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Synthesis and biological evaluation of 2-methyl-1*H*-benzimidazole-5-carbohydrazides derivatives as modifiers of redox homeostasis of *Trypanosoma cruzi*

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ABSTRACT

Twelve novel benzimidazole derivatives were synthesized and their *in vitro* activities against epimastigotes of *Trypanosoma cruzi* were evaluated. Two derivatives (**6** and **7**), which have 4-hydroxy-3-methoxyphenyl moiety in their structures, proved to be the most active in inhibiting the parasite growth. Compound **6** showed a trypanocidal activity higher than benznidazole (IC_{50} = 5 μM and 7.5 μM, respectively) and less than nifurtimox (IC_{50} = 3.6 μM). In addition, the ability of **6** and **7** to modify the redox homeostasis in *T cruzi* epimastigote was studied; cysteine and glutathione increased in parasites exposed to both compounds, whereas trypanothione only increased with **7** treatment. These results suggest that the decrease in viability of *T. cruzi* may be attributed to the change in cellular redox balance caused by compound **6** or **7**. Furthermore, compounds **6** and **7** showed CC_{50} values of 160.64 and 160.66 μM when tested in mouse macrophage cell line J774 and selectivity indexes (macrophage/parasite) of 32 and 20.1, respectively.

Chagas disease, caused by the protozoa *Trypanosoma cruzi*, affects approximately 6 million inhabitants on the American continent. It is listed by the World Health Organization as one of the 17 Neglected Tropical Diseases (NTD).[1] The parasite is transmitted to humans either by blood-sucking triatomine vectors ("kissing" bug), by blood transfusion or by congenital transmission. This ailment is manifested in two clinical phases: acute and chronic. The acute infection is usually asymptomatic, but the ensuing chronic infection has been associated with high rates of morbidity and mortality.[2]

Despite over 100 years have passed since the clinical description of Chagas disease, and the tremendous efforts in the development of vaccines, so far chemotherapy remains as the only economical and practical means to achieve its control. The current treatment against this infection rely on two nitroheterocyclic drugs, benznidazole (**Bnz**) and nifurtimox (**Nfx**); which were introduced to the clinic over thirty years ago.[3] Both compounds are effective for acute infections, but controversy exists on their use for chronic patients due to undesirable side effects frequently forcing the abandonment of the treatment, besides their poor indices of apparent cure, and a lack of consensus about criteria for parasitological cure. Because **Bnz** and **Nfx** are far from being considered ideal trypanocidal drugs, the search for new compounds effective against *T. cruzi*, with low toxicities and increased efficacies during the chronic phase, continues. Trypanocidal effect of **Bnz** and **Nfx** depends on the formation of reactive metabolites which mediate parasite killing.[4] In fact, the reduction of **Bnz** by type I nitroreductase activity leads to formation of glyoxal, a well-known toxic metabolite.[5]

On the other hand, **Nfx** acts via reduction of its nitro group to an unstable nitroanion radical, which reacts to produce highly toxic oxygen reduced metabolites (superoxide anion, hydrogen peroxide and hydroxyl radical.[6,7]

Even though these mechanisms are effective to kill the epimastigote and trypomastigote stages of *T. cruzi*, unfortunately both

Abbreviations: **Nfx**, nifurtimox; **Bnz**, benznidazole; GR, glutathione reductase; MDA, malondialdehyde; GSH, glutathione; GSSG, glutathione disulfide; $T(SH)_2$, trypanothione.

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drugs are not very active in the chronic phase of the disease. This failure is attributed to adverse pharmacokinetic properties.[8] Therefore, doses of 5–12 mg/kg/day are administered during 30–90 days, which propitiates several adverse effects that compromise the health of patients.[8,9] Moreover, *T. cruzi* is an organism that, depending on the strain, manifests different degrees of susceptibility to **Bnz** and **Nfx**.[8,10]

These facts emphasize the need to develop new more effective and safe drugs against *T. cruzi*. Within this search, the benzimidazole core has been identified as an important pharmacophore and a privileged structure in medicinal chemistry having a broad range of pharmacological activities,[11–13] among them, antiprotozoal activity.[14–17]

After evaluating several derivatives, it was established that the structural requirements for the presence of antiparasitic activity are mainly the substituents at 2 and 5(6) positions of the heterocyclic nucleus.[14,15,18,19] In previous reports, it has been demonstrated that benzimidazole derivatives show activity against NINOA, INC5 and MR strains of *T. cruzi*.[15,18] As part of our research, it is reported herein the synthesis and trypanocidal activity of novel derivatives containing as scaffold 2-methyl-1*H*-benzimidazole. Their trypanocidal activity was evaluated against epimastigotes of *T. cruzi* Queretaro strain. The cytotoxicity of some compounds was tested using mouse macrophages cell line J774. In order to determine the possible mechanism of action of the most active compounds (**6** and **7**), the changes in thiol metabolites were evaluated in *T. cruzi*.

Twelve molecules were designed as antiparasitic ones, retaining the 2-methyl-1*H*-benzimidazole core and modifying the substituents at the 5-position of the heterocycle (Table 1). For this purpose, it was considered to use phenolic residues (compounds **2**, **4**–**7**) due to the fact that they have been recognized to have antioxidant/prooxidant properties under certain conditions. Phenolic compounds could exert prooxidants effects by autoxidation, leading to formation of semiquinone and superoxide anion. The hydrogen peroxide is generated and immediately converted to hydroxyl radical (in a Fenton-type reaction) that may damage macromolecules such as proteins, DNA or lipids. The antioxidant/prooxidant activity of phenolic derivatives may depend on factors such as metal-reducing potential, chelating behavior, pH, and solubility characteristics.[20] Consequently, they could alter cellular redox status, action mechanism validated to kill *T. cruzi*. In addition several authors have reported that *T. cruzi* is particularly sensitive to compounds that can produce free radicals for example 5-nitrofuran derivatives,[21] as in compound **12**. Substituents in compounds **1**, **3**, **9**, **10** and **11** were placed in order to obtain information from the structural requirements for presenting trypanocidal activity. While as a linker, one *N*-acylhydrazone is a versatile moiety in medicinal chemistry that is able to interact selectively with different biological targets[22] and it has been widely used in various compounds that show trypanocidal activity[23]

The synthetic strategy followed for the preparation of the benzimidazole derivatives (**1**–**12**) is showed in Scheme 1. 2-Methyl-1*H*-benzimidazole-5-carboxylic acid (**13**) was prepared from 3, 4-diaminobenzoic acid with excess of acetic acid and catalytic amounts of hydrochloric acid according to Philips' method.[24] The benzimidazole derivative **13** was reacted with *tert*-butyl carbazate using 1,1′-carbonyldiimidazole (CDI) as coupling agent to give **14**. The BOC group was removed using trifluoroacetic acid, resulting in 2-methyl-1*H*-benzimidazole-5-carbohydrazide (**15**). Afterwards, condensation of this carbohydrazide with the appropriate aromatic aldehydes (**16**–**27**; see methods) in isopropyl alcohol constructed the new series of *N*-acylhydrazone benzimidazole derivatives (**1**–**12**). The overall yield of compounds was in the range of 60–83%.

The proposed structures of compounds (**1**–**12**) are aligned with the experimental data obtained from IR, MS and NMR experiments. In ^{1}H NMR spectrum, the signal corresponding to the imine proton (N=CH) was observed as a singlet at 8.6–8.2 ppm for compounds (**1**–**12**). The assignment of isomers (*Z*) or (*E*) was performed as results of the experiments of NMR (^{1}H NMR and NOESY) and the characteristic fragmentation in mass spectrometry, showing a McLafferty rearrangement peak. Derivatives (**1**–**12**) were obtained as *E*-isomers[25] (see Supplementary information). Additionally, the single crystals of compound **6** were obtained by slow evaporation from a DMSO solution. The *E* geometry was corroborated by X-ray analyses (CCDC Number 1457310) (Fig. 1).[26].

The effect of derivatives **1**–**12** and two intermediaries (**13** and **15**) on the growth of *T. cruzi* epimastigotes of the Queretaro strain was evaluated.[27] The concentration that inhibits by 50% the parasite growth (IC_{50}) was determined as describe in Supplementary information and the results are summarized in Table 1. Most compounds showed an IC_{50} greater than the reference drugs (**Bnz**, IC_{50} = 7.5 μM and **Nfx**, IC_{50} = 3.6 μM), except for compound **6**; which exhibited an IC_{50} = 3 μM, similar to **Nfx**, and benzimidazole **7** which showed an IC_{50} = 8 μM, similar to **Bnz**.

Remarkably, the related compound **8** (substituent: 3,4-dimethoxyphenyl) showed a loss of biological activity (IC_{50} >500 μM) and compound **5** (substituent: 3,4-dihydroxyphenyl) showed a poor activity (IC_{50} = 380 μM). The above results lead us to believe that the presence of 4-hydroxy-3-methoxyphenyl moiety, in **6** and **7**, promotes the interaction of both compounds with some receptor or transport system to enhance its trypanocidal action. Compounds **2** and **5**, which have a hydroxyl group in the same position, do not show the same behavior because they lack the methoxy group.

Moreover, cytotoxicity of compounds **6**, **7**, **13** and **15** was determined in (J774) mouse macrophages by measurement of resazurin reduction; according to the methodology previously described,[28] with some modifications.[29] **Nfx** was included as the reference drug. The results are shown in Table 1. Derivatives **6** and **7** showed no significant cytotoxic effect on mouse macrophages. The selectivity index (CC_{50}/IC_{50}) calculated was 53.5 and 20.1 for **6** and **7**, respectively.

In order to test if the trypanocidal activity of **6** and **7** was related to its ability to modify redox balance, thiol metabolites were determined in parasite exposed to these compounds.[30,31] Cysteine (Cys), glutathione (GSH) as major cellular thiol participating in cellular redox reactions[32] and trypanothione [$T(SH)_2$] have been found directly and indirectly involved in oxidative stress response. Particularly $T(SH)_2$ is considered the main antioxidant metabolite for the parasite since it provides the reducing equivalents for its antioxidant machinery.[30] Changes in these thiol metabolites have been observed in *T. cruzi* treated with **Bnz** or **Nfx**.[3,7] In parasites exposed to **6** and **7**, Cys and GSH showed a tendency to increase their contents at increasing drug concentrations (Fig. 2). On the other hand, different patterns of changes for $T(SH)_2$ were observed with **6**; the pool of this metabolite remain relatively constant despite of accumulation of its precursor GSH, whereas for **7**, a tendency to increase the metabolite was observed. A possible explanation for the not increase content of $T(SH)_2$ with **6** is that the rates of its synthesis and its usage to contend the oxidative stress are similar, preventing its accumulation and therefore making the parasites more susceptible to this drug. In contrast, the lower cytotoxic effect of **7** may be due to a slower kinetics of cytotoxicity which may allow the parasite increase the $T(SH)_2$ content. These hypotheses remain to be elucidated.

The epimastigotes were used as a proper model for studying the mode of action of **6** or **7** due to the fact the low molecular weight thiol content in epimastigote form of *T. cruzi* is higher than amastigote and trypomastigote forms.[6]

In a nut shell, only compounds **6** and **7** showed an interesting trypanocidal activity. Compound **6** showed intermediate potency