1 **_In vitro_ antileishmanial activity and iron superoxide dismutase**
2 **inhibition of arylamine Mannich base derivatives.**
3
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25 SUMMARY

26 Leishmaniasis is one of the world's most neglected diseases, and it has a worldwide
27 prevalence of 12 million. There are no effective human vaccines for its prevention, and
28 treatment is hampered by outdated drugs. Therefore, research aiming at the
29 development of new therapeutic tools to fight Leishmaniasis remains a crucial goal
30 today. With this purpose in mind, we present twenty arylaminoketone derivatives with a
31 very interesting *in vitro* and *in vivo* efficacy against *Trypanosoma cruzi* that have now
32 been studied against promastigote and amastigote forms of *L. infantum*, *L. donovani* and
33 *L. braziliensis* strains. Six out of the twenty Mannich base-type derivatives showed
34 Selectivity Index between 39 and 2337 times higher in the amastigote form than the
35 reference drug glucantime. These six derivatives affected the parasite infectivity rates;
36 the result was lower parasite infectivity rates than glucantime tested at a IC_{25} dose. In
37 addition, these derivatives were substantially more active against the three Leishmania
38 species tested than glucantime. The mechanism of action of these compounds has been
39 studied, showing a greater alteration in glucose catabolism and leading to greater levels
40 of Fe-SOD (iron superoxide dismutase) inhibition. These molecules could be potential
41 candidates for Leishmaniasis chemotherapy.

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43

44 KEY WORDS: *Leishmania infantum*, *Leishmania donovani*, *Leishmania braziliensis*,
45 iron superoxide dismutase, arylamine derivatives, Mannich base derivatives.

46

47 KEY FINDINGS

48 1. Arylaminoketone Mannich base-type derivatives have been studied as potential
49 candidates for Leishmania therapy.
50 2. The tested compounds showed less cytotoxicity in macrophages than glucantime
51 3. Compounds showed higher intracellular activity than glucantime in the promastigote
52 and amastigote forms of three *Leishmania spp.*
53 4. The lead compounds used against three *Leishmania spp.* affected the parasite
54 infectivity rates; the result was lower parasite infectivity rates than glucantime tested
55 at a IC_{25} dose.
56 5. Compounds produced a greater alteration in glucose catabolism and Fe-SOD
57 inhibition; this could be related to mitochondrial malfunction.
58

59 INTRODUCTION

60 Leishmaniasis caused by the intracellular protozoan *Leishmania* is one of the world's
61 most neglected diseases. (WHO, 2016).

62 Although the immunology, biology and genetics of the parasites causing these diseases
63 have been studied extensively, there are no effective human vaccines for their
64 prevention, and treatment of kinetoplastid infections is hampered by outdated drugs.
65 (Uliana *et al.* 2017). The use of these drugs has been limited due to their elevated cost,
66 side effects, variable degree of efficacy, route of administration, long treatment duration
67 and the emergence of drug-resistant strains. Therefore, research aiming at the
68 development of new therapeutic tools to fight Leishmaniasis remains a crucial goal
69 today (Menezes *et al.* 2015).

70 The design of new potential drugs for Leishmania treatment claims to understand the
71 essential metabolic biochemical pathways and crucial parasite- specific enzymes. In this
72 context, enzymes that can help to avoid the damage caused by oxidative stress have
73 emerged as interesting targets (Coimbra *et al.* 2016; Singh *et al.* 2016). The most
74 interesting ones are those that present biochemical and structural differences with their
75 human counterparts (Menna-Barreto and de Castro, 2014; Hunter *et al.* 2003; Piacenza
76 *et al.* 2006). It has been shown that superoxide dismutase (Fe-SOD) enzyme plays an
77 important role in the defense of trypanosomatids against oxidative agents. It is exclusive
78 to the parasite, and parasitic protozoan survival is closely related to the ability of this
79 enzyme to evade toxic radical damage originated by their host. (Sanz *et al.* 2008;
80 Sánchez-Moreno *et al.* 2011; Turrens 2004; Bodyl and Mackiewicz, 2008).

81 From a chemical point of view, thiophene entity is a promising scaffold in medicinal
82 chemistry due to its broad spectrum as an anti-inflammatory, analgesic or antibacterial
83 (Arun *et al*. 2010; Issa *et al*. 2009; Puterová and Krutosilová 2010). Moreover, the

84 potential of thiophene derivatives as leishmanicidal agents only or in combination with
85 other moieties has also been reported (Félix *et al*. 2016), and the leishmanicidal
86 properties for a range of benzodioxole derivatives have also been described (Fernandes
87 *et al*. 2015; Parise-Filho 2012). Naphthalene derivatives have already been described for
88 their antileishmanial activity (Manzano *et al*. 2016; Mori-Yasumoto *et al*. 2012). The
89 interest in Mannich base-type derivatives as drugs or drug candidates is well known and
90 their antitrypanosomal action has been reported (Lee *et al.* 2005; Wenzel *et al*. 2009;
91 Mahal *et al*. 2017). Moreover Mannich reaction is an important tool for C-C bond
92 formation in organic chemistry, widely used for the preparation of β-aminoketones used
93 as antiparasitic agents. So, taking into account the potential of these scaffolds we
94 decided to explore the antitrypanosomal capacity of a new family of Mannich base
95 derivatives.
96 Recently, our research group has described the *in vitro* and *in vivo* anti *T. cruzi* activity
97 of 20 arylaminoketone Mannich base-type compounds obtained by condensation of the
98 corresponding arylamines and different aromatic rings with interest in medicinal
99 chemistry including thiophene, benzothiophene, benzodioxole and naphthalene (see
100 supplementary information) (Moreno-Viguri *et al.* 2016). This family of compounds has
101 shown promising activity in the infective forms of the parasites, and no genotoxicity or
102 mutagenicity was observed in the primary screening. The mechanism of action of these
103 compounds has been studied at metabolic levels by ^{1}H NMR (Nuclear Magnetic
104 Resonance), and the study has been completed by testing their activity against Fe-SOD.
105 These molecules could be potential candidates for Leishmania therapy (Turrens *et al.*
106 2004; Sanchez-Moreno *et al*. 2015) because they show selectivity over FeSOD.
107 Therefore, we decided to test these molecules against promastigote and amastigote
108 forms of *L. infantum*, *L. donovani* and *L. braziliensis* strains.

109 MATERIALS AND METHODS

110 *Chemistry*

111 The synthesis of the arylaminoketone Mannich base compounds (**1-20**) was previously
112 described (Moreno-Viguri *et al* 2016). The desired compounds were prepared by
113 condensation of the corresponding methylketone with the appropriate arylamine via
114 Mannich reaction in acidic medium and using 1,3-dioxolane as the solvent and the
115 formaldehyde source. Purification of the compounds was performed in all cases using
116 Flash column chromatography eluting in gradient with CH_2Cl_2/methanol. Spectroscopic
117 data were the same as those described in reference (Moreno-Viguri *et al.* 2016) and the
118 adequate purity of the compounds was confirmed by the analytical data.

119 *Parasite strain and culture*

120 Promastigote forms of *L. infantum* (MCAN/ES/2001/UCM-10), *L. braziliensis*
121 (MHOM/BR/1975/M2904) and *L. donovani* (LCR-L 133 LRC, Jerusalem (Israel) were
122 cultured *in vitro* in medium trypanosomes liquid (MTL) supplemented with 10%
123 inactive fetal calf serum (FCS) and kept in an air atmosphere at 28ºC in Roux flasks
124 (Corning, USA) with a surface area of 75 cm^2, following the methodology described by
125 González, P. *et al.* 2005.

126 *In vitro activity assays*

127 The tested compounds were first dissolved in dimethyl sulfoxide (DMSO, Panreac,
128 Barcelona, Spain) at a final concentration of 0.1% and then assayed for toxicity and
129 inhibitory effects on parasite and mammalian cell growth as previously described by
130 González *et al.* 2005.

131 *Cell culture and cytotoxicity tests*

132 The macrophage line J774.2 [European collection of cell cultures (ECACC) number
133 91051511] was used for the cytotoxicity test. The macrophages were cultured and the

134 cytotoxicity testing was performed by flow cytometry analysis according to a method
135 previously described (Kirkinezos and Moraes, 2001)
136 *Promastigote and amastigote assay*
137 The compounds were dissolved in the culture medium to give final concentrations of
138 100, 50, 25, 10 and 1 μM. The effects of each compound against the promastigote forms
139 at the different concentrations were tested according to the methodology described by
140 González *et al.* 2005. The inhibition effect was expressed as the IC_{50} value, i.e. the
141 concentration required to result in 50% inhibition, calculated by linear regression
142 analysis.
143 In the case of amastigote forms, J774.2 macrophage cells were cultured and seeded at a
144 density of $1x10^4$ cells per well in 24-well microplates (Nunc) with rounded coverslips
145 on the bottom and cultured for 2 days, according to the method described by Sánchez-
146 Moreno *et al*. 2012.
147 *Infectivity assay*
148 Adherent macrophage cells grown as described above were infected *in vitro* with
149 promastigote forms of *L. infantum*, *L. braziliensis* and *L. donovani* at a ratio of 10:1.
150 The tested compounds (IC_{25} concentrations) were added immediately after infection,
151 and incubated for 12 h at 37ºC in 5% CO_2 (Gonzalez *et al.* 2005). Compounds and
152 nonphagocytosed parasites were removed by washing, and then the infected cultures
153 were cultured for 10 days in fresh medium. Cultures were washed every 48 h and fresh
154 culture medium was added. Compound activity was determined on the basis of both the
155 percentage of infected cells and the number of amastigotes per infected cell in treated
156 and untreated cultures in methanol-field and Giemsa-stained preparations. The
157 percentage of infected cells and the mean number of amastigotes per infected cell were

158 determined by analyzing more than 200 host cells distributed in randomly chosen
159 microscopic fields.

160 *Metabolite excretion*

161 Cultures of *L. infantum*, *L. braziliensis* and *L. donovani* promastigotes (initial
162 concentration 5 x 10^5 cells per mL) received the IC_{25} dose of each compound (except
163 for control cultures). The methodology used was described by Fernandez-Becerra *et al.*
164 1997.

165 *Superoxide Dismutase (SOD) Inhibition Studies*

166 Promastigotes of *Leishmania* spp. were grown in tissue-culture flasks and an axenic
167 medium, as described above, until reaching a population of approximately 1×10^7
168 parasites/mL. Cells were harvested at the logarithmic growth phase by centrifugation
169 (1500 ×g for 10 min at room temperature). The pellet of cells was washed twice in MTL
170 medium without serum, and the cells were counted, distributed into aliquots of 5×10^9
171 parasites/mL in MTL medium without serum, and llowed to grow for 24 h.

172 After 24 h, the promastigote culture was centrifuged (1500 ×g for 10 min) and the
173 supernatant was filtered (Minisart®,Φ 20 μm). The filtered supernatant was subjected to
174 ice-cold ammonium sulphate precipitation at 35% salt concentration. Following
175 centrifugation, the resultant supernatant was then treated with 85% ice-cold ammonium
176 sulphate and the second precipitate was collected. The resulting precipitate was finally
177 dissolved in 2.5 mL of distilled water and desalted by chromatography in a Sephadex G-
178 25 column (GE Healthcare Life Sciences®, PD 10 column), previously equilibrated
179 with 25 mL of distilled water, bringing it to a final volume of 3.5 mL (Fraction P85e).

180 The protein content was quantified using the Sigma Bradford test, which uses bovine
181 serum albumin (BSA) as a standard (Bradford, 1976). Iron and copper-zinc superoxide
182 dismutases activities were determined using a previously described method (Beyer and

183 Fridovich, 1987) that measures the reduction in nitroblue tetrazolium (NBT) by
184 superoxide ions. According to the protocol, 845 μL of stock solution [3 mL of L-
185 methionine (300 mg, 10 mL^{-1}), 2 mL of NBT (1.41 mg, 10 mL^{-1}) and 1.5 mL of Triton
186 X-100 1% (v/v)] were added to each well, along with 30 μL of the parasite homogenate
187 fraction, 10 μL of riboflavin (0.44 mg, 10 mL^{-1}), and an equivalent volume of the
188 different concentrations of the compounds being tested. Seven different concentrations
189 were used for each agent, from 0.1 to 100 μM. In the control experiment, the volume
190 was made up to 1000 μL with 50 mM potassium phosphate buffer (pH 7.8, 3 mL), and
191 30 μL of the parasite homogenate fraction were added to the mixtures containing the
192 compounds. Next, the absorbance (A0) was measured at 560 nm in a UV
193 spectrophotometer. Afterward, each well was illuminated with UV light for 10 min
194 under constant stirring and the absorbance (A1) was measured again. The human CuZn-
195 SOD and substrates used in these assays were obtained from Sigma-Aldrich®. The
196 resulting data were analyzed using the Newman-Keuls test.
197 RESULTS
198 *In vitro antileishmanial evaluation*
199 In a first step we assayed the *in vitro* antileishmanial activity of compounds **1–20** on
200 both extra- and intracellular forms of the parasites. **Table 1** shows the IC_{50} values
201 obtained after 72 h of exposure when compounds **1–20** were tested on extra- and
202 intracellular forms of *L. infantum*, *L. braziliensis* and *L. donovani*. Toxicity values
203 against J774.2 macrophage after 72 h of culture were also calculated and Selectivity
204 Index (SI) values for the amastigote form have also been included in **Table 1**. Results
205 obtained for the reference drug glucantime were included in all cases for comparison.
206 An overall analysis of the biological data evidenced that nine of the screened
207 compounds (**3**, **4**, **7**, **11**, **12**, **14**, **17**, **18** and **19**) showed high activity against at least one

208 of three Leishmania species in both promastigote and amastigote forms. For example,
209 the SI of compound **3** exceeded that of the reference drug in *L. infantum* by 150-fold, by
210 2337-fold in *L. braziliensis* and by 1215-fold in *L. donovani*. Different authors have
211 claimed that compounds having SI values greater than 20 can be considered ideal
212 candidates for further development as leishmanicidal drugs (Nwaka and Hudson, 2006).
213 This requirement is satisfied by compounds **3**, **4**, **6**, **7**, **10** and **17** (**17** only in *L.*
214 *donovani*).

215 **Table 1**

216 *Infectivity assay*

217 In order to gain a better insight into the activities of the lead compounds **3**, **4**, **6**, **7**, **10**
218 and **17**, their effect on the infectivity and intracellular replication of amastigotes was
219 subsequently determined. Macrophage cells were grown and infected with
220 promastigotes in the stationary phase. The parasites invaded the cells and underwent
221 morphological conversion to amastigotes within 1 day after infection. On day 10, the
222 rate of host cell infection reached its maximum (control experiment). We used the IC_{25}
223 of each product as the test dosage. **Figure 1** shows the effect of the studied derivatives
224 on the infection and growth rates of the three Leishmania species. A measure of the
225 average number of amastigotes per infected macrophage led to similar conclusions: in
226 the case of *L. infantum* (**Figure 1A**), all compounds were more effective than
227 glucantime. Amastigote numbers obtained on *L. braziliensis* (**Figure 1B**) also showed
228 that all compounds were clearly more effective than glucantime under the tested
229 conditions. It was also observed that the infection rate decreased with respect to the
230 control and, furthermore, the six compounds (**3**, **4**, **6**, **7**, **10** and **17**), were also
231 remarkably more effective in decreasing parasite infectivity than glucantime at a IC_{25}
232 dose.

233 **Figure 1**

234 *Metabolite excretion*

235 Trypanosomatids are unable to completely degrade glucose to CO_2 so that they excrete
236 part of the hexose skeleton into the medium as partially oxidized fragments. The nature
237 and percentage of the oxidized fragments depend on the pathway used for glucose
238 metabolism (Turrens, 2004). The catabolism products in Leishmania species are
239 principally succinate, acetate, D-lactate and L-alanine (Kirkinezos and Moraes, 2001).
240 In order to acquire information regarding the effects of **3**, **4**, **6**, **7**, **10** and **17** on the
241 glucose metabolism of the parasite, we obtained the ^{1}H NMR spectrum of three species
242 of Leishmania (*L. infantum*, *L. braziliensis* and *L. donovani*) promastigotes treated with
243 the test compounds (compound **17** only in *L. donovani*); the final excretion products
244 were qualitatively and quantitatively identified. **Figure 2** shows the results obtained and
245 the comparison with those found for untreated control promastigotes.
246 All the compounds induce an increase in succinate production in the three species of
247 Leishmania ranging from 16.1 to 251.3% (Compound **17** only in *L. donovani*) as can be
248 observed in **Figure 2**. This effect is observed in *L. donovani* to a lesser extent (**Figure 2**
249 **C**) except for compound **10** that presents a higher accumulation of succinate in *L.*
250 *donovani* than in *L. infantum* and *L. braziliensis*.

251 *SOD enzymatic inhibition in the Leishmania parasites and in human erythrocytes*

252 Considering the obtained results, we decided to test the effects of these compounds on
253 Fe-SOD isolated from *L. infantum*, *L. braziliensis* and *L. donovani* over a range of
254 concentrations, from 0.1 to 100 μM. We used promastigote forms of both species,
255 which excrete Fe-SOD when cultured in a medium lacking inactive FBS ((Kirkinezos
256 and Moraes, 2001). The inhibition data obtained are shown in **Figures 3 (A, B and C),**
257 and the corresponding IC_{50} values are included for easier evaluation of the displayed

258 graphs; for comparison, **Figure 3A** shows the effects of the same compounds on CuZn-
259 SOD obtained from human erythrocytes.
260 Regarding the SOD enzymatic inhibition in the Leishmania parasites and in human
261 erythrocytes (**Figure 3**), the most remarkable result was the inhibitory effect on Fe-SOD
262 found for the highly antileishmanial compounds **3**, **4** and **7** in the three species tested,
263 whereas their inhibition of human CuZn-SOD was clearly lower. If we consider the IC_{50}
264 calculated for *L. infantum*, inhibition of Fe-SOD by compounds **3**, **4** and **7** was 25- , 11-
265 and 29-fold higher, respectively, than inhibition of CuZn-SOD. Compound **3** showed a
266 Fe-SOD inhibition 25-, 66- and 10- times higher than CuZn-SOD inhibition in *L.*
267 *infantum, L. braziliensis and L. donovani*, and compound **7** showed the respective
268 values of 29-, 14- and 36. Therefore, compounds **3** and **7** could be considered the most
269 selective inhibitors of Fe-SOD.
270 **Figure 2**
271 **Figure 3**
272 DISCUSSION
273 As explained above (Moreno-Viguri *et al* 2016), previous studies have indicated that
274 arylaminoketone Mannich base-type compounds may be considered prospective
275 chemotherapeutic drugs in the treatment of Chagas disease caused by *T. cruzi* parasites
276 (Turrens, 2004). We now comment on the results obtained regarding the antiparasitic
277 activity of compounds **1–20** (**Table 1**) against three significant species of Leishmania:
278 *L. infantum, L. braziliensis* and *L. donovani.* It was shown that the inhibition activities
279 against intracellular forms of the parasites of studied compounds (**17** with efficacy only
280 against *L. donovani*) were higher than those found for the reference drug glucantime,
281 whereas the effect on extracellular forms was more random. Regarding the toxicity in
282 mammalian cells, the tested compounds were found to be much less toxic for

283 macrophages than the reference drug. Therefore, compounds **3**, **4**, **6**, **7**, **10** and **17** were
284 considered the lead ones due to their excellent antileishmanial activity and were selected
285 for subsequent studies.
286 Interestingly, the best SI results for the more representative intracellular forms were
287 obtained in *L. infantum* and *L. donovani*, two species forming part of the *L. donovani*
288 complex, pointing towards a greater specificity towards parasites causing the
289 particularly harmful visceral leishmaniasis in both its European and American versions.
290 With regard to the structure-activity relationship, in general, derivatives with the
291 benzo[*b*]thiophene scaffold are less cytotoxic than the rest of the derivatives.
292 In the infectivity assay (**Figure 1**) all the compounds were more effective in relation to
293 IC_{25} than glucantime. The infection rates decreased with respect to the control and the
294 reference drug glucantime. The measure of the average number of amastigotes per
295 infected macrophage led to similar conclusions. All these data seem to be in line with
296 results previously described for *T. cruzi* (Moreno-Viguri *et al.* 2016).
297 Regarding the studies to elucidate the possible mechanism of action, the studied
298 compounds produce greater glucose metabolism alteration because they increase
299 succinate excretion (Figure 2). Detection of large amounts of succinate as a major end
300 product is a usual feature, because it relies on glycosomal redox balance, enabling re-
301 oxidation of the NADH produced in the glycolytic pathways. It is interesting to mention
302 that the increase in succinate with these compounds indicates catabolic changes that
303 could be related to mitochondria malfunction, due to the redox-stress produced by
304 inhibition of the mitochondrion-resident Fe-SOD enzyme (Marín *et al.* 2011).
305 In addition, these compounds led to greater levels of Fe-SOD inhibition. All these data
306 appear to confirm some type of relation between the antileishmanial activity and the Fe-
307 SOD inhibition, coinciding with the results described in previous work (Ginger, 2005).

308 Fe-SOD inhibition could also, at another level, be related to the catabolic changes
309 discussed above because a mitochondrial malfunction, originated from the redox stress
310 produced by inhibition of the mitochondrion-resident Fe-SOD enzyme, (Marin *et al.,*
311 2011) should result in severe alteration of pyruvate metabolism and consequently, a
312 decrease in the production of succinate. Because the Fe-SOD present in mitochondria is
313 an essential part of the antioxidant protective response of the parasite, its inhibition
314 would be related to a decrease in the rate of survival for the parasite.

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483 **Figure 1.** Effect of arylaminoketone derivatives **3**, **4**, **6**, **7** and **10** on the infection and

484 growth rates and mean numbers of amastigotes per infected J774.2 macrophage cell (at

485 IC_{25} concentration) of *L. infantum*. **(A)**, *L. braziliensis* **(B)** *L. donovani* **(C).** Values are

486 the means of three separate experiments.

487 All compounds are statistically significant against glucantime at a p-value <0.05, except

488 compounds labeled as NS.

489 **Figure 2.** Variation percentages in the area of the peaks corresponding to excreted

490 catabolites by *L. infantum* **(A)**, *L. brasilienzis* **(B)** and *L. donovani* **(C)** promastigotes in

491 the presence of compounds **3**, **4**, **6**, **7** ,**10** and **17** at their IC_{25} compared to a control

492 sample after 96 h of incubation.

493 **Figure 3. (A)** *In vitro* inhibition of CuZn-SOD in human erythrocytes by compounds **3**,

494 **4**, **6**, **7**, **10** and **17**. **(B–D)** *In vitro* inhibition (%) of Fe-SOD of *L. infantum* **(B)**, *L.*

495 *braziliensis* **(C)** and *L. donovani* **(D)** promastigotes by compounds **3**, **4**, **6**, **7**, **10** and **17**.

496 Values are the average of three separate determinations. Differences between the

497 activities of the control homogenate and those incubated with the tested compounds

498 were obtained according to the Newman–Keuls test. IC_{50} was calculated by linear

499 regression analysis.