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Synthesis and biological evaluation of trifluralin analogues as antileishmanial agents

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ABSTRACT

A series of new analogues of trifluralin (**TFL**) were synthesized and characterized in view of changing the unfavorable properties that limits its use as antileishmanial agent. Some of the **TFL** analogues display more activity than a standard drug (miltefosine) against the promastigote forms of *Leishmania infantum* and *Leishmania donovani* and the intracellular form (THP-1 infected with *L. infantum*). All analogues showed a clear advantage over miltefosine, as they are not hemolytic. Some analogues can conjugate these characteristics with reduced cell toxicity and improved intracellular activity.

1. Introduction

Leishmaniasis is a protozoan disease that constitutes a major public health problem. It affects about 12 million people worldwide, particularly in tropical and sub-tropical regions. In 2001 leishmaniasis was responsible for almost 60,000 deaths and it represents an increased risk of infection among the immunosuppressed patients.[1,2] It attracted a special attention from WHO as it is considered the second most important protozoan disease.[3] *Leishmania infantum* is the parasite responsible for the visceral form of the disease in the Mediterranean region. This disease is reemerging in Europe in association with HIV but also poses a major veterinary problem in areas where domestic dogs serve as reservoir host. Inefficient treatment and the absence of vaccine is the major cause for the high rates of infected dogs that results in the continuous circulation of the parasite in endemic areas and in the evolution of resistant phenotypes.

The first line drugs recommended for the treatment of this disease, such as the pentavalent antimonial agents, have serious side effects. This and the emergence of resistant strains are responsible for their progressive abandon. Several other classes of drugs have been used for the treatment of leishmaniasis, such as pentamidine, paramomycin, amphotericin B, liposomal amphotericin B, and miltefosine. In spite of the battery of antileishmanial drugs, none of the current therapeutic schemes exhibits high efficiency and low cytotoxicity together with an affordable cost.[2,4,5] For these reasons the development of new effective and less toxic therapies is urgently needed.

Dinitroanilines are tubulin-binding agents than have shown selective antileishmanial properties in vitro. A member of this class of compounds, trifluralin (**TFL**) was effective against the cutaneous form of the disease, as an ointment.[6] However, its use for other forms of leishmaniasis, namely by parenteral administration, has been limited by its low water solubility and easy sublimation.[7] These two features have created several drawbacks in the development of pharmaceutical formulations of dinitroanilines as antiparasitic agents.

To overcome the handling problems associated with dinitroanilines different strategies can be adopted: one is to introduce chemical modification that will change the water solubility of the compound; another is to incorporate the compound in liposomes or both. **TFL** in its liposomal formulation, showed to be active in mice and dogs that were experimentally infected with *Leishmania donovani* and *L. infantum*, respectively.[8,9] In order to improve the antiparasitic activity, some dinitroanilines (**TFL** and oryzalin analogues) were used in different in vitro models and proved to be active against *C. parvum*[10] and *L. mexicana*.[6]

In this work we have synthesized and characterized a series of new **TFL** analogues (Fig. 1) in which the amine group was modified with different kind of substituents, with increased either water or lipidic solubility to allow in future better incorporation in

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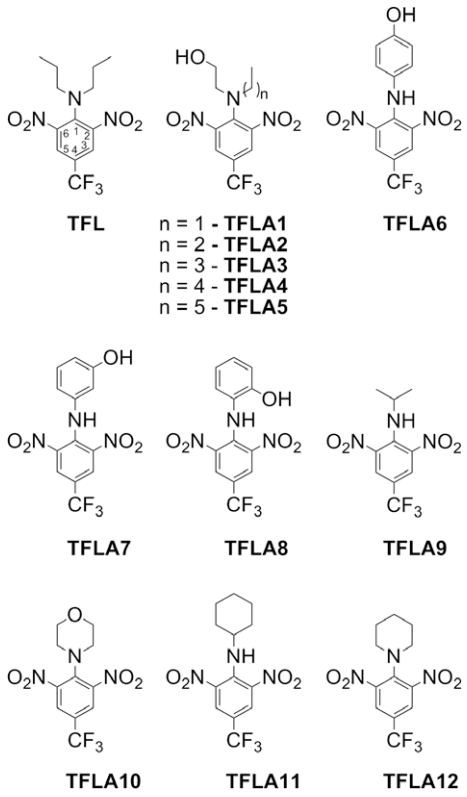


Figure 1. Trifluralin **TFL** and analogues **TFLA1–TFLA12**.

liposomes. The cytotoxicity and hemolytic activity of the new compounds was assessed against THP1 and whole blood. Several **TFL** analogues showed better activity and less toxicity than miltefosine, a reference compound usually used for the treatment of leishmanial infections. The new compounds were also tested in vitro against the promastigote forms of *L. infantum* and *L. donovani* and the intracellular form (THP1 infected with *L. infantum*).

2. Results and discussion

2.1. Chemistry

The synthesis of the **TFL** analogues **TFLA1–TFLA12** was accomplished by a general procedure[10,11] starting from a commercially available chlorinated precursor, chloralin (Scheme 1).

The method consisted of reacting chloralin, with primary or secondary amines in the presence of triethylamine using ethanol as solvent. The substituents R_1 and R_2 in the amines were aryl and alkyl groups. All the new compounds were obtained in 70–80% yield and are stable and easily-handled crystalline solids, except **TFLA4** and **TFLA5** which were obtained as oils that solidified to waxy solids upon storage in a refrigerator. The compounds were fully characterized by FTIR, MS, NMR and elemental analysis and their water solubility determined by UV spectrophotometry.[10] Most of the analogues showed improved water solubility in comparison with **TFL**, but the values are still very low (Table 1). The larger increase in water solubility was obtained for **TFLA1**, resulting from the introduction of a hydroxyethyl group in substitution of one of the propyl groups in the **TFL** structure. Moreover, in the series **TFLA1–TFLA5** it can be observed a decrease in water solubility with the increase of the length of the alkyl substituent, as expected.

2.2. In vitro biological evaluation

The compounds were evaluated in vitro for their toxicity to human cells, for their hemolytic activity to human red blood cells (RBCs) and for their antileishmanial activity. The antileishmanial activity was evaluated against both *L. infantum* and *L. donovani* promastigote form as well as against the *L. infantum* intracellular form. These parameters were systematically evaluated for all **TFL** analogues and compared with **TFL** and miltefosine as reference drugs.

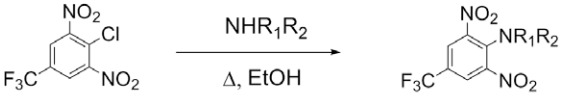
2.2.1. Cytotoxicity assays

The compounds were evaluated in vitro for their toxicity to human cells using the monocytic THP-1 cell line also used as a host cell line of the determination of the antiparasitic activity. The hemolytic activity of the compounds was also assessed using human RBCs. The ability of **TFL** and some **TFL** analogues to kill THP-1 is displayed in Figure 2. From these data we calculated the concentration that was able to kill 50% of the cells (CC_{50}) and the results are displayed in Table 1.

From the 12 analogues tested only three **TFLA5–TFLA7** revealed cytotoxicity at concentrations lower than 50 μM, whereas the rest of the compounds tested showed some cytotoxic potential only at concentrations above 100 μM.

2.2.2. Hemolytic activity

The hemolytic activity against RBCs was used as a marker of a general membrane toxicity effect of the compounds. For concentrations up to 100 μM, **TFL** and the analogues did not exhibit any



Chloralin

TFLA1 - $R_1 = CH_2CH_3$, $R_2 = CH_2CH_2OH$
TFLA2 - $R_1 = (CH_2)_2CH_3$, $R_2 = CH_2CH_2OH$
TFLA3 - $R_1 = (CH_2)_3CH_3$, $R_2 = CH_2CH_2OH$
TFLA4 - $R_1 = (CH_2)_4CH_3$, $R_2 = CH_2CH_2OH$
TFLA5- $R_1 = (CH_2)_5CH_3$, $R_2 = CH_2CH_2OH$
TFLA6 - $R_1 = H$, $R_2 = 4\text{-}C_6H_4OH$
TFLA7 - $R_1 = H$, $R_2 = 3\text{-}C_6H_4OH$
TFLA8 - $R_1 = H$, $R_2 = 2\text{-}C_6H_4OH$
TFLA9 - $R_1 = H$, $R_2 = CH(CH_3)_2$
TFLA10 - $R_1 = R_2 = (CH_2CH_2)O$
TFLA11 - $R_1 = H$, $R_2 = C_6H_5$
TFLA12 - $R_1 = R_2 = (CH_2CH_2)CH_2$

Scheme 1.

Table 1
In vitro biological evaluation of **TFL** and **TFL** analogues

Compound name	Water solubility (ppm)	Cytotoxicity CC_{50} ± SEM (μM)	Hemolysis HC_{50} ± SEM (μM)	% Hemolysis at 100 μM	Potency promastigotes IC_{50} ± SEM (μM) *L. infantum*	Potency promastigotes IC_{50} ± SEM (μM) *L. donovani*	Intracellular A1 (*L. infantum*) IC_{50} ± SEM (μM)
Miltefosine	—	28.6 ± 2.5	38.3 ± 2.8	96.1	23.9 ± 4.2	8.7 ± 0.7	2.7
TFL	0.5	>50	>100	1.3	>100	>100	>50
TFLA1	157.4	>50	>100	0.4	>100	>100	>50
TFLA2	72.3	>50	>100	0.2	>100	>100	>50
TFLA3	22.6	>50	>100	0.5	>100	>100	1.2 ± 0.7
TFLA4	6.0	>50	>100	0.8	nd	nd	>50
TFLA5	5.7	10	>100	0.7	nd	nd	>50
TFLA6	11.4	40	>100	1.4	2.2 ± 1.1	0.60	1.8 ± 1.3
TFLA7	9.5	35	>100	1.4	nd	nd	2.2 ± 0.5
TFLA8	20.0	>50	>100	1.2	nd	nd	1.1 ± 0.4
TFLA9	4.6	>50	>100	0.0	>100	>100	7.3 ± 2.8
TFLA10	18.6	>50	>100	0.3	>100	>100	0.5 ± 0.1
TFLA11	0.1	>50	>100	1.1	nd	nd	9.1 ± 0.9
TFLA12	0.8	>50	>100	1.2	nd	nd	>50

nd—not determined.

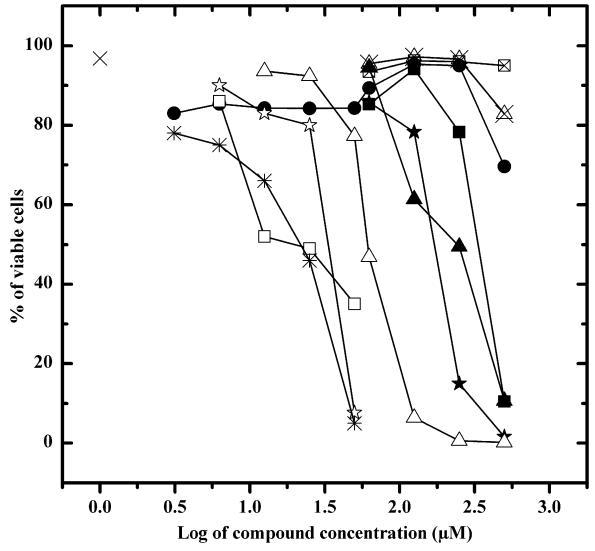


Figure 2. Cytotoxic effect of **TFL**, **TFL** analogues and miltefosine against THP-1 cells. Cells were exposed to increasing concentrations of compounds for 48 h and the percentage of live/dead cells was measured by flow cytometry after staining with SYBR14/PI. The control represents non-treated cells. –●–, **TFL**; –■–, **TFLA1**; –▲–, **TFLA2**; –★–, **TFLA3**; –□–, **TFLA5**; –△–, **TFLA6**; —☆—, **TFLA7**; —⊠—, **TFLA9**; —※—, **TFLA10**; —✳—, miltefosine; –×–, control.

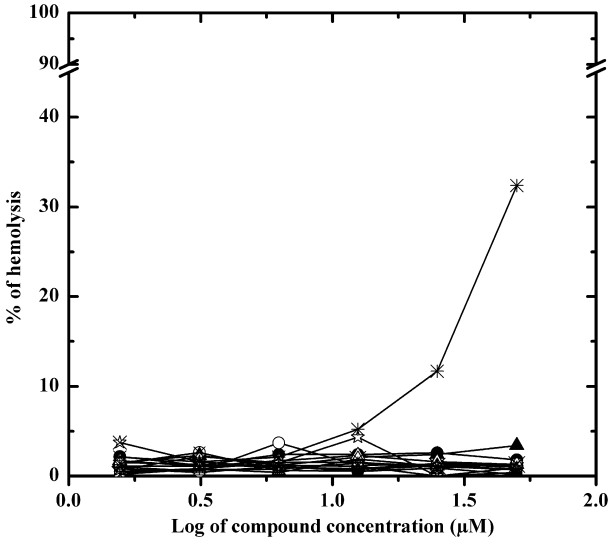


Figure 3. Hemolytic activity induced by **TFL**, **TFL** analogues and miltefosine at different concentrations. RBCs were exposed to different concentrations of the compounds and hemolysis was calculated as described in Section 4 by measuring the absorbance of the cell supernatants at 550 nm with reference filter at 625 nm. One hundred percentage of hemolysis was induced by incubating RBCs with H_2O. –●–, **TFL**; –■–, **TFLA1**; –▲–, **TFLA2**; –★–, **TFLA3**; –○–, **TFLA4**; –□–, **TFLA5**; –△–, **TFLA6**; —☆—, **TFLA7**; —⊠—, **TFLA8**; —⊠—, **TFLA9**; —※—, **TFLA10**; —※—, **TFLA11**; —+—, **TFLA12**; —✳—, miltefosine.

hemolytic activity (Fig. 3 and Table 1) whereas miltefosine lysed almost 100% of RBCs.

2.2.3. Activity of TFL and TFL analogues against *Leishmania* promastigotes

The first screening of antileishmanial activity was performed against the free-living promastigote form of *L. infantum* and *L. donovani* using selected compounds. We have incubated the promastigotes with different concentrations of the compounds and we measured the cell viability by means of flow cytometry. In Fig. 4a and b are displayed the kinetics of the parasite killing at different concentrations and in Table 1 is shown the IC_{50} for the tested compounds. We observed that only **TFLA6** had an antileishmanial activity significantly higher than that of miltefosine (IC_{50} 2.2 vs 23.9 μM for *L. infantum* promastigotes and 0.6 vs 8.7 μM for *L. donovani* promastigotes), whereas **TFL** and the other tested analogues are inactive at concentrations up to 100 μM.

As it will be discussed in Section 2.3, no clear correspondence was found between promastigotes and intracellular amastigotes assays, being the later test identified as the most relevant for the biological evaluation of the new compounds.

2.2.4. Activity of TFL and TFL analogues against the intracellular form of *Leishmania infantum*

The antileishmanial activity of all analogues against the intracellular amastigote form of leishmania was assessed in vitro by infecting THP-1 cells with a naturally occurring *L. infantum* strain (A1), isolated from the bone marrow of a dog with kala-azar. After treatment with increasing concentrations of the compounds for 48 h, the percentage of the infected cells was measured and plotted on a semi-logarithmic plot (Fig. 5). The IC_{50} for each compound was calculated and displayed in Table 1. The potency of the analogues

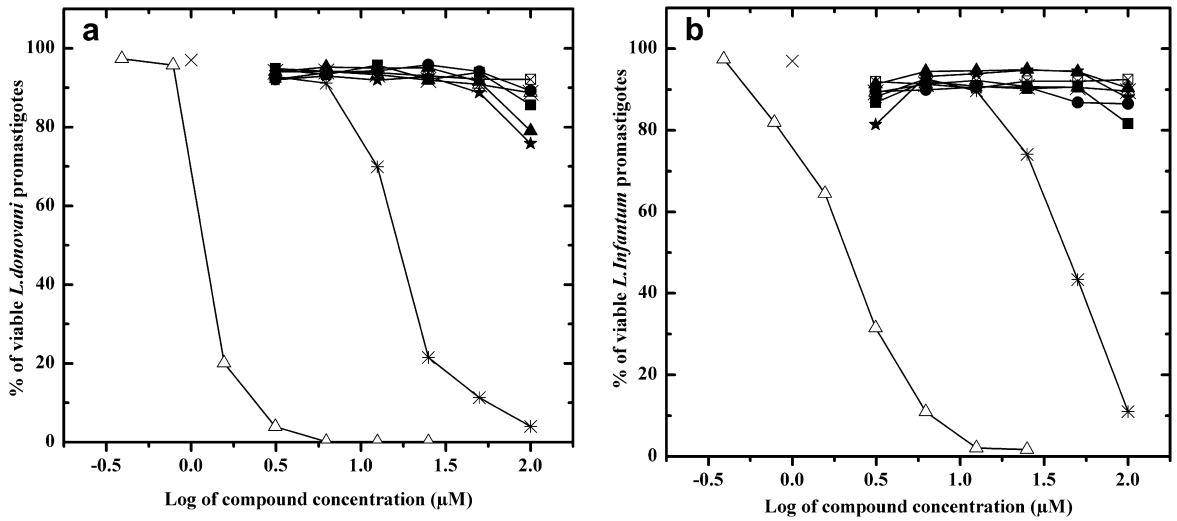


Figure 4. Antileishmanial activity of **TFL**, **TFL** analogues and miltefosine against promastigotes of *L. donovani* (LEM703) (a) and of *L. infantum* (LEM 235) (b). Drug concentrations ranged from 0.08 μM to 50 μM were incubated with the cells for 48 h. The percentage of live/death cells was quantified by flow cytometry after staining with SYBR14/PI. The control represents non-treated promastigotes. –●–, **TFL**; –■–, **TFLA1**; –▲–, **TFLA2**; –★–, **TFLA3**; –□–, **TFLA5**; –△–, **TFLA6**; –☆–, **TFLA7**; –⊠–, **TFLA9**; –⊛–, **TFLA10**; –✳–, miltefosine; –×–, control.

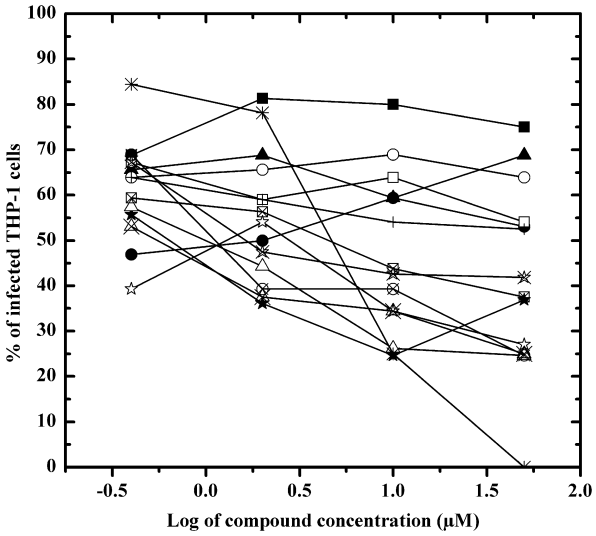


Figure 5. Antiparasitic activity of **TFL**, **TFL** analogues and miltefosine against THP-1 cell line infected with *L. infantum* A1 isolate. –●–, **TFL**; –■–, **TFLA1**; –▲–, **TFLA2**; –★–, **TFLA3**; –○–, **TFLA4**; –□–, **TFLA5**; –△–, **TFLA6**; –☆–, **TFLA7**; –⊠–, **TFLA8**; –⊠–, **TFLA9**; –⊛–, **TFLA10**; –✳–, **TFLA11**; –+–, **TFLA12**; –✳–, miltefosine.

was compared with that of **TFL** and miltefosine. We observed that **TFLA3** and **TFLA6**–**TFLA11** were active against the intracellular form of the parasite and in particular the analogues **TFLA3**, **TFLA8**, and **TFLA10**, significantly reduced the parasite load of the cells exhibiting IC_{50} values 2.5–5-fold lower than the IC_{50} of miltefosine. However, none of the analogues achieved a complete clearance of the parasite in this in vitro assay.

2.3. Selection of most potent TFL analogues

Table 1 summarizes the results of the in vitro evaluation of **TFL** analogues as compared to **TFL** and to miltefosine. A common feature of all dinitroanilines tested was the absence of hemolytic activity (0–1.4% hemolysis), at concentrations as high as 100 μM that make them substantially less hemolytic than miltefosine (HC_{50} values of analogues are higher than 100 vs 38 μM for miltefosine). Similarly, most dinitroanilines showed 2-fold lower cytotoxic potential than miltefosine.

It was found that the antiparasitic activity of the compounds was different when tested against promastigotes in culture or against the intracellular form. Against promastigotes all the tested dinitroanilines (except **TFLA6**) display IC_{50} higher than 100 μM and higher than miltefosine. Against the intracellular form **TFL** analogues behave differently, same displaying comparable or even smaller IC_{50} values than miltefosine and others being inactive. Interestingly **TFLA6** that displayed lower IC_{50} against the promastigote form, proved to be also potent against the intracellular form. The opposite is true with analogues, **TFLA3, TFLA9**, and **TFLA10**, showing antiparasitic activity against the intracellular form but not against the promastigote form. This different behavior could not be attributed to the solubility of the analogues, and consequently to the different ability to cross cell membranes, as they

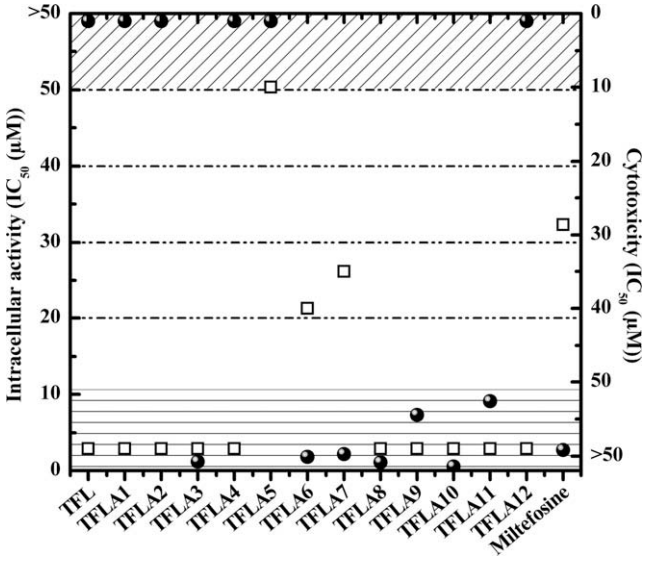


Figure 6. Comparative study of **TFL** and **TFL** analogues. ●, Intracellular activity; □, cytotoxicity. ▨, High cytotoxicity and low intracellular activity; ▤, low cytotoxicity and high intracellular activity regions.

display water solubility in the same range. For this reason, and as one of the most relevant assays are those against the intracellular form, the activity against promastigotes was not tested for all the analogues systematically. Another relevant assay to take into consideration in the selection of the analogues for further tests, namely in vivo, is the cytotoxicity. The results of these two parameters for all analogues were compared in Figure 6.

The compounds can be assembled in different groups with respect to their antileishmanial activity and cytotoxicity. The upper shadowed level comprises those analogues with low activity and high toxicity, whereas in the lower shadowed level are grouped analogues displaying high activity and low toxicity. According to these results the analogues **TFLA3**, **TFLA8** to **TFLA10** are the most promising for further investigations.

3. Conclusion

Due to the absence of effective alternatives for the treatment of leishmania infections it is of high priority to develop derivatives from new classes of chemical compounds that exhibit a potent antiparasitic activity combined with reduced cytotoxic effects.

Dinitroanilines were identified as promising antileishmanial drugs, based on their antiparasitic activity, their mechanism of action and their reduced toxicity to human cells. In this context, we recently reported the successful use of a dinitroaniline, **TFL**, as an antileishmanial agent in animal models.[8,9] In order to improve the physicochemical properties of this compound such as solubility, and to enhance the antiparasitic activity we designed and synthesized **TFL** chemical derivatives.

Twelve **TFL** analogues were synthesized and tested for their antileishmanial activity and their cytotoxicity to human cells as compared with the parent compound, **TFL** and one standard drug, miltefosine that is currently used as antileishmanial agent.

Most of the new compounds were substantially less cytotoxic to human cells when compared to miltefosine with exception of **TFLA5**–**TFLA7**. Seven of them (**TFLA3, TFLA6**–**TFLA11**) exhibited antileishmanial activity against the intracellular form of the parasite. In particular, three compounds (**TFLA3**, **TFLA8**, and **TFLA10**) showed 2.5–5-fold higher potency than miltefosine.

The design of the derivatives allowed us to elucidate some structural features with important contribution to the antileishmanial activity of the compounds. For the analogues with a linear alkyl substituent in the amino group (**TFLA1**–**TFLA5**), the length of the alkyl chain seems to be critical for the potency against the intracellular parasite. An optimal length of the alkyl chain, specifically four carbons (**TFLA3**), was identified. The analogue with six carbons (**TFLA5**) was the most toxic and less active compound.

The three isomers (**TFLA6**–**TFLA8**) showed similar intracellular activity which suggests that the position of the hydroxy group in the aryl ring in this case is not relevant. On the contrary, the position of that substituent seems to have some influence in the cytotoxicity since only the *ortho*-isomer (**TFLA8**) is not cytotoxic.

It was also observed, by comparing **TFLA10** and **TFLA12**, a remarkable improvement in the intracellular activity due to the introduction of an oxygen atom in the heterocyclic ring (**TFLA10**).

All the tested analogues were inactive against the promastigote form of *Leishmania*, except for **TFLA6**. This analogue is the only one that shows activity in both forms of the parasite. This could probably be due to the differences in membrane transport between the two parasite forms and differences in solubility in cell medium. Surprisingly, **TFLA6** also shows cytotoxicity.

Taking into consideration that the targets of the disease are infected macrophages, the results on the intracellular form of the parasite are more critical for the assessment of the compounds that should be studied further, namely in vivo. In order to overcome the toxicity problem of some compounds with promising intracellular activity (**TFLA6** and **TFLA7)** and to further increase cellular accessibility, these compounds could benefit from the association with macrophage targeted drug delivery systems such as liposomes. This strategy already proved to be beneficial for **TFL** by improving the therapeutic index of this compound in vivo.[8,9] In this respect, of special concern was the incomplete clearance of the intracellular parasite even by the most active compounds, (**TFLA3**, **TFLA8**, and **TFLA10**) and the low activity of others (**TFLA9** and **TFLA11**) that may be due to the reduced accessibility of the parasitophorous vacuole or to efficient removal of the drug from the host cell.

We believe that the association to liposomes will be of great interest to further improve the pharmacological characteristics of this class of compounds. Therefore, the most promising analogues will be tested in appropriated animal models, both in the free form and in the respective liposomal formulations.

4. Experimental

Reagents and solvents were of the purest grade available, and generally were used without further treatment. The starting material, chloralin was purchased from Aldrich. 2-Pentylaminoethanol and 2-hexylaminoethanol were prepared by a previously described procedure.[12]

Melting points were determined in a Reichert Thermovar apparatus and are uncorrected. FTIR spectra were recorded on a Perkin–Elmer Spectrum BX v5.3.1 spectrometer. UV–vis spectra were recorded on a Hitachi U-2800A spectrophotometer. Fourier transform (FT) NMR spectra were run on a Brucker QE-300 spectrometer with resonance frequency of 300.65 MHz for ^{1}H using an appropriate solvent. The chemical shifts are reported in δ (ppm, TMS) and coupling constants in Hz. Electronic impact (EI) mass spectra (MS) and EI gas-chromatography–mass spectra (GC–MS) were determined on a Kratos MS 25RF instrument at 70 eV and electron spray ionization (ESI) mass spectra were performed on a Brucker Daltonics Apex-Qe instrument at 300.0 V at CACTI of Vigo University. Microanalyses were performed on a Fisons EA-1108 microanalyzer at CACTI of Vigo University. Thin-layer chromatography (TLC) was performed on Silica Gel 60 F_{254} plates with 0.2 mm layer thickness from Macherey-Naguel, and the compounds visualized by illumination under UV light at 254 nm. Column chromatography (CC) was carried out with Macherey-Naguel Si Gel 60 (230–400 mesh).

4.1. General procedure for trifluralin analogues synthesis

To a solution of chloralin (1 mmol) in ethanol (5 mL) under an nitrogen atmosphere was added simultaneously triethylamine (1.1 mmol) and the amine (1.1 mmol) via a syringe. The mixture was refluxed until complete consumption of amine (0.5–1 h), as monitored by TLC, cooled to room temperature, poured into ice-water and extracted with ethyl acetate. The organic extracts were dried over magnesium sulfate, filtered and concentrated in vacuum to obtain a solid or oily residue. The residues were purified by silica column chromatography and/or recrystallization to afford the products as crystalline solids.

4.1.1. 2-((2,6-Dinitro-4-trifluoromethyl-phenyl)-ethylamino)-ethanol (TFLA1)

The general method was used with chloralin (0.273 g, 1 mmol), triethylamine (0.15 mL, 1.1 mmol), and 2-ethylamino-ethanol (0.10 mL, 1.1 mmol) to afford the product as white crystals (diethyl ether/hexane, η = 62%). Mp 59–60 °C. FTIR (KBr): ν 2926, 1458, 1411, 1317 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ (ppm) 8.12 (2H, s, H3 and H5), 3.73 (2H, m, CH_2OH), 3.19 (4H, m, CH_2N), 2.62 (1H, t,

J = 6 Hz, O*H*), 1.22 (3H, t, *J* = 7 Hz, C*H*$_3$CH$_2$). MS (EI) *m*/*z* 323 [M$^+$] (3), 292 (100), 264 (23), 145 (16), 43 (25). Anal. Calcd for $C_{11}H_{12}F_3N_3O_5$: C, 40.87; H, 3.74; N, 13.00. Found: C, 40.92; H, 3.92; N, 13.01.

4.1.2. 2-((2,6-Dinitro-4-trifluoromethyl-phenyl)-propylamino)-ethanol (TFLA2)

The general method was used with chloralin (0.273 g, 1 mmol), 2-propylaminoethanol (0.13 mL, 1.1 mmol), and triethylamine (0.15 mL, 1.1 mmol) to afford the product as orange crystals (diethyl ether/hexane, η = 76%). Mp 50–52 °C. FTIR (KBr): ν 3560, 2971, 1445, 1411, 1320 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ (ppm) 8.12 (2H, s, H3 and H5), 3.74 (2H, m, C*H*$_2$OH), 3.21 (2H, t, *J* = 5 Hz, NC*H*$_2$CH$_2$OH), 3.02 (2H, m, NC*H*$_2$CH$_2$CH$_3$), 2.67 (1H, t, *J* = 7 Hz, O*H*), 1.64 (2H, m, C*H*$_2$CH$_3$), 0.89 (3H, t, *J* = 7 Hz, C*H*$_3$). GC-MS (EI) *m*/*z* 338 [M$^+$+1] (2), 306 (65), 264 (100), 206 (30), 160 (19). Anal. Calcd for $C_{12}H_{14}F_3N_3O_5$: C, 42.74; H, 4.18; N, 12.46. Found: C, 42.72; H, 4.44; N, 12.46.

4.1.3. 2-((2,6-Dinitro-4-trifluoromethyl-phenyl)-butylamino)-ethanol (TFLA3)

The general method was used with chloralin (0.273 g, 1 mmol), 2-butylaminoethanol (0.14 mL, 1.1 mmol), and triethylamine (0.15 mL, 1.1 mmol) to afford the product as orange crystals (diethyl ether/hexane, η = 62%). Mp 56–58 °C. FTIR (KBr): ν 3398, 2962, 1447, 1412, 1321 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ (ppm) 8.12 (2H, s, H3 and H5), 3.73 (2H, m, C*H*$_2$OH), 3.20 (2H, t, *J* = 5 Hz, NC*H*$_2$CH$_2$OH), 3.06 (2H, t, *J* = 7 Hz, NC*H*$_2$CH$_2$CH$_2$), 2.66 (1H, t, *J* = 7 Hz, O*H*), 1.57 (2H, m, NCH$_2$C*H*$_2$CH$_2$), 1.28 (2H, m, C*H*$_2$CH$_3$), 0.90 (3H, t, *J* = 7 Hz, C*H*$_3$). MS (EI) *m*/*z* 351 [M$^+$] (3), 320 (57), 264 (38), 251 (36), 57 (100). Anal. Calcd for $C_{13}H_{16}F_3N_3O_5$: C, 44.45; H, 4.59; N, 11.96. Found C, 44.40; H, 4.58; N, 11.96.

4.1.4. 2-((2,6-Dinitro-4-trifluoromethyl-phenyl)-pentylamino)-ethanol (TFLA4)

The general method was used with chloralin (0.273 g, 1 mmol), 2-pentylaminoethanol (0.14 g, 1.1 mmol), and triethylamine (0.15 mL, 1.1 mmol) to afford the product as a orange oil which solidified to a waxy orange solid upon storage in a refrigerator (η = 83%). FTIR (KBr): ν 3324, 2963, 2938, 2876, 1630, 1549, 1309, 1140, 909, 711 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ (ppm) 8.12 (2H, s, H3 and H5), 3.74 (2H, d, *J* = 3.5 Hz, C*H*$_2$OH), 3.21 (2H, t, *J* = 4.7 Hz, NC*H*$_2$CH$_2$OH), 3.05 (2H, t, *J* = 7.6 Hz, NC*H*$_2$CH$_2$CH$_2$), 2.66 (1H, s, O*H*), 1.56–1.66 (2H, m, NCH$_2$C*H*$_2$CH$_2$), 1.22–1.31 (4H, m, C*H*$_2$C*H*$_2$CH$_3$), 0.88 (3H, t, *J* = 6.5 Hz, C*H*$_3$). HRMS (ESI-TOF) *m*/*z* calcd for [$C_{14}H_{18}F_3N_3O_5$+H]$^+$ 366.1278, found 366.1271.

4.1.5. 2-((2,6-Dinitro-4-trifluoromethyl-phenyl)-hexylamino)-ethanol (TFLA5)

The general method was used with chloralin (0.273 g, 1 mmol), 2-hexylaminoethanol (0.16 g, 1.1 mmol), and triethylamine (0.15 mL, 1.1 mmol) to afford the product as a orange oil which solidified to a waxy orange solid upon storage in a refrigerator (η = 68%). FTIR (KBr): ν 3461, 2933, 2860, 1630, 1542, 1314, 1137, 909, 713 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ (ppm) 8.12 (2H, s, H3 and H5), 3.74 (2H, d, *J* = 3.8 Hz, C*H*$_2$OH), 3.21 (2H, t, *J* = 4.7 Hz, NC*H*$_2$CH$_2$OH), 3.05 (2H, t, *J* = 7.5 Hz, NC*H*$_2$CH$_2$CH$_2$), 2.66 (1H, t, *J* = 6.6 Hz, O*H*), 1.57–1.60 (2H, m, NCH$_2$C*H*$_2$CH$_2$), 1.18–1.31 (6H, m, C*H*$_2$C*H*$_2$C*H*$_2$CH$_3$), 0.86 (3H, t, *J* = 6.7 Hz, C*H*$_3$). HRMS (ESI-TOF) *m*/*z* calcd for [$C_{15}H_{20}F_3N_3O_5$+H]$^+$ 380.1434, found 380.1428.

4.1.6. 4-(2,6-Dinitro-4-trifluoromethyl-phenylamino)-phenol (TFLA6)

The general method was used with chloralin (0.273 g, 1 mmol), 4-aminophenol (0.12 g, 1.1 mmol), and triethylamine (0.15 mL, 1.1 mmol) to afford the product as dark red crystals (diethyl ether/hexane, η = 79%). Mp 180–183 °C. FTIR (KBr): ν 3483, 3311, 1305, 730 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ (ppm) 9.90 (1H, s, O*H*), 8.42 (2H, s, H3 and H5), 6.95 (2H, d, *J* = 9 Hz, C*H*COH), 6.80 (2H, d, *J* = 9 Hz, C*H*CN), 5.02 (1H, s, N*H*). MS (EI) *m*/*z* 343 [M$^+$] (100), 296 (18), 263 (22), 251 (36), 43 (50). Anal. Calcd for $C_{13}H_8F_3N_3O_5$: C, 45.49; H, 2.35; N, 12.24. Found: C, 45.44; H, 2.12; N, 12.24.

4.1.7. 3-(2,6-Dinitro-4-trifluoromethyl-phenylamino)-phenol (TFLA7)

The general method was used with chloralin (0.273 g, 1 mmol), 3-aminophenol (0.12 g, 1.1 mmol), and triethylamine (0.15 mL, 1.1 mmol) to afford the product as dark red crystals (dichloromethane/hexane, η = 90%). Mp 156–157 °C. FTIR (KBr): ν 3461, 3340, 3088, 1638, 1602, 1541, 1311, 1276, 1131, 773 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ (ppm) 9.88 (1H, s, O*H*), 8.48 (2H, s, H3 and H5), 7.20 (1H, t, *J* = 8.1 Hz, CHC*H*CH), 6.59 (1H, d, *J* = 8.1 Hz, C(NH)C*H*CH), 6.69 (1H, dd, *J* = 2.1 Hz, CHC*H*C(OH)), 6.54 (1H, d, *J* = 2.1 Hz, C(NH)C*H*C(OH), 5.31 (1H, s, N*H*). MS (ESI) *m*/*z* 343 [M$^+$] (100), 269 (12), 245 (11), 231 (55). Anal. Calcd for $C_{13}H_8F_3N_3O_5$, C, 45.49; H, 2.35; N, 12.24. Found: C, 45.44; H, 2.27; N, 12.17.

4.1.8. 2-(2,6-Dinitro-4-trifluoromethyl-phenylamino)-phenol (TFLA8)

The general method was used with chloralin (0.273 g, 1 mmol), 2-aminophenol (0.12 g, 1.1 mmol), and triethylamine (0.15 mL, 1.1 mmol) to afford the product as dark red crystals (dichloromethane/hexane, η = 52%). Mp 157–159 °C. FTIR (KBr): ν 3448, 3351, 3090, 1638, 1533, 1517, 1142, 756 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ (ppm) 9.77 (1H, s, O*H*), 8.47 (2H, s, H3 and H5), 7.17 (1H, dt, *J* = 2.4 Hz, C(NH)CHC*H*), 6.87-6.97 (3H, m, C(NH)C*H* + C(OH)C*H* + -C(OH)CHC*H*), 5.36 (1H, s, N*H*). MS (ESI) *m*/*z* 343 [M$^+$] (100), 269 (20), 245 (17). Anal. Calcd for $C_{13}H_8F_3N_3O_5$: C, 45.49; H, 2.35; N, 12.24. Found: C, 45.28; H, 2.20; N, 12.02.

4.1.9. *N*-(2,6-Dinitro-4-trifluoromethyl-phenyl)-isopropylamine (TFLA9)

The general method was used with chloralin (0.273 g, 1 mmol), triethylamine (0.15 mL, 1.1 mmol), and isopropylamine (0.10 mL, 1.1 mmol) to afford the product as yellow crystals (petroleum ether/hexane, η = 78%). Mp 68–72 °C. FTIR (KBr): ν 3304, 2999, 1468, 1414, 1296, 729 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ (ppm) 8.52 (1H, s, N*H*), 8.37 (2H, s, H3 and H5), 3.56 (1H, hept, *J* = 9 Hz, CH$_3$C*H*CH$_3$), 1.25 (6H, d, *J* = 9 Hz, C*H*$_3$CH). GC–MS (EI) *m*/*z* 294 [M$^+$+1] (16), 275 (81), 232 (76), 202 (100), 186 (70). Anal. Calcd for $C_{10}H_{10}F_3N_3O_4$: C, 40.96; H, 3.44; N, 14.33. Found: C, 40.71; H, 3.64; N, 14.18.

4.1.10. 4-(2,6-Dinitro-4-trifluoromethylphenyl)-morpholine (TFLA10)

The general method was used with chloralin (0.273 g, 1 mmol), morpholine (0.10 mL, 1.1 mmol), and triethylamine (0.15 mL, 1.1 mmol) to afford the product as yellow crystals (diethyl ether/hexane, η = 82%). Mp 141–142 °C (139–140 °C).[1] FTIR (KBr): ν 2993, 1458, 1312, 1243 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ (ppm) 8.07 (2H, s, H3 and H5), 3.79 (4H, t, *J* = 4 Hz, C*H*$_2$O), 3.14 (4H, t, *J* = 4 Hz, C*H*$_2$N). GC–MS (EI) *m*/*z* 321 [M$^+$+1] (14), 200 (90),186 (100), 159 (69), 145 (58). Anal. Calcd for $C_{11}H_{10}F_3N_3O_5$: C, 41.13; H, 3.14; N, 13.08. Found: C, 41.08; H, 3.02; N, 13.03.

4.1.11. *N*-(2,6-Dinitro-4-trifluoromethylphenyl) cyclohexylamine (TFLA11)

The general method was used with chloralin (0.273 g, 1 mmol), cyclohexylamine (0.13 mL, 1.1 mmol), and triethylamine (0.15 mL, 1.1 mmol) to afford the product as yellow crystals (dichloromethane/hexane, η = 73%). Mp 83–84 °C. FTIR (KBr): ν 3313, 2942, 2924, 2857, 1646, 1526, 1509, 1310, 1255, 1128, 915, 764 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ (ppm) 8.62 (1H, d, *J* = 7.2 Hz, N*H*), 8.36 (2H, s, H3 and

H5), 3.20-3.25 (1H, m, NC*H*), 1.28–1.98 (10H, m, 5 × CH_2). MS (ESI) *m*/*z* 334 [M^++1] (100), 202 (7). Anal. Calcd for $C_{13}H_{14}F_3N_3O_4$: C, 46.85; H, 4.23; N, 12.61. Found: C, 46.76; H, 4.18; N, 12.50.

4.1.12. 1-(2,6-Dinitro-4-trifluoromethyl-phenyl)-piperidine (TFLA12)

The general method was used with chloralin (0.273 g, 1 mmol), piperidine (0.11 mL, 1.1 mmol), and triethylamine (0.15 mL, 1.1 mmol) to afford the product as yellow crystals (diethyl ether/hexane, η = 82%). Mp 109–110 °C. FTIR (KBr): ν 3080, 2954, 2865, 1634, 1526, 1361, 1160, 1123, 910, 712 cm^{-1}. ^1H NMR ($CDCl_3$): δ (ppm) 8.01 (2H, s, H3 and H5), 3.05 (4H, d, J = 4.8 Hz, 2 × NCH_2), 1.66 (6H, s, 3 × CH_2). MS (ESI) *m*/*z* 320 [M^++1] (100), 252 (4), 209 (5), 187 (7). Anal. Calcd for $C_{12}H_{12}F_3N_3O_4$: C, 45.15; H, 3.79; N, 13.16. Found: C, 45.21; H, 3.69; N, 13.09.

4.2. Determination of water solubility

The water solubility values were obtained from the UV spectra recorded for each compound. The samples were prepared according to the following general procedure: a suspension of each compound (5 mg) in water (10 mL) was stirred at room temperature for 1 h, then it was filtered and the UV spectra of the filtrate (3 mL) was recorded and the value of the absorbance at the maximum wavelength (λ_{max}) was obtained. In order to determine the value of the absorptivity at λ_{max}, a standard solution of known concentration was prepared by dissolving the compound (4 mg) in methanol (10 mL) and then diluting this solution in water (5, 12.5, and 25 times) followed by recording the UV spectra for the three dilutions. The value of absorptivity obtained is the average of the absorptivity values calculated for each dilution by applying the Beer law to the UV data. With this value of absorptivity it was then calculated the concentration of each compound in the water sample (water solubility).

4.3. Evaluation of in vitro antiparasitic activity

4.3.1. Cell culture and parasite strains

The human monocytic leukemia THP-1 cell line was used as a host for leishmania parasites. Infection rate of this cell line ranged between 35% and 45% and was significantly lower than the infection rate achieved with mouse peritoneal macrophages (85–95%). However, the use of THP-1 as the parasite host cell for the evaluation of the compounds' antileishmanial activity against the intracellular parasite, proved to be highly reproducible and convenient for the high number of compounds to be tested in three independent experiments. THP-1 cells were maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine and antibiotics at 37 °C, in 5% CO_2 atmosphere. Promastigotes of *L. infantum* MHOM/TN/80/IPT1/LEM 235 and *L. donovani* MHOM/IN/80/DD8/LEM 703, were grown in RPMI 1640 supplemented with 10% FCS, L-glutamine and antibiotics, at 26 °C.

4.3.2. Determination of in vitro antileishmanial activity in promastigote cultures

All compounds were dissolved in DMSO/ethanol 50:50 v/v to a final concentration of 65 mM and linear 2-fold dilutions ranging from 100 to 0.8 μM were done in the culture medium. Promastigotes of *L. infantum* and *L. donovani* at a final concentration of 1×10^6 cells/mL, were incubated with the appropriate compound concentration in 24-well tissue culture plates (Cellstar, Greiner) at 26 °C. After 72 h, cells were harvested, resuspended in Hepes-buffered saline solution, containing 10% BSA, and stained with 12 μM PI and 100 nM SYBR-14, using the LIVE/DEAD Sperm Viability kit (Molecular Probes) according to the manufacturer recommendations. Cells were analyzed by flow cytometry as described in the following section. The $IC_{50} \pm \sigma$ values were calculated using sigmoidal regression analysis[13,14] from the data of three independent experiments.

4.3.3. Determination of in vitro antileishmanial activity against the intracellular *L. infantum*

THP-1 cells were differentiated with 1 μM retinoic acid (Sigma) for 72 h at 37 °C/5% CO_2. Cells were then washed twice in PBS and once in plain RPMI medium, and incubated overnight with promastigotes at 8:1 parasite/cell ratio at 37 °C/5% CO_2. Following incubation, cells were harvested, collected in RPMI medium and carefully layered on 4 mL of Histopaque 1077 (Sigma). Free promastigotes were removed by centrifugation at 1000*g* for 20 min. The opaque cell layer containing the mononuclear cells was collected, washed thrice in PBS and once in plain RPMI, and resuspended at a concentration of 4×10^5cells/mL. Cells were then incubated with the appropriate compound concentrations in 24-well tissue culture plates (Cellstar, Greiner) for 48 h at 37 °C/5% CO_2. The percentage of infected cells was assessed microscopically after Giemsa staining. The $IC_{50} \pm \sigma$ values were calculated as described in Section 4.3.2.

4.3.4. Assessment of hemolytic potential

EDTA-preserved peripheral blood from healthy volunteers was centrifuged in order to remove serum and red blood cells were washed twice in PBS. After the final wash, cells were distributed in 96-well microplates (100 μL/well) and an equal volume of the appropriate compound concentration was added. The tested compounds were diluted in PBS in concentrations ranging from 100 to 6.25 μM. Incubation proceeded at 37 °C for 1 h, and the microplates were centrifuged at 800*g* for 10 min. Absorbance of the supernatants were measured at 550 nm with reference filter at 620 nm. The percentage of hemolytic activity of each drug at different concentrations was estimated as the $(A - A_0/A_{max} - A_0) \times 100$ where A_0 is the background hemolysis obtained by incubation with PBS and A_{max} is the 100% hemolysis achieved after incubation in water. Each compound was assayed in triplicate.

4.3.5. Assessment of cytotoxicity in THP-1 monocytic cells

As a quantitative measurement of the cell damage after incubation with different concentrations of drugs, dual staining with SYBR-14 and PI was carried out, using the LIVE/DEAD Sperm Viability kit (Molecular Probes). THP-1 cells were incubated at a final concentration of 1×10^6 cells/mL with different concentrations of the compounds ranging from 50 to 1.56 μM. After an incubation period of 72 h, cells were stained with 12 μM PI and 100 nM SYBR-14, as described for promastigotes (Section 4.3.2). The ratio live/dead cells was assessed by flow cytometry as described below. Each compound was assayed in triplicate.

4.3.6. Flow cytometric analysis

Cell samples were analyzed on an Epics Elite model flow cytometer (Coulter, Miami, FL), equipped with a 488 nm argon laser. Differential monitoring of the dyes was achieved by detecting green fluorescence of SYBR-14 at 545 nm and red fluorescence of PI at 645 nm. At least 10,000 cells were analyzed per sample, and data analysis was performed on fluorescence intensities that excluded cell autofluorescence and cell debris.

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