UNIVERSIDADE FEDERAL DE ALFENAS

ELDA GONÇALVES DOS SANTOS

ANTIPARASITIC AND CYTOTOXIC EFFECT OF BENZNIDAZOLE AND NEW METRONIDAZOLE/EUGENOL HYBRIDS ON *Trypanosoma cruzi*-INDUCED INFECTION *IN VITRO* AND *IN VIVO*

Alfenas/MG

2023

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Tese apresentada como parte dos requisitos para obtenção do título de Doutora em Biociências Aplicadas à Saúde pela Universidade Federal de Alfenas. Área de concentração: Fisiopatologia.

Orientador: Prof. Dr. Rômulo Dias Novaes
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Alfenas/MG

2023

Sistema de Bibliotecas da Universidade Federal de Alfenas
Biblioteca Central

Santos, Elda Gonçalves dos.
Antiparasitic and cytotoxic effect of benznidazole and new metronidazole/eugenol/ hibrids on Trypanosoma cruzi-induced infection in vitro and in vivo / Elda Gonçalves dos Santos. - Alfenas, MG, 2023.
99 f. : il. -

Orientador(a): Rômulo Dias Novaes.
Tese (Doutorado em Biociências Aplicadas à Saúde) - Universidade Federal de Alfenas, Alfenas, MG, 2023.
Bibliografia.

1. Quimioterapia experimental . 2. Doença de Chagas. 3. Trypanosoma cruzi. I. Novaes, Rômulo Dias , orient. II. Título.

Ficha gerada automaticamente com dados fornecidos pelo autor.

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A Banca examinadora abaixo-assinada, aprova a Tese apresentada como parte dos requisitos para obtenção do título de Doutora em Biociências Aplicadas à Saúde pela Universidade Federal de Alfenas. Área de concentração: Fisiopatologia.

Aprovada em: 17 de Fevereiro de 2023

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Documento assinado eletronicamente por **Manuella Carvalho da Costa**, **Professor do Magistério Superior**, em 17/02/2023, às 11:19, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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Dedico este trabalho a Deus, o orientador da minha vida. E ao meu filho, Abner Vinícius Gonçalves dos Santos, meu maior incentivo para lutar e viver.

AGRADECIMENTOS

*A **Deus**, pelo dom da vida e por me proporcionar oportunidades inimagináveis. Senti sua presença ao meu lado durante toda a execução deste projeto.*

*À minha mãe, **Rita**, que esteve em constante oração e se alegrou junto comigo em cada vitória recebida, mesmo sem entender uma vírgula desta pesquisa.*

*Ao meu filho, **Abner Vinícius**, pelos abraços tão aconchegantes que me encorajavam a acreditar e continuar na caminhada. Você é meu coração batendo fora do peito.*

*Ao meu esposo, **Kelsen de Souza e Leite**, pelo amor, apoio, carinho, palavras de incentivo e por acreditar tanto em mim, mais que eu mesma.*

*Ao professor Drº **Rômulo Dias Novaes** por me orientar com tanta maestria e dedicação.*

*Ao meu coorientador, professor Drº **Ivo Santana Caldas**, não apenas pela orientação científica, mas também pela amizade construída ao longo destes nove anos de parceria.*

*Aos servidores do **Departamento de Patologia e Parasitologia** da Universidade Federal de Alfenas, pelo acolhimento, apoio e amizade.*

*À **Universidade Federal de Alfenas** e ao **Programa de Pós-Graduação em Biociências Aplicadas à Saúde**, por mais esta oportunidade na minha carreira acadêmica.*

*Às instituições de fomento, pelo incentivo, em especial à **FAPEMIG**.*
A todos os que se fizeram importantes para a conclusão deste trabalho, minha gratidão!

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001

RESUMO

Apesar de sua descoberta há mais de um século, a escassez de fármacos e os efeitos colaterais das terapias disponíveis permanecem como desafios para o tratamento da doença de Chagas. Nossa investigação primária foi por meio de uma revisão crítica detalhada da evidência sobre a relevância e a aplicabilidade do benznidazol em diferentes estágios da doença. Nossos achados demonstraram que o benznidazol exibe alta biodisponibilidade após administração oral em adultos e crianças. Porém, a posologia e o tempo de duração do tratamento recomendados muitas vezes são dissociados dos achados farmacodinâmicos. Além disso, os múltiplos métodos analíticos utilizados em acompanhamento pós-terapêutico de longo prazo suportam a evidência de que a maior eficácia terapêutica é obtida quando o tratamento com benznidazol é administrado em infecções agudas, piorando gradualmente à medida que a infecção se torna crônica. Diante de falha terapêutica, o tratamento com benznidazol nem sempre garante melhor prognóstico, podendo o paciente desenvolver a Cardiomiopatia Chagásica assim como pacientes não tratados. Como a evidência atual indica a necessidade de novas estratégias para o tratamento da doença de Chagas, em nossa abordagem secundária avaliamos a relevância antiparasitária *in vitro* e *in vivo* de dois novos híbridos moleculares de metronidazol e eugenol na infecção por *T. cruzi*. Nossos achados mostraram que os híbridos possuem efeito antiparasitário direto sobre o parasito atenuando o a taxa de infecção celular, a biossíntese de espécies reativas e o estresse oxidativo em cardiomiócitos infectados *in vitro*. Além disso, os híbridos foram bem tolerados em camundongos infectados por *T. cruzi*, não sendo relacionados à mortalidade ou hepatotoxicidade. Essas novas moléculas também induziram um efeito antiparasitário relevante ao atenuar a parasitemia e a miocardite *in vivo*. Diante dos resultados, um dos híbridos, denominado AD07 (híbrido metronidazol/dihidroeugenol nitrado na posição orto à hidroxila), destacou-se como candidato potencialmente relevante para o desenvolvimento de novos regimes medicamentosos mais seguros e eficazes para o tratamento da infecção por infecção *T. cruzi*.

Palavras-chave: Quimioterapia experimental; Doença de Chagas; *Trypanosoma cruzi*.

ABSTRACT

Despite being discovered more than a century ago, the scarcity of drugs and the side effects of available therapies are among the greatest challenges for the Chagas disease treatment. Our primary investigation was through a detailed critical review of the evidence to assess benznidazole relevance and applicability indifferent stages of the disease. Our findings demonstrated that benznidazole exhibits high bioavailability after oral administration in adults and children; however, the recommended dosage and duration of treatment are often dissociated from pharmacodinamic findings. In addition, the multiple analytical methods used in long-term post-therapeutic follow-up support the evidence that the greatest therapeutic efficacy is obtained when benznidazole-based treatment is administered in acute infections, gradually worsening as the infection becomes chronic. In the face of confirmed therapeutic failure, treatment with benznidazole does not always guarantee a better prognosis, and the patient may develop Chagas Cardiomyopathy as well as untreated patients. Based on these observations, indicating the need for new strategies for Chagas disease treatment, our secondary approach evaluated antiparasitic relevance of two new metronidazole/eugenol molecular hybrids on *T. cruzi* infection *in vitro* and *in vivo*. Our findings showed that the hybrids have a direct antiparasitic effect on *T. cruzi*, attenuating cellular parasitism, reactive species biosynthesis and oxidative stress in *T. cruzi*-chalenged cardiomyocytes *in vitro*. In addition, the hybrids were well tolerated in *T. cruzi*-infected mice, not being related to mortality or hepatotoxicity. These new molecules also induced a relevant antiparasitic effect by attenuating parasitemia and myocarditis *in vivo*. In view of the results, one of the hybrids, called AD07 (metronidazole/dihydroeugenol hybrid nitrated at ortho position to hydroxyl), stood out as a potentially relevant candidate for the development of new, safer and more effective drug regimens for the treatment of *T. cruzi* infection.

Keywords: Experimental chemotherapy; Chagas disease; *Trypanosoma cruzi*.

SUMÁRIO

1 GENERAL INTRODUCTION

Although the centenary of Chagas disease discovery has been celebrated recently, this parasitic infection remains considered as a neglected tropical disease by the World Health Organization (WHO, 2022). Accordingly, Chagas disease is still associated with important limitations mainly related to the availability of screening and diagnostic tools, as well as effective and less toxic trypanocidal drugs (ÁLVAREZ-HERNÁNDEZ et al., 2021). Chagas disease is an anthropozoonosis caused by *Trypanosoma cruzi*, a flagellated protozoan that affects approximately 7 million people worldwide and causes about 50,000 deaths annually, specially in South and Central Americas (WHO, 2022).

Mainly found in 21 endemic continental countries in Latin America, the disease has been object of concern in the last four decades due the intense migratory flow from Latin America to Europe, United States, Canada and Japan (ANTINORI et al. 2017). We can take the United States as an example. In that country, despite the wild transmission cycles been well described, envolving several triatomines species and mammalian hosts like opossums, dogs and rodents; the chronic *T. cruzi* infections imported in Latin America migrants surpasses the localy aquired human cases (BERN et al., 2019). According to Arnal and Audrey et al. (2019), over 100.000 *T. cruzi* infected immigrants lives in Europe, and 300.000 lives in the United States. In this context, human migration has significant role to the disease spread, taking it from a Latin American problem to a global health concern with a high morbimortality rate and substantial social impact (CONNERS et al., 2016; LIDANI et al., 2019). Accordingly, this desease determines a global economic burden surpassing US$ 7,2 billion per year, which is mainly associated with hospital care for patients in more advanced infection stages (CONNERS et al., 2016; LIDANI et al., 2019).

In endemic regions, *T. cruzi* transmission is predominantly vectorial, which occurs through contact with infected feces of hematophagous insects called triatomines, of Reduviidae family. However, the parasite can also be transmitted by non vectorial ways, as the ingestion of parasite-contaminated foods, vertically or congenitally, through blood transfusion or transplantation of infected organs (GUARNER, 2019; PÉREZ-MOLINA and MOLINA, 2018). While vector and non-vector forms of contamination overlap in endemic areas, non-vector transmission is predominant or exclusive in non-endemic countries (LIDANI et al., 2019). Interestingly, oral transmission of *T. cruzi* plays a leading role in some endemic areas, including Brazil (FILIGHEDDU et. al., 2017).

Although not yet well understood, the clinical course of Chagas disease is divided in

two successive phases, acute and chronic. The acute phase starts in the first two weeks after the vectorial transmision and lasts about eight weeks (KEEGAN, ROBERTO et al., 2020). This phase is characterized by high parasitemia levels and usually exhibits a difficult diagnosis, being subclinical or oligossymptomatic. In addition, patients often manitest unspecific symptoms like eyelid edema, fever, malaise and hepatosplenomegaly (CUCUNUBÁ et al., 2016). The acute phase symptoms are mainly caused by the generalized immunological reaction and the intense tissue parasitism, generating inflammation in heart and gastrointestinal organs, meninges and nervous system (NUNES et al., 2018). At acute phase, some patients can also manifest a worse prognosis and develop a severe myocarditis. Usually, the symptoms of acute phase cease spontaneously over weeks to months, even without specific treatment. However, cardiac manifestations are more usual in the symptomatic chronic form of Chagas disease (CHADALAWADA, SINDHU et al., 2020). Accordingly, the disease evolves to the chronic phase and the infected patient can manifestan undetermined infection, characterized by the absence of symptoms and undetectable parasitemia, as result of the quiescent invasion of host's target tissues (ECHAVARRÍA et al., 2021). About 50% of infected people stays life long at undetermined form showing no progressive damage to the parasitized tissues and organs, mainly due to a balanced host-pathogen interation between by means of especific activation of defense cells and anti-*T.cruzi* antibodies (MALIK et al., 2015; RASSI et al., 2017). Between ten to thirty years after staying in undetermined form, 30-50% of patients evolves with a symptomatic infection, developing detectable organ damage (CARVALHO et al., 2019; HENAO-MARTÍNEZ et al., 2019). In chronic disease, cardiac, digestive, neurologic and mixed infectionsforms are recognized, being the cardiac form the most severe and frequent manifestation. This form manifests in 30-40% cases, followed by 15-20% patients showing digestive disturbs, including megaesophagus and megacolon (CHAO et al., 2020). Nearly 1/3 of patients displaying the cardiac form develops chronic Chagas cardiomyopathy, involving parasite-dependent myocardial injury and immune-mediated tissue damage. In this case, ventricular disfunction, thromboembolism, arrhythmia, and congestive heart failure are common morbid events, which often results in sudden death (CALDAS et al., 2019; RASSI et al., 2017).

In addition to representing an important public health problem, the early diagnosis and treatment of Chagas disease still do not present desirable effectiveness (MILLS, 2020). Currently, Chagas disease treatment is based on the drugs benznidazole (N-benzil-2-nitro-1-imidazoleacetamida) and nifurtimox ((RS)-3-metil-N-[(1E)-(5-nitro-2-furil)metileno]tiomorfolin-4-amina 1,1-dióxido). Although they are the only drugs available,

benznidazole and nifurtimox cause several side effects and show no complete efficiency in chronic infections, achieving cure in only 20% of infected patients (ZUMA; SOUZA, 2021).

Due to its systemic toxicity, nifurtimox commecialization was suspended since the 80's in some Latin American countries, including Brazil (COURA, 2009; COURA and DE CASTRO, 2002). Thus, benznidazole is considered the first choice drug for Chagas disease treatment. Administred in the early stage of infection, this drug can exhibits good antiparasitic effieciency. However, offers little to no beneficial therapeutic effect when applied just at the chronic phase (CALDAS et al., 2019). Accordingly, limitations related to time-dependent efficacy, toxicity, pharmacokinetics properties, and drug resistance of several *T.cruzi* strains; indicates the urgent need for safer and more effective drugs.

It is known that nitroimidazoles have several biological activities, including immunosuppressive, antiparasitic and antimicrobial properties (KAPOOR, et al, 2003). Several nitroimidazole derivatives are widely used in different medical conditions such as giardiasis, amoebiasis (HERNÁNDEZ CERUELOS et al, 2019), bacterial vaginosis (FAUGHT and SONIA REYES, 2019), and trichomoniasis (PALADINE; URMI, 2018). The literature also reports the pharmacological properties of eugenol (4-allyl-2-methoxyphenol or 2-methoxy-4-(2-propenyl) phenol), a substance present in several plants with antibacterial, antifungal, antiparasitic, cytotoxic and antioxidant potential (MORRISON and BOYD, 1961; KOTHARI et al., 2004). Considering the therapeutic properties of metronidazole and substances from the eugenol family, widely described in the literature, we synthesizetwo new molecular hybrids, one containing a unit of metronidazole coupled to the natural phenol eugenol, and the other coupled to dihydroeugenol, an analogous unit to eugenol. Accordingly, we evaluated the relevance of thees two new molecular hybrids and demonstrated their effects on *in vitro* and *in vivo T. cruzi* infections.

CHAPTER 1

AN EVALUATION OF BENZNIDAZOLE AS A CHAGAS DISEASE THERAPEUTIC

Manuscript published in: Ivo S. Caldas, Elda G. Santos & Rômulo D. Novaes. Expert Opinion on Pharmacotherapy, 2019 Oct;20(15):1797-1807. doi: 10.1080/14656566.2019.1650915. Epub 2019 Aug 28

GENERAL OBJECTIVE

Critically analyze the evidence available in the literature on the relevance and applicability of the benznidazole in different stages of Chagas disease.

Specific objectives

a) To evaluate the pharmacological potential of benznidazole in patients with Chagas disease of different ages and at different stages of infection;

b) To demonstrate the current evidence of benznidazole-based chemotherapy for Chagas disease, with a specific focus on the mechanism of action, clinical recommendations, cure criteria and therapeutic efficacy at different stages of the disease.

AN EVALUATION OF BENZNIDAZOLE AS A CHAGAS DISEASE THERAPEUTIC

ABSTRACT

Backgound: As benznidazole is the first-line treatment for patients with Chagas disease, rational chemotherapy strategies are required based on the critical analysis of the evidence on the relevance and applicability of this drug at different disease stages.

Areas covered: The authors discuss the current understanding of benznidazole-based chemotherapy for Chagas disease, focusing specifically on epidemiology, pharmacokinetics, mechanism of action, clinical recommendations, cure criteria, and therapeutic efficacy in different phases of the disease.

Expert opinion: Benznidazole shows high bioavailability after oral administration. Benznidazole at 5–8 mg/kg/day and 5–10 mg/kg/day for 30–60 days are consistent clinical recommendations for children and adults, respectively. A high correlation between negative parasitological, serological, and polymerase chain reaction (PCR) assays in long-term post-therapeutic follow-up has been consistently used to evaluate therapeutic efficacy. These methods support the evidence that the success of benznidazole-based chemotherapy is closely correlated with the phase of infection in which the treatment is administered. The greater therapeutic efficacy is obtained in acute infections, gradually worsening as the infection becomes chronic. When therapeutic failure is confirmed by any diagnostic assay, benznidazole treatment does not always ensure better long-term prognosis, and Chagas cardiomyopathy may develop as well as in untreated patients.

KEYWORDS: American trypanosomiasis; antiparasitic chemotherapy; etiological therapy; nitroimidazole; therapeutic efficacy

1. INTRODUCTION

American trypanosomiasis or Chagas disease is a neglected tropical anthropozoonose caused by the parasite protozoan *Trypanosoma cruzi*. At least seven million people are infected by this parasite and 25 million people are at risk of infection worldwide [1,2]. Chagas disease is endemic in Latin America, and most cases of infection are reported in areas with low socioeconomic development [1]. In the last decades, this disease has been more consistently detected in non-endemic areas, and at least 181,181 cases in Europe and 350,000 cases in North America were recently confirmed [3,4]. While oral and vector routs are responsible for most cases of *T. cruzi* infection in endemic countries, blood donation and transplantation of infected organs, congenital transmission (mother to fetus), and laboratory accidents are the main routes of infection in non-endemic countries [5]. The Chagas disease develops into acute and chronic stages. Acute infections are asymptomatic in about 95% of the cases and are characterized by high parasitemia easily detectable by direct microscopic observation of *T. cruzi* trypomastigotes in fresh blood [4,6]. About 5–10% of patients die in the acute phase and those who survive progress to the chronic phase of the disease [7,8]. Due to low or undetectable parasitemia, the diagnosis of chronic infections are based on conventional serological methods and polymerase chain reaction (PCR)-based molecular methods, which exhibit more acceptable profiles of sensitivity and specificity compared to conventional parasitological methods [5,9]. About 70% of *T. cruzi*-infected patients develop a chronic indeterminate infection, which is asymptomatic. Conversely, 30% of patients evolve to a chronic symptomatic disease, developing mega syndromes (megacolon and megaesophagus) and Chronic Chagas Cardiomyopathy (CCC), the main causes of morbidity and mortality in Chagas disease [1,10]. Complex and multifactorial processes are involved in the pathogenesis of mega syndromes and CCC, especially parasite persistence, autonomic denervation, microvascular insufficiency, oxidative damage, and autoimmunity [4,10]. The mega syndromes are caused by the parasitism and persistent inflammation in the digestive organs, determining severe loss of smooth muscle cells and destruction of intramural parasympathetic neurons [11,12]. Esophagus and colon dilatation, thickening of their walls, segmental disturbances of peristalsis of the digestive tract, dysphagia, regurgitation, and constipation are the main manifestations of mega syndromes [11,12]. Chronic Chagas cardiomyopathy (CCC) is the most severe and incapacitating manifestation of Chagas disease that occurs years or decades after acute infection [5,10]. CCC develops as a dilated cardiomyopathy in response to extensive heart microstructural remodeling associated with cardiomyocyte parasitism, autonomic denervation, persistent low-grade inflammation, redox

imbalance and oxidative stress, thromboembolic events, necrotizing arteriolitis, cardiomyocytolysis, myonecrosis, and progressive myocardial fibrosis [10,13]. As a functional consequence, Chagasic patients develop potentially fatal electromechanical cardiac abnormalities, mainly characterized by conduction defects, frequent and complex ventricular arrhythmias and systolic ventricular dysfunction [1,10]. In Latin America, *T. cruzi* infection is the leading cause of non-ischemic cardiomyopathy and the third cause of indication for heart transplantation [5,14]. CCC is also associated with a worst prognosis and risk of death 2.48 times higher than noninfectious cardiomyopathies [5,15].

2. ETIOLOGICAL TREATMENT OF CHAGAS DISEASE

Although the nitroheterocyclic drugs nifurtimox (4[(5-nitrofurfurylidene)amino]-3-methylthiomorpholine-1,1-dioxide) and benznidazole (N-benzyl-2-nitroimidazole-1-acetamide) have been used for more than four decades, they remain as the reference etiological treatment of Chagas disease in the absence of more efficient drugs [5,16]. These drugs have been associated with high therapeutic efficacy in the acute phase of infection, while the efficacy of etiological treatment remains debatable in chronic diseases [17–20]. Due to the high toxicity and side effects such as hypersensitivity reactions, anorexia, vomiting, polyneuritis, and depression of the production of cells by the bone marrow, nifurtimox has become inconvenient for clinical use [5,21]. This drug is no longer marketed in most countries of Central and South America, and benznidazole (Bz – Box 1) is the only drug available and clinically prescribed for the treatment of patients with Chagas disease [5,16]. Although the associated toxicity and side effects (e.g. hepatitis, peripheral polyneuropathy, digestive intolerance, and anorexia) represent important limitations to the use of benznidazole [18], the benefit-risk ratio of benznidazole treatment is still favorable, especially in the acute phase of the disease, since clinical studies have demonstrated acceptable therapeutic efficacy in reducing parasitemia, parasitism, and parasitic load even in those cases where parasitological cure is not achieved [16,18].

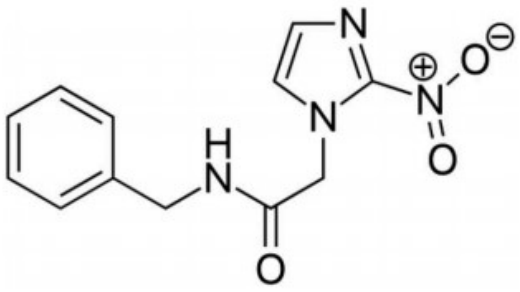
3. BENZNIDAZOLE: PHARMACOKINETICS

As benznidazole pharmacokinetic in humans was historically neglected, the current evidence is scarce. However, there is now an increasing interest in characterizing the pharmacokinetic profile of this drug in children and adults [23–25]. Part of this interest is motivated by the hypothesis that problems in the absorption, distribution, metabolism, and

elimination of this drug could be related to the limited effects of etiologic treatment. As available data indicate that benznidazole exhibits high bioavailability after oral administration in children and adults, intestinal absorption does not appear to be a limitation to the therapeutic efficacy of benznidazole treatment [24,25]. In the first meta-analysis of pharmacokinetics, Wiens et al. [25] investigated clinical single-dose studies published between 1979 and 2016. Within the acceptance criteria for the bioequivalence described by the Food and Drug Administration [26], the authors reported mean pharmacokinetic parameters under fasting conditions for a single 100 mg dose of benznidazole in adults. Thus, was encountered an area under the concentration-time curve (AUC) of 51.31 mg·h/liter (45.01–60.28 mg·h/L; 95% credible interval [CrI]), and maximum concentration of drug in plasma (Cmax) of 2.19 mg/L (2.06–2.33 mg/L; 95% CrI). In the same study, the absorption rate constant (Ka) was registered at 1.16 h−1 (0.59–1.76 h−1 ; 95% CrI), and elimination rate constant (kel) at 0.052 h−1 (0.045–0.059 h−1 ; 95% CrI); with an absorption half-life corresponding to 0.60 h (0.38–1.11 h; 95% CrI), elimination halflife of 13.27 h (11.79–15.42 h; 95% CrI), oral clearance of 2.04 liters/h (1.77–2.32 L/h; 95% CrI), and a volume of distribution (V) at 39.19 L (36.58–42.17 L; 95% CrI).Pharmacokinetics parameters for adults receiving a single oral dose of 100 mg of benznidazole was additionally reported by Molina et al. [24], which encountered AUC at 46.4 μg h/ml, plasma benznidazole concentrations peaked at 3.5 h; with maximal concentrations of 2.2 μg/ml, terminal half-life of 12.1 h, Cmax between 1.6 and 2.9 μg/ml (men vs. women), and V between 125.9 and 88.6 L (men vs. women).Pharmacokinetics data were also reported by Altcheh et al. [23] in children receiving 5–8 mg/kg/day benznidazole for 60 days.In this pediatric population, the authors observed median values of Ka at 0.638 h−1 (0.34–1.52; 95% confidence interval [CI]), V at 23.2 L (18.23–42.6; 95% CI), and apparent total clearance of the drug from plasma after oral administration (CL/F) of 1.54 L/h (1.29–1.91; 95% CI). Over decades of research and use of benznidazole in clinical practice, the pharmacokinetic profile of this drug has not been consistently considered in the treatment of patients with Chagas disease. In addition to hampering the development and evaluation of the pharmacological characteristics of novel benznidazole formulations [27], the paucity of pharmacokinetic evidence often encourages the application of adult dosages to children by using simple and inadvisable weight based adjustments [23]. However, understand the pharmacokinetic profile of benznidazole can contribute to the rational design of specific protocols for adult and pediatric populations [23,24]. In this sense, information about absorption and elimination half-life, maximal concentrations, and total clearance are useful for adjusting the doses administered and reduce

the risk of bioaccumulation and systemic toxicity [23,24], which are factors known to be associated with treatment discontinuation and lower therapeutic efficacy [8,27].

Box 1. Drug summary.

Drug name	Benznidazole (N-benzyl-2-nitroimidazole-1-acetamide)
Phase	Launched
Indication	Antiparasitic used in the treatment of Chagas disease (American trypanosomiasis)
Mechanism of action	Partly elucidated: Induction of reactive stress; inhibition of protein, DNA, and RNA biosynthesis in *Trypanosoma cruzi*.
Route of administration	Oral
Chemical structure	
Pivotal trial(s)	Phase III: [20], NCT00123916 [86]; Phase II: NCT01377480 [95], NCT01489228 [22]

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4. BENZNIDAZOLE: MECHANISM OF ACTION

Currently, the mechanism of action of benznidazole in not completely understood. However, there is evidence that as a prodrug, benznidazole exerts its trypanocidal effects after enzymatic activation by trypanosomal type I nitroreductases (NTRs), a group of oxygen-insensitive enzymes expressed in protozoan parasites [28]. In the bioactivation process, NADHdependent NTRs reduce the 2-nitroimidazole residue of benznidazole to hydroxylamine in a two-step/two-electron transfer reaction. The hydroxylamine is converted to dialdehyde glyoxal, a highly reactive product that forms molecular complexes from covalent bonds with DNA, RNA, proteins, and low molecular weight thiols (e.g. involved trypanothione reductase system) [29,30]. From glyoxal-guanosine adducts formation, the synthesis of new DNA strands is blocked, while glyoxal-thiols adducts inhibit the rudimentary *T. cruzi* antioxidant system, making the parasite highly susceptible to oxidative damage [31]. When processed by NADPH cytochrome P450 reductase and mitochondrial dihydrolipoamide

dehydrogenase, benznidazole metabolism also produces nitro radical anion derivatives (R–NO2•-), which together with dialdehyde glyoxal exerts potent cytotoxic effects on all evolutionary forms of *T. cruzi* [29]. However, these antiparasitic potentials of benznidazole are even more complex, since the etiological treatment is also able to inhibit protein, DNA, and RNA synthesis in *T. cruzi*, induce nucleic acid fragmentation and enhance the phagocytic activity of macrophages and dendritic cells, potentiating the parasitological clearance in infected hosts [32].

5. CLINICAL RECOMMENDATIONS AND EFFICACY OF BENZNIDAZOLE-BASED CHEMOTHERAPY

From a comprehensive literature search using electronic databases (Table S1), we developed a structured screening procedure following the PRISMA statement [33] to gather the available evidence on clinical recommendations for benznidazole-based chemotherapy in children and adults with Chagas disease. The evidence was based on 56 clinical reports (17 children and 39 adults) published between 1994 and 2018 involving patients originating from the Latin America countries (Argentina, Brazil, Bolivia, Colombia, El Salvador, Paraguay, and Venezuela) and Spain. In general, clinical protocols of benznidazole-based chemotherapy prescribed to children/adolescents (0 months to 17 years old) and adults (18 to 73 years old) were highly consistent. Benznidazole administration of 5–8 mg/kg/day for children or 5–10 mg/kg/day for adults during 30–60 days were the most frequent recommendations. From these protocols, parasitological, serological, and molecular markers of therapeutic efficacy were analyzed in a post-treatment follow-up ranging from 1 month to 20 years in children and two-month to 49 years in adults (Table 1). Until the year 1999, data on the therapeutic efficacy was exclusively based on parasitological and serological methods. Only from the year 2000, sensitive and specific molecular methods based on polymerase chain reaction (PCR) were incorporated in the evaluation of patients receiving the etiological treatment (Figure 1). Despite the methodological and technological advances, the absence of a feasible and consensual criterion still represents the greatest challenge for an accurate and reliable evaluation of therapeutic efficacy [9,67–69]. According to the diagnostic method used, variable rates of parasitological detection, seroconversion, and PCR negativation are clearly observed for children and adults treated with benznidazole (Figure 1, Table 1).

For each population group, the results of the therapeutic efficacy obtained from specific diagnostic methods showed a similar behavior. In general, the highest rates of therapeutic efficacy in children and adults were reported from parasitological methods (95.5%

children vs. 97.1% acute adults vs. 72.8% chronic adults) followed by PCR-based techniques (82.6% children vs. 86.1% acute adults vs. 57.6% chronic adults) and serological assays (61.7% children vs. 44.5% acute adults vs. 15.2% chronic adults) (Figure 1). This tendency remains consistent when the treatment is administered in different phases of Chagas disease, which are stratified into congenital, acute, recent chronic, and late chronic infection according to the classical criteria [70]. Considering this classification and the parasitological, serological, and molecular negative rates as primary outcomes, it is clear that as soon the infection is detected and benznidazole is administered, better chemotherapy effectiveness is achieved (Table 1).

Table 1. Summary of therapeutic efficacy obtained in children and adults treated with benznidazole-based chemotherapy, according different diagnostic methods and phase of Chagas disease (congenital, acute, recent chronic and late chronic phase) when the treatment was carried out.

Study	Country	Study design	No. of Patients*	Age	Treatment protocol	Follow-up	Cured patients / total, n (%)
Congenital infection							
Russomando et al. [38]	Paraguay	Case study	6	0-22m	7 mg/kg 60d	48m	6/6 (100%) ELISA, IF, PCR, Hc
Blanco et al. [39]	Argentina	Case series	32	0m-12y	10 mg/kg60d	24m	30/32 (93.8%) IH, ELISA, MIC
Burgos et al. [40]	Argentina	Case study	2 Bz	0-1m	5 mg⁄kg 60d	7m	2/2 (100%) MIC, ELISA, IF, PCR
Chippaux et al. [41]	Bolivia	Case series	59 Bz 52 Bz	1d	2.5 mg⁄kg 60d 7.5 mg⁄kg 30d	9-16m	58/58 (100%) ELISA, IC 50/50 (100%) ELISA, IC
Acute infection							
Shikanai-Yasuda et al. [42]	Brazil	Case series	16 Bz	0-62y	4-10 mg/kg 30-60d	156y	12/16 (75%) XD, IF, IH, IF, CoML
Andrade et al. [43]	Brazil	Case study	9 Bz	6-40y	5-10 mg/kg 60d	6m	6/9 (66.7%) CF, IF, XD
Galvão et al. [44]	Brazil	Cohort	101 Untreated 7 Bz	>16y	7 mg/kg 30-60d	144m	2/101 (2%) 7/7 (100%) Hc, IF
Bahia-Oliveira et al.[45]	Brazil	Case series	25 Bz	18-75y	not informed	168-360m	17/25 (68%) CoML, ELISA, IH, IF, Hc, PCR

* Were reported only patients included in the follow-up. Bz= benznidazole, †= mean value, ? = unknown in retrospective study. Serological methods: IC= immunochromatography; CF= complement fixation, CoML= complement mediated lysis;IF= immunofluorescence, IH= indirect hemagglutination, DH= direct hemagglutination, MIC= microhematocrit, PPA= passive particle agglutination, #= not specified. Parasitological methods: MIC= fresh blood microscopic examination;Hc= hemoculture, XD= xenodiagnosis. Molecular method: PCR= polymerase chain reaction.ND= not described, m= months, y= years.

Table 1 (*continuation*).Summary of therapeutic efficacy obtained in children and adults treated with benznidazole-based chemotherapy, according different diagnostic methods and phase of Chagas disease (congenital, acute, recent chronic and late chronic phase) when the treatment was carried out.

Study	Country	Study design	No. of Patients*	Age	Treatment protocol	Follow-up	Cured patients / total, n (%)
Acute infection							
Cançado et al. [46]	Brazil	Case series	21 Bz	0-60y	5-10 mg/kg 32d	156-252m	16/21 (76.2%) ELISA, IF, IH, CF
Schijman et al. [47]	Argentina	Case series	10 Bz 6 Bz 24 Bz	<3m 7m-12y >3y	5-8 mg/kg 60d	6m-2y 30d-3y 30d-3y	10/10 (100%) ELISA, IH, PPA, PCR 4/6 (66.7%) ELISA, IH, PPA 6/6 (100%) PCR 3/24 (12.5%) ELISA, IH, PPA 24/24 (100%) PCR
Pinto et al. [48]	Brazil	Case series	179 Bz	2-72y	5-7 mg/kg 60-90d	67.2m†	168/170 (97.7%) XD 140/145(96.5%) Hc 47/179 (26.3%) IF, IH 65/72 (90.2%) PCR
Añez et al. [49]	Venezuela	Cohort	52 Bz	1-60y	5-10 mg/kg 60d	12-276m	50/52 (97%) MIC, HC, XD 16/52 (30%) ELISA, IF 43/52 (82%) PCR

* Were reported only patients included in the follow-up. Bz= benznidazole, †= mean value, ? = unknown in retrospective study. Serological methods: IC= immunochromatography; CF= complement fixation, CoML= complement mediated lysis;IF= immunofluorescence, IH= indirect hemagglutination, DH= direct hemagglutination, MIC= microhematocrit, PPA= passive particle agglutination, #= not specified. Parasitological methods: MIC= fresh blood microscopic examination;Hc= hemoculture, XD= xenodiagnosis. Molecular method: PCR= polymerase chain reaction.ND= not described, m= months, y= years.

Table 1 (*continuation*).Summary of therapeutic efficacy obtained in children and adults treated with benznidazole-based chemotherapy, according different diagnostic methods and phase of Chagas disease (congenital, acute, recent chronic and late chronic phase) when the treatment was carried out.

Study	Country	Study design	No. of Patients*	Age	Treatment protocol	Follow-up	Cured patients / total, n (%)
Recent chronic infection							
Andrade et al. [43]	Brazil	Case study	2 Bz	6-42y	6-8mg/kg 60d	6m	1/2 (50%) CF, IF, XD
De Andrade et al. [50]	Brazil	Double-blind, randomized	64 Bz	7-12y	7.5 mg/kg 60d	36m	37/64 (57.81%)ELISA, IH, IF
Sosa Estani et al. [51]	Argentina	Double-blind, randomized	55 Bz	6-12y	5 mg/kg 60d	48m	27/44 (61.4%) ELISA, IH, IF 40/44 (90.9%) XD
Silveira et al. [52]	Brazil	Case series	10 Bz	7-12y	5-7 mg/kg 60d	96-240m	10/10 (100%) ELISA, IF, IH, XD, PCR
Streiger et al. [53]	Argentina	Cohort	64 Bz	4-14y 1-14y	5 mg/kg 30d	96-288m 160.7y†	23/37 (62%) DH, IH, IF
Vera de Bilbao et al. [54]	Paraguay	Case study	5 Bz	<12y	5-7 mg/kg 60d	60-122	2/5 (40%) ELISA, IF
Escribà et al. [55]	Spain	Case series	231 Bz	<13y	7.5mg/kg 60d	18-36m	215/229 (93.9%) ELISA

* Were reported only patients included in the follow-up. Bz= benznidazole, †= mean value, ? = unknown in retrospective study. Serological methods: IC= immunochromatography; CF= complement fixation, CoML= complement mediated lysis;IF= immunofluorescence, IH= indirect hemagglutination, DH= direct hemagglutination, MIC= microhematocrit, PPA= passive particle agglutination, #= not specified. Parasitological methods: MIC= fresh blood microscopic examination;Hc= hemoculture, XD= xenodiagnosis. Molecular method: PCR= polymerase chain reaction.ND= not described, m= months, y= years.

Table 1 (*continuation*).Summary of therapeutic efficacy obtained in children and adults treated with benznidazole-based chemotherapy, according different diagnostic methods and phase of Chagas disease (congenital, acute, recent chronic and late chronic phase) when the treatment was carried out.

Study	Country	Study design	No. of Patients*	Age	Treatment protocol	Follow-up	Cured patients / total, n (%)
Recent chronic infection							
Sosa-Estaniet al. [56]	Argentina	Case series	16 Bz	6-15y	6 mg⁄kg 60d	168m	10/16 (62.5%) ELISA, IH, IF 16/16 (100%) XD 14/16 (87.5%) PCR
Rumi et al. [57]	Argentina	Case series	57 Bz	3-15y	5 mg⁄kg 60d	24-60m	0/45 (0%) ELISA, IH, IF 32/44 (72.7%) PCR
Sánchez et al. [58]	Argentina	Case series	28 Bz	5-16y	Not reported	4m	0/28 (0%) ELISA, IH, IF 10/28 (35.7%) PCR
Brum-Soares et al. [59]	Brazil	Case series	98 Bz	6-16y	5 mg/kg 56d	72m	95/98 (97%)ELISA, IH
Andrade et al. [60]	Brazil	Double-blind, randomized	64 Bz	7-12y	7.5 mg/kg 60d	36m 72m	37/58 (63.8) ELISA, IH, IF 47/53 (88.7) ELISA, IH, IF
Late chronic infection							
Ferreira [61]	Brazil	Case series	50 Bz	Not described	5-8 mg/kg 60d	84-204m	35/50 (70%) XD 5/50 (10%) CF, IH,IF
Galvão et al. [62]	Brazil	Cohort	68 Bz	>16y	5-7mg/kg30-60d	144m	12/75 (16%) Hc 7/75 (9.3%) CF, IF

* Were reported only patients included in the follow-up. Bz= benznidazole, †= mean value, ? = unknown in retrospective study. Serological methods: IC= immunochromatography; CF= complement fixation, CoML= complement mediated lysis;IF= immunofluorescence, IH= indirect hemagglutination, DH= direct hemagglutination, MIC= microhematocrit, PPA= passive particle agglutination, #= not specified. Parasitological methods: MIC= fresh blood microscopic examination;Hc= hemoculture, XD= xenodiagnosis. Molecular method: PCR= polymerase chain reaction.ND= not described, m= months, y= years.

Table 1 (*continuation*).Summary of therapeutic efficacy obtained in children and adults treated with benznidazole-based chemotherapy, according different diagnostic methods and phase of Chagas disease (congenital, acute, recent chronic and late chronic phase) when the treatment was carried out.

Study	Country	Study design	No. of Patients*	Age	Treatment protocol	Follow-up	Cured patients / total, n (%)
Late chronic infection							
Viotti et al. [63]	Argentina	Cohort	131 Bz	20-59y	5 mg/kg 30d	60-156m	19/70 (19.1%) IF, IH, CF
Fragata Filhoet al. [64]	Brazil	Case series	71 Bz	8-52y	5 mg/kg 60d	84m†	43/71 (60%) XD 2/71 (2.8%) Serology[#]
Levi et al. [65]	Brazil	Case series	41 Bz	25-62y	5-8 mg/kg 60d	240m	26/41 (63.4%) XD
Coura et al. [66]	Brazil	Double-blind, randomized	26 Bz	Not reported	5 mg/kg 30d	12m	24/26 (92.3%) XD
Brittoet al. [67]	Brazil	Case series	76 Bz	Adults	Not reported	240m	64/76 (84%) XD 51/76(67%) PCR
Fabbro De Suasnábar et al. [68]	Argentina	Case series	36 Bz	13-52y	5 mg/kg 30d	96-276m	1/34 (2.9%) DH,IH, IF 36/36 (100%) XD
Lauria-Pires et al. [69]	Brazil	Cohort	41 Uninfected 17 Bz	31-60y	10 mg/kg 60d	120m	0/41 (0%) ELISA, IH, IF, PCR 0/17 (0%) ELISA, IH, IF, PCR
Cançado et al. [70]	Brazil	Case series	113 Bz	9-69y	5-10 mg/kg 40d	72-216	9/113 (8%) ELISA, IF, IH,CF

* Were reported only patients included in the follow-up. Bz= benznidazole, †= mean value, ? = unknown in retrospective study. Serological methods: IC= immunochromatography; CF= complement fixation, CoML= complement mediated lysis;IF= immunofluorescence, IH= indirect hemagglutination, DH= direct hemagglutination, MIC= microhematocrit, PPA= passive particle agglutination, #= not specified. Parasitological methods: MIC= fresh blood microscopic examination;Hc= hemoculture, XD= xenodiagnosis. Molecular method: PCR= polymerase chain reaction.ND= not described, m= months, y= years.

Table 1 (*continuation*).Summary of therapeutic efficacy obtained in children and adults treated with benznidazole-based chemotherapy, according different diagnostic methods and phase of Chagas disease (congenital, acute, recent chronic and late chronic phase) when the treatment was carried out.

Study	Country	Study design	No. of Patients*	Age	Treatment protocol	Follow-up	Cured patients / total, n (%)
Late chronic infection							
De Castro et al. [71]	Brazil	Cohort	27 Bz	23-88y	5 mg/kg/60d	24m	24/27 (88.8%) Hc
Fabbro et al. [72]	Argentina	Cohort	27 Bz	17-46y	5 mg/kg 30d	252m†	0/27 (0%) XD 9/27 (33.3%) DH, IH, IF
Negrette et al. [73]	Argentina	Case series	18 Bz	19-41y	5 mg/kg60d	36m	14/18 (77.7 %) ELISA,IH
Fernandes et al. [74]	Brazil	Cohort	80 Bz	17-42y	5 mg/kg 60d	36m	80/80 (100%) Hc 4/80 (5%) ELISA, IF 9/80 (11.7%) PCR
Lana et al. [75]	Brazil	Case series	28 Bz	6-37y	5-10 mg/kg 40-60d	Not described	0/28 (0%) ELISA, IH, PCR
Viotti et al. [76]	Argentina	Cohort	53 Bz	>20y	5 mg/kg 30d	36m	21/53 (40%) ELISA, IH, IF
Machado-de-Assis et al. [35]	Brazil	Case series	94 Bz	2-60y	5 mg/kg 60d	120m	8/94 (8.5%) ELISA, IH, IF 48/94 (51%) Hc 18/94 (19.2%) PCR

* Were reported only patients included in the follow-up. Bz= benznidazole, †= mean value, ? = unknown in retrospective study. Serological methods: IC= immunochromatography; CF= complement fixation, CoML= complement mediated lysis;IF= immunofluorescence, IH= indirect hemagglutination, DH= direct hemagglutination, MIC= microhematocrit, PPA= passive particle agglutination, #= not specified. Parasitological methods: MIC= fresh blood microscopic examination;Hc= hemoculture, XD= xenodiagnosis. Molecular method: PCR= polymerase chain reaction.ND= not described, m= months, y= years.

Table 1 (*continuation*).Summary of therapeutic efficacy obtained in children and adults treated with benznidazole-based chemotherapy, according different diagnostic methods and phase of Chagas disease (congenital, acute, recent chronic and late chronic phase) when the treatment was carried out.

Study	Country	Study design	No. of Patients*	Age	Treatment protocol	Follow-up	Cured patients / total, n (%)
Late chronic infection							
Aguiar et al. [77]	Brazil	Cohort	10 Untreated 29 Bz	20-39y	5 mg⁄kg 60d	60m	29/29 (100%) Hc 0/29 (0%) ELISA, IF 12/29 (41.4%) PCR
Bertocchiet al. [78]	Argentina	Cohort	545 Bz	>20y	5 mg/kg 30d	120m†	82/545 (15.1%) ELISA, IH, IF
Machado-de-Assis et al. [79]	Brazil	Cohort	29 Bz	22-37y	5 mg/kg 60d	156m	27/29 (93.1%) Hc 16/29 (55.2%) PCR
Gomes et al. [80]	Brazil	Case series	17 Bz	27-59y	5-7 mg/kg 30d	120m	17/17 (100%) Hc 10/17 (58.8%) PCR
Morillo et al. [21]	Canada	Double-blind, randomized	1431 Bz	18-75y	300 mg 40-80d	64.8m†	668/1431 (46.7%) PCR
Niborski et al. [81]	Argentina	Case series	203 Bz	15-55y	5 mg/kg60d	36m	34/45 (75.5%) PCR
Murcia et al. [82]	Spain	Case series	455 Bz	36.2 y†	100 mg/day 3 × day / 60d	24m 84m	120/125 (96%) PCR 6/6 (100%) PCR

* Were reported only patients included in the follow-up. Bz= benznidazole, †= mean value, ? = unknown in retrospective study. Serological methods: IC= immunochromatography; CF= complement fixation, CoML= complement mediated lysis;IF= immunofluorescence, IH= indirect hemagglutination, DH= direct hemagglutination, MIC= microhematocrit, PPA= passive particle agglutination, #= not specified. Parasitological methods: MIC= fresh blood microscopic examination;Hc= hemoculture, XD= xenodiagnosis. Molecular method: PCR= polymerase chain reaction.ND= not described, m= months, y= years.

Table 1 (*continuation*).Summary of therapeutic efficacy obtained in children and adults treated with benznidazole-based chemotherapy, according different diagnostic methods and phase of Chagas disease (congenital, acute, recent chronic and late chronic phase) when the treatment was carried out.

Study	Country	Study design	No. of Patients*	Age	Treatment protocol	Follow-up	Cured patients / total, n (%)
Late chronic infection							
Antunes et al.[83]	Brazil	Cohort	28 Bz 15Bz	47.2y† 45.2y† 42.7y†	? mg/kg 50-60d ? mg/kg > 60d	120m	21/27 (77.8%) PCR 10/15 (66.7%) PCR
Álvarezet al. [84]	Argentina	Case series	20 Bz	43 y†	5 mg/kg 2 × day every 5 days 60d	2m	16/17 (94%) PCR
Morillo et al. [85]	Canada	Double-blind, randomized	30 Bz	18-50y	200mg/day2 × day / 60d	12m	29/30 (96%) PCR
Sartor et al. [86]	Argentina	Case series	78 Bz	<18y	5-8 mg/kg 60d	6m	56/66 (97%) PCR
Cardoso et al. [87]	Brazil	Cohort	493 Bz	59.4 y 54.1y	Not reported	24m	411/493 (83.4%)PCR
Torrico et al. [88]	Bolivia	Double-blind, randomized	45 Bz	18-50y	5 mg/kg60d	12m	5/44 (11.4%) ELISA 0/44 (0%) PCR

* Were reported only patients included in the follow-up. Bz= benznidazole, †= mean value, ? = unknown in retrospective study. Serological methods: IC= immunochromatography; CF= complement fixation, CoML= complement mediated lysis;IF= immunofluorescence, IH= indirect hemagglutination, DH= direct hemagglutination, MIC= microhematocrit, PPA= passive particle agglutination, #= not specified. Parasitological methods: MIC= fresh blood microscopic examination;Hc= hemoculture, XD= xenodiagnosis. Molecular method: PCR= polymerase chain reaction.ND= not described, m= months, y= years.

6. POST-THERAPEUTIC CURE CRITERIA IN CHAGAS DISEASE

The high variability in the efficacy of benznidazole-based chemotherapy is a constant clinical concern [9,68,70]. There is no doubt that part of this variability is associated with parasites characteristics, especially its genetic groups classified from discreet typing units (DTU), which group *T. cruzi* stocks with similar genetic, molecular and immunological patterns [71,72]. Thus, variable clinical forms of Chagas disease and divergent profiles of drug resistance has been closed correlated with specific *T. cruzi* DTU, which predominates in distinct geographical areas [71,72]. Patients' characteristics such as age, health status, phase of infection, and adherence to treatment also exert an important impact on therapeutic efficacy, representing a clinical challenge since these variables are difficult to control in different populations infected by *T. cruzi* [73,74]. However, the absence of a feasible and consensual parasitological cure criterion and the use of analytical methods with different profiles of specificity and sensitivity represent the greatest challenge for an accurate and reliable evaluation of cure after etiological treatment [9,68] Thus, the relevancy and applicability of parasitological, serological, and molecular methods have been subjected to systematic criticism when applied as post-therapeutic cure criteria at the different stages of infection and clinical forms of Chagas disease [9,68]. The effective cure of infection requires negative results for clinical, parasitological, serological and molecular tests after a long period of post-therapeutic monitoring [9,75]. Parasitological tests such as xenodiagnoses and hemoculture are very limited for the monitoring of therapeutic efficacy since they require a long period of evaluation besides presenting limited negative predictive value due to their low sensitivity [76,77]. As the evidence prior to the year 2000 has been consistently influenced by xenodiagnostic and blood culture methods, it is possible that the therapeutic efficacy rates are underestimated, requiring a careful analysis of cases with an apparent parasitological cure. On the other hand, the diagnostic value of parasite blood tests and xenodiagnoses cannot be disregarded in case of positive results, whose microscopic detection of circulating parasites represent a definitive and unequivocal marker of therapeutic failure [78–81].

Compared to parasitological methods, serological assays exhibit marked diagnostic relevance due to higher sensitivity and specificity. Clinical trials have demonstrated that conventional serology becomes negative after the effective treatment of congenital Chagas disease with benznidazole. In most cases, negative seroconversion occurs between 2 to 24 months after treatment, and as later the treatment is initiated the longer it takes for seroconversion to occur [82–84]. Differently, a complete parasite clearance is more rapidly

and accurately detected by PCR than serological methods [84–88]. However, serology exhibits a limited relevance as a cure criterion after the etiologic treatment of the chronic phase of Chagas disease, since negative seroconversion usually occurs Table 1. only in a small percentage of individuals after several years [89–91]. This limitation is clear in clinical trials performed in Brazil and Argentina, in which longitudinal evaluations showed that negative seroconversion occurs between three to 10 years after treatment [90,91]. The introduction of PCR for the detection of *T. cruzi* mitochondrial DNA in blood samples from Chagasic patients opened the possibility of its use in a more accurate diagnosis of therapeutic failure, since it is significantly more sensitive than traditional methods such as blood culture, xenodiagnoses, and serology [78,79,92]. This applicability was clearly demonstrated in patients treated with benznidazole during the recent [93,94] and late [95] chronic phases of Chagas disease. Even after negative seroconversion and negative parasitological diagnosis, these studies showed that PCR was a remarkable tool for the early detection of therapeutic failure. However, it is important to emphasize that the diagnostic value of PCR-based methods is true only in positive results. According to Britto et al. [77], PCR is safer and more sensitive to detect cure following specific treatment, but the negative PCR results after the specific treatment indicate the absence of parasite DNA at that particular time, which cannot be assumed as a definitive cure when only one time-point is analyzed. This is particularly worrisome in late infections, since parasite load may be below the limit of PCR detection [77]. Although each method has a particular limitation, when a high correlation between negative results obtained by PCR and serological tests is consistently observed in post-therapeutic follow-up, definitive cure diagnosis can be more securely established [96].

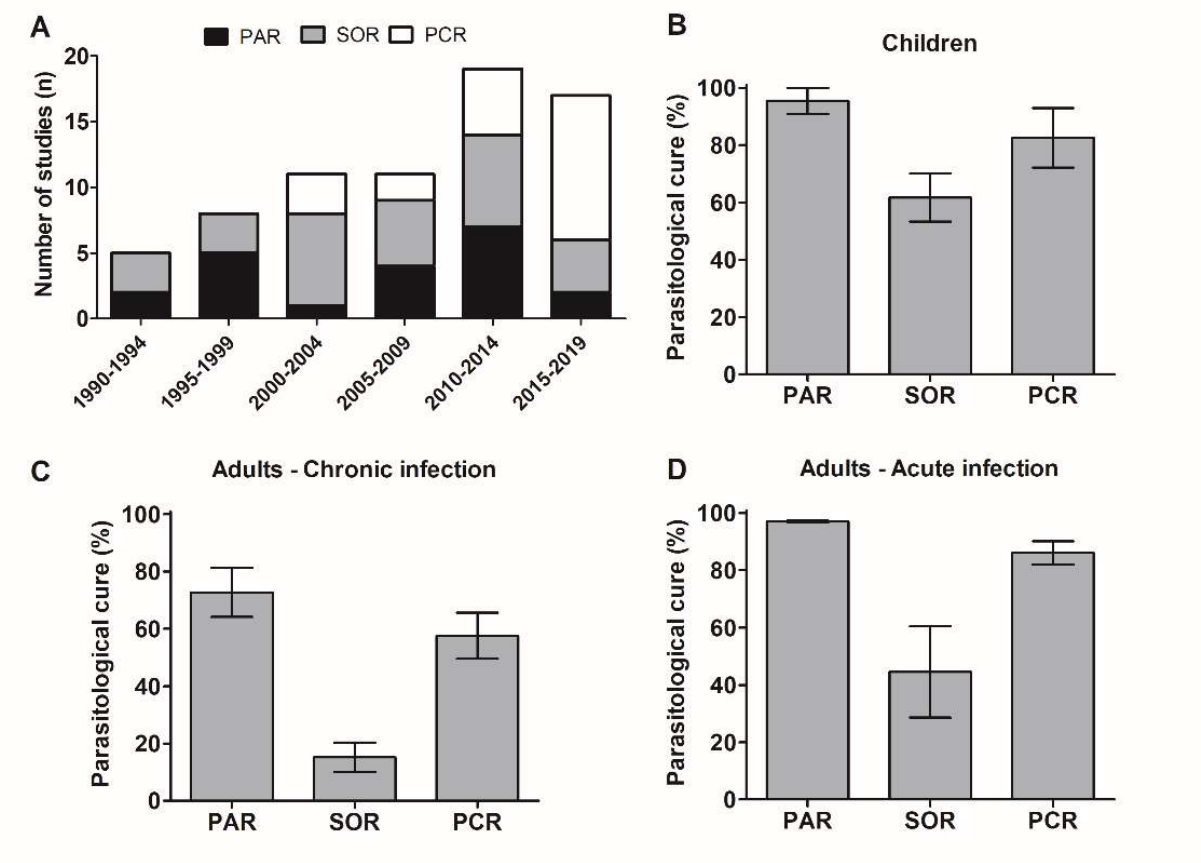

Figure 1. Diagnostic methods used to estimates post-therapeutic efficacy and rates of therapeutic efficacy obtained in children and adults treated with benznidazole-based chemotherapy, according different diagnostic methods. (A) The x-axis indicates the chronological period in which different curing control methods were used. (B, C and D) The data are presented as mean values. Diagnostic methods: PAR, parasitological; SOR, serological, PCR, polymerase chain reaction.

7. CONCLUSION

Currently, the data available indicates that benznidazole exhibits high bioavailability after oral administration in adults and children. Benznidazole at 5–8 mg/kg/day and 5–10 mg/ kg/day for 30-60 days are consistent clinical recommendations for children and adults, respectively. However, this recommendation is often dissociated from pharmacokinetic findings, and is essentially based on the clinical experience of different heath centers. In long-term post-therapeutic followup, a high correlation between negative parasitological, serological, and polymerase chain reaction (PCR) assays has been useful and advisable to evaluate therapeutic efficacy. Together, these methods have consistently shown that the success of benznidazole-based chemotherapy is associated with the phase of infection in which the treatment is initiated. Thus, greater therapeutic efficacy is often obtained in acute infections, gradually worsening as the infection progresses to a chronic stage. However, when therapeutic failure is confirmed by any diagnostic method, benznidazole treatment does not

always ensure better long-term prognosis, and Chagas heart disease may develop as well as in untreated patients.

8. EXPERT OPINION

From the detailed analysis of the evidence, it becomes clear that the characteristics of the Chagasic population, genotypic, and phenotypic variability of parasites strains, heterogeneity of methodological design in clinical trials, as well as the multiple analytical methods used to evaluate parasitological cure, have a significant impact on the results of therapeutic efficacy attributed to the etiological treatment of Chagasic patients. As the establishment of methods and criteria to express a definitive cure after benznidazole-based chemotherapies has not yet been overcome and constitutes a worrying clinical challenge, there is a growing and prudent trend in replacing the term parasitological cure by the broader concept of therapeutic efficacy. This concept is assembled covering several integrated parasitological, serological, molecular, pathological, and clinical markers, which allow a broader characterization of the general impact of the etiological treatment on Chagasic patients. When the analysis of therapeutic efficacy is exclusively based on parasitological, serology and PCR negativation, the evidence indicates that the success of benznidazole-based chemotherapy is closely correlated with the phase of infection in which the treatment is administered. In this sense, the clinical findings reported during decades of research are consistent in demonstrating that the therapeutic results worsen gradually as the infection becomes chronic. This aspect expresses a relevant clinical challenge, indicating that early diagnosis and treatment of infection may be decisive for obtaining favorable therapeutic outcomes. There is no doubt that parasitological clearance and seroconversion are important and desirable clinical endpoints of benznidazole treatment. However, the concept of therapeutic efficacy requires a more in-depth assessment of the impact of etiologic therapy, especially in cases where seroconversion and/or parasitological negativation have not been achieved. From this perspective, there is an understanding that the effect of treatment may also manifest as an independent but complementary response to the parasitological cure, which is linked to the prevention and attenuation of pathological cardiovascular processes directly associated with the highest morbidity and mortality rates of Chagas disease. Thus, the cardiovascular benefit of benznidazole-based chemotherapy is still debatable in uncured patients. Previous studies indicated that the treatment of acute infection with benznidazole was unable to prevent or attenuate heart inflammation and fibrosis months or years after the end of treatment in patients whose cure was not detected [97,98]. Although these aspects

suggest that benznidazole treatment could have no or limited protective effect on the development of Chagasic cardiomyopathy in these patients, the high rates of therapeutic success are undeniably relevant in preventing the onset of Chagas heart disease. The current evidence indicates that benznidazole treatment is also associated with the attenuation of heart disease progression in patients with recent chronic infections. In this case, there are consistent indications that chronic Chagasic patients without treatment exhibit higher risk to develop cardiovascular abnormalities and mortality rates compared to benznidazoletreated patients. This benefit was most clearly demonstrated in adolescents [86,99], reinforcing the understanding that the treatment of young people is highly recommended because of the great potential to achieve favorable therapeutic outcomes. Considering all phases of Chagas disease, the available evidence leaves no doubt that the greatest current controversy is whether and to what extent the treatment may bring cardiovascular benefits in patients with late chronic infections. Thus, the relevance of benznidazole in attenuating electromechanical heart deterioration in patients with chronic Chagas cardiomyopathy already installed remains controversial. Although some studies support the efficacy of etiologic treatment in late chronic infections [100,101], a recent clinical trial has demonstrated persistent post-treatment infection [20]. Accordingly, as treated and untreated Chagasic patients with prolonged infections exhibit similar progressive functional heart deterioration, the therapeutic efficacy attributed to the chemotherapy has been worryingly put in check. However, although the current evidence indicates that benznidazole treatment will not necessarily lead the patient to a better prognosis, the etiological treatment is still advisable even in cases of late chronic Chagas disease. As long as we do not modify the chemotherapy paradigms using doses and times of treatment other than those traditionally practiced, and expand the evaluation of therapeutic efficacy, the pharmacological potential of benznidazole will remain poorly understood. Thus, we cannot disregard the possibility of clinical benefit even in cases of patients with late chronic infection.

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CHAPTER 2

CYTOTOXICITY AND ANTIPARASITIC EFFECT OF NEW METRONIDAZOLE/EUGENOL HYBRIDS ON *Trypanosoma cruzi*-INDUCED INFECTION *IN VITRO* AND *IN VIVO*

GENERAL OBJECTIVE

To develop two new metronidazole/eugenol-based molecular hybrids and to compared the cytotoxic and antiparasitic potential of these hybrids whith benznidazole on *in vitro* and *in vivo T. cruzi* infection.

Specific objectives

a) To compare the cytotoxic profile of metronidazole/eugenol-based molecular hybrids and benznidazole on cardiomyocytes *in vitro*;

b) To compare the antipatasitic effect of metronidazole/eugenol-based molecular hybrids and benznidazole on *T. cruzi in vitro*;

c) To investigate the impact of metronidazole/eugenol-based molecular hybrids and benznidazole on antioxidant and prooxidant markers in *T. cruzi*-infected cardiomyocytes *in vitro*;

d) To compare the impact of metronidazole/eugenol-based molecular hybrids and benznidazole on *T. cruzi*-infection *in vivo*.

CYTOTOXICITY AND ANTIPARASITIC EFFECT OF NEWMETRONIDAZOLE/ EUGENOL HYBRIDS ON *Trypanosoma cruzi*-INDUCED INFECTION *IN VITRO* AND *IN VIVO*

Abstract

Aims: From well-delimited immunomodulatory, redox and antimicrobial properties; metronidazole and eugenol were used as structural platforms to assembly two new molecular hybrids (AD06 and AD07), whose antiparasitic relevance was analyzed on *T. cruzi* infection *in vitro* and *in vivo*. *Methods*: Uninfected, *T. cruzi*-infected H9c2 cardiomyocytes and mice untreated and treated with benznidazole (Bz – reference drug), AD06 and AD07 were investigated. Parasitological, prooxidant, antioxidant, microstructural, immunological, and hepatic function markers were analyzed. *Results*: Our findings indicated that in addition to having a direct antiparasitic effect on *T. cruzi*, metronidazole/eugenol hybrids (especially AD07) attenuated cellular parasitism, reactive species biosynthesis and oxidative stress in infected cardiomyocytes *in vitro*. Although AD06 and AD07 exerted no relevant impact on antioxidant enzymes activity (CAT, SOD, GR and GPx) in host cells, these drugs (especially AD07) exerted an inhibitory effect on *T. cruzi* trypanothione reductase, which may have increased the parasite's susceptibility to *in vitro* pro-oxidant challenges. AD06 and AD07 were well toleratedand do not determined mortality or hepatotoxicity in mice, as indicated by transaminases plasma levels. AD07 also induced a relevant *in vivo* antiparasitic effect, attenuating parasitemia (peak and mean) and myocarditis in *T. cruzi*-infected mice. Although this cardioprotective response is potentially related to AD07 antiparasitic effect, a direct anti-inflammatory effect of this molecular hybrid cannot be ruled out. *Conclusion*: Due to the greater similarity to the antiparasitic effect obtained with Bz, AD07 stood out as a potentially relevant candidate for the development of new, safe and more effective drug regimens for *T. cruzi* infection treatment.

Keywords: Antiparasitic chemotherapy; Eugenol; Chagas disease; Experimental parasitology, *Trypanosoma cruzi*.

1. Introduction

Trypanosoma cruzi infection is a tropical parasitic disease highly disabling, whose diagnosis is often late and associated with severe cardiovascular and digestive disturbances (WHO, 2023). This disease is closely correlated to poverty and is endemic in 21 continental Latin America countries, accounting for6 to 7 million people infected worldwide (WHO, 2023). *Trypanosoma cruzi* infection is on the rise in non-endemic countries such as the United States and Europe, whose spread is mainly determined by the migratory flow of infected people from endemic areas, donation of infected tissues and organs, vertical transmission, and laboratory accidents (Nogueira et al., 2018; Suárez et al., 2022).This infection accounts for about 69,000 annual deaths worldwide, and is a leading cause of heart failure and transplantation in endemic areas (Nogueira et al., 2018).

Currently, the nitro-heterocyclic drugs nifurtimox (NFx) and benznidazole (Bz) are the only specific treatment for *T. cruzi* infection (Caldas et al., 2019; Mills et al. 2020). Both drugs have marked toxicity, inducing serious dose-dependent side effects (e.g., bone marrow depression, peripheral neuropathy, dermatitis and gastrointestinal disturbances) that oftendetermine chemotherapy discontinuation and therapeutic failure (Caldas et al., 2019; Zuma and de Souza, 2021). Although therapeutic efficacy is high in acute disease (about 90% cure) (Santana Nogueira et al., 2022), parasitological cure in chronic infections is consistently low (about 20%) (Zuma and de Souza, 2021).Thus, NFx- and Bz-based chemotherapy is questionable in chronic diseases without parasitemia recrudescence (Caldas et al., 2019). In addition, cases of parasitic tolerance to the reference drugs provide an alert about the loss of effectiveness of the etiological treatment available, reinforcing the urgent need to develop safer and more efficient anti-*T. cruzi* drugs (Murtaet al., 1998; Campos et al., 2014).

The development of new antiparasitic chemical entities has benefited from the combination of drug repositioning and medicinal chemistry strategies (Pelozo et al., 2021; Reis et al., 2022). Accordingly, antifungal, antibacterial and antihypertensive drugs have demonstrated promising antiparasitic effects in preclinical studies (Santos et al., 2015; Mazzeti et al., 2019; Machado et al., 2020). Objectively, the antiparasitic properties of the respective nitroimidazole and allylbenzenederivatives, metronidazole (Simões-Silva et al., 2017; Dingsdag and Hunter et al., 2018) and eugenol (Leite et al., 2013; Taleuzzaman et al., 2021); revealed lead compounds whose structural platforms may be useful for the assembly of new trypanocidal drugs from chemical synthesis (Pelozo et al., 2021; Reis et al., 2022). Metronidazole (1-(β-hydroxyethyl)-2-methyl-5-nitroimidazole) exhibits a broad spectrum of biological properties, including immunomodulatory, cardioprotective and genotoxicity-

mediated antiparasitic effectsin preclinical and clinical trials (Levi and Amato Neto, 1970; Blandón et al., 1993; Simões-Silva et al., 2017; Pelozo et al., 2021) of *T. cruzi* infection. In addition, eugenol (4-allyl-2-methoxyphenol or 2-methoxy-4-(2-propenyl) phenol) is a natural secondary metabolite with anti-inflammatory, antifungal, trypanostatic and trypanocidal potential often associated to prooxidant effects (Nisar et al., 2021; Taleuzzaman et al., 2021); whose *in vitro* efficacy has indicated potential relevance for *T. cruzi* infection management (Leite et al., 2013; Pelozo et al., 2021).

Although promising, the isolated use of metronidazole and eugenol does not always overcome the antiparasitic effect achieved from Bz-based chemotherapy, the reference trypanocidal treatment. Thus, metronidazole and eugenol combination from chemical synthesis can generate a new set of less toxic molecules with optimized selectivity and potentially relevant antiparasitic properties. Accordingly, from a battery of chemical hybridization reactions, we developed and purified two new molecular hybrids containing a metronidazole unit coupled to analogous eugenol units. Thus, the present study was designed to explore the *in vitro* and *in vivo* preclinical cytotoxic and antiparasitic potential of these new metronidazole-eugenol hybrids in experimental *T. cruzi* infection.

2. Methods

2.1. In vitro assays

2.1.1 Chemical synthesis of the new metronidazole-eugenol hybrids AD06 and AD07

Chemical synthesis was developed following the protocol reported by (Pelozo et al., 2021). Briefly, 1 mmol nitroeugenol or nitro-dihydroeugenol and 1 mmol NaHCO3 were added in a 25 mL round bottom flask containing 5 mL N,N-Dimethylformamide (DMF). The reaction mixture was kept under magnetic stirring and heated at 80 ºC for 20 minutes. Then, 1mmol methanessulfony-metronidazole derivative was added, and left to react for 12 hours at 80°C. The crude product obtained was purified via column chromatography (Hexane 7:3 AcOEt). The pure products were obtained with 85% yield. Two hybrids of metronidazole connected to natural phenols were synthesized: AD06, hybrid of metronidazole connected to eugenol and nitrated in meta position to hydroxyl and AD07, hybrid of metronidazole connected to dihydroeugenol and nitrated in ortho position to hydroxyl. The products were characterized by infrared spectroscopy (IR), ^{1}H NMR and ^{13}C NMR spectrometry, as shown in Fig. 1.

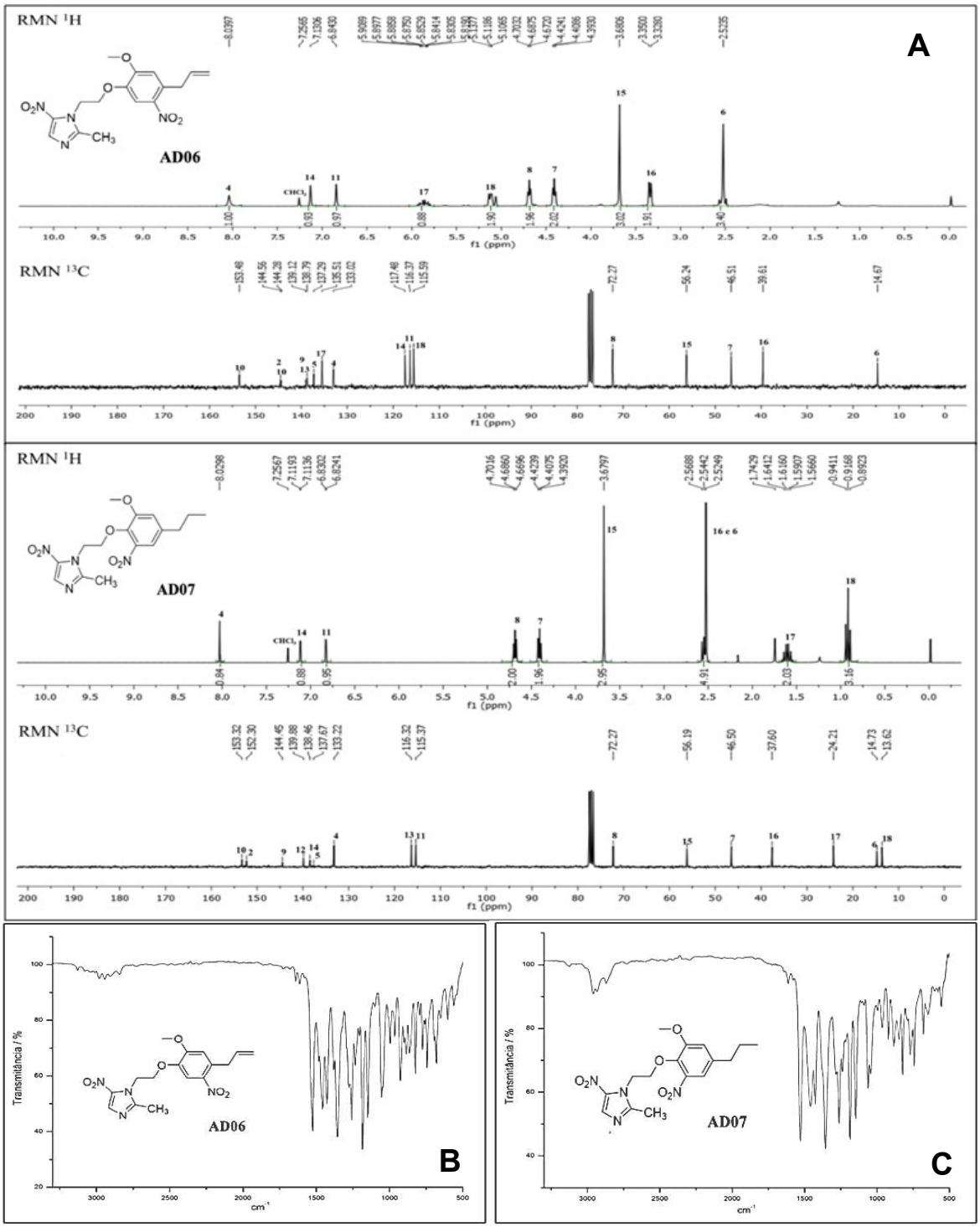

Fig. 1. Molecular structure of hybrid of metronidazole connected to eugenol and nitrated in meta position to hydroxyl (AD06) and hybrid of metronidazole connected to dihydroeugenol and nitrated in ortho position to hydroxyl (AD07). (A) Proton nuclear magnetic resonance (^{1}H NMR, 300 MHz) and Carbon-13 nuclear magnetic resonance (^{13}C NMR, 75 MHz) spectrum of AD06and AD07($CDCl_3$). Infrared spectrum obtained from KBr pellet of AD06 (B) and AD07(C).

2.1.2. Drugs and Parasites

Benznidazole (reference antitrypanosomal drug) (LAFEPE, Pernambuco, RE, Brazil) and metronidazole (Sigma-Aldrich, St Louis, MO, USA) were commercially obtained (Fig 2). The metronidazole hybrids were synthesized as previously reported. The stock solutions with both drugs were prepared in Mili-Q water and dimethyl sulfoxide (DMSO) and stored at -20 ºC. For *in vitro* assays, all drugs were diluted in fresh culture medium and DMSO (concentration in the solution was ≤ 0.06%) (Souza-Silva et al., 2020). The Y strain (DTU II) of *Trypanosoma cruzi*, was used in all assays considering its high virulent, pathogenicity and partially resistant to benznidazole (Diniz et al., 2018).

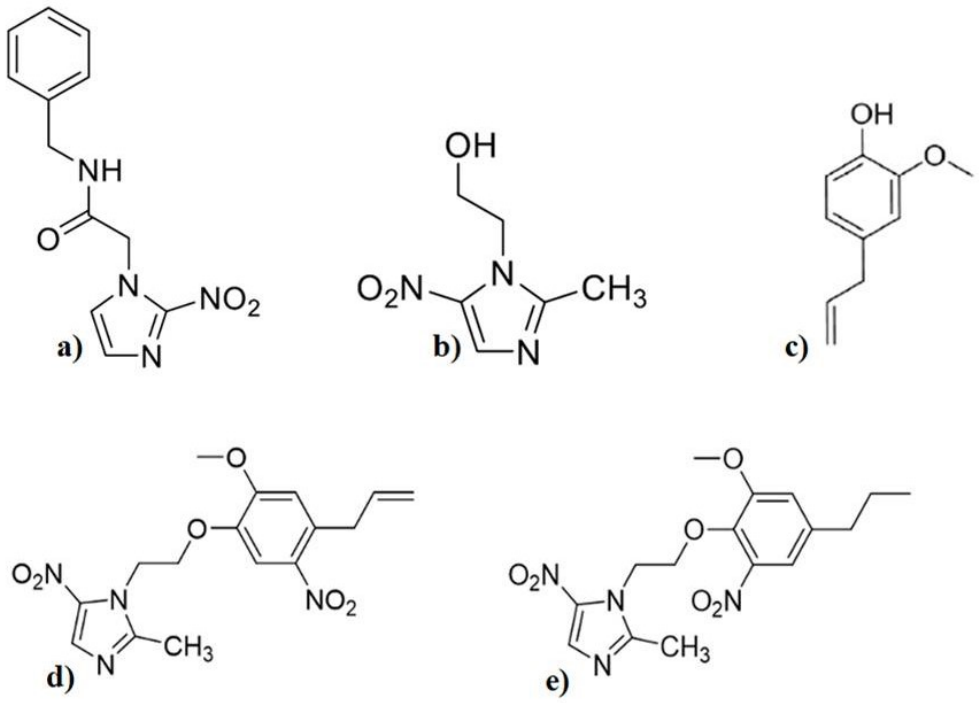

Fig. 2. Molecular structure of (a)benznidazole, (b)metronidazole, (c)eugenol, (d) hybrid of metronidazole connected to eugenol and nitrated in meta position to hydroxyl (AD06), and (e) hybrid of metronidazole connected to dihydroeugenol and nitrated in ortho position to hydroxyl (AD07).

2.1.3. Parasites and Cardiomyocytes Cultures

Trypanosoma cruzi epimastigotes were cultured in hepatic tryptose infusion (LIT) supplemented with 10% fetal bovine serum (FBS) and maintained in a Bio-Oxygen Demand (BOD) incubator at 28 ºC for exponential growth. Trypomastigotes were isolated by

centrifugation of the peripheral blood collected from venous puncture in previously infected mice. The parasites were collected from mice blood at the peak of parasitemia, which occurred 8 days post-infection (Gonçalves-Santos et al., 2019). Parasites were propagated in monolayers of H9c2 cardiomyocytes (American Type Culture Collection, ATCC: CRL 1446), which were cultured in Dulbecco's modified Eagle medium (DMEM) containing 2% fetal bovine serum (FBS). Propagated trypomastigotes were harvested after centrifuging the supernatant as previously reported (Tardieux et al., 1992; Petersen and Burleigh, 2003). Briefly, cardiomyocytes were inoculated with fresh blood trypomastigotes. After 3 days, propagated trypomastigotes were collected together with the culture supernatant, which was centrifuged for 5 min at 4 ºC and 2500×g. The supernatant was removed and the parasites were resuspended in fresh DMEM. Trypomastigotes were used in toxicity assays. H9c2 Cardiomyocytes were kept in DMEM culture medium (Gibco, Thermo Fisher Scientific, USA) containing0.1% penicillin (200 µg/mL), 1% 2mM glutamine (2%Hepes), and 10% fetal bovine serum (FBS). Culture flasks with 75 cm² containing the cardiomyocytes were incubated at 5% CO_2 (37 ºC) and trypsinized when 100% cell confluence was obtained (Tardieux et al., 1992; Gonçalves-Santos et al., 2019).

2.1.4. Drug-induced Cardiomyocytes Toxicity Assay

After cell cultivation in DMEM medium, 3×10^3 cardiomyocytes/well was incubated in a sterile 96-well polystyrene plate for 24 hours at 37ºC and 5% CO_2. The medium was replaced by 200µL/well of DMEM medium containing seven different concentrations of AD06, AD07 hybrids and Bz with a 1:2 dilution (initial concentration of 200 µg/mL and final concentration of 1.56 µg/mL). Negative controls (medium only) and positive controls (medium + cells at the same concentration as the other wells) were added and incubated for 72 hours. Resazurin (20µL/well at 1mM) was added and after 12 hours a reading was performed in a spectrophotometer at 570 nm and 600 nm. Proliferation inhibition rate was calculated using the formula: Resazurin reduction = $[A_{570} - (A_{600} \text{ X } R_0)]$. Where A_{570} and A_{600} are absorbance readings at 570 nm and 600 nm, respectively, and R_0 = correction factor. The R_0 factor was calculated from the absorbance values of the negative control, which contained the medium and resazurin in the absence of cells [$R_0 = (A_{570} / A_{600}C\text{-})$]. After calculating the inhibition percentage of each treatment using the *Compuyn* software, the CC_{50} (concentration of substances and drug that reduces 50% of cell viability) was determined (Brancaglion et al., 2018; Gonçalves-Santos et al., 2019).

2.1.5. Drug-induced Epimastigotes Toxicity Assay

Epimastigotes (1.5×10^6 parasites/mL) were added to a sterile 96-well polystyrene plate containing seven serial dilutions of AD06, AD07 hybrids and Bz at 1:2 dilution (initial concentration of 200 µg/mL and final concentration of 1.56 µg/mL), in duplicate. Resazurin (20µL/well at 1mM) was added after 72h incubation at 28 ºC in a BOD oven. After 12 hours, the reading was performed in a spectrophotometer at 570 nm and 600 nm wavelength. Proliferation inhibition (%) induced by the hybrids was calculated using the formula described above and the *Compusyn* software to calculate the IC_{50} (inhibitory concentration of the drug that induces 50% of the analyzed effect) Brancaglion et al., 2018.

2.1.6. Drug-induced Trypomastigotes Toxicity

Trypomastigotes (5×10^5/well/mL) were added to a sterile 96-well polystyrene plate containing seven serial dilutions of AD06, AD07 hybrids and Bz at 1:2 dilution (initial concentration of 100 µg/mL and final concentration of 1.56 µg/mL), in duplicate. Negative controls (containing DMEM medium only) and positive controls (containing DMEM medium and the parasite, without treatment) were added to the plate. After incubation at 37ºC, 5% CO_2 for 24 hours, the parasite were quantified using a Neubauer chamber and bright field microscope. To calculate the percentage of proliferation inhibition, the following formula was used: % inhibition = $\overline{X}$ of duplicates ÷ $\overline{X}$ of controls × 100 – 100. After calculating the percentage of inhibition, the *Compusyn* program was used to calculate the IC_{50} (inhibitory concentration of the drug that induces 50% of the analyzed effect) (Mazzeti et al., 2019).

2.1.7. Cardiomyocytes Parasitism Assay

H9c2 cardiomyocytes (12×10^4 cells/well) were incubated for 24 hours at 37ºC and 5% CO_2 in a 24-well polystyrene plate containing 13 mm glass coverslips. Then, the supernatant was discarded and the wells were washed three times with DMEM medium. Five different dilutions of Bz, AD06 and AD07 hybrids were made in 10% DMEM medium with an initial concentration of 10 µg/mL and a final concentration of 0.75 µg/mL at 1mL/well. Negative controls (uninfected untreated cells) and positive controls (infected untreated cells) were used. After 72 hours incubation, cells were washed with fresh, the coverslips were fixed with methanol, stained with Giemsa (10% in distilled water), mounted on histological slides and observed at ×100 objective lens using a bright field microscope (AxioScope A1, Carl Zeiss, Germany). Cardiomyocytes infection rate were carried out from the count of 100 cells per coverslip. Cell parasitism was determines using the endocytic index method calculating the

average number of infected cells multiplied by the average number of intracellular amastigotes per cell as previously reported (Souza-Silva, etal., 2020).

2.1.7. Total ROS, Superoxide, and Hydrogen Peroxide Assay

Total ROS and superoxide levels were estimated in cardiomyocyte cultures using a cellular ROS assay kit (ab186029 Abcam, Cambridge, UK) and a mitochondrial superoxide assay kit (ab219943 Abcam, Cambridge, UK) following the manufacturer's instructions (ab186029 Abcam, Cambridge, UK). In both analysis, cardiomyocytes were seeded at 10^6 cells/well/100 µL and incubated for 12h at 37ºC and 5% CO_2 in a 96 well black wall/clear bottom plate. Cells were treated with Bz, AD06 and AD07 at 25%, 50% and 100% IC_{50} (parasites) and challenged with trypomastigotes at 20:1 ratio (parasites:cardiomyocytes). After 48h incubation, culture supernatant was collected and non-internalized parasites were removed by washing the cells with fresh culture medium. Uninfected and infected cells receiving culture medium alone were used as control. After treatment period, cells were incubated in the dark with 50µL ROS deep red dye (total ROS) and MitoROS 580 (mitochondrial superoxide) working solutions for 1 hour. The reactions were read by spectrofluorimetry (Varioskan Flash, Thermo Scientifics, USA) at 650/675 nm (Excitation/Emission) for total ROS and 540/590 nm (Excitation/Emission) for mitochondrial superoxide estimation. ROS and mitochondrial superoxide content was estimated as percentage of control after background subtraction.

Hydrogen peroxide (H_2O_2) was measured cardiomyocytes homogenate using a commercial assay kit (ab102500 Abcam, Cambridge, UK). Briefly, cardiomyocytes were seeded at 10^6 cells/well/100 µL and incubated for 12h at 37ºC and 5% CO_2. Then, cells submitted to the same protocol described below were resuspended in assay buffer, homogenized using a Dounce homogenizeron ice, and centrifuged for 15 min at 8000×*g* and 4ºC. Supernatant samples (50 µL) were mixed with an equal volume of OxiRed-HRP (horse radish peroxidase) working solution and incubated in the dark for 10 min at room temperature. The reactions were read by spectrophotometry (Varioskan Flash, ThermoScientifics, USA) at 535/587 nm (Excitation/Emission). Hydrogen peroxide content was estimated as percentage of control after background subtraction.

2.1.8. Lipid and ProteinOxidation Assay

Lipid oxidation was estimated from malondialdehyde (MDA) levels. For such, MDA was quantified by high-performance liquid chromatography (HPLC), as previously reported

(Brown and Kelly, 1996). Briefly, cardiomyocytes were seeded at 10^6 cells/well/100 µL, treated with Bz, AD06 and AD07 at 25%, 50% and 100% IC_{50} (parasites), and challenged with trypomastigotes at 20:1 ratio (parasites:cardiomyocytes). After 48h incubation at 37ºC and 5% CO_2, culture supernatant was collected and non-internalized parasites were removed by washing the cells with fresh culture medium. Uninfected and infected cells receiving culture medium alone were used as control. Cells were treated with lysis buffer (2% Triton X-100, 50 mMTris [pH 7.5], 40 mM HEPES, and 1 mM EDTA supplemented with 1 mM phenylmethanesulfonyl fluoride) at 4ºC, and for 1 min and centrifuged for 15 min at 13000×*g*. The supernatant was treated with acetonitrile to precipitate proteins, filtered through a 0.2 pm filter and separated on a 250 mm × 4.6 mm i.d. VC-ODS RP18 column with 25 mM phosphate buffer (pH 6.5). Methanol was used as the mobile phase and a flow rate of 0.8 mL min^{-1}. Fluorometric detection was performed at 532nm/553 nm excitation/emission using a RF-10AXL detector coupled to the HPLC system (Shimadzu Scientific Instruments, Kyoto, Japan) to ensure the sensitivity for low concentrations of the MDA-TBA2 adduct. Tetraethoxypropane was processed in the same way as the cell samples and was applied to calibrates MDA-TBA peak (Brown and Kelly, 1996).

Protein carbonyl (PCN) content was concurrently measured using a modified colorimetric biochemical assay based on 2,4-dinitrophenylhydrazine (DNPH) (Mesquita et al., 2014). Briefly, cell pellets obtained after centrifuging the cardiomyocytes lysate were incubated for 15 min with 0.4 mL of 10 mM2,4-dinitrophenylhydrazine (DNPH) prepared in in 0.5 M H_3PO_4 solution. Then, 200 µLNaOH (6 M) was added and incubated for 10 min. The reaction for oxidized proteins involved derivatization of the carbonyl group with DNPH, which generates a stable 2,4-dinitrophenyl (DNP) hydrazone metabolite. The reaction was read by spectrophotometry at 450 nm (Anthos Zenyth 200, Biochrom, Cambridge, UK). The results were corrected considering the cell viability rates determined in the cytotoxicity assay for cardiomyocytes treated with the selected doses of Bz, AD06 and AD07 hybrids.

2.1.9. Trypanosoma cruzi Trypanothione Reductase Activity Assay

Trypanothione reductase activity was investigated in trypomastigotes by using a colorimetric biochemical method previously reported (van den Bogaart et al., 2014). Briefly, 2×10^6 trypomastigotes in culture were treated with Bz, AD06 and AD07 at 25%, 50% and 100% IC_{50}, and incubated at 37ºC and 5% CO_2 for 1h. Then, parasites were lysed by 15 min incubation with 100 µl/well lysis buffer (2% Triton X-100, 50 mM Tris [pH 7.5], 40 mM HEPES, and 1 mM EDTA supplemented with 1 mM protease inhibitor

phenylmethanesulfonyl fluoride). TryR activity was measured in 96-well microplates at 75 µl lysate sample/well. Thus, 25 µl/well NADPH, 75 µl/well $T[S]_2$, and 25 µl/well DTNB were sequentially added to the sample lysate to respectively obtain 200, 75, and 100 µM (final concentrations). A blank was obtained for each sample, which was prepared from sample lysate supplemented with the reaction mixture with 0.05 M Tris buffer (pH 7.5) replacing $T[S]_2$ substrate. The mixtures were incubated for 3h at 27°C, and the absorbance was measured by spectrophotometry at 412 nm. The blank signal subtracted from the sample absorbance corresponded to TryR activity, which developsa colored reaction from 2-nitro-5-thiobenzoate production. The results were corrected considering the cell viability rates determined in the cytotoxicity assay for trypomastigotes treated with the selected doses of Bz, AD06 and AD07 hybrids.

2.1.10. Total Antioxidant Capacity Assay

Antioxidant markers were analyzed in 10^6 cardiomyocytes treated with Bz, AD06 and AD07 at 25%, 50% and 100% IC_{50} (parasites), and challenged with trypomastigotes at 20:1 ratio (parasites:cardiomyocytes). After 48h incubation at 37ºC and 5% CO_2, cells were treated with lysis buffer at 4ºC, sonicated for 1 min and centrifuged for 15 min at 8000×*g*. Total antioxidant capacity in celllysates was measuredusing a colorimetric biochemical assay following the manufacturer's instructions (Abcam, Cambridge, UK). In this method, Cu^{2+} ions are converted to Cu^{+} by protein and non-protein antioxidants. Protein masks are used to prevent Cu^{2+} reduction by proteins, allowing isolating the effect of these molecules on the cellular antioxidant capacity. The reduced Cu^{+} ion was chelated with a colorimetric probe giving a broad absorbance peak near to 570 nm, which is proportional to the cellular antioxidant capacity. The results were corrected considering the cell viability rates determined in the cytotoxicity assay for cardiomyocytes treated with the selected doses of Bz, AD06 and AD07 hybrids.

2.1.12. Antioxidant Enzymes Assay

Enzymatic antioxidant activity were investigated in *T. cruzi*-challenged cardiomyocytes submitted to the same treatments reported. Catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and glutathione peroxidase (GPx) activities were analyzed from 96-wells commercial kits and the manufacturer’s instructions. Catalase (ThermoFisher Scientific, Waltham, Massachusetts, USA), SOD (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and GP (Abcam, Cambridge, UK) activities were measured from

colorimetric kits. Catalase, SOD and GPx activities were respectively monitored at 560 nm, 450 nma nd 340 nm, and the assays sensitivities were 0.052 U/ml (CAT), 0.044 U/ml (SOD) and 0.5 U/ml (GPx). Glutathione reductase activity was measured by fluorimetry at 390 nm/510 nm excitation/emission usinga 96-wells kit following the manufacturer's instructions (ThermoFisher Scientific, Waltham, Massachusetts, USA). The assays sensitivity for GR was 0.009 mU/ml.

2.1.13. Hydrogen Peroxide-Mediated Cardiomyocytes and Trypanosoma cruzi Toxicity Assay

Cardiomyocytes (10^6 cells/well) and trypomastigotes (2×10^6 parasites/well) were plated in 96-well polystyrene microplates and incubated for 48h at 37 ºC and 5% CO_2 with culture medium containing 0.67µg/ml Bz, 1.28µg/ml AD06 and 1.33µg/ml AD07,doses immediately below the IC_{50} for trypomastigotes. Then, culture medium was replaced with 200µl fresh culture medium supplemented or not with different H_2O_2 concentrations for cardiomyocytes (0,50, 100, 200, and 300 µMH_2O) (Qiu et al., 2019) and *T. cruzi* (0, 100, 200, 300 and 400 µM H_2O) (de Moura et al., 2009). Cardiomyocytes and trypomastigotes cytotoxicity was investigated from three independent assays. Control cardiomyocytes and parasites were treated with culture medium alone without H_2O_2 (vehicle). After 48h incubation, 20µL of 1mM resazurin solution was added to each well, and cell viability was measured according to the absorbance obtained from a microplate reader calibrated to 600nm and 570nm wavelength (Mazzeti et al., 2019).

2.1.14. Antioxidant and Prooxidant Regulatory Genes

Cardiomyocytes gene expression was analyzed by quantitative reverse transcription PCR (qPCR) as previously described (Guo et al., 2018). First, cardiomyocytes were seeded at 10^6 cells/well/100 µL, treated with Bz, AD06 and AD07 at 25%, 50% and 100% IC_{50} (parasites), and challenged with trypomastigotes at 20:1 ratio (parasites:cardiomyocytes). After 48h incubation atat 37ºC and 5% CO_2, total RNA was extracted using TRIzol reagent, resuspended in nuclease-free water, and quantified by UV spectrophotometry (NanoDrop 2000, ThermoFisher Scientific, Waltham, MA, USA). cDNA was synthesized with 1µg of total RNA using a commercial reverse-transcription kit following the manufacturer's instructions (ThermoFisher Scientific, Waltham, MA, USA).Validated primers for *HO-1*, *NFR2*, *NQO-1* and β-actin were used (Guo et al., 2018). The nucleotide sequences of the forward and reverse primers are shown in Table 1. Quantitative PCR reactions used SYBR Green PCR Mastermix, and were performed according to the manufacturer's instructions

(Applied Biosystems, Carlsbad, CA, USA). Reactions were performed using the 7900HT Real-Time PCR Detection System (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA). Data were standardized to the *β-actin* housekeeping gene. The relative expression level of each mRNA was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

Table 1. Primers used to analyze the expression of genes involved in oxidative stress using quantitative polymerase chain reaction (qPCR).

HO-1	Forward	5′-GCGAAACAAGCAGAACCCA-3′
	Reverse	5′-GCTCAGGATGAGTACCTCCCA-3′
NFR2	Forward	5′-CCATGCCTTCTTCCACGAA-3′
	Reverse	5′-AGGGCCCATGGATTTCAGTT-3′
NQO-1	Forward	5′-AACGTCATTCTCTGGCCAATTC-3′
	Reverse	5′-GCCAATGCTGTACACCAGTTGA-3′
B-actin	Forward	5′-TACAACCTCCTTGCAGCTCC-3′
	Reverse	5′-GGATCTTCATGAGGTAGTCAGTC-3′

All primers were validated: ExpTher Med. 2018; 16(4): 3333–3344.

2.1.15. Cardiac Troponin I Assay

Cardiac troponin I (cTnI) levels were quantified culture supernatant as a marker of microstructural cardiomyocytes damage (Bonney et al., 2013). For such, cardiomyocytes were seeded at 10^6 cells/well/100 µL, treated with Bz, AD06 and AD07 at 25%, 50% and 100% IC_{50} (parasites), and challenged with trypomastigotes at20:1 ratio (parasites:cardiomyocytes). After 48h incubation at 37ºC and 5% CO_2, culture medium was collected and centrifuged at 8000 ×*g*and 4ªC for 15 minutes in the presence of protease inhibitor (Sigma-Aldrich, San Luis, MO, USA). The supernatant was collected and cTnI levels were quantified in 50 µL samples by using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (MyBioSource, San Diego, CA, USA). Cardiac troponin I was quantified by spectrophotometry at 450 nm by using a 99-wells microplate reader (Anthos Zenyth 200, Biochrom, Cambridge, UK). This method has detection limits ranging from 7.813 to 500pg/ml.

2.2 *In vivo assays*

2.2.1Animals, Infection, Drugs and Treatments

Five groups with 30-days-old female Swiss mice (20-25g) were kept in a room with temperature (21±2 ºC) and air humidity (60-70%) controlled, and access to water and food *ad libitum*. After 1-week acclimatization, mice were inoculated intraperitoneally with 5×10^3 *T. cruzi* trypomastigotes (Y-strain) obtained from the blood of previously infected animals. Uninfected untreated animals were used as negative control, while infected untreated mice were admitted as positive control. The others groups were treated with Bz (reference antiparasitic drug) or AD06 and AD07 hybrids, which were administered at 100 mg/kg of body weight for seven consecutive days, starting on the fifth day after infection in which infection was microscopically confirmed (positive parasitemia) (Filardi and Brener, 1987). All drugs were solubilized in 0.9% NaCl solution containing 5% cremophor (Merck, São Paulo, SP).The Institutional Animal Research Ethics Committee approved this study as (Protocol number 59/2017).

2.2.2 Blood parasitism and Toxicity Assay

The assessment of blood parasitemia was performed daily by analyzing 5µL of peripheral blood collected from the tail of each infected animal (Brener, 1962). The blood was distended on a histological slide and was observed under abright field microscope (AxioScope A1, Carl Zeiss, Germany) to count trypomastigotes in fifty random fields using a ×40 objective lens. The results of parasite counting were used to calculate the peak parasitemia, mean parasitemia and area under the parasitemia curve (AUC) (Novaes et al., 2017). At the end of the experiment, the animals were euthanized using anesthesia (150 mg/kg ketamine and 16 mg/kg xylazine, i.p.) and cardiac puncture. Blood was collected and centrifuged with EDTA at 3500 ×*g* and 4°C for 15 minutes. Circulating levels of the hepatic function enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were quantified in plasma samples from an automatic method using the LabmaxPlenno device following the manufacturer's guidelines (Labtest, Lagoa Santa, MG, Brazil).

2.2.3 Immunoglobulin G Immunoassay

Plasma samples were also and used for detection of class IgG anti-*T. cruzi* antibodies. Antibodies were analyzed by enzyme-linked immunosorbent assay (ELISA), according

protocol previously reported (Voller et al., 1976). Briefly, 96-wells polystyrene ELISA plates were coated with *T. cruzi* antigen previously prepared with epimastigotes in exponential growth in LIT medium. Then, plates were incubated with 50µl plasma samples of control and infected animals for 12h at 4º C. Then, each well was treated with anti-mouse peroxidase-conjugated IgG, IgG1, IgG2a and IgG2b antibodies (PeproTech, RibeirãoPreto – SP, Brazil), which were used as detection probes. After 4h incubation at room temperature, the plates were treated with the peroxidase substrate ABTS (2,2'-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid) Diammonium Salt) for 30 minutes and the reaction was stopped with 1H HCl. The optical density was read at 490 nm using a microplate spectrophotometer (Anthos Zenyth 200; Biochrom, Cambridge, UK). To discriminate between positive and negative results, mean absorbance was used as the cutoff point (Vilas-Boas et al., 2022).

2.2.4 Necropsy and Heart Microstructure

After euthanasia (day 13 post-infection) the heart was removed. Heart samples were fixed in10% buffered formalin (pH 7.2) solution for 24 hours. The samples were dehydrated in ethanol, clarified in xylene and embedded in paraffin. Paraffin blocks were cutat 5-µm thickness slices using a rotary microtome, stained with haematoxylin and eosin (H&E) (Sequettoet al., 2014), mounted with coverslips, and used for quantitative histopathological and microstructural analysis (Novaes et al., 2018). Histological slides were observed and digital images were captured using a bright field photomicroscopecoupled to the AxioVision image analysis software (AxioScope A1, Carl Zeiss, Germany). The occurrence of myocarditis was assessed by counting interstitial/inflammatory nuclei in twenty random histological images for each animal. (Carl Zeiss, Germany) (Rodrigues et al., 2017).

2.3. Statistical Analysis

The results were presented as mean and standard deviation (mean ± S.D.). Data distribution was verified according to the D'Agostino-Pearson's K2 normality method. Data with normal distribution were compared using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post-hoc* test. Non-parametric data were compared using the Kruskal-Wallis one-way ANOVA on Ranks test followed by the Student-Newman-Keuls method. Results with $P \leq 0.05$ were statistically significant.

3. Results

3.1. In vitro findings

Table 2 indicate that AD06 and AD07 hybrids exhibited potent inhibitory activity against *T. cruzi* trypomastigotes with 2.57µg/mL $IC_{50}$and 2.66µg/mLIC_{50}, respectively. Although these results were not superior to those obtain with Bz (1.34µg/mLIC_{50}), it is possible to see that molecular hybridization conferred greater trypanocidal activity, inducing IC_{50} below that presented by metronidazole and eugenol administered alone. Although dihydroeugenol, which is a synthetic phenol analogous to eugenol, initially showed a better percentage of inhibition on *T. cruzi* epimastigotes and trypomastigotes, it showed higher toxicity than all other molecules, indicating an undesirable toxic characteristic.

Table 2. Antiparasitic, cytotoxic and selective potential of metronidazole hybrids compared to benznidazole on*Trypanosoma cruzi*and cardiomyocyres *in vitro.*

	IC_{50} Epi.(µg/mL)	**IC_{50} Tryp.(µg/mL)**	**CC_{50} (µg/mL)**	**SI Epi.**	**SI Tryp.**
Benznidazole	5.06±0.08	1.34 ± 0.01	>200	39.50	149.00
Metronidazole	32.81 ± 1.33	30.83 ± 5.42	133.95 ± 10.25	4.08	4.34
Eugenol	10.20 ± 3.07	45.03 ± 4.34	41.32 ± 12.71	4.05	0.91
Dihydroeugenol	3.32 ± 0.73	1.95 ± 0.18	14.19 ± 9.40	4.27	7.27
AD06	11.58 ± 0.57	2.57 ± 0.34	149.90 ± 18.70	12.94	58.13
AD07	9.49 ± 0.55	2.66 ± 0.07	108.87 ± 8.77	11.47	40.92

AD06: Hybrid of metronidazole connected to eugenol and nitrated in meta position to hydroxyl. AD07: Hybrid of metronidazole connected to dihydroeugenol and nitrated n ortho position to hydroxyl. IC_{50}: Half maximal inhibitory concentration on different parasite forms, CC_{50}: Half maximal cytotoxic concentration on H9c2 cardiomyocytes. SI: Selectivity index. Epi: Epimastigotes. Tryp: Trypomastigotes. IC_{50} and CC_{50} are represented as mean and standard deviation.

In the cell invasion assay (Fig. 2), untreated *T. cruzi*-challenged cardiomyocytes (group UUC) showed a high infection rate, cell hypertrophy, and marked amastigotes cytoplasmic accumulation.All treatments attenuated cell parasitism and parasite load in a dose-dependent way. This antiparasitic effect was more evident in Bz- and AD07-treated cardiomyocytes,

whose infection rate and amastigotes accumulation was mainly attenuated in cell receiving the higher dose (10 µg/mL) of these drugs.

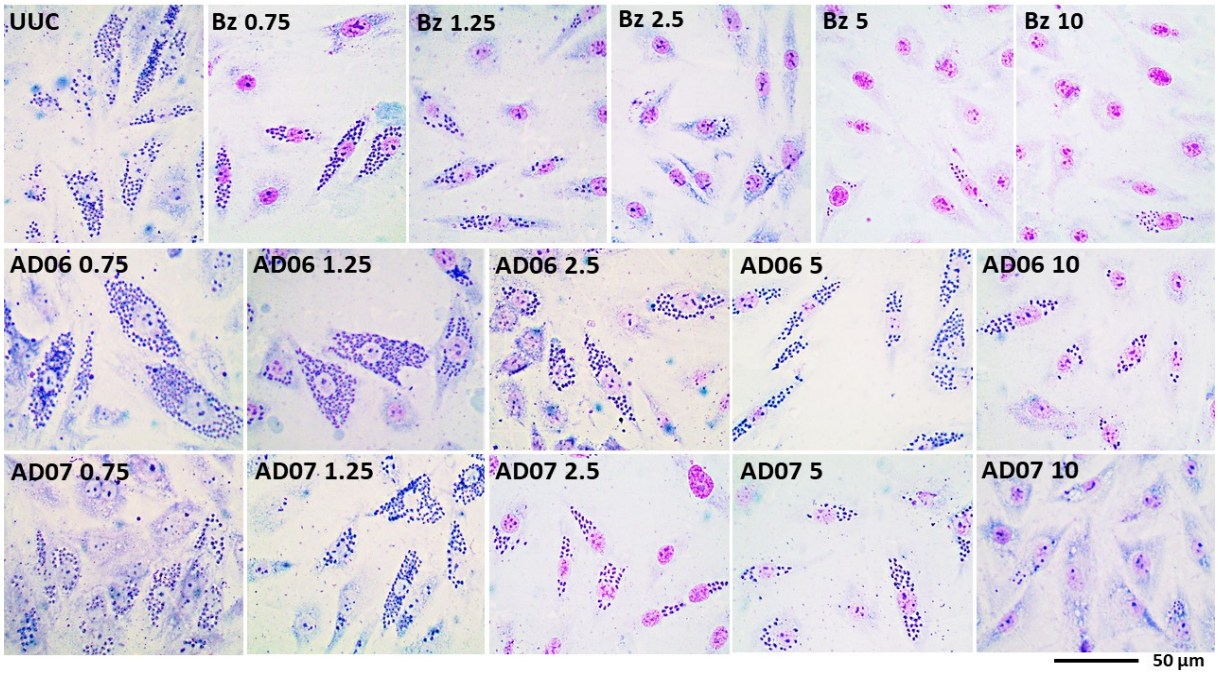

Fig. 2. Representative photomicrography's of H9c2 cardiomyocytes cultures 48h after *Trypanosoma cruzi* challenge and treatment with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz) at 0.75, 1.25, 2.5, 5, and 10 µg/mL (Bright field microscopy, Giemsa staining). UUC= cardiomyocytes untreated infected with *T. cruzi*. Each basophilic point in cardiomyocytes cytoplasm represents *T. cruzi* amastigotes.

Infection rate and cell parasitismin in *T. cruzi*-challenged H9c2 cardiomyocytes treated with Bz, AD06 and AD07 hybrids are shown in Fig. 4. Although none of the treatments eliminated intracellular parasites; Bz, AD06 and AD07 reduced cardiomyocyte infection rate in all doses investigated compared to infected untreated cells ($P<0.05$). This response exhibited a dose-dependence behavior, with the best results achieved in the highest concentration of all treatments investigated. The endocytic index revealed a high parasitic load in infected untreated cells, demonstrating that all treatments also attenuated cardiomyocytes parasitism, especially in the higher concentrations used ($P< 0.05$).

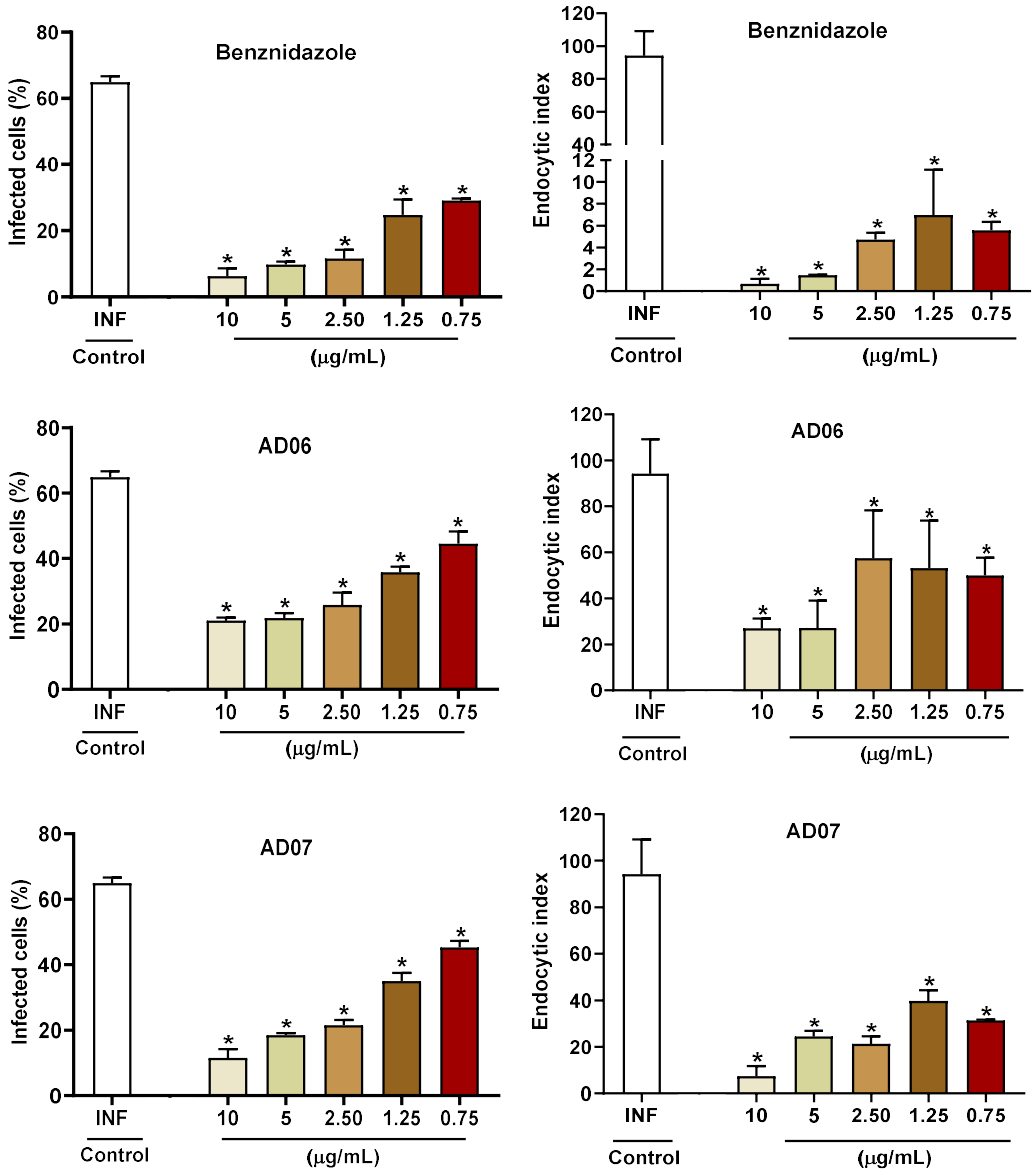

Fig. 4. Infection rate and cell parasitism (endocytic index) in *Trypanosoma cruzi*-challenged H9c2 cardiomyocytes treated with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz). INF= cardiomyocytes untreated challenged with *T. cruzi*, BZ, AD06 and AD07= cardiomyocytes respectively treated with benznidazol, AD06 and AD07 hybrids. Data are expressed as mean and standard deviation. *Statistical difference ($P \leq 0.05$) between the groups in relation to the control group INF.

As indicated in Fig. 5, total ROS, mitochondrial superoxide and hydrogen peroxide levels were increased in infected untreated cardiomyocytes compared to uninfected untreated cardiomyocytes ($P<0.05$). These parameters were reduced in *T. cruzi*-challenged cells

receiving all Bz and AD07 doses compared to infected untreated cardiomyocytes ($P<0.05$). Bz and AD07 treatment also exhibited a dose-dependent effect on these pro-oxidant parameters. AD06 was effective in reducing total ROS, mitochondrial superoxide and hydrogen peroxide levels only in the highest dose compared to infected untreated cardiomyocytes ($P<0.05$). Total ROS and hydrogen peroxide levels were similar, while mitochondrial superoxide was increased in *T. cruzi*-challenged cells treated with the highest Bz and AD07 doses compared to uninfected untreated cardiomyocytes ($P>0.05$).

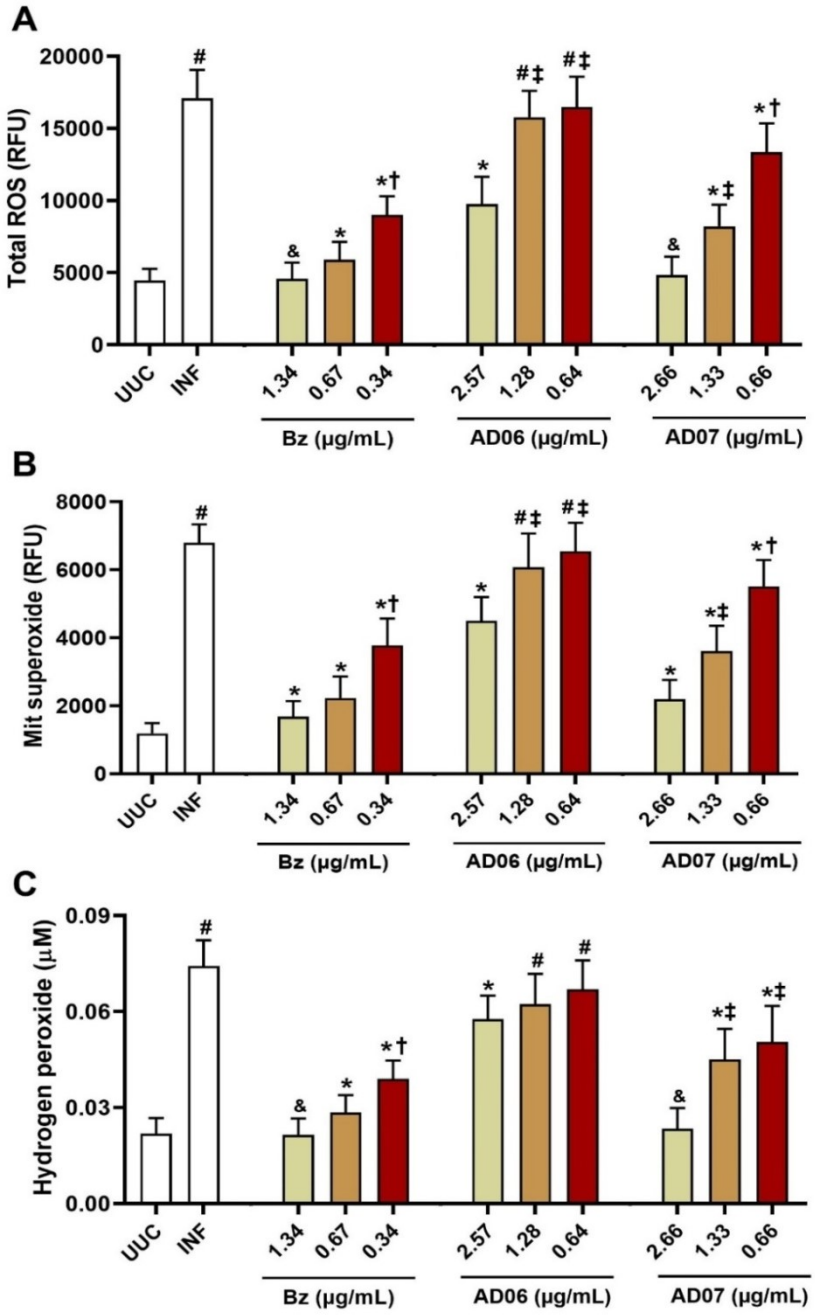

Fig. 5. Total reactive oxygen species (ROS - A), mitochondrial superoxide (B), and hydrogen peroxide (C) production in *Trypanosoma cruzi*-challenged H9c2 cardiomyocytes treated with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz). Groups: UUC= uninfected untreated cardiomyocytes, INF= cardiomyocytes untreated challenged with *T. cruzi*, AD06 and AD07= cardiomyocytes treated with AD06 and AD07 at 100%, 50% and 25% IC_{50} for *T. cruzi* and challenged with this parasite .Data are expressed as mean and standard deviation. Statistical difference ($P \leq 0.05$) among different drugs compared to *UUCand INF;& INF; # UUC; † Bz 1.34 / 0.67, AD06 2.57 / 1.28, and AD07 2.66 / 1.33; ‡ Bz 1.34, AD06 2.57, and AD07 2.66.

As indicated in Fig. 6, lipid (MDA) and protein (PCN) oxidation were increased in infected untreated cardiomyocytes compared to uninfected untreated control cardiomyocytes (P<0.05). A dose-dependent attenuation in these parameters was observed in *T. cruzi*-challenged cells receiving Bz and AD07 compared to infected untreated cardiomyocytes (P<0.05). AD06 was effective in reducing MDA and PCN levels only the highest dose compared to infected untreated cardiomyocytes (P<0.05). PCN levels in uninfected untreated control cardiomyocytesand*T. cruzi*-challenged cells treated with the highest Bz and AD07 doses were similar(P>0.05). Only the highest AD07 doses prevented MDA increase in *T. cruzi*-challenged cells compared to uninfected untreated control cardiomyocytes (P>0.05).

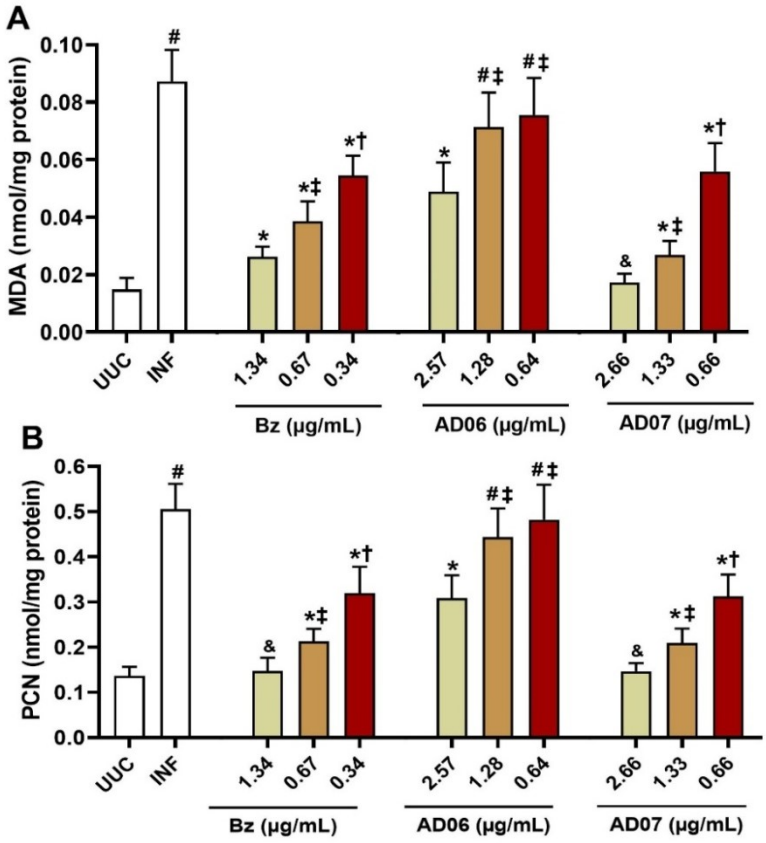

Fig. 6. Malondialdehyde (MDA) and protein carbonyl (PCN) heart levels in *Trypanosoma cruzi*-challenged H9c2 cardiomyocytes treated with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz). Groups: UUC= uninfected untreated cardiomyocytes, INF= cardiomyocytes untreatedchallenged with *T. cruzi*, AD06 and AD07= cardiomyocytes treated with AD06 and AD07 at 100%, 50% and 25% IC_{50} for *T. cruzi* and challenged with this parasite. Data are expressed as mean and standard deviation. Statistical difference (P≤0.05) among different drugs compared to *UUC and INF; & INF; # UUC; † Bz 1.34 / 0.67, AD06 2.57 / 1.28, and AD07 2.66 / 1.33; ‡ Bz 1.34, AD06 2.57, and AD07 2.66.

As shown in Fig. 7, the enzymatic assay indicated that Bz exerted no effect on *T. cruzi* trypanothione reductase activity compared to untreated parasites ($P>0.05$). This activity was attenuated in parasites treated with AD06 at 100% IC_{50}, as well as AD07 at 100% and 50% IC_{50} for *T. cruzi* trypomastigotes. This response revealed a dose-dependent effect of AD07 on trypanothione reductase activity. In addition, TAC was attenuated in untreated *T. cruzi*-challenged cardiomyocytes compared to uninfected untreated control cells ($P<0.05$). A dose-dependent attenuation in TAC was observed in *T. cruzi*-challenged cells receiving Bz and AD07 compared to infected untreated cardiomyocytes ($P<0.05$). TAC was similar in *T. cruzi*-challenged cells treated with the highest Bz and AD07 doses compared to uninfected untreated control cardiomyocytes ($P>0.05$). Only *T. cruzi*-challenged cells treated with the highest AD06 dose presented increased TAC compared to infected untreated cardiomyocytes ($P<0.05$).

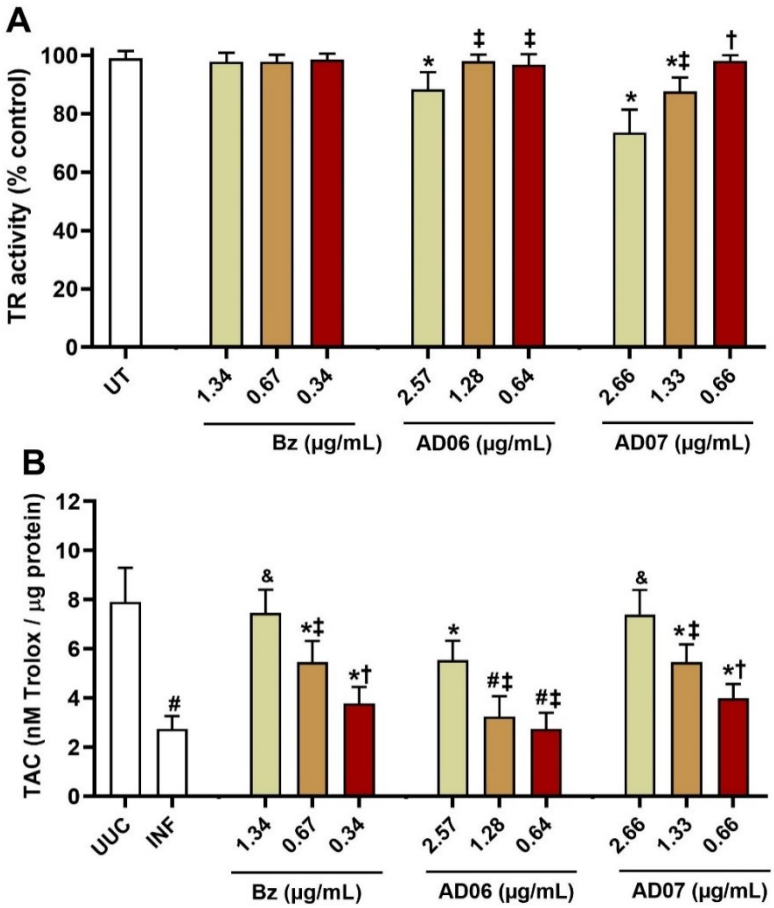

Fig. 7. Trypanothione reductase activity in *Trypanosoma cruzi*(A) and total antioxidant in *T. cruzi*-challengedH9c2 cardiomyocytes (B) treated with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz). Groups: UT= untreated parasites, UUC= uninfected untreated cardiomyocytes, INF= cardiomyocytes untreated challenged with *T. cruzi*, AD06 and AD07= AD06 and AD07 administered at 100%, 50% and 25% IC_{50} calculated for *T. cruzi.* Data are expressed as mean and standard deviation. Statistical difference ($P \leq 0.05$) among different drugs compared to *UT, UUC and INF; ‡ Bz 1.34, AD06 2.57, and AD07 2.66; † Bz 1.34 / 0.67, AD06 2.57 / 1.28, and AD07 2.66 / 1.33; & INF; # UUC.

As indicated in Fig. 8, CAT, SOD, GR and GPx activities were reduced in untreated *T. cruzi*-challenged cardiomyocytes compared to uninfected untreated cardiomyocytes ($P<0.05$). Bz and AD07 exerted dose dependent effects on CAT, SOD, GR and GPx activities in *T. cruzi*-challenged cardiomyocytes. In general, the enzymatic antioxidant activities were improved in *T. cruzi*-challenged cells receiving Bz, AD06 and AD07 at 100% IC_{50}, as well as Bz and AD07 at 50% IC_{50} calculated for *T. cruzi* trypomastigotes compared to infected untreated cardiomyocytes ($P<0.05$). Cat and SOD activities were similar, in *T. cruzi*-

challenged cells treated with the highest Bz and AD07 doses compared to uninfected untreated control cells (P>0.05).

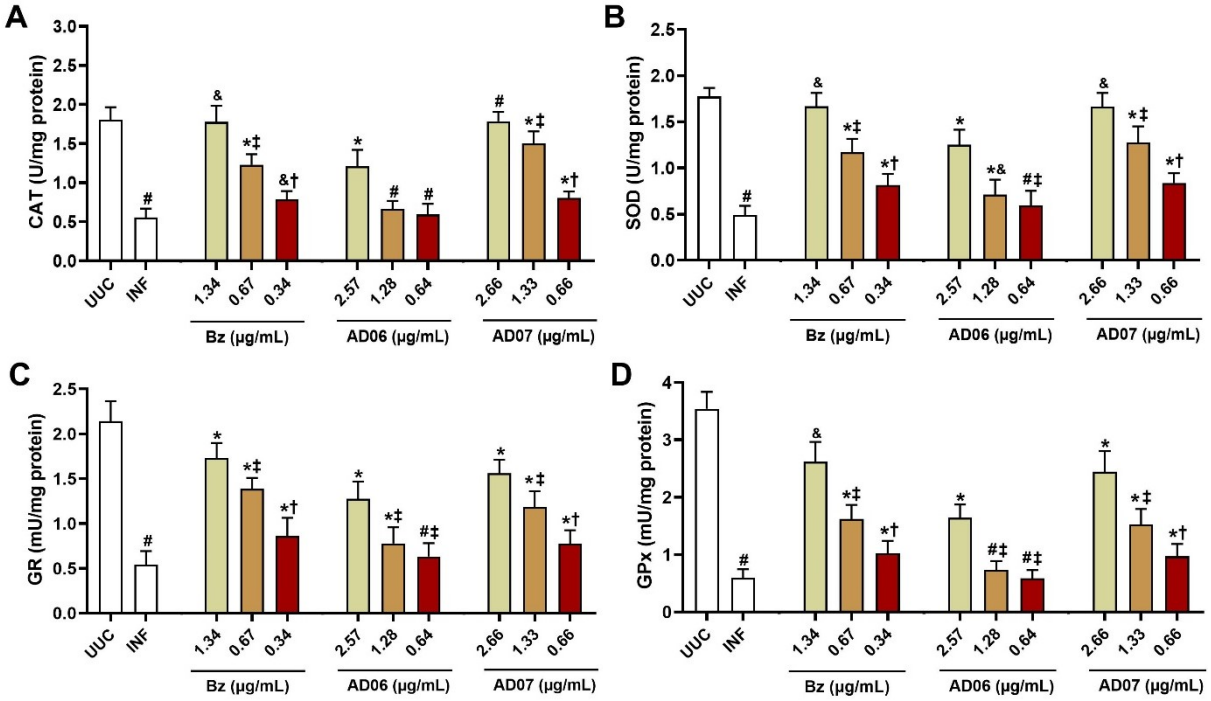

Fig. 8. Activity of the antioxidant enzymes catalase (CAT – A), superoxide dismutase (SOD – B), glutathione reductase (GR – C) and glutathione peroxidase (GPx – D) in *Trypanosoma cruzi*-challenged H9c2 cardiomyocytes treated with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz). Groups: UUC= uninfected untreated cardiomyocytes, INF= cardiomyocytes untreated challenged with *T. cruzi*, AD06 and AD07= cardiomyocytes treated with AD06 and AD07 at 100%, 50% and 25% IC_{50} for *T. cruzi* and challenged with this parasite. Data are expressed as mean and standard deviation. Statistical difference (P≤0.05) among different drugs compared to *UUC and INF; & INF; # UUC; † Bz 1.34 / 0.67, AD06 2.57 / 1.28, and AD07 2.66 / 1.33; ‡ Bz 1.34, AD06 2.57, and AD07 2.66.

As indicated in Fig. 9, HO-1, NFR2 and NQO-1 gene expression was increased in untreated *T. cruzi*-challenged cardiomyocytes compared to uninfected untreated cells (P<0.05). Bz and AD07 exerted dose dependent effects on gene expression in *T. cruzi*-challenged cardiomyocytes. In general, gene expression was downregulated in *T. cruzi*-challenged cells receiving Bz, AD06 and AD07 at 100% IC_{50}, as well as Bz and AD07 at 50% and 25% IC_{50} calculated for *T. cruzi* compared to infected untreated cardiomyocytes (P<0.05). HO-1 and NFR2 gene expression was similar in *T. cruzi*-challenged cells treated with AD06

at 50% and 25% IC_{50} compared to infected untreated cells (P>0.05). No treatment determined gene expression levels similar to uninfected untreated control cardiomyocytes (P<0.05).

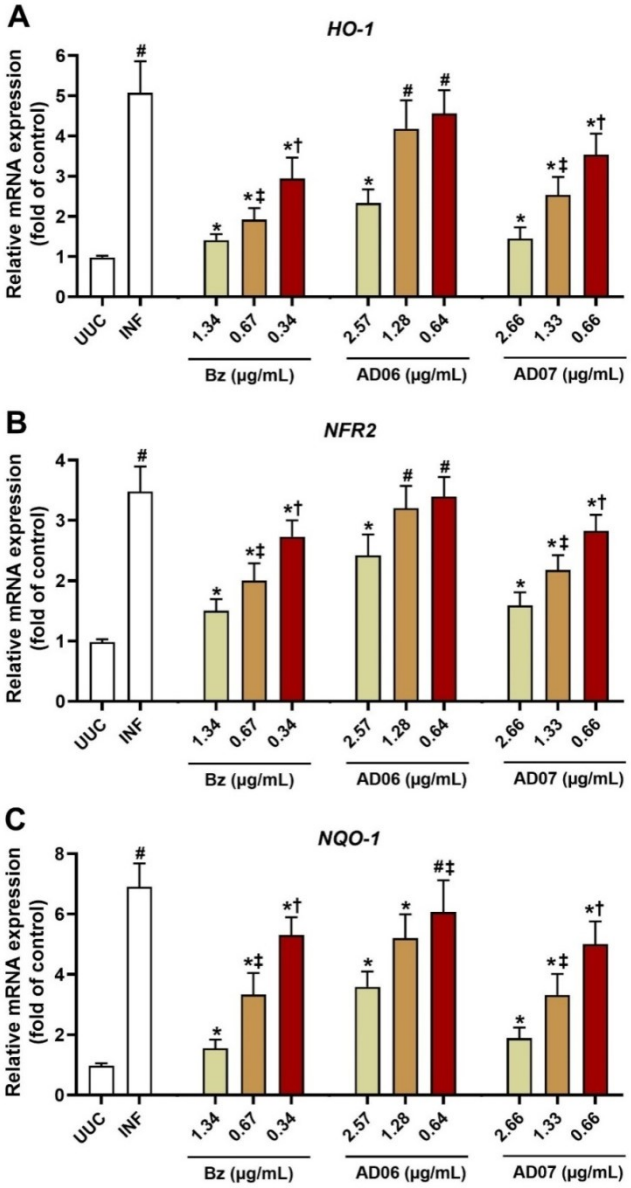

Fig. 9. Heme oxygenase-1 (HO-1), nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and NAD(P)H dehydrogenase (quinone) 1 (NQO-1) gene expression in *Trypanosoma cruzi*-challenged H9c2 cardiomyocytes treated with newmetronidazole/eugenol-based hybrids(AD06 and AD07) and benznidazole (Bz). Groups: UUC= uninfected untreated cardiomyocytes, INF= cardiomyocytes untreated challenged with *T. cruzi*, AD06 and AD07= cardiomyocytes treated with AD06 and AD07 at 100%, 50% and 25% IC_{50} for *T. cruzi* and challenged with this parasite. Data are expressed as mean and standard deviation. Statisticaldifference (P≤0.05) among different drugs compared to *UUC and INF; & INF; # UUC; † Bz 1.34 / 0.67, AD06 2.57 / 1.28, and AD07 2.66 / 1.33; ‡ Bz 1.34, AD06 2.57, and AD07 2.66.

Cardiomyocytes and *T. cruzi* trypomastigotes exhibited a dose-dependent susceptibility to H_2O_2-based pro-oxidant challenge (Fig. 10). Reduced cardiomyocytes viability was detected only in cells treated with Bz at 50% IC_{50} calculated for *T. cruzi* and exposed to the highest H_2O_2 concentration (300 μM) compared to untreated parasites ($P<0.05$). At 50% IC_{50}, Bz and AD07 reduced trypomastigotes viability in all H_2O_2 concentrations analyzed compared to untreated and AD06-treated parasites ($P<0.05$). This effect was most prominent in Bz-treated compared to AD07-treated trypomastigotes exposed to the highest H_2O_2 concentrations (200-400 μM) ($P<0.05$).

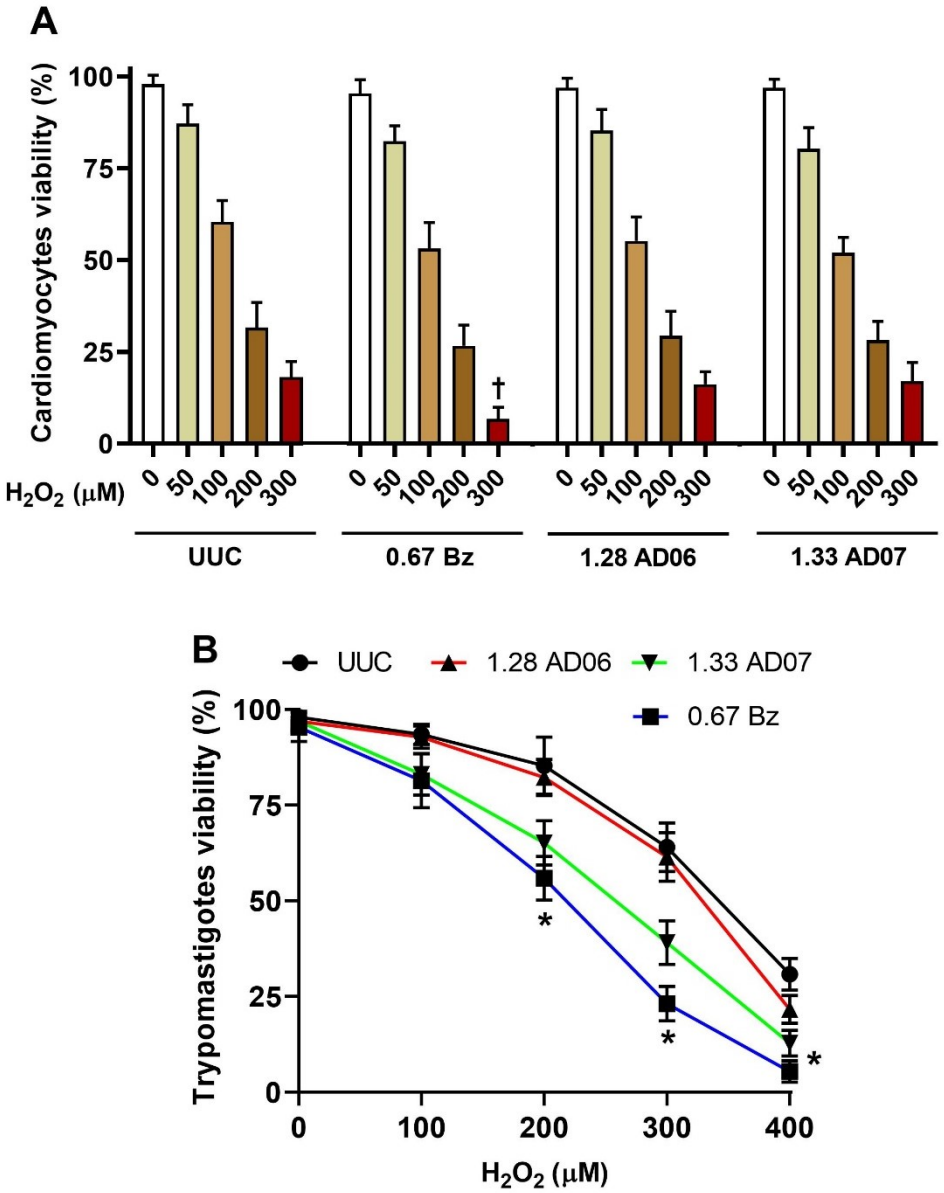

Fig. 10. Hydrogen peroxide (H_2O_2) resistance in H9c2 cardiomyocytes (A) and trypomastigotes (B) treated with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz). Groups: UUC= uninfected untreated cardiomyocytes, AD06 and AD07= cardiomyocytes treated with AD06 and AD07 at 50% IC_{50} calculated for *T. cruzi*. Data are expressed as mean and standard deviation. Statistical difference ($P \leq 0.05$) among different drugs compared in (A) to † UUC, AD06 and AD07 at 300 μM H_2O_2; and in (B) to * UUC and AD06 at 200, 300 and 400 μM H_2O_2.

As shown in Fig. 11, cardiac troponin I was markedly increased in untreated *T. cruzi*-challenged cardiomyocytes compared to uninfected untreated cells ($P<0.05$). Bz and AD07

exerted dose dependent effects on cardiac troponin I extracellular levels in *T. cruzi*-challenged cardiomyocytes. This molecule was reduced in *T. cruzi*-challenged cells receiving Bz, AD06 and AD07 at 100% IC_{50}, as well as Bz and AD07 at 50% IC_{50} calculated for *T. cruzi* compared to infected untreated cardiomyocytes ($P<0.05$). Troponin I levels were similar in *T. cruzi*-challenged cells treated with AD06 and AD07 at 25% IC_{50} compared to infected untreated cells ($P>0.05$). No treatment determined similar troponin I extracellular levels comparedto uninfected untreated control cardiomyocytes ($P<0.05$).

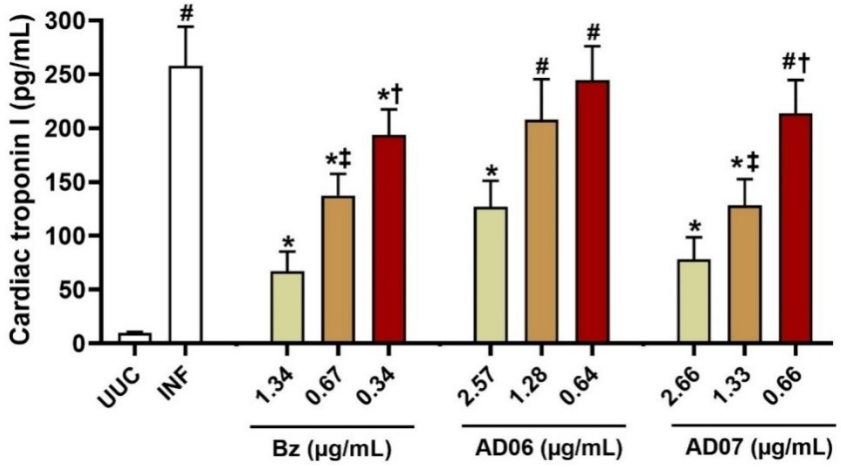

Fig. 11. Cardiac troponin I levels in *Trypanosoma cruzi*-challenged H9c2 cardiomyocytes treated with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz). Groups: UUC= uninfected untreated cardiomyocytes, INF= cardiomyocytes untreated challenged with *T. cruzi*, AD06 and AD07= cardiomyocytes treated with AD06 and AD07 at 100%, 50% and 25% IC_{50} for *T. cruzi* and challenged with this parasite. Data are expressed as mean and standard deviation. Statistical difference ($P\leq0.05$) among different drugs compared to *UUC and INF; # UUC; † Bz 1.34 / 0.67, AD06 2.57 / 1.28, and AD07 2.66 / 1.33; ‡ Bz 1.34, AD06 2.57, and AD07 2.66.

3.2. In vivo findings

Parasitemia and mortality findings in mice are shown in Fig. 12 and the Table 3. Uninfected animals were also investigated during the entire experiment, but circulating parasites were not identified, confirming the absence of infection. All mice inoculated with *T. cruzi* developed the infection and presented a prepatent period of five days, confirmed by the

microscopic fresh blood test. Parasitemia peak was anticipated and attenuated in the groups receiving Bz and AD07. This parameter was late in AD06-treated animals. Bz and AD07 were effective in attenuating parasitemia peak and mean parasitemia compared to untreated and AD06-treated infected animals. A complete trypomastigotes clearance was obtained only in Bz-treated animals.

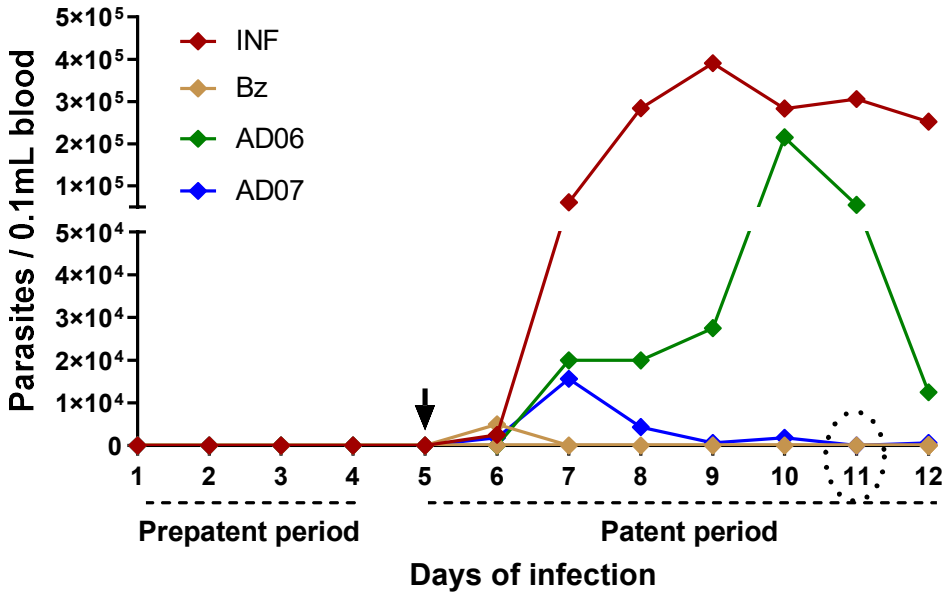

Fig. 12. Parasitemia curve obtained from *Trypanosoma cruzi*-infectedmice treated with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz). INF: Infected and untreated (n=10); Bz: infected and treated with 100 mg/kg benznidazole (n=8); AD06: infected and treated with 100 mg/kg AD06 (n=8); AD07: infected and treated with 100 mg/kg AD07 (n=8). The lines represent the variation in mean blood parasitism observed in all experimental groups during the experimental period.Treatment was administered daily orally for seven consecutive days (5-11). Arrow: infection confirmation and treatment initiation (day 5). Dotted ellipse last day of treatment (day 11).

In Table 3, quantitative analysis confirmed Bz and AD07 as the most effective in attenuating mean and peak of parasitemia compared to the groups INF and AD06 ($P<0.05$). AD06 presented the some antiparasitic effect relative to the mean parasitemia obtained in the group INF ($P<0.05$). No deaths were recorded during the experimental period, indicating the good tolerability of all treatments.

Table 3. Mortality rate, peak of parasitemia and area under the parasitemia curve (AUC) obtained from *Trypanosoma cruzi*-infected mice treated with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz).

Groups	Mortality rate	Mean Parasitemia*	Parasitemia Peak*	AUC
INF	0%	22.56×10^{4a}	3.91×10^{5a}	14.54×10^{5a}
Bz	0%	0.89×10^{2b}	5.00×10^{3b}	5.00×10^{3b}
AD06	0%	78.66×10^{c}	2.15×10^{5a}	54.00×10^{c}
AD07	0%	3.57×10^{b}	1.56×10^{4c}	25.00×10^{d}

* Mean values of Parasites/0.1 mL of freshblood. INF: Infected untreated (n=10); Bz: infected treated with 100 mg/kg benznidazole (n=8); AD06: infected and treated with 100 mg/kg AD06 (n=8); AD07: infected and treated with 100 mg/kg AD07 (n=8). a, b, c Groups marked with different letters have statistical difference ($P<0.05$), and groups marked with any letter in common have no statistical difference ($P>0.05$) by the Kruskal-Wallis One Way Analysis of Variance on Ranks followed by the Tukey post-hoc test.

As shown in Fig. 13, the three treated groups had lower AST levels than the PC group ($P<0.05$). Hybrid AD06 induced AST level in animals similar to that presented by the group treated with BZ. Incredibly, the AD07 hybrid showed a result superior to that presented by the reference drug Bz, which, in addition to the lowest AST level, was also the only treatment to confer an AST level equal to that presented by the animals in the NC group ($P<0.05$). Animals treated with the AD06 hybrid showed similar ALT levels to the animals in the PC group ($P<0.05$) and above the ALT levels of animals treated with Bz and the AD07 hybrid ($P>0.05$). In this parameter, the animals that received the treatment with Bz and with the hybrid AD07 had levels similar to the NC group ($P<0.05$). Comparing these results with the somatic index, it is possible to perceive a profile very similar to that presented by the measurement of ALT levels, where animals treated with Bz and with the AD07 hybrid were similar ($P<0.05$). Although these two therapeutic regimens had a somatic index higher than the NC ($P>0.05$), they were the only ones that were lower than the PC group ($P<0.05$).

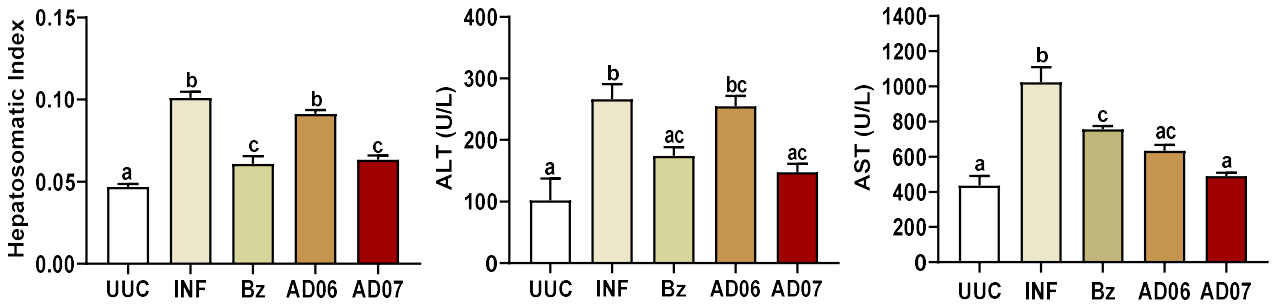

Fig. 13. Hepatosomatic index and hepatic function enzymes plasma levels in control uninfected and *Trypanosoma cruzi*-infected mice treated with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz).UUC: Uninfected untreated control (n=10); INF: infected untreated (n=8); Bz: infected treated with 100 mg/kg benznidazole (n=8); AD06: infected treated with 100 mg/kg AD06 (n=8); AD07: infected treated with 100 mg/kg AD07 (n=8). AST: aspartate aminotransferase, ALT: alanine aminotransferase. a, b, c Groups marked with different letters have statistical difference ($P<0.05$), and groups marked with any letter in common have no statistical difference ($P>0.05$) by the Kruskal-Wallis One Way Analysis of Variance on Ranks followed by the Tukey post-hoc test.

As shown in Figure 14, all animals infected with *T.cruzi*, treated or untreated, have increased levels of total IgG reactivity index, IgG1 and IgG2a in relation to non-infected animals, whose values are related to non-specific reactions in the ELISA method. In general, these parameters were similar ($P>0.05$) in the Bz, AD06 and AD07 groups in relation to untreated infected animals. Only the Bz group showed a similar IgG2a reactivity index to the group of non-infected and untreated animals ($P>0.05$).

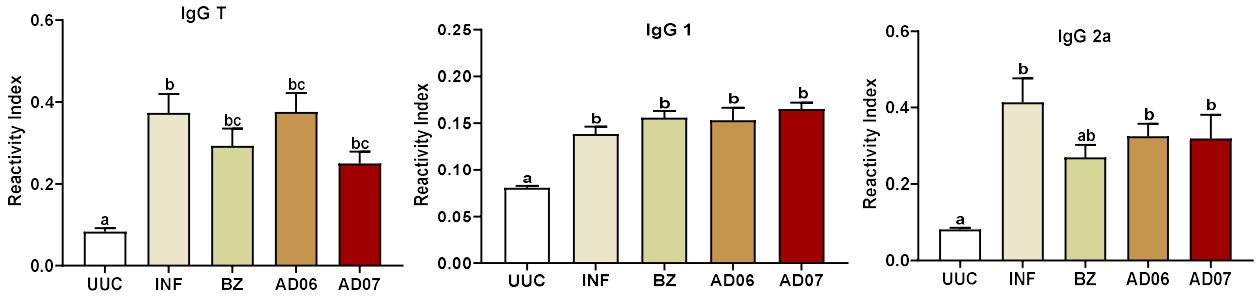

Fig. 14. Total, IgG1and IgG2a anti-*Trypanosoma cruzi* immunoglobulin G reactivity index in plasma samples from control uninfected and *Trypanosoma cruzi*-infected mice treated with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz). UUC: Uninfected untreated control (n=10); INF: infected untreated (n=8); Bz: infected treated with 100 mg/kg benznidazole (n=8); AD06: infected treated with 100 mg/kg AD06 (n=8); AD07: infected treated with 100 mg/kg AD07 (n=8). Data are expressed as mean and standard deviation. a, b, c Groups marked with different letters have statistical difference ($P<0.05$), and groups marked with any letter in common have no statistical difference ($P>0.05$) by the Kruskal-Wallis One Way Analysis of Variance on Ranks followed by the Tukey post-hoc test.

As indicated in Fig. 15 (myocardial images), the histopathological analysis indicated that control uninfected animals and *T. cruzi*-infected mice receiving Bz and AD07 exhibited a typical myocardial structure with parallel and wee defined cardiomyocytes, scarce connective tissue and interstitial cellularity with no evidence of myocarditis and cell degeneration. Conversely, infected untreated mice and those receiving AD06 exhibited marked myocarditis with evident connective tissue expansion and inflammatory infiltrate with predominance on mononuclear cells. The quantitative analysis (Fig. 16, graphic) indicated that Bz and AD07 were effective in attenuating myocardial interstitial cellularity compared to infected untreated animals ($P<0.05$). The reference drug Bz and the AD07 hybrid determined a similar myocardial cellularity profile ($P>0.05$). Bz-treated and uninfected untreated animals exhibited a similar myocardial cellularity ($P>0.05$).

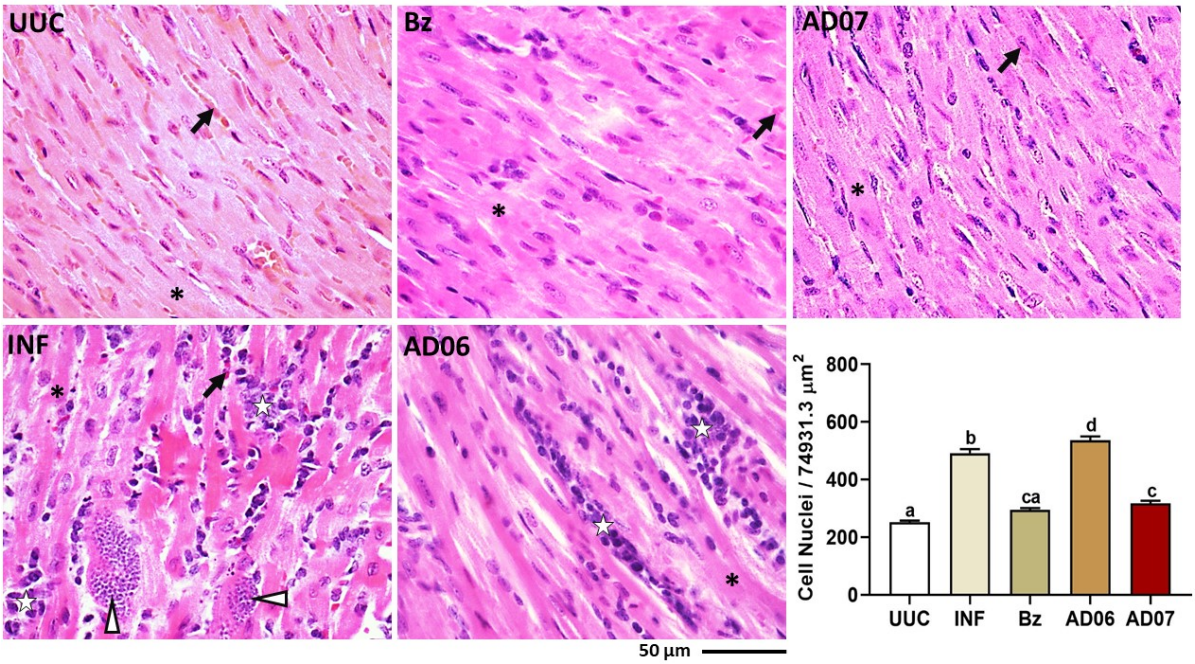

Fig. 15. Microscopic images and interstitial cellularity (graphic) of the heart from uninfected and *Trypanosoma cruzi*-infected mice treated with new metronidazole/eugenol-based hybrids (AD06 and AD07) and benznidazole (Bz). (Bright field microscopy, hematoxylin and eosin staining, scale bar= 50 µm). UN: undetectable. UUC: Uninfected untreated control (n=10); INF: infected untreated (n=8); Bz: infected treated with 100 mg/kg benznidazole (n=8); AD06: infected treated with 100 mg/kg AD06 (n=8); AD07: infected treated with 100 mg/kg AD07 (n=8). Microscopic images: Asterisk= cardiomyocytes, arrowhead= amastigote nests, arrow= blood vessels, star= inflammatory infiltrate. In the graphic, data are expressed as mean and standard error. a, b, c Groups marked with different letters have statistical difference ($P<0.05$), and groups marked with any letter in common have no statistical difference ($P>0.05$) by the Kruskal-Wallis One Way Analysis of Variance on Ranks followed by the Tukey post-hoc test

4. Discussion

This study investigated the antiparasitic effect of metronidazole/eugenol/dihydroeugenol hybrids on *T. cruzi* infection *in vitro* and *in vivo*. From a drug repositioning and chemical synthesis perspective, we developed two novel molecular hybrids (AD06 and AD07) using structural chemical platforms with previously described antiparasitic evidence (Dingsdag and Hunter et al., 2018; Reis et al., 2022). As expected, our assays were consistent in demonstrating that the hybridization method used potentiated the antiparasitic effect of the constructed molecules in comparison with the results obtained when metronidazole and phenols were administered separately. Primarily, the antiparasitic advantage of molecular hybrids was objectively demonstratedby the lower IC_{50} for *T.cruzi* epimastigotes and trypomastigotes forms. Both Bz AD06 and AD07 hybrids exhibited a dose-dependent effect in reducing cells infection rate. Despite the treatments not having eradicated the infection, an expressive attenuation in amastigotes proliferation was observed, determining a marked reduction in parasite load in infected cardiomyocytes. Furthermore, AD06 and AD07 hybrids showed lower toxicity to host cells and higher selectivity index compared to their constitutive molecules, suggesting better cellular tolerability and pharmacological specificity against *T. cruzi.*

The antiparasitic effect of several drugs has been frequently evaluated on different evolutionary forms of *T. cruzi* (Assíria et al., 2015; Martinez-Peinado et al., 2022). However, trypomastigotes and amastigotes are clinically more relevant, since they are mammalian infective forms (Jansen et al., 2018; Siqueira et al., 2022; Shikanai Yasuda et al., 2022). As each evolutionary form exhibits a distinct profile of drugs resistance and susceptibility (Quebrada et al., 2018), the antiparasitic potential of AD06 and AD07 identified in the present study suggests a desirable pharmacological profile for new anti-*T. cruzi* drugs with *in vivo* applicability. Although metronidazole and eugenol antiparasitic mechanisms is not fully known, this effect may be associated with prooxidant events, as well as proteins, DNA and mitochondrial damage in *T. cruzi*; compromising parasite replication and survival (Turrens et al., 2004; Schamber-Reis et al., 2012; Andrioli et al., 2012). However, these effects should not be extrapolated to molecular hybrids, since the biological properties of these new chemical entities may differ from those described for their precursor molecules. Accordingly, we contextualize AD06 and AD07 antiparasitic effects in relation to antioxidant and prooxidant processes that develop during host-pathogen interaction, since they are centrally involved in cellular parasitism and in the mechanism of action of anti-*T.cruzi* reference drugs (Caldas et al., 2019; Wilkinson et al., 2000).

As expected, our findings indicated that untreated *T.cruzi*-challenged cardiomyocytes exhibited marked ROS production, which was also expressed as mitochondrial superoxide and hydrogen peroxide upregulation. The classical role of these molecules in oxidative stress (Pisoschi et al., 2015) was clearly associated with increased MDA and PCN levels, indicating lipid and protein oxidation in *T. cruzi*-infected cells (Felizardo et al., 2018; Mendonça et al., 2020). Apparently, reactive species exert a paradoxical effect on *T. cruzi* infection. Although they are produced as an antiparasitic cellular defense mechanism (Felizardo et al., 2018; Bombaça et al., 2019), they can also activate signaling pathways and cellular mechanisms that potentiate parasitism, acting as a potential "fuel" for *T. cruzi* infection (Goes et al., 2016). Interestingly, marked ROS, superoxide and H_2O_2 downregulation was identified in *T. cruzi*-challenged cardiomyocytes treated with Bz and AD07 at 100%, 50% and 25% IC_{50} for trypomastigotes, an effect obtained with AD06 only at 100% IC_{50}. These effects were accompanied by reduced lipid and protein oxidation (MDA and PCN levels), indicating that Bz and AD07 were more efficient in controlling *T. cruzi*-triggered oxidative stress. Particularly, AD07 exerted a remarkable effect at 100% IC_{50}, since was the only treatment effective in preventing lipid oxidation. The mechanisms associated with the antioxidant effects of the investigated treatments are not completely understood. However, it seems related to the direct antiparasitic effect, since Bz and AD07 were potent inhibitors of *T. cruzi*replication and survival. Although direct antioxidant property is not reported for Bz, this effect cannot be ruled out for AD06 and AD07, since there is antioxidant evidence for eugenol (Fujisawa et al., 2016; Chatterjee et al., 2015), one of the precursors of these hybrids. Even in the presence of some antioxidant effect, this seems not to be equally relevant for AD06, since it exerted a limited effect on reactive species biosynthesis and oxidative molecular damage even when used at doses similar to those used for AD07.

Interestingly, we identified that TR activity in *T. cruzi* was not influenced by Bz, which was effectively attenuated by AD06 and especially by AD07. The dose-dependent inhibitory effect of these hybrids on TR was consistent with ROS, MDA and PCN production, indicating that AD07 may exhibit a broader antioxidant protective spectrum than AD06. Trypanothione reductase is essential to Trypanosomatids homeostasis and survival, playing a central protective role in *T. cruzi* redox system against oxidative stress (González-González et al., 2022). Accordingly, AD07 antiparasitic effect may be partially associated with the inhibition of central antioxidant effectors in *T. cruzi*, a mechanism that is not consistently attributed to Bz. Complementarily, we identified that infected cardiomyocytes showed a marked attenuation of the total antioxidant capacity, which was totally or partially restored

with Bz and AD07 treatment. Thus, it is possible that the antiparasitic damage caused by these drugs attenuated the potential of *T. cruzi* to disrupt the redox balance in infected cells. This proposition is reinforced by evidence that *T. cruzi*-induced mitochondrial dysfunction is the main source of ROS in parasitized cells (Wen et al., 2008). Thus, drugs that alter parasite metabolism and viability may be relevant in modulating oxidative stress in host cells (Mendonça et al., 2020; Rose et al., 2021).

Similar to total antioxidant capacity, CAT, SOD, GR and GPx activities were markedly attenuated in untreated*T. cruzi*-infected cardiomyocytes. In general, this effect was attenuated by Bz, AD06 and AD07 in a dose dependent effect. These findings reinforce the influence of parasite control on the activity of protein effectors essential to host cell antioxidant defenses (Florentino et al., 2021; Rose et al., 2021). The production and activity of these enzymes exhibit bidirectional behavior associated with the intensity of cellular metabolic demand, increasing during moderate stress, but suffering significant exhaustion in response to intense oxidative stress (Lushchak et al., 2014). Accordingly, even with the improvement in the activity of these enzymes and in the total antioxidant capacity in Bz- and especially AD07-treated infected cells, only the highest doses of these drugs were efficient in attenuating cellular oxidative damage (e.g., MDA and PCN).This finding also reinforces a potential intrinsic antioxidant property in AD07 partially dissociated from its trypanocidal effect, especially considering that the antiparasitic efficacy of this drug did not surpass that achieved with Bz. Oxidative/redox effectors play important roles in several cell signaling pathways, modulating energy metabolism, immune functions, antimicrobial resistance, cell replication and survival (Gregory et al., 2005 ; Campo et al., 2014). However, excessive ROS production is often associated with infection worsening, since the non-specificity of these molecules favors damage to both parasites and host cells (Paiva et al., 2018; Mendonça et al., 2020). Although Bz is a reference for *T. cruzi* infection treatment, this drug has *in vivo* prooxidant characteristics, which is often associated with antiparasitic chemotherapy toxicity (Novaes et al., 2015; Rigalli et al., 2016). Thus, antiparasitic drugs with an antioxidant profile such as AD07 may be relevant to increase treatment tolerability, while at the same time attenuating oxidative damage secondary to *T. cruzi* infection.

An extraordinary finding was the apparent antagonism between antioxidant effectors responses and the expression of critical regulatory genes to redox metabolism. As expected, *T. cruzi* infection upregulated HO-1, NFR2 and NQO-1 gene expression, indicating the increased oxidative metabolic stress in parasitized cardiomyocytes. However, the lower enzymatic activity reinforces the proposition of metabolic exhaustion, a process in which

antioxidant effectors are consumed in a counterregulatory mechanism to oxidative stress (Maldonado et al., 2021; Estrada et al., 2018). Accordingly, activation of regulatory genes indicates a protective cellular response (Loboda et al., 2016), which combined with adequate parasitological control induced by Bz and AD07 seems viable to reduce parasite load and restore cellular redox balance. Considering the cellular response to oxidative stress, only the highest dose of Bz increased the sensitivity of cardiomyocytes to H_2O_2 prooxidant challenge, indicating that the investigated hybrids did not impair the antioxidant defenses in host cells. Conversely, Bz and AD07 increased *T. cruzi* susceptibility to H_2O_2 in a dose-dependent manner, a relevant effect potentially linked to the antiparasitic properties of these drugs. This effect is more predictable for Bz, since it generates glyoxal when metabolized by *T. cruzi* nitroreductases, which reacts with thiols, proteins and nucleic acids, increasing the parasite's susceptibility to oxidative stress (Caldas et al., 2019). However, the mechanism attributed to AD07 needs to be better elucidated, although TR inhibition may contribute to greater susceptibility to prooxidant challenges, including H_2O_2.

The protective effect of the investigated drugs was also observed by the extracellular levels of cardiac troponin I, a marker of microstructural cardiomyocytes injury (Park et al. 2017). Accordingly, significant reduction in this marker was achieved fromBz and AD07 treatment compared untreated infected cells. These findings were aligned with the infection rate, parasite load and cellular oxidative damage, indicating that parasitological and oxidative stress control is essential to ensure morphological integrity in cardiomyocytes (Maldonado et al., 2020, 2021). This finding is not trivial since *T. cruzi* have a natural tropism for these cells, which are direct targets of parasitism and morphofunctional dysfunction (Bonney et al., 2019; Martinez et al., 2020). Mitigating cardiomyocyte infection is a central goal of antiparasitic chemotherapy, especially considering that cardiomyopathy is the most serious and disabling consequence of *T. cruzi* infection (Nunes et al., 2021; Montalvo-Ocotoxtle et al., 2022). Thus, it was relevant to compare AD06 and AD07 therapeutic potential with Bz effectiveness *in vivo* in *T. cruzi*-infected animals.

Similar to what has been demonstrated in other studies (Gonçalves-Santos et al., 2019; Caldas et al., 2009), Bz was effective in quickly suppress parasitemia at low doses. This effect was not equally achieved in animals treated with AD06 and AD07. Accordingly, we identified a more pronounced effect in animals receiving AD07, which determined a marked reduction in the peak and mean parasitemia. In fact, parasite killing and reduction in parasite load is an expected and desirable effect in effective antiparasitic drugs (Brener et al., 1962; Santos et al., 2015). In general, there is a direct correlation between parasitemia, organs parasitism and

morphofunctional damage (Mazzeti et al. 2021). Thus, parasitological control is a primary objective of antiparasitic chemotherapy (Lascano et al. 2022), which should ideally be associated with low toxicity of the treatment for the host (Caldas et al. 2019). Accordingly, animal mortality was not identified, which may be related to the good tolerability of the drugs evaluated and the short period of treatment. Good tolerability was also demonstrated by the hepatosomatic index, ALT and AST levels in treated animals compared to untreated infected animals. These findings indicated that the infection itself causes liver overload, which was not increased or even reduced by treatments, especially Bz and AD07.

As expected, *T. cruzi* infection was associated with high plasma titers of anti-*T.cruzi* immunoglobulin G, while IgG results obtained for uninfected control mice is related to unspecific results inherent of the ELISA method. *Trypanosoma* cruzi infection activates a broad spectrum of cellular and humoral antiparasitic defense mechanisms (Acevedo et al. 2018; Puerta et al. 2023). Although these mechanisms are not enough to eliminate the parasite (Magalhães et al. 2022), immunosuppression aggravates infection (Pinazo et al. 2013), and non-lethal infections course with greater parasitemia and become lethal in the absence of B cells, which orchestrate the humoral response (Bryan et al., 2010). This response is especially relevant against extracellular *T. cruzi* forms (trypomastigotes), which are essential to propagate the parasite to other cells after completing its proliferative cycle in previously infected cells (Pérez-Molina et al., 2018). It is widely recognized that the immune response acts synergistically with antiparasitic chemotherapy (Albareda et al., 2015). Thus, the maintenance of high anti-*T. cruzi* IgG levels indicated that the new metronidazole/eugenol hybrids do not induce an immunosuppressive response, and may be a relevant component linked to the improved parasitological control in animals receiving different treatments, especially Bz and AD07. Accordingly, it was demonstrated that immune response inhibition attenuates Bz antiparasitic effects (Sztein et al., 1993), a response that remains to be proven for the molecular hybrids tested. Furthermore, adequate parasitological control is often associated with attenuation in IgG titers due to lower antigenic load and immune activation (Murphy et al., 2021). However, IgG clearance is slow, and significant variations in this marker can be detectedonly months after the end of treatment, even in cases of parasitological cure (Llaguno et al., 2021).

In addition to improving parasitemia control without interfering with the humoral response, Bz and AD07 were also efficient in modulating the cardiac inflammatory response. It is recognized that in addition to its antiparasitic potential, Bz still exhibits a direct anti-inflammatory effect by inhibiting the NF-kB pathway (Cevey et al., 2021). This effect is

relevant, since exacerbated inflammatory responses aggravate tissue damage and the risk of host death (Talvani et al., 2011). Thus, attenuating inflammation is an additional goal of antiparasitic chemotherapy (Guimarães-Pinto et al., 2022), which was achieved in AD07-treated animals. However, myocarditis attenuation may be predominantly dependent on the antiparasitic action of this drug, since the direct anti-inflammatory potential of AD07 still needs to be clarified. Even so, there is evidence that eugenol inhibits prostaglandin synthesis, neutrophils and macrophageschemotaxis; exerting an anti-inflammatory effect (Estevão-Silva et al.,2014; Barboza et al., 2018) with potential expression in the hybridized molecule.

Taken together, our findings indicated that metronidazole and eugenol derivatives may be relevant molecular platforms for the rational development of new molecules against *T. cruzi*. Accordingly, the combination of drug repositioning and molecular hybridization was effective in generating chemical entities with enhanced antiparasitic effect, lower cytotoxicity, and better selectivity in relation to their precursor molecules. In addition to having a direct antiparasitic effect on *T. cruzi*, metronidazole/eugenol hybrids (especially AD07) attenuated cellular parasitism, production of reactive species and oxidative stress in infected cardiomyocytes. Although they did not have a relevant impact on the activity of antioxidant enzymes in host cells, AD06 and especially AD07 exerted an inhibitory effect on the *T. cruzi* antioxidant enzyme, which may be related to the increased parasite susceptibility to prooxidant challenges *in vitro*. Complementarily, treatment with AD07 was well tolerated and induced a relevant *in vivo* antiparasitic effect, attenuating parasitemia and myocarditis in mice infected with *T. cruzi*. Although this cardioprotective response is potentially related to the direct antiparasitic effect of AD07, a direct anti-inflammatory effect of this molecular hybrid cannot be ruled out. Thus, further mechanistic studies are required to expand the understanding of the therapeutic potential of AD07 to treat *T. cruzi* infection.

Acknowledgements

The authors are grateful to the support provided by Fundação do Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, processes APQ-01895-16, PPM-00687-17 and PPM-00077-18), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, processes 303972/2017-3, 423594/2018-4, 305093/2017-7 and MCTIC 408503/2018-1), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES, finance code 001).

Conflict of interest

None to declare.

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3 CONSIDERAÇÕES FINAIS

A relevância e a aplicabilidade do benznidazol em diferentes estágios da doença foram investigadas por meio de uma revisão crítica detalhada. Em nossas análises demonstramos que apesar de o benznidazol exibir alta biodisponibilidade após administração oral em adultos e crianças, a posologia e o tempo de duração do tratamento recomendados muitas vezes são dissociados dos achados farmacodinâmicos. Além disso, maior eficácia terapêutica é obtida quando o tratamento com benznidazol é administrado em infecções agudas, piorando gradualmente à medida que a infecção se torna crônica. Sendo assim, diante de falha terapêutica, o tratamento com benznidazol nem sempre garante melhor prognóstico, podendo o paciente desenvolver a Cardiomiopatia Chagásica assim como pacientes não tratados. Uma vez que é evidente a necessidade de novas estratégias para o tratamento da doença de Chagas, em nossa abordagem secundária avaliamos a relevância antiparasitária *in vitro* e *in vivo* de dois novos híbridos moleculares de metronidazol e eugenol na infecção por *T. cruzi*. Nossos resultados indicaram que os híbridos possuem efeito antiparasitário direto sobre o parasito atenuando a taxa de infecção celular, a biossíntese de espécies reativas e o estresse oxidativo em cardiomiócitos infectados *in vitro*. Além disso, os híbridos foram bem tolerados em camundongos infectados por *T. cruzi*, não sendo relacionados à mortalidade ou hepatotoxicidade. Essas novas moléculas também induziram um efeito antiparasitário relevante ao atenuar a parasitemia e a miocardite *in vivo*. Diante dos resultados, um dos híbridos, denominado AD07 (híbrido metronidazol/dihidroeugenol nitrado na posição orto à hidroxila), destacou-se como candidato potencialmente relevante para o desenvolvimento de novos regimes medicamentosos mais seguros e eficazes para o tratamento da infecção por infecção *T. cruzi*.

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