM de CPA, em cotratamento com diferentes

concentrações de AVA observamos uma redução da morte induzida por dano e do número de células com DNA danificado (micronucleadas), além da redução nas subpopulações Sub-G1, em comparação com os grupos expostos apenas ao MMS ou à CPA, o que representa uma diminuição dos níveis de morte celular, como no ensaio de viabilidade celular. Observamos também redução na subpopulação com poliploidia após tratamento com AVA, fato que pode estar relacionado ao seu efeito antigenotóxico, que foi o desfecho observado no ensaio de micronúcleo. É importante ressaltar que não houve alterações importantes nas fases G1, S e G2, mesmo após graves danos celulares, e a manutenção do ciclo celular é um aspecto fundamental para a confiabilidade do ensaio de micronúcleos (FENECH, 2007).

A resposta mais precoce do AVA frente a indução de micronúcleos pelo MMS está relacionada à capacidade de compostos heterocíclicos de nitrogênio em reduzir a reatividade de sulfonatos (ISHITAWA, 2005) e provavelmente a posterior resposta contra CPA foi devido à neutralização de epóxidos radicalares de CPA oriundos das reações de metabolização por CYP2C9 (PARVATHY; NEGI; SRINIVAS, 2010). Assim, AVA foi capaz de atuar como agente quimiopreventivo, protegendo o DNA de mutações pontuais mediadas por alquilação direta e indireta, genotoxicidade e morte celular, reduzindo o status redox e a instabilidade genômica. Esses efeitos protetores podem evitar a catástrofe mitótica (ZHANG et al., 2008) e são esperados para um bom antimutágeno. Em resumo, nossos dados mostraram que AVA reduz o dano ao DNA mediado pela alquilação em diferentes modelos experimentais *in vitro*.

CONCLUSÃO

Diante dos desafios que constituem os novos paradigmas de saúde pública do século XXI, a busca por alternativas terapêuticas para o tratamento de doenças órfãs, como a Doença de Chagas, representa uma necessidade e urge por prioridade no cenário mundial e principalmente na América Latina. Os compostos heterocíclicos, representados neste estudo pelas classes das arilimidamidas, dos nitroimidazois e das estatinas, são apontadas como promissoras alternativas terapêuticas para o tratamento da Doença de Chagas. Muitos compostos testados neste estudo foram ativos contra as formas de *T. cruzi*, outros tantos demonstraram evidências de toxicidade e ainda foi possível inferir sobre as relações de estrutura-atividade envolvidas na toxicidade e a atividade dessas moléculas. Por fim, a estratégia de reposicionamento da atorvastatina tanto para a terapia combinada com benzonidazol, quanto para a prevenção de danos ao DNA induzidos por agentes alquilantes também se mostrou promissora e merece destaque em futuras observações.

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ANEXO – Carta de aceite da Comissão de Ética para o Cuidado e Uso de Animais Experimentais

UNIVERSIDADE DO ESTADO DO RIO DE JANEIRO
INSTITUTO DE BIOLOGIA ROBERTO ALCANTARA GOMES

COMISSÃO DE ÉTICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS
(CEUA – IBRAG - UERJ)

CERTIFICADO

Certificamos que o Protocolo nº **CEUA/058/2010** sobre **"Estudo de análogos do megazol"**, sob a responsabilidade de **Israel Felzenszwalb (UERJ),** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética Para o Cuidado e Uso de Animais Experimentais (CEUA) do Instituto de Biologia Roberto Alcântara Gomes da UERJ em **10/12/2010**. Este certificado expira em **10/12/2014**.

Rio de Janeiro, 10 de Dezembro de 2010.

Profa. Patricia Cristina Lisboa
CEUA/IBRAG/UERJ

Profª Dra. Patrícia Cristina Lisboa da Silva
Profª Adj. do Depto. Ciências Fisiológicas / IBRAG / UERJ
Matrícula 34765-8

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Instituto Oswaldo Cruz

Comissão de Ética no Uso de Animais - CEUA/ IOC

LICENÇA L-032/2016

Certificamos que o protocolo (CEUA/IOC-009/2016), intitulado "Novas abordagens terapêuticas no tratamento da infeção experimental pelo *Trypanosoma cruzi*: efeitos de inibidores da biossíntese de esteróis em associação ou não ao uso de sangue autólogo", sob a responsabilidade de **MARIA DE NAZARÉ CORREIA SOEIRO** atende ao disposto na Lei 11794/08, que dispõe sobre o uso científico no uso de animais, inclusive, aos princípios da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL). A referida licença não exime a observância das Leis e demais exigências legais na vasta legislação nacional.

Esta licença tem validade até 31/03/2019 e inclui o uso total de:

Camundongo, cepa:

Mus musculus / Swiss Webster – 336 animais machos – 11-13 gramas

Observação: Esta licença não substitui outras licenças necessárias, como Certificado de Qualidade em Biossegurança para animais geneticamente modificados, certificado do IBAMA para captura de animais silvestres ou outros.

Rio de Janeiro, 11 de julho de 2016.

Flávio Alves Lara

Coordenador da CEUA/Instituto Oswaldo Cruz

Fundação Oswaldo Cruz

FIOCRUZ-Fundação Oswaldo Cruz/IOC-Instituto Oswaldo Cruz
Av. Brasil, 4365 - Manguinhos - Rio de Janeiro - RJ - Brasil
CEP: 21040-360 Tel: (21) 2562-1056

Instituto Oswaldo Cruz

Comissão de Ética no Uso de Animais - CEUA/ IOC

LICENÇA L-038/2016

Certificamos que o protocolo (CEUA/IOC-026/2016), intitulado "O reposicionamento de medicamentos para o tratamento da doença de Chagas associada à comorbidades: impacto de terapias isoladas e combinadas no curso de coinfecção esquistossomótica e pelo *Trypanosoma cruzi*", sob a responsabilidade de **MARIA DE NAZARÉ CORREIA SOEIRO** atende ao disposto na Lei 11794/08, que dispõe sobre o uso científico no uso de animais**,** inclusive, aos princípios da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL). A referida licença não exime a observância das Leis e demais exigências legais na vasta legislação nacional.

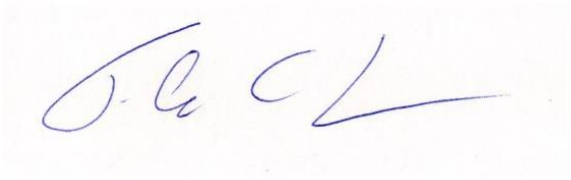
Esta licença tem validade até 31/03/2019 e inclui o uso total de:

Camundongo, cepa:

Mus muscullus / Swiss webster – 72 animais fêmeas – 21 dias de gestação

Observação: Esta licença não substitui outras licenças necessárias, como Certificado de Qualidade em Biossegurança para animais geneticamente modificados, certificado do IBAMA para captura de animais silvestres ou outros.

Rio de Janeiro, 24 de agosto de 2016.



Flávio Alves Lara

Coordenador da CEUA/Instituto Oswaldo Cruz

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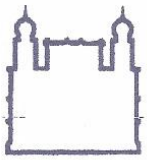

Instituto Oswaldo Cruz
Comissão de Ética no Uso de Animais - CEUA/ IOC

LICENÇA L-038/2017

Certificamos que o protocolo (CEUA/IOC-030/2017), intitulado "Estudo sobre a utilização de novos quimioterápicos experimentais no curso da infecção pelo *Trypanosoma cruzi* e *Leishmania amazonensis* em camundongos", sob a responsabilidade de **MARIA DE NAZARÉ CORREIA SOEIRO** atende ao disposto na Lei 11794/08, que dispõe sobre o uso científico no uso de animais, inclusive, aos princípios da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL). A referida licença não exime a observância das Leis e demais exigências legais na vasta legislação nacional.

Esta licença tem validade até 31/01/2022 e inclui o uso total de:

Animal	Espécie ou linhagem	Quant (total)	♂	♀	Idade	Peso	Origem
camundongo	*M. musculus* Swiss Webster	13.920 (Procedimentos: 1,2,3,5,6,7 e 17)	480 (Procedimento 3) 13.440 (Procedimentos 5,6 e 7)			13-18g	ICTB
	M. musculus Swiss Webster	1.152 (Procedimentos 1,2,4 e 17)		1.152 (Procedimento 4)	Grávidas com 21 dias de gestação		ICTB
	M. musculus Swiss Webster	800 (Procedimentos 1,2,8 e 17)		800 (Procedimento 8)		18-20g	ICTB
	M. musculus Swiss Webster	8.832 (Procedimentos 1,2,5,9,10,11,1213 e 17)	8.832 (Procedimentos 1,2,5,9,10,11,1213 e 17)			11-13g	ICTB
	M. musculus BALB/c An	1.392 (Procedimento 1,2,11,12,13,14,15,16 e 17)	Proc 14: 288 (Procedimento 14) 1.104 (Procedimento 11,12,1315, 16 e 17)		6 semanas de vida		ICTB

Observação: Esta licença não substitui outras licenças necessárias, como Certificado de Qualidade em Biossegurança para animais geneticamente modificados, certificado do IBAMA para captura de animais silvestres ou outros.

Rio de Janeiro, 29 de novembro de 2017.

Flávio Alves Lara
Coordenador da CEUA/Instituto Oswaldo Cruz
Fundação Oswaldo Cruz

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