Expanding the chemical space of aryloxy-naphthoquinones as potential anti-Chagasic agents: synthesis and trypanosomicidal activity

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Abstract
In continuation our effort to research the chemical space of aryloxy-naphthoquinones as potential anti-Chagas agents, we synthesized nine derivatives and these compounds were evaluated in vitro against the epimastigote and trypomastigote forms of Mexican strains of Trypanosoma cruzi (T. cruzi). Most of these derivatives are highly active against epimastigote forms (IC50 < 1.0 µM) compared to the reference drug benznidazole (Bzn). Then these were evaluated on trypomastigotes, which is showing better potency results than Bzn for compounds 3b and 3g. In addition, the cytotoxicity of these compounds was determined on the murine macrophage cell line J774. 3b and 3i were the most selective compounds against NINOA trypomastigote and INC-5 epimastigote forms, respectively. Further these compounds also have good oral bioavailability according to theoretical predictions. Finally, we were able to determine optimal substitution patterns using pharmacophoric models. All these results are provided very useful structural information to continue our designing of naphthoquinone derivatives against T. cruzi.

Graphical Abstract

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Introduction

Chagas disease or American trypanosomiasis is one of the 20 “Neglected Tropical Diseases” (NTD) (WHO, 2020). The disease is caused by Trypanosoma cruzi (T. cruzi), a protozoan parasite endemic in Latin America. It affects ~7 million people and causes more than 10,000 deaths a year [1]. There are two drugs approved for the treatment of this disease, benznidazole (Bzn) and nifurtimox (Nfx) [2]. Both are effective in the early stages of the disease (acute stage), but their efficacy is limited in advanced stages (chronic stage). These drugs also present serious adverse effects and require long-term therapy leading to treatment suspension [3, 4]. Considering the above and the fact the pharmaceutical industry does not invest in research for NTDs, it is of great importance to find new alternatives for the treatment of trypanosomiasis. For this reason, many academic research groups are searching for new trypanosomicidal therapeutic alternatives through the design and synthesis of small molecules [5, 6].

Compounds derived from a naphthoquinone scaffold appear to be an interesting alternative for the Chagas disease pharmacotherapy. Lapachol I and α-lapachone II (Fig. 1) are important naphthoquinone derivatives in nature with trypanosomicidal activity [7, 8]. Considering this background, a library of 2-aryloxy-1,4-naphthoquinone compounds were synthesized by Bolognesi et al. 2-phenoxy-1,4-naphthoquinone III was the most prominent derivative in this series, with an IC50 of 1.70 μM against T. cruzi amastigotes [9].

In recent years, our research group has focused on the search for new anti-T. cruzi agents containing the structure of the aryloxy-quinone scaffold. Our first approach was through the synthesis of aryloxy-indolequinone derivatives and their evaluation against T. cruzi epimastigotes culture. Of these, 6-phenoxyindolequinone derivative IV (Fig. 1) showed excellent inhibitory activity in the nanomolar range against Y strain epimastigote (IC50 = 20 nM) [10].

In a subsequent article, our group reported the synthesis and trypanosomicidal activity of 2-aryloxy-naphthoquinones, 7-aryloxyquinolinquinones and 6-aryloxyfuronaphthoquinones derivatives [11]. In this work compound 2-(3-nitrophenyloxy)-naphthoquinone V (Fig. 1) showed an IC50 = 20 nM and a selectivity index (SI) of 625 against J774 murine macrophage cells, which showed low toxicity against non-neoplastic cells. In our last report on aryloxy-naphthoquinones with trypanosomicidal activity, we obtained good results against epimastigote cultures with an IC50 = 230 nM for 2-(4-methyl-3-nitrophenoxy)-naphthoquinone VI [12].

From these results, we observed the presence of an aryloxy group attached to the naphthoquinone core is important for anti-T. cruzi activity. Furthermore, we have noticed the addition of a nitro or a methyl group on the

![Fig. 1 Chemical structures of lapachol (I), α-lapachone (II) and aryloxy-quinones (III-VI) with trypanosomicidal activity, and new aryloxy-naphthoquinones studied in this work](image)
aryloxy moiety gives rise to compounds with equal or better trypanosomicidal activity. It remarked that Nfx and Bzn, drugs have been used in the chemotherapy of Chagas disease, have a nitro group in their structure. Interestingly, in 2017 the FDA has approved Bzn as pharmacological treatment for the Chagas disease [13]. Therefore, considering the aforementioned, in this work, we carried out the synthesis of new naphthoquinones (Fig. 1), which was functionalized according to four criteria, to evaluate the effect of these modifications on the trypanosomicidal effect on epimastigote and trypomastigote forms from Mexican strains of T. cruzi.

These modifications were: (a) the substitution of the hydrogen atom on C-3 by bromine or chlorine because it has been reported that this change modulates the trypanosomicidal activity on Tulahuén, NINOA, INC-5 and Y strains [14]. In the aryloxy moiety: (b) the substitutions of a nitro group in the aryloxy moiety at meta- or para-position; (c) as shown in VI, a methyl group at para-position and meta-position; (d) a substitution at para-position by chlorine atom. With these new derivatives, our group expanded the library of compounds with trypanosomicidal activity and contributed to the chemical space of naphthoquinones with anti-Chagasic activity.

Results and discussion

Chemistry

We synthesized new aryloxy-naphthoquinones by nucleophilic substitution reaction of phenols with halo or dihaloquinones in a basic medium at room temperature (Scheme 1) [11, 12, 15]. These aryloxy-naphthoquinones were obtained from low to moderate yields (23–67%). The chemical structures of the compounds 3a–i were established based on their spectral properties (IR, 1H-NMR, 13C-NMR and MS). The chemical structures and the yield for the synthesis of 3a–i are shown in the Table 1.

Biology

Trypanosomicidal effect against on the epimastigote form

First, a screening assay was performed on the INC-5 and NINOA strains at a fixed concentration of 10 µM to determine the trypanosomicidal effect on epimastigote cultures. The results indicated that all compounds had a percentage of growth inhibition (% GI) in both strains greater than 70%. Considering these results, a dose-dependent assay was carried out using different concentrations (5, 2.5, 1.25, 0.625 µM) to determine the half-maximal inhibitory concentration (IC50) in each strain (Table 2).
The half-maximal lytic concentration (LC50) in the trypomastigote form of both strains was measured for the compounds showed the lowest IC50 values. The best results were obtained in the NINOA strain with LC50 values of 9.38 and 9.75 µM for compounds 3b and 3g, respectively (Table 2). These compounds were four-fold more active than Bzn (LC50 = 40.67 µM). On the other hand, in the INC-5 strain, two compounds, 3c and 3g showed approximately two-fold greater potency than Bzn (LC50 = 46.67 µM). However, it was not possible to establish a structure-activity relationship in trypomastigotes because the assayed compounds were limited in number. It is remarkable that in NINOA, the most active compounds were those with a chlorine atom at 3-position on the naphthoquinone core.

The important results are obtained from these strains. The trypomastigote is the infective flagellated form of the parasite in the blood. This form is responsible for the acute and chronic stages of Chagas disease in mammalian hosts [16]. Epimastigotes were used as a primary screening to handle and maintain in the laboratory [17].

Cytotoxicity in murine macrophages

The cytotoxicity of the most promising compounds 3b, 3c, 3f, 3g and 3i was determined on the murine macrophage J774 cell line (Table 3). In general, the five derivatives had greater selectivity for the epimastigote form of both strains, with 3i being the compound with the highest selectivity index (SI = 320). Moreover, it is important to point out the high selectivity of compound 3b against the NINOA strain in trypomastigotes. The SI for 3b was comparable with Bzn but with a better LC50 on this strain than this reference drug.

Pharmacophoric elucidation

Compounds were aligned by a flexible alignment technique. This technique overlaps 3D structures minimizing the
relative distances between equivalent atoms (in each molecule), including flexibility that allows all degrees of freedom in the rotatable angles, covering all possible dihedral angles. The pharmacophoric model was built using the Polar-Charged-Hydrophobic (PCH) scheme in the MOE program [18]. The model was built with all features present in at least 60% of aligned molecules, resulting in the selection of the following features: Aromatic rings (Aro); Hydrophobic center (Hyd); Hydrogen bond acceptor capacity (Acc); Metal ligation site (ML), and a combination of these. Some combinations could be defined (Acc&ML).

Considering that there are more IC₅₀ values for the epimastigote form in both strains, a pharmacophoric model was carried out.

**Pharmacophoric models for INC-5 epimastigote *T. cruzi* growth inhibitors**

In order to understand the chemical features that were essentials for the trypanosomicidal activity of these quinones, a pharmacophoric model was built. The molecules were classified according to their degree of inhibition of INC-5 epimastigote growth (Fig. 2). Using this at home designed pharmacophore, differential features associated with bioactivities were found and described. The main difference observed between both non-active/active pharmacophoric descriptors were sites allowing bound to metals (meta-position, red spheres) Then, the “metal ligation” pharmacophoric characteristic in this space region could be proposed as a descriptor of a non-favouring bioactivity. On the other hand, looking for substructures related to a favored bioactivity, in the same place of three-dimensional space, a nitro group will favor the desired bioactivity. All in all, the desired bioactivity is related once with the electronic density of the aromatic ring sustaining this group because of the high electronegativity of nitrogen and oxygen of the nitro substituent. The association of the electron density with the desired trypanosomicidal activity is not a discovering but confirm once again the classical theory that this characteristic must be essential to this activity. The discovering of a differential pharmacophoric characteristic, that could be very helpful for the rationalization of our result as well as for future design of new trypanosomicidal compounds.

**Pharmacophoric models of NINOA epimastigotes *T. cruzi* growth inhibitors**

Using the same strategy above described, the molecules were classified according to their trypanosomicidal (NINOA epimastigotes) high or low growth inhibition (Fig. 3); their differential features were also detected. In this case, bioactivity is favored with the presence of acceptor groups of metallic atoms at para-position on the phenoxy group (cyan spheres) and non-favored when these features are placed at meta-position (red spheres).
Physicochemical properties

Drug-likeness properties such as the pharmacokinetic (ADME) and pharmacodynamic (e.g., toxicological) profiles are important during the process of discovery and development process of a drug. These properties are shown the optimization of a leading compound to a successful candidate for pre-clinical stages [19].

ADMET properties are important to determine some chemical descriptors such as the polar surface area (PSA) and the molecular weight (MW) of molecules, which are useful to determine the oral absorption of drugs. Small and hydrophilic molecules are undergoing rapid renal clearance, while large and hydrophobic compounds are undergoing extensive hepatic metabolism and poor absorption [20]. Therefore, finding a suitable hydrophilic-hydrophobic drug balance is a great challenge for medicinal chemists. Thus, to evaluate these properties and predict good oral bioavailability, two sets of rules, Lipinski and Veber, should be followed to make a good prediction [20, 21].

Lipinski’s rule of five states that an orally bioavailable molecule should not violate the following criteria: ≤5 hydrogen bond donors (HBD); ≤10 hydrogen bond acceptors (HBA); a MW ≤500, and a log P value ≤5. On the other hand, Veber et al. described the role of PSA and the number of rotatable bonds as criteria to estimate oral bioavailability. Veber’s rule states that the compound to be orally bioavailable, it should have either a PSA ≤ 140Å and ≤10 rotatable bonds. As shown in Table 4, compounds 3a–i achieved the Drug-likeness criteria described by Lipinski and Veber; therefore, they are expected to have good oral bioavailability.

Conclusions

In this study, a set of nine aryloxy-naphthoquinones were synthesized and evaluated in vitro and ex vivo against the epimastigote and trypomastigote forms in two Mexican T. cruzi strains. The trypanosomicidal activity of these compounds against both forms and were better than the reference drug, benznidazole (Bzn). The most promising naphthoquinone derivative is compound 3b due to its highest trypanosomicidal activity against epimastigotes (INC-5 and NINOA strains) and trypomastigote (NINOA strain) and SI values. From the theoretical studies, important features from pharmacophoric models were identified to develop the more active naphthoquinone derivatives. Predictions about their ADME properties indicated that these aryloxy-naphthoquinones would have good bioavailability.

Experimental section

General

Melting points were determined on a Kofler Thermogerate apparatus and were uncorrected. IR spectra were recorded.
were recorded, unless otherwise speci
on a JASCO FT/IR-400 spectrophotometer. NMR spectra

Table 4 Molecular properties of

| Compound | MW (Da)  | HBA  | HBD  | MiLogP  | TPSA (Å²)  | NRB   |
|----------|----------|------|------|----------|------------|-------|
| Desirable value | ≤500 | ≤10 | ≤5 | ≤5 | ≤140 | ≤10 |
| 3a | 309.28 | 6 | 0 | 3.69 | 89.20 | 3 |
| 3b | 343.72 | 6 | 0 | 4.29 | 89.20 | 3 |
| 3c | 388.17 | 6 | 0 | 4.42 | 89.20 | 3 |
| 3d | 329.69 | 6 | 0 | 3.91 | 89.20 | 3 |
| 3e | 364.14 | 6 | 0 | 4.52 | 89.20 | 3 |
| 3f | 408.59 | 6 | 0 | 4.65 | 89.20 | 3 |
| 3g | 329.69 | 6 | 0 | 3.91 | 89.20 | 3 |
| 3h | 343.72 | 6 | 0 | 4.29 | 89.20 | 3 |
| 3i | 388.17 | 6 | 0 | 4.42 | 89.20 | 3 |

MW molecular weight, HBA number of hydrogen bond acceptors, HBD number of hydrogen bond donors, MiLogP Log P value predicted by Molinspiration (www.molinspiration.com), TPSA topological polar surface, NRB number of rotatable bonds

on a JASCO FT/IR-400 spectrophotometer. NMR spectra were recorded, unless otherwise specified, on a Bruker AM-400 instrument using deuterochloroform (CDCl₃) or deutero(dimethyl)sulfoxide (DMSO-d₆) solutions containing tetramethylsilane as an internal standard. Samples were analyzed using an Advion (Itaca, NY, USA) Expression-L mass spectrometer equipped with an electrospray ionization source (ESI). Sample were directly injected (5 µL) using LC-grade methanol as mobile phase. Mass spectrometry analysis was carried out using the following settings: ESI (−) voltage of 2.5 kV, nebulizer gas (N₂) flow: 3.0 L min⁻¹, drying gas flow: 10 L min⁻¹, desolvation line temperature 200 °C and heat block temperature 250 °C. Analytes were evaluated in Full Scan mode (m/z) 100–1500. Data was acquired by means of Advion Mass Express and processed applying Data Express Software. Thin layer chromatography (TLC) was performed using Merck GF-254 type 60 silica gel. Column chromatography was carried out using Merck type 9385 silica gel. The purity of the compounds was determined by TLC. All the spectra of aryloxy-naphthoquinones synthesized are shown in the Supporting information.

General procedure for the synthesis of aryloxy-naphthoquinones

In a reaction flask, the suitable phenol (1.1 mmol) and K₂CO₃ (2 mmol) were suspended in DMF (5 mL). The mixture was stirred for 10 min, and then, the corresponding naphthoquinone (1 mmol) was added. Later, the reaction mixture was stirred for 2–3 h at room temperature. The solvent was then removed under vacuum, and the solid residue was purified by column chromatography on silica gel and using dichloromethane as the mobile phase.

2-(4-Methyl-3-nitrophenoxy)naphthalene-1,4-dione

3a Yellow solid, yield 63%, mp 163–165 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.21–8.15 (m, 1H), 8.10–8.03 (m, 1H), 7.81 (d, J = 2.5 Hz, 1H), 7.79–7.75 (m, 2H), 7.46 (d, J = 8.4 Hz, 1H), 7.32 (dd, J = 8.4, 2.5 Hz, 1H), 6.01 (s, 1H), 2.64 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 184.5, 179.3, 159.5, 151.1, 149.7, 134.7, 134.6, 133.8, 132.0, 131.8, 131.0, 126.9, 126.4, 125.7, 117.5, 114.4, 20.1. MS (ESI) for (C₁₇H₁₁NO₅ [M–]): 309.3. Found 309.0.

2-Chloro-3-(4-methyl-3-nitrophenoxy)naphthalene-1,4-dione 3b Yellow solid, yield 25%, mp 150–152 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.22 (d, J = 7.0 Hz, 1H), 8.05 (d, J = 6.8 Hz, 1H), 7.85–7.74 (m, 2H), 7.61 (d, J = 1.6 Hz, 1H), 7.33 (d, J = 8.4 Hz, 1H), 7.24–7.17 (m, 1H), 2.58 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 179.0, 177.6, 154.5, 152.7, 149.3, 134.9, 134.7, 134.5, 134.0, 131.2, 130.4, 129.4, 127.6, 127.3, 121.66, 112.8, 20.0. MS (ESI) for (C₁₇H₁₀ClNO₅ [M–]): 343.0. Found 343.0.

2-Bromo-3-(4-methyl-3-nitrophenoxy)naphthalene-1,4-dione 3c Yellow solid, yield 24%, mp 188–190 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.16–8.08 (m, 1H), 7.95 (dd, J = 7.2, 1.7 Hz, 1H), 7.76–7.64 (m, 2H), 7.52 (d, J = 2.6 Hz, 1H), 7.24 (d, J = 8.5 Hz, 1H), 7.13 (dd, J = 8.4, 2.6 Hz, 1H), 2.49 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 178.1, 177.1, 155.2, 154.4, 149.3, 134.8, 134.6, 134.0, 131.1, 130.4, 129.3, 128.6, 127.8, 127.4, 121.6, 112.8, 20.0. MS (ESI) for (C₁₇H₁₀BrNO₅ [M–]): 386.9. Found 387.0.

2-(4-Chloro-3-nitrophenoxy)naphthalene-1,4-dione

3d Yellow solid, yield 54%, mp 164–165 °C. ¹H NMR (400 MHz, CDCl₃) δ 8.18–8.14 (m, 1H), 8.09–8.05 (m, 1H), 7.83–7.74 (m, 2H), 7.72 (d, J = 2.8 Hz, 1H), 7.65 (d, J = 8.8 Hz, 1H), 7.35 (dd, J = 8.8, 2.8 Hz, 1H), 6.11 (s, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 184.2, 179.1, 158.7, 151.8, 148.5, 134.8, 134.0, 133.7, 131.8, 130.9, 127.0, 126.5, 125.7, 124.7, 118.3, 115.6. MS (ESI) for (C₁₆H₁₅ClNO₅ [M–]): 329.0. Found 328.9.
2-Chloro-3-(4-chloro-3-nitrophenoxy)naphthalene-1,4-dione 3e
Yellow solid, yield 43%, mp 199–201 °C. $^1H$ NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.14 (dd, $J = 7.3, 1.4$ Hz, 1H), 8.09 (d, $J = 2.9$ Hz, 1H), 8.02 (dd, $J = 7.1, 1.7$ Hz, 1H), 7.79–7.92 (m, 2H), 7.80 (d, $J = 8.9$ Hz, 1H), 7.67 (dd, $J = 8.9, 3.0$ Hz, 1H). $^{13}$C NMR (101 MHz, DMSO-$d_6$) $\delta$ 178.5, 178.0, 155.5, 152.0, 148.9, 135.2, 135.0, 134.9, 133.1, 131.9, 127.1, 127.0, 122.2, 119.3, 113.4. MS (ESI) for (C$_{16}$H$_7$ClNO$_5$ [M-]): 363.0. Found 363.0.

2-Chloro-3-(4-nitrophenoxy)naphthalene-1,4-dione 3g
Yellow solid, yield 61%, mp 169–198 °C. $^1H$ NMR (400 MHz, CDCl$_3$) $\delta$ 8.40–8.31 (m, 3H), 8.21–8.15 (m, 1H), 8.00–7.86 (m, 2H), 7.28–7.13 (m, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 177.9, 177.3, 160.7, 152.4, 143.9, 135.0, 134.8, 131.1, 130.3, 127.7, 127.4, 126.0, 116.8. MS (ESI) for (C$_{16}$H$_7$NO$_5$ [M-]): 329.0. Found 328.9.

2-Chloro-3-(4-nitrophenoxy)naphthalene-1,4-dione 3h
Yellow solid, yield 37%, mp 170–171 °C. $^1H$ NMR (400 MHz, CDCl$_3$) $\delta$ 8.19–8.10 (m, 1H), 8.06 (d, $J = 9.0$ Hz, 1H), 8.01 (dd, $J = 7.1, 1.5$ Hz, 1H), 7.98–7.87 (m, 2H), 7.38 (d, $J = 2.5$ Hz, 1H), 7.31 (dd, $J = 9.0, 2.7$ Hz, 1H), 2.51 (s, 2H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 178.5, 177.9, 159.5, 152.1, 144.6, 137.0, 135.2, 135.0, 135.0, 131.2, 131.0, 127.7, 127.2, 127.0, 119.7, 115.2, 20.5. MS (ESI) for (C$_{16}$H$_7$NO$_5$ [M-]): 343.0. Found 343.0.

2-Chloro-3-(3-methyl-4-nitrophenoxy)naphthalene-1,4-dione 3i
Yellow solid, yield 32%, mp 174–176 °C. $^1H$ NMR (400 MHz, CDCl$_3$) $\delta$ 8.27–8.11 (m, 1H), 8.11–7.95 (m, 2H), 7.85–7.71 (m, 2H), 6.94–6.87 (m, 2H), 2.59 (s, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 178.0, 176.9, 158.9, 155.0, 144.74, 137.3, 134.9, 134.7, 131.1, 130.4, 129.1, 127.9, 127.5, 127.5, 119.9, 114.4, 21.3. MS (ESI) for (C$_{17}$H$_{10}$BrNO$_3$ [M-]): 386.9. Found 387.0.

Trypanosomical effect

*T. cruzi* NINOA and INC-5 strain epimastigotes were grown at 28 °C in an axenic medium (BHI), as previously described [22], and complemented with 5% fetal bovine serum. Epimastigotes from a 10-day-old culture (stationary phase) were inoculated into 50 mL of fresh culture medium to reach an initial concentration of $1 \times 10^6$. Cell growth was monitored by measuring culture absorbance of the culture at 600 nm in an ELISA Epoch reader (Epoch 2 Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) every day. Before inoculation, the media was supplemented with a given amount of the drug from a stock solution in DMSO (25 mM). The final DMSO concentration in the culture medium never exceeded 0.4% and the control was run in the presence of 0.4% DMSO and in the absence of drugs. The percentage of growth inhibition (% GI) and half-maximal inhibitory concentration values (IC$_{50}$), and parasite growth were followed in the absence (control), and in the presence, of a range of concentrations of the corresponding drug. On day 5, the absorbance of the culture was measured and related to the control. The IC$_{50}$ value was determined as the concentration of drug needed to reduce the absorbance ratio by 50%. The experiments of each compound were carried out in triplicate; Bzn was used as a reference compound.

**Ex vivo evaluation of INC-5 and NINOA strain tryptomastigotes**

CD1 mice, 6–8 weeks old, were peritoneally infected with bloodstream tryptomastigotes of the INC-5 and NINOA strains, respectively. After 4–6 weeks, at the maximum peak of parasitaemia, the parasitized blood was obtained by cardiac puncture using sodium heparin as an anticoagulant. Blood was adjusted to 1 × 10$^6$ tryptomastigotes/mL. In a 96-well plate, 90 µl of infected blood and 10 µl of 1,4-naphthoquinone derivatives or reference drug dilutions were deposited for a final volume of 100 µl per well. Each evaluation was carried out in triplicate. The compounds with the best IC$_{50}$ in the epimastigotes of each strain were evaluated in tryptomastigotes of both strains at 20, 10 and 5 µM. The reference drug benznidazole was used as a positive lysis control and wells with untreated blood tryptomastigotes were used as a negative lysis control; the microplates were incubated at 4 °C for 24 h. Subsequently, mobile tryptomastigotes were quantified using the Brener–Pizzi method, for this, 5 µL of blood was deposited between a slide and a 13 × 13 coverslip. The tryptomastigotes were counted in 15 microscope fields in an optical microscope with ×40 magnification. The percentage of lysis of each treatment was calculated by comparing the viable tryptomastigotes with the negative control [16]. The LC$_{50}$ was determined by linear regression. The experiments were carried out in accordance with the recommendations and approval of the local Ethics and Research Committee (Approval number: ENCB/CEI/078/2020).
Cytotoxicity assays

A 96-well plate was seeded with 50,000 cells/well with RPMI medium and 2% fetal bovine serum and incubated for 24 h at 37 °C. The compound from stock was dissolved in DMSO for subsequent serial dilutions with PBS until a concentration in the plate that did not exceed 1% of DMSO was reached. After 24 h, the culture medium was removed from the plate and fresh culture medium was added together with the compounds reaching a final volume of 100 µl per well. The plate was then incubated for 24 h at 37 °C in 5% CO₂ where the following controls were added: cells with the medium as a positive control; cells without culture medium as a negative control; and last, the reference drug, Bzn at a concentration of 20 µM.

After 24 h of incubation, the cell morphology was observed with a microscope and the MTT viability assay was performed. The culture medium was removed and the MTT solution was prepared and later added to the cells; it was incubated for 1 h and the absorbance was read at 570 nm. The following formula was used to obtain the percentage: % cytotoxicity = (100 – (mean number of cells with treatment/cells without treatment) *100) [23].

Pharmacophore

A flexible alignment strategy was used, consisting of the overlap of 3D structures, and minimizing the relative distances between equivalent atoms, including the flexibility for all freedom degrees for all rotatable dihedral bonds. Polar-Charged-Hydrophobic (PCH) of the MOE program was used and evaluated over a centroid position (an averaged position of all atoms relates to the feature). Pharmacophoric features in this scheme are annotated as Aromatic (aromatic), hydrophobic (Hyd), acceptor hydrogen bonding (Acc), and capacity to bond to metallic centers (ML). Afterward, databases were spotted in groups of high and low diversity (Acc), and capacity to bond to metallic centers (ML). For each group, a pharmacophore was built, and all features present in a minimum of 60% of molecules were retained. Finally, all pharmacophores were compared.

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Compliance with ethical standards

Conflict of interest  The authors declare no competing interests.

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