Accepted Manuscript

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Please cite this article as: A. Salerno, A.M. Celentano, J. López, V. Lara, C. Gaozza, D.E. Balcazar, C. Carrillo, F.M. Frank, M.M. Blanco, Novel 2- arylazoimidazole derivatives as inhibitors of *Trypanosoma cruzi* proliferation: Synthesis and evaluation of their biological activity, *European Journal of Medicinal Chemistry* (2016), doi: 10.1016/j.ejmech.2016.09.045.

Novel 2- arylazoimidazole derivatives as inhibitors of *Trypanosoma cruzi* proliferation: synthesis and evaluation of their biological activity

NOVEL 2- ARYLAZOIMIDAZOLE DERIVATIVES AS INHIBITORS OF *Trypanosoma cruzi* PROLIFERATION: SYNTHESIS AND EVALUATION OF THEIR BIOLOGICAL ACTIVITY

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Abstract

In this work, the synthesis of a series of 2-arylazoimidazole derivatives **6-20** has been achieved through the reaction of imidazole with aryldiazonium salts, followed by ultrasound-assisted alkylation. This approach has important advantages including higher yield, shorter reaction times and milder reaction conditions. The structures of the compounds obtained were determined by MS, IR; and ^{1}H and ^{13}C NMR. The anti-*Trypanosoma cruzi* activity of the 15 compounds obtained was evaluated. Two compounds with piperidino substituents in the carboxamide moiety proved to be effective inhibitors of epimastigote proliferation, obtaining inhibition values comparable to those achieved with the reference drug Benznidazole. Besides, these compounds displayed low cytotoxicity on mammalian cells. *In vivo,* both compounds protected mice against a challenge with a lethal *Trypanosoma cruzi* strain. These results allow us to propose 2-arylazoimidazoles as lead compounds for the design of novel drugs to treat Chagas' disease.

Keywords: 2-arylazoimidazoles, ultrasound-assisted alkylation, Chagas' disease, biological activity.

1. Introduction

The World Health Organization lists Chagas' disease as one the most neglected tropical diseases. This infectious disease, also known as American Trypanosomiasis, represents a major public health problem in the Americas, where more than 7 million people are infected with *Trypanosoma cruzi,* the aetiological agent [1]. It is estimated that 25% of the population of Latin America is at risk of acquiring the infection [2] and it is currently considered an emerging disease in non-endemic areas due to globalization, immigration and non-vectorial transmission routes [3].

T. cruzi has a complex life cycle involving several developmental stages. Epimastigotes are the replicative form in the reduviidae vector, while trypomastigote and amastigote forms are the stages found in the bloodstream and in the host cell of the infected mammalian. Epimastigotes are usualy employed as a first step for the screening of anti-*T. cruzi* new drugs, because they can be easily obtained in axenic culture. However, it is noteworthy that these assays may not truly reflect the effectiveness of compounds on mammals. In addition to vector-associated transmission, the disease can also be transmitted by blood transfusion, organ transplantation, contaminated food or beverages and vertically from mother to child [4,5]. The infection comprises two phases: the acute and the chronic one. A 40%-50% of patients who resolve the acute phase enter a lifelong chronic phase that can progress to a severe cardiac or gastrointestinal disease after 10-30 years of the primary infection [2].

The chemotherapy employed to control this parasite infection is based on the administration of the nitroderivatives Nifurtimox (Nfx) (nitrofuran) or Benznidazole (Bnz) (nitroimidazol), which have been discovered empirically more than four decades ago. These drugs are considered far from being ideal, mainly due to the fact that they cause multiple side effects and have limited efficacy. These limitations may be related to unfavorable pharmacokinetic properties, such as a relatively short half-life and limited tissue penetration, which limit its action in the chronic phase, when the parasites are mostly confined to tissues where replication occurs [6]. Therefore, there is a need for the development of safer and more effective molecules to treat this disease [7].

Imidazole derivatives (i.e., mebendazole and albendazole) have been used for many years to treat parasitic diseases caused by either protozoa or helminthes [8-10]. The imidazole ring is commonly found in highly significant endogenous biomolecules including biotin, the essential amino acid histidine, and the autacoid histamine [11]. Additionally, the imidazole nucleus is of significant importance in medicinal chemistry research since many imidazole-containing compounds exhibit biological activities such as antiparasitic [12], antifungal [13], antibacterial [14,15], and antidepressant [16], among others [17].

In connection with our work related to the synthesis of heterocyclic compounds with potential anti-trypanosomal activities [12a], In this work we describe the synthesis and the anti-*T. cruzi* activity of a novel series of 2-arylazoimidazole derivatives **I** (**6-20**) (Bnz analogues).

2. Materials and Methods

2.1. *Chemistry*

Melting points were taken on a Büchi capillary apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded in DCCl$_3$ or DMSO-d_6 solutions on a Bruker Avance II 500 MHz spectrometer at room temperature in 5 mm tubes. Standard concentration of the samples was 10 and 30 mg/mL for ^{1}H and ^{13}C NMR, respectively. Chemical shifts were reported in ppm (δ) relative to TMS employed as internal standard. Coupling constant (*J*) values are given in Hz. Splitting multiplicities are reported as singlet (s), broad signal (bs), doublet (d), triplet (t), broad triplet (bt) and multiplet (m). High resolution mass spectra (HMRS) were acquired with a model GCT (Waters, Milford, MA, USA), operating at 8000 resolving power (50% valley definition) using heptacose (*m/z* 219) as the reference compound. IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer. Ultrasonic irradiation experiments were carried out in an ultrasonic bath (Arcano Model PS-10A) operating at 200 W with a frequency of 40 kHz. HIU irradiation was performed with a VCX 750 Vibra-Cell high intensity ultrasonic processor (Sonics & Materials, USA) equipped with an immersion ultrasonic probe made of titanium alloy T1-6AL-4V, and with the tip diameter of 13 mm. The frequency employed was 20 KHz and the net power output was 750 W. The variable power output control allows the ultrasonic vibrations at the probe tip to be set to any desired amplitude. With the amplitude control set at 100%, the amplitude at the tip with diameter of 13 mm is 124 nm. In our work, the amplitude control was set at 35%.

2-Arylazoimidazoles **1-5** were prepared following literature procedures [18].

2.1.1. *2-((4-(Trifluoromethyl)phenyl)diazenyl)-1H-imidazole (1)*

Yield: 87%; orange crystals, mp 148-150 ºC (2-propanol); IR (KBr): 3368,1590, 1343, 1110, 859 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 7.46 (2H, bs), 7.97 (2H, d, *J*= 8.3 Hz), 8.02 (2H, d, *J*= 8.3 Hz), 13.50 (1H, bs) ppm; ^{13}C-NMR (DMSO-d_6) δ: 120.0, 122.9, 123.5, 124.4 (c, *J*= 272.5 Hz), 127.2 (c, *J*= 3.6 Hz), 131.0 (c, *J*= 31.9 Hz), 154.6, 155.0 ppm; HRMS-EI: *m/z* calcd for C$_{10}$H$_7$F$_3$N$_4$: 241.07010; found: 241.07015 [M+H].

2.1.2. *2-((4-Nitrophenyl)diazenyl)-1H-imidazole (2)*

Yield: 92%; orange crystals, mp 150-151 ºC (2-propanol); IR (KBr): 3429, 1640, 1321, 843 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 7.50 (2H, bs), 8.02 (2H, bs), 8.42 (2H, bs) ppm; ^{13}C-NMR (DMSO-d_6) δ: 123.2, 125.6, 126.2, 126.6, 136.0, 148.5, 156.1ppm; HRMS-EI: m/z calcd for $C_9H_7N_5O_2$: 218.06780; found: 218.06742 [M+H].

2.1.3. 2-((4-Chlorophenyl)diazenyl)-1H-imidazole (3)

Yield: 88%; orange crystals, mp 173-175 ºC (2-propanol); IR (KBr): 3434, 1642, 1360, 1082, 832, 767 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 7.38 (2H, s), 7.65 (2H, d, J= 8.7 Hz), 7.86 (2H, d, J= 8.7 Hz), 13.50 (1H, bs) ppm; ^{13}C-NMR (DMSO-d_6) δ: 123.9, 124.3, 129.7, 130.1, 135.9, 151.2, 155.8 ppm; HRMS-EI: m/z calcd for $C_9H_7ClN_4$: 207.04375; found: 207.04354 [M+H].

2.1.4. 2-((4-Methoxy-2-nitrophenyl)diazenyl)-1H-imidazole (4)

Yield: 85%; orange crystals, mp 195-197 ºC (2-propanol); IR (KBr): 3429, 1639, 1364, 1239, 1068, 828 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 3.94 (3H, s), 7.37 (1H, dd, J= 9.1, 2.7 Hz), 7.39 (2H, bs), 7.67 (1H, d, J= 2.7 Hz), 7.78 (1H, d, J= 9.1) ppm; ^{13}C-NMR (DMSO-d_6) δ: 56.3, 108.4, 118.2, 118.9, 119.1, 137.5, 150.1, 154.7, 162.2 ppm; HRMS-EI: m/z calcd for $C_{10}H_9N_5O_3$: 248.07836; found: 248.07812 [M+H].

2.1.5. 2-((2,4-Dichlorophenyl)diazenyl)-1H-imidazole (5)

Yield: 95%; orange crystals, mp 167-169 ºC (2-propanol); IR (KBr): 3419, 2873, 1634, 1366, 1101, 819, 691 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 7.40 (1H, bs), 7.50 (1H, bs), 7.85 (1H, dd, J= 8.6, 2.2 Hz), 7.88 (1H, d, J= 8.6 Hz), 8.00 (1H, d, J= 2.2 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 121.4, 123.3, 123.5, 132.1, 132.8, 133.0, 133.9, 151.8, 154.8 ppm; HRMS-EI: m/z calcd for $C_9H_6Cl_2N_4$: 241.00478; found: 241.00450 [M+H].

2-Arylazoimidazoles **1-5** (1 mmol), potassium carbonate (1.3 mmol), the corresponding chloroamide (1.1 mmol) and DMF (5 mL) were added to a flask. The reaction mixture was sonicated by an ultrasonic probe at room temperature for the specified period until complete consumption of starting materials (60-90 min.) monitored by TLC (DCM:MeOH 4.7:0.3). When the reaction was completed, the mixture was poured over ice-water. The resulting solid, compounds **6-20**, were filtered, washed with water and purified by chromatographic methods.

2.1.6. 1-(Piperidin-1-yl)-2-(2-((4-(trifluoromethyl)phenyl)diazenyl)-1H-imidazol-1-yl)ethanone (6)

Yield: 92%; reddish crystals, mp 215-217 °C (2-propanol); IR (KBr): 3102, 2945, 1651, 1321, 1161, 1062, 789 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 1.43 (2H, bs), 1.62 (4H, bs), 3.41 (2H, bs), 3.53 (2H, bs), 5.42 (2H, s), 7.36 (1H, s), 7.59 (1H, s), 7.96 (2H, d, J= 8.9 Hz), 7.98 (2H, d, J= 8.9 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 24.3, 25.7, 26.4, 43.0, 45.6, 48.5, 123.3, 124.2 (c, J= 274 Hz), 127.2 (c, J= 3.8 Hz), 130.0 (c, J= 29 Hz), 131.1, 152.3, 155.2, 165.0 ppm; HRMS-EI: m/z calcd for C$_{17}$H$_{18}$F$_3$N$_5$O: 241.00478; found: 241.00450 [M+H].[note]

2.1.7. 1-Morpholino-2-(2-((4-(trifluoromethyl)phenyl)diazenyl)-1H-imidazol-1-yl)ethanone (7)

Yield: 85%; reddish crystals, mp 254-255 °C (2-propanol); IR (KBr): 3079, 2931, 1657, 1322, 1113, 1063, 847 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 3.44-3.46 (2H, m), 3.57-3.60 (4H, m), 3.60-3.69 (2H, m), 5.45 (2H, s), 7.37 (1H, s), 7.59 (1H, s), 7.97 (2H, d, J= 8.7 Hz), 8.00 (2H, d, J= 8.7 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 42.4, 45.1 48.5, 66.5, 123.4, 124.2 (c, J= 265 Hz), 127.2 (c, J= 3.8 Hz), 127.6, 130.9 (c, J= 30 Hz), 131.1, 152.2, 155.1, 165.7 ppm; HRMS-EI: m/z calcd for C$_{16}$H$_{16}$F$_3$N$_5$O$_2$: 368.13343; found: 368.13273 [M+H].[note]

2.1.8. 1-(Pyrrolidin-1-yl)-2-(2-((4-(trifluoromethyl)phenyl)diazenyl)-1H-imidazol-1-yl)ethanone (8)

Yield: 88%; reddish crystals, mp 245-246 °C (2-propanol); IR (KBr): 3120, 2930, 1650, 1327, 1112, 858 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 1.79-1.85 (2H, m), 1.95-2.01 (2H, m), 3.30-3.33 (2H, m), 3.57-3.59 (2H, m), 5.32 (2H, s), 7.36 (1H, s), 7.60 (1H, s), 7.95 (2H, d, J= 8.7 Hz), 7.98 (2H, d, J= 8.7 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 24.2, 26.1, 45.6, 46.3, 49.0, 123.9, 124.2 (c, J= 268 Hz), 127.2 (c, J= 3.8 Hz), 130.0 (c, J= 29 Hz), 131.1, 152.2, 155.2, 165.0 ppm; HRMS-EI: m/z calcd for C$_{16}$H$_{16}$F$_3$N$_5$O: 352.13852; found: 352.13870 [M+H].[note]

2.1.9. N-Cyclohexyl-2-(2-((4-(trifluoromethyl)phenyl)diazenyl)-1H-imidazol-1-yl)acetamide (9)

Yield: 90%; reddish crystals, mp 241-244 °C (2-propanol); IR (KBr): 3283, 3092, 1651, 1323, 850 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 1.12-1.27 (6H, m), 1.64-1.71 (4H, m), 3.50 (1H, bm), 5.06 (2H, s), 7.35 (1H, d, J= 0.9 Hz), 7.64 (1H, d, J= 0.9 Hz), 7.95 (2H, d, J= 8.4 Hz), 8.03 (2H, d, J= 8.4 Hz) 8.22 (1H, d, J= 7.8 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 24.8, 25.6, 32.7, 48.1, 49.6, 124.4 (c, J= 268.1 Hz), 127.2 (c, J= 3.9 Hz), 127.7, 130.8 (c, J= 30 Hz), 131.8, 152.1, 155.1, 165.8 ppm; HRMS-EI: m/z calcd for C$_{18}$H$_{20}$F$_3$N$_5$O: 380.16982; found: 380.17031 [M+H].[note]

2.1.10. 2-(2-((4-Nitrophenyl)diazenyl)-1H-imidazol-1-yl)-1-(piperidin-1-yl)ethanone (10)

Yield: 87%; reddish crystals, mp 168-170 °C (2-propanol); IR (KBr): 3012, 2935, 1651, 1515, 1341, 857 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 1.43 (2H, bs), 1.63 (4H, bs), 3.42 (2H, bs), 3.52 (2H, bs),

5.44 (2H, s), 7.40 (1H, s), 7.64 (1H, s), 8.00 (2H, d, J= 8.8 Hz), 8.42 (2H, d, J= 8.8 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 24.3, 25.7, 26.4, 43.0, 45.6, 48.6, 123.6, 125.5, 128.3, 131.6, 148.6, 152.4, 156.3, 164.9 ppm; HRMS-EI: m/z calcd for $C_{16}H_{18}N_6O_3$: 343.15186; found: 343.15107 [M+H].[note]

2.1.11. 1-Morpholino-2-(2-((4-nitrophenyl)diazenyl)-1H-imidazol-1-yl)ethanone (11)

Yield: 97%; reddish crystals, mp 128-130 ºC (2-propanol); IR (KBr): 3106, 2914, 1660, 1520, 1343, 1112, 858 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 3.45 (2H, bs), 3.59 (4H, bs), 3.70 (2H, bs), 5.47 (2H, s), 7.42 (1H, s), 7.64 (1H, s), 8.02 (2H, d, J= 8.9 Hz), 8.43 (2H, d, J= 8.9 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 42.4, 45.1, 48.5, 66.5, 123.7, 125.4, 128.2, 131.7, 148.7, 152.4, 156.3, 165.2 ppm; HRMS-EI: m/z calcd for $C_{15}H_{16}N_6O_4$: 345.13113; found: 345.13142 [M+H].[note]

2.1.12. 2-(2-((4-Nitrophenyl)diazenyl)-1H-imidazol-1-yl)-1-(pyrrolidin-1-yl)ethanone (12)

Yield: 93%; reddish crystals, mp 186-189 ºC (2-propanol); IR (KBr): 3045, 2975, 1654, 1519, 1341, 857 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 1.83 (2H, bs), 1.98 (2H, bs), 3.34 (2H, bs), 3.59 (2H, bs), 5.34 (2H, s), 7.41 (1H, s), 7.65 (1H, s), 8.01 (2H, d, J= 8.1 Hz), 8.41 (2H, d, J= 8.1 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 24.2, 26.1, 45.6, 46.3, 49.1, 123.7, 125.5, 128.1, 131.7, 148.7, 152.4, 156.3, 164.9 ppm; HRMS-EI: m/z calcd for $C_{15}H_{16}N_6O_3$: 329.13621; found: 329.13664 [M+H].[note]

2.1.13. 2-Amino-5-(2-(2-((4-nitrophenyl)diazenyl)-1H-imidazol-1-yl)acetamido)pentanoic acid (13)

Yield: 95%; reddish crystals, mp 130-132 ºC (methanol); IR (KBr): 3297, 2943, 1656, 1544, 854 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 1.20-1.63 (8H, m), 5.11-5.20 (3H, m), 7.37 (1H, d, J= 5.2 Hz), 7.69 (1H, d, J= 5.2 Hz), 8.03 (2H, d, J= 8.0 Hz), 8.38 (1H, d, J= 8.0 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 22.9, 23.0, 29.0, 49.4, 54.2, 123.7, 125.4, 128.2, 131.7, 148.6, 152.3, 156.3, 166.3, 166.5 ppm; HRMS-EI: m/z calcd for $C_{17}H_{21}N_7O_5$: 404.16824; found: 404.16856 [M+H].

2.1.14. N-Cyclohexyl-2-(2-((4-nitrophenyl)diazenyl)-1H-imidazol-1-yl)acetamide (14)

Yield: 89%; reddish crystals, mp 162-164 ºC (2-propanol); IR (KBr): 3266, 2922, 1651, 1524, 1340, 1300, 856 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 1.12-1.27 (6H, m), 1.64-1.71 (4H, m), 3.56 (1H, m), 5.11 (2H, s), 7.42 (1H, s), 7.71 (1H, s), 8.08 (2H, d, J= 8.8 Hz), 8.40 (1H, d, J= 2.2 Hz), 8.43 (2H, d, J= 8.8 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 23.7, 24.4, 31.6, 47.0, 48.6, 122.6, 124.3, 127.2, 130.6, 147.6, 151.1, 155.1, 164.6 ppm; HRMS-EI: m/z calcd for $C_{17}H_{20}N_6O_3$: 357.16751; found: 357.16722 [M+H].[note]

2.1.15. 2-(2-((4-Chlorophenyl)diazenyl)-1H-imidazol-1-yl)-1-(piperidin-1-yl)ethanone (15)

Yield: 93%; reddish crystals, mp 185-188 ºC (ethanol); IR (KBr): 3104, 2939, 1651, 1368, 1086, 836, 793 cm^{-1} among others; ^{1}H-NMR (DCCl$_3$) δ: 1.55-1.61 (4H, m), 1.67-1.72 (2H, m), 3.51 (2H, bt, J= 5.5 Hz), 3.60 (2H, bt, J= 5.5 Hz), 5.26 (2H, s), 7.29 (1H, d, J= 1.1 Hz), 7.38 (1H, d, J= 1.1 Hz), 7.47 (2H, dd, J= 8.5, 2.5 Hz), 7.88 (2H, dd, J= 8.5, 2.5 Hz) ppm; ^{13}C-NMR (DCCl$_3$) δ: 24.3, 25.4, 26.4, 43.5, 46.6, 47.0, 124.3, 124.4, 129.4, 131.0, 137.5, 151.3, 151.9, 164.1 ppm; HRMS-EI: m/z calcd for C$_{16}$H$_{18}$ClN$_5$O: 332.12781; found: 332.12712 [M+H].[note]

2.1.16. 2-(2-((4-Chlorophenyl)diazenyl)-1H-imidazol-1-yl)-1-(pyrrolidin-1-yl)ethanone (**16**)

Yield: 90%; reddish crystals, mp 160-162 ºC (ethanol); IR (KBr): 3102, 2935, 1648, 1368, 837, 751 cm^{-1} among others; ^{1}H-NMR (DCCl$_3$) δ: 1.89-1.95 (2H, m), 2.03-2.08 (2H, m), 3.52-3.56 (4H, m), 5.19 (2H, s), 7.33 (1H, s), 7.37 (1H, s), 7.47 (2H, d, J= 8.7 Hz), 7.87 (2H, d, J= 8.7 Hz) ppm; ^{13}C-NMR (DCCl$_3$) δ: 24.1, 26.2, 46.0, 46.4, 47.7, 124.1, 124.5, 129.4, 131.1, 137.5, 151.4, 152.3, 164.2 ppm; HRMS-EI: m/z calcd for C$_{15}$H$_{16}$ClN$_5$O: 318.11216; found: 318.11260 [M+H].[note]

2.1.17. 2-(2-((4-Methoxy-2-nitrophenyl)diazenyl)-1H-imidazol-1-yl)-1-(piperidin-1-yl)ethanone (**17**)

Yield: 85%; reddish crystals, mp 126-129 ºC (methanol); IR (KBr): 3079, 2940, 1655, 1608, 1533, 1369, 1237, 1168 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 1.44 (2H, bs), 1.61 (4H, bs), 3.40 (2H, bs), 3.46 (2H, bs), 3.94 (3H, s), 5.29 (2H, s), 7.31 (1H, s), 7.36 (1H, d, J= 8.8 Hz), 7.52 (1H, s), 7.64 (1H, s), 7.82 (1H, d, J= 8.8 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 24.3, 25.6, 26.2, 43.0, 45.5, 48.6, 57.0, 109.0, 110.2, 120.7, 127.5, 131.0, 137.9, 149.4, 152.1, 162.0, 164.7 ppm; HRMS-EI: m/z calcd for C$_{17}$H$_{20}$N$_6$O$_4$: 373.16243; found: 373.16222 [M+H].[note]

2.1.18. 2-(2-((4-Methoxy-2-nitrophenyl)diazenyl)-1H-imidazol-1-yl)-1-morpholinoethanone (**18**)

Yield: 88%; reddish crystals, mp 178-180 ºC (ethanol); IR (KBr): 3110, 2915, 1663, 1608, 1369, 1238, 1114 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 3.42 (2H, bt), 3.53 (2H, bt), 3.58 (2H, bt), 3.70 (2H, bt), 3.94 (3H, s), 5.31 (2H, s), 7.33 (1H, d, J= 1.0 Hz), 7.37 (1H, dd, J= 8.0, 2.7 Hz), 7.51 (1H, d, J= 1.0 Hz), 7.66 (1H, d, J= 2.7 Hz), 7.85 (1H, d, J= 8.0 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 42.4, 45.0, 57.1, 66.2, 66.4, 109.1, 119.3, 120.0, 127.4, 131.2, 137.8, 149.5, 152.1, 162.1, 165.4 ppm; HRMS-EI: m/z calcd for C$_{16}$H$_{18}$N$_6$O$_5$: 375.14169; found: 375.14175 [M+H].[note]

2.1.19. N-Cyclohexyl-2-(2-((4-methoxy-2-nitrophenyl)diazenyl)-1H-imidazol-1-yl)acetamide (**19**)

Yield: 91%; reddish crystals, mp 245-248 ºC (ethanol); IR (KBr): 3399, 29302, 1650, 1322, 1064, 849 cm^{-1} among others; ^{1}H-NMR (DMSO-d_6) δ: 1.12-1.22 (6H, m), 1.64-1.71 (4H, m), 3.51 (1H, m), 3.93 (3H, s), 5.00 (2H, s), 7.29 (1H, s), 7.33 (1H, dd, J= 8.9, 2.5 Hz), 7.60 (1H, s), 7.67 (1H, d, J= 2.5 Hz),

7.81 (1H, d, J= 8.9 Hz), 8.17 (1H, d, J= 8.2 Hz) ppm; ^{13}C-NMR (DMSO-d_6) δ: 24.8, 25.6, 32.7, 48.2, 49.1, 57.1, 108.8, 119.2, 120.0, 127.3, 130.9, 138.0, 152.1, 153.2, 162.1, 165.6 ppm; HRMS-EI: *m/z* calcd for $C_{18}H_{22}N_6O_4$: 386.17025; found: 386.17085 [M+H].[note]

*2.1.20. 2-(2-((3,4-Dichlorophenyl)diazenyl)-1H-imidazol-1-yl)-1-(pyrrolidin-1-yl)ethanone (**20**)*

Yield: 89%; reddish crystals, mp 200-203 ºC (2-propanol); IR (KBr): 3101, 2935, 1653, 1416, 1369, 788 cm^{-1} among others; ^{1}H-NMR (DCCl$_3$) δ: 1.92-1.97 (2H, m), 2.06-2.12 (2H, m), 3.57-3.59 (4H, m), 5.19 (2H, s), 7.35 (1H, d, J= 1.0 Hz), 7.40 (1H, d, J= 1.0 Hz), 7.61 (1H, d, J= 8.5 Hz), 7.85 (1H, dd, J= 8.5, 2.8 Hz), 7.95 (1H, d, J= 2.8 Hz) ppm; ^{13}C-NMR (DCCl$_3$) δ: 24.1, 26.2, 46.1, 46.5, 47.7, 122.7, 124.1, 125.1, 131.0, 131.4, 133.5, 135.3, 151.8, 151.9, 164.1 ppm; HRMS-EI: *m/z* calcd for $C_{15}H_{15}Cl_2N_5O$: 352.07319; found: 352.07347 [M+H].[note]

[Note] In **6-12** and **14-20** spectra the presence of diasterotopic nucleous, was observed.

2.2. *Biological evaluation*

2.2.1. *Trypanosoma cruzi cultures*

Trypanosoma cruzi epimastigotes employed in the screening of the compounds were from the TCC strain, which derived from the Tulahuen strain that had undergone attenuation through years of *in vitro* passages [19]. Epimastigotes were grown at 28 ℃ wi th slightly shaking in T25 culture flasks containing 3 mL of Diamond medium [20], supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 20 µg/ml haemin (Sigma) and 10 % v/v heat-inactivated foetal bovine serum (Natocor).

Bloodstream trypomastigotes belonged to the RA strain, which is a strain extremely virulent for mice and highly replicative in macrophages [21, 22]. This strain is routinely maintained by weekly passages in 21-day-old CF1 mice. Bloodstream trypomastigotes were obtained from infected CF1 mice by cardiac puncture at the parasitaemia peak and employed for both *in vitro* trypanocidal activity and mouse infection.

2.2.2. *Epimastigote proliferation inhibition assay*

The effect of the synthesized compounds was first evaluated on epimastigote proliferation assays, as previously described [20]. Briefly, an epimastigote suspension (10 x10^6 parasites/mL) was seeded in duplicate onto 24-well culture plates and incubated with each compound at of 1, 5, 10, 15 µM. In parallel, epimastiogote cultures were also performed without the addition of the compounds. Benznidazole (Bnz; Roche) was used as positive control. Viability and proliferation was monitored daily through parasite counts performed in a haemocytometer. Every ~9 - 10 days, cultures were re-diluted

with fresh medium supplemented with each compound to initiate a new culture round with 5 x10^6 parasites/mL.

2.2.3. Bloodstream trypomastigotes viability assay

The trypanocidal effect of the selected compounds and Bnz was tested on bloodstream trypomastigotes according to a standard WHO protocol with minor modifications [23] and in which blood bank conditions are reproduced. Mouse blood, obtained at the parasitaemia peak (5 days p.i), was diluted in culture medium to adjust the trypomastigote concentration to 1.5 x 10^6 tryp/ml. Parasites were seeded (150μL/well) in duplicate onto a 96-well microplate in the presence of 125 to 1000 μM of the compounds or 0.39 to 100 μM of crystal violet. Plates were incubated at 4ºC for 24h and the remaining viable parasites were counted in a haemocytometer chamber. Results were calculated as the percentage of lysed parasites (%L) relative to the number of parasites in the control (without adding the drug) and expressed as IC$_{50}$ as previously described [24].

2.2.4. Cytotoxicity assay

RAW cells were employed to determine the cytotoxic effect of each compound by means of the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) method, as previously described (Sulsen et al. 2016). Briefly, cells (1x 10^5) were settled in a final volume of 100 μL in a flat-bottom 96-well microplate and cultured at 37℃ in a 5% CO_2 atmosphere in absence or presence of increasing concentrations of compounds 6 and 10 (125 to 1000 μM). After 24 h, MTT was added at a final concentration of 1.5 mg/mL. Plates were incubated for 4 h at 37℃. The purple formazan crystals were dissolved by adding 100 mL of 1% SDS in 0.01M HCl and the absorbance was read at 540 nm in a microplate reader. The 50% cytotoxic concentration (CC$_{50}$) was calculated for each compound. All experiments were performed in duplicate.

2.2.5. Animals

Outbred CF1 male mice and inbred Balb/c male mice were nursed at the Biotery of Facultad de Medicina, Universidad de Buenos Aires. Animals were handled in accordance with the guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC).

2.2.6. In vivo trypanocidal activity assay

Groups of six Balb/c mice (6 to 8 weeks old) were infected with 100 bloodstream *T. cruzi* trypomastigotes of the virulent RA strain by the intraperitoneal route. Treatment of mice began as soon

as the presence of circulating parasites was confirmed microscopically in all groups. Mice were treated orally for five consecutive days with compound 6, compound 10 or Bnz (100 mg/kg of body weight/day) according to standard recommendations [25]. Bnz was prepared by pulverization of tablets containing 50mg of the active principle; all drugs were suspended in 0.1M phosphate buffered saline (PBS, pH 7.2), and this vehicle was also employed as negative control. All suspensions were prepared daily, homogenized extensively before administration and administered immediately after preparation. Levels of parasitaemia were monitored in 5 µl of blood taken from the tail vein and diluted 1:10 in lysis buffer (0.75% NH_4Cl, 0.2% Tris, pH 7.2). Parasites were counted in a haemocytometer. Parasitaemia was monitored every other day throughout the acute period until 50% of mortality of the control group. Mortality was recorded daily up to 45 days post-infection.

2.2.7 Statistical analysis

To calculate the IC_{50} and CC_{50} values, a linear regression was performed from the log of %L or optical density values plotted against the log of drug concentration (µM) (GraphPad Prism 5.01software, GraphPad Software Inc., San Diego, CA). $IC_{50,}$ CC_{50} and values and parasitaemia values are expressed as means ± standard error of the mean (SEM). The significance of differences between IC_{50} was assessed by the Student's *t* test. The two-way ANOVA followed by the Bonferroni's post-test was employed to evaluate differences in parasitaemia levels. The Log-rank test was used to evaluate mouse Kaplan-Meier's survival curves. P values <0.05 were considered significant.

Supplementary data related to this article can be found at

3. Results and discussion

3.1. *Chemistry*

Based on the molecular structure of the Bzn prototype, a novel class of imidazole derivatives **I** (**6-20**) was synthesized. The modifications introduced to the Bnz structure are shown in the Scheme 1:

- the nitro group on the C-2 imidazole nucleus was substituted by an arylazo group, which mimics the electron-withdrawing properties of the nitro group;

- the benzylamino group on the carboxamide moiety was replaced by hydrophilic and lypophilic amino substituents.
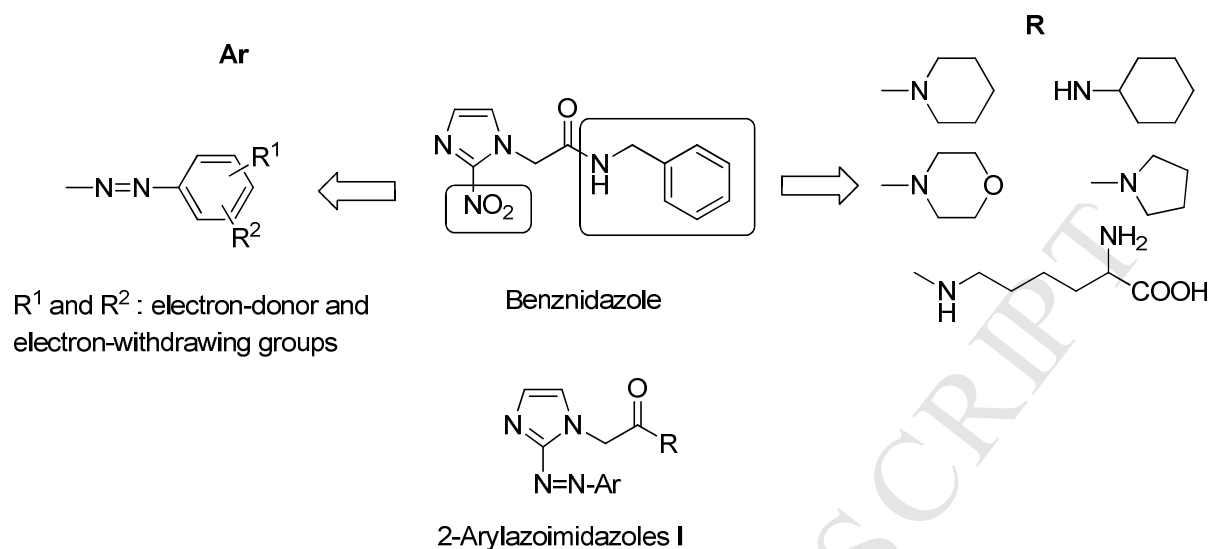
Scheme 1. Design concept to the novel 2-arylazoimidazoles **I**

The synthetic route used for the preparation of title compounds **1-20** is outlined in Scheme 2. 2-Arylazoimidazoles **1-5**, were synthesized by coupling imidazole with the corresponding aryldiazonium salts in aqueous sodium carbonate solution (pH 7).

N-alkylation reactions were carried out by addition of the appropriate chloroamide in dimethylformamide (DMF) to a mixture of the corresponding arylazoimidazole and potassium carbonate in DMF at room temperature. Under these conditions, the reactions proceeded with low yields (30-40%) and longer times were required (24-60 h). In addition, when ω-chloropropan- and butanamides were utilized as precursors, no products were detected after 70 h and starting materials were recovered without transformation.

On the other hand, the first step comprised the imidazole alkylation followed by the coupling reactions with aryldiazonium salts (Scheme 2). The latter sequence of reactions led to lower overall yields. Therefore, the first methodology was employed for the synthesis of compounds **I** (**6-20**) (Scheme 2).
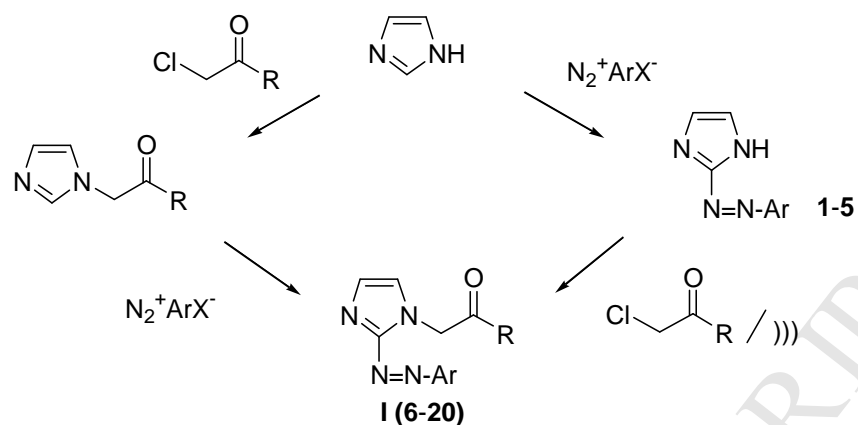
Scheme 2. Synthetic procedure for the development of derivatives **1-20**

In order to optimize the *N*-alkylation reaction, we explored the use of ultrasound (US) irradiation as promoting agent. US irradiation has proved to be an efficient technique for reagent activation in organic reactions and, over the last years, it has been considered a clean and useful methodology in organic synthesis, in accordance with green chemistry requirements [26]. The remarkable advantages of this methodology are the simple experimental procedures, high product yields, shorter reaction times, mild conditions and easy work-ups. Taking into account these advantages, a large number of organic reactions can be carried out under US irradiation as an alternative to the classical synthetic procedures [27].

The *N*-alkylation reactions were carried out at room temperature in a low intensity ultrasonic (LIU) laboratory cleaning bath (which is more economic) and with a high intensity ultrasonic (HIU) probe system (sonicator). It is well known that a LIU cleaner is considerably less powerful and that the energy of ultrasound radiation is not uniformly delivered when compared with a HIU equipment. Reactions were easy to perform when both ultrasonic instruments were employed; however, excellent yields after a shorter reaction time with reproducible results were achieved using a sonicator (HIU) (60-90 min, 85-97%).

3.2 Biological evaluation

The trypanocidal activity of some of the novel compounds was assayed against *T. cruzi* epimastigotes, and those that exhibited high activity were further tested *in vivo*.

3.2.1 Screening of trypanocidal activity of arylimidazoles on epimastigotes proliferation

The 2-arylimidazoles derivatives **6-20** were screened as possible trypanocidal compounds by measuring the inhibition of epimastigotes proliferation as described in Materials and Methods. While thirteen compounds did not show significant effects on parasite proliferation (data not shown), compounds **6** and **10**, both derived from carboxamides bearing a piperidino moiety, showed inhibitory activity similarly to that induced by Bnz (Figure 1). As soon as the first round of culture, compound **10** showed a progressive dose-response effect on proliferation, causing a significant growth inhibition at 15 µM. At this concentration, the parasite duplication time was ~4 days and a maximal density achieved was ~20 x10^6 epimastigotes/mL, while in control cultures, these parameters were ~2 days and ~45 x10^6 epimastigotes/mL, respectively and for Bnz, ~3 days and ~25 x10^6 epimastigotes/mL, respectively. At the second and third replication rounds, both compound **10** and compound **6** caused a reduction in the proliferation values, showing at the third week a duplication time of 9-12 days and maximal parasite densities of 5 x10^6 - 6 x10^6 /mL, an effect that was comparable to Bnz. Moreover, these values were 7 - 8 times lower than those obtained in control cultures. Similarly to the effect induced by Bnz, in the third round, the epimastigote motility and viability were also deeply affected by compounds **6** and **10**.
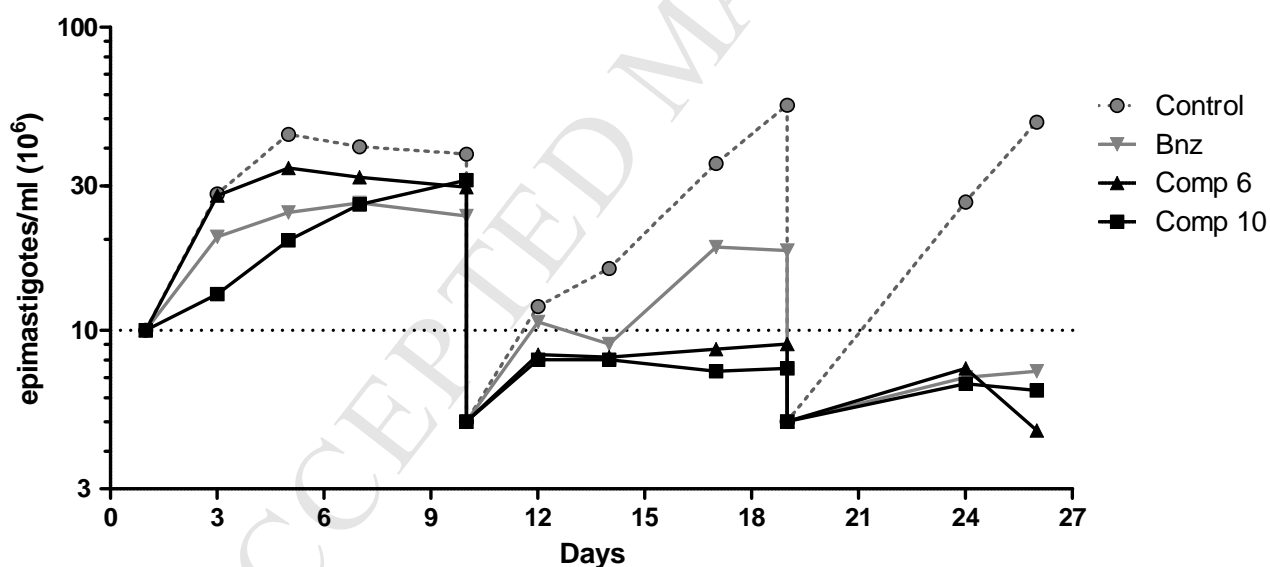

Figure 1. *In vitro* effect of compounds **6** and **10** (15 µM) on epimastigote growth curves

*3.2.2. Trypanocidal activity of compounds **6** and **10** on bloodstream trypomastigotes*

Selected compounds (**6** and **10)** were tested for *in vitro* trypanocidal activity against bloodstream trypomastigotes in blood kept at 4°C, as in blood banks. Although compound **10** was more active than compound **6** (p<0.05), IC$_{50}$ values were higher than expected (10: 645 ± 116 µM *vs.* 6: 1080± 0.09 µM

n=3). Besides, IC_{50} values were two orders of magnitude less active against bloodstream forms than the reference compound crystal violet, (2.8 ± 1.6, μM n=4, p<0.001). These results indicate that crystal violet is still the best choice for the treatment of infected blood in an emergency situation when no other donor is available.

3.2.3. In vitro cytotoxicity of compounds **6** and **10**

Inhibitory activity of compounds **6** and **10** found on epimastigotes led us to analyse their activity on an *in vivo* model of infection. In order to determine the selectivity of the compounds before treating animals, the cytotoxic effect of such compounds was evaluated on mammalian macrophages. Neither of the two arylazoimidazoles had significant cytotoxic activity on RAW cells (CC_{50}6: 414.6± 24.7 and CC_{50}10: 205.9± 34.4 μM). These results allowed us to continue with the study in the animal model.

*3.2.4 Therapeutic activity of compounds **6** and **10** in experimental mouse infection*

Groups of mice infected with *T. cruzi* trypomastigotes (RA strain) were treated for 5 consecutive days beginning on day 9 post infection (p.i.), when parasitaemia levels were significant. When animals were treated with either Comp **6** or **10,** a significant reduction in the number of circulating parasites during the acute phase of *T. cruzi* infection was observed, as compared to control group (p<0.001, Figure 2). Trypomastigotes were detected in all animals, peaking on day 18 p.i) and declining gradually to low values by day 35 p.i. in the surviving animals. The mean peak parasitaemia for untreated mice was 60.8 ± 13.7 $\times 10^{5}$ parasites/mL. Mice treated with Comp **6** or **10** presented a 60% and 71% reduction respectively, with no significant differences between them (Figure 2A).

Animals with higher parasitaemia values also presented a higher body weight loss (Figure 2B); particularly, untreated animals presented a 9.6% of body weight loss and an abnormal hunched posture during peak infection, a moment after which they died.

Although the compounds did not suppress parasitaemia as Bnz did, they prevented mice death (p< 0,005). By the end of the experiment (day 60 p.i.), 100% of Bnz-treated animals and 67% of compound.6- and compound.10-treated mice survived (Figure 2C p<0.05 *vs.* Control) with no significant differences in survival values as compared with Bnz or between them.

In summary, although compound **6** and compound **10** did not induce a suppression of parasitaemia and a reduction of mortality rates as Bnz did, they effectively reduced parasite blood levels and partially prevented mice death with respect to the control group, a result that encourages further evaluation of related compounds.
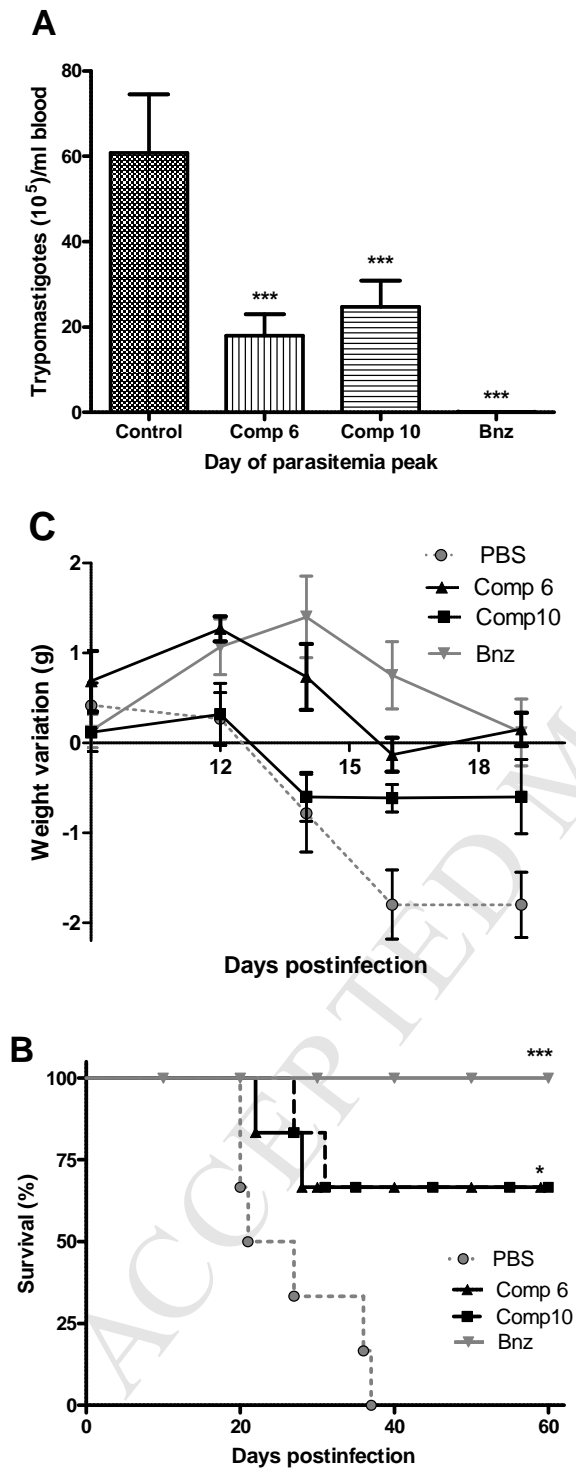
Figure 2. *In vivo* trypanocidal activity of compounds **6** and **10**: parasitaemia (peak levels) (A), body weight variation (B) and survival curve (C) during the acute infection period. *** p<0.001 parasitaemia

of all compound groups *vs* control (PBS). Bnz group was significantly different from compound-treated groups only in parasitaemia peak levels (p<0.05 *vs.* Compound 6 and p<0.01 *vs.* Compound 10, No differences were found in parasitaemia or survival between compounds **6** and **10**.

4. Conclusion

In conclusion, we have developed a simple and efficient procedure for the synthesis of a series of imidazole derivatives analogous to Bnz. 2-Arylazoimidazoles were alkylated under ultrasound irradiation at room temperature. When compared to the conventional method, this approach has several and important advantages including milder conditions, shorter reaction times and higher yields. The screening of the synthesized compounds on the epimastigote form of *T. cruzi* showed that those with inhibitory activity were compounds derived from carboxamides bearing a piperidino moiety. The evaluation in an *in vivo* model confirmed the protective capacity of these derivatives from the challenge with a virulent *T. cruzi* strain, in spite of the poor lytic activity on the extracellular bloodstream trypomastigote stage. These results indicate that 2-arylazoimidazoles series are suitable candidates for further evaluation as lead compounds to treat Chagas' disease.

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Green synthesis of 2-aryazolimidazole derivatives

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