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Original article

Selenocyanates and diselenides: A new class of potent antileishmanial agents

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ABSTRACT

Thirty five selenocyanate and diselenide compounds were subjected to *in vitro* screening against *Leishmania infantum* promastigotes and the most active ones were also tested in an axenic amastigote model. In order to establish the selectivity indexes (SI) the cytotoxic effect of each compound was also assayed against Jurkat and THP-1 cell lines.

Thirteen derivatives exhibit better IC_{50} values than miltefosine and edelfosine. Bis(4-aminophenyl) diselenide exhibits the best activity when assayed in infected macrophages and one of the lowest cytotoxic activities against the human cell lines tested, with SI values of 32 and 24 against Jurkat and THP-1 cells, respectively. This compound thus represents a new lead for further studies aimed at establishing its mechanism of action.

1. Introduction

The leishmaniases are a group of diseases with a broad range of clinical manifestations. These diseases are caused by different species of the kinetoplastid parasite *Leishmania* and affect man and other mammals in tropical and subtropical regions of the world. The World Health Organization (WHO) estimates that the disease results in 2 million new cases a year, threatens 350 million people in 88 countries and that there are 12 million people currently infected worldwide [1,2]. This pathology is transmitted by blood-feeding female sandflies. Parasites cycle between two different forms: extracellular flagellated promastigotes in the vector and intracellular non-flagellated amastigotes within mononuclear phagocytes in the mammalian host [3,4].

Maintenance of parasites at dermal sites or subsequent dispersal to internal tissues is responsible for the different pathologies of the disease, giving rise to cutaneous, mucocutaneous, diffuse cutaneous and visceral leishmaniases (CL, MCL, DCL and VL, respectively) [5]. Conventional therapies are based on treatment with pentavalent antimonials (sodium stibogluconate and meglumine antimonate), pentamidine, or amphotericin B [6]. The high resistance rates developed against pentavalent antimonials, their high-dose regimens, and the long treatment courses of parentheral administration required – as well as their high renal and cardiac toxicity – are all major drawbacks. On the other hand, the high toxicity and declining efficacy of pentamidine have restricted its use. Amphotericin B and its lipid formulation proved to be very effective in the treatment of leishmaniasis, but the cost of this drug is still prohibitively high and out of the reach of poor people. More recently, the alkyl-phospholipid miltefosine [7] has emerged as an oral drug that is highly effective in children but occasionally presents gastrointestinal complications and also shows teratogenic effects. Edelfosine [8], another alkyl-phospholipid, has also been tested and was found to display a higher *in vitro* activity than miltefosine. Other drugs, such as paromomycin and sitamaquine, have been reported to give variable cure rates [9].

The serious problems still associated with the treatment of leishmaniasis have led many research groups to search for novel, more efficient and safe chemotherapeutic agents against *Leishmania* infections.

Recently, the development and use of metal complexes as potent chemotherapeutic agents against leishmaniasis has been explored. Thus, some organometallic and coordination complexes of palladium, gold, iridium, rhodium, platinum and zinc sulfate have been tested against the *Leishmania* parasite [10]. In addition, some metallointercalators of DNA based on platinum, copper and silver have been evaluated and were shown to possess remarkable antileishmanial activity [11]. Another relevant trace element is selenium, whose increased concentration in plasma has been recognised as a new defensive strategy against *Leishmania* infection

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[12,13]. Recently, a study has implicated the human selenium-containing selenoprotein P (Sepp1) in the protection against illness caused by *Trypanosoma* due to its protective action against oxidative damage [14]. The biochemical pathways that constitute the cellular targets for selenium are still under investigation.

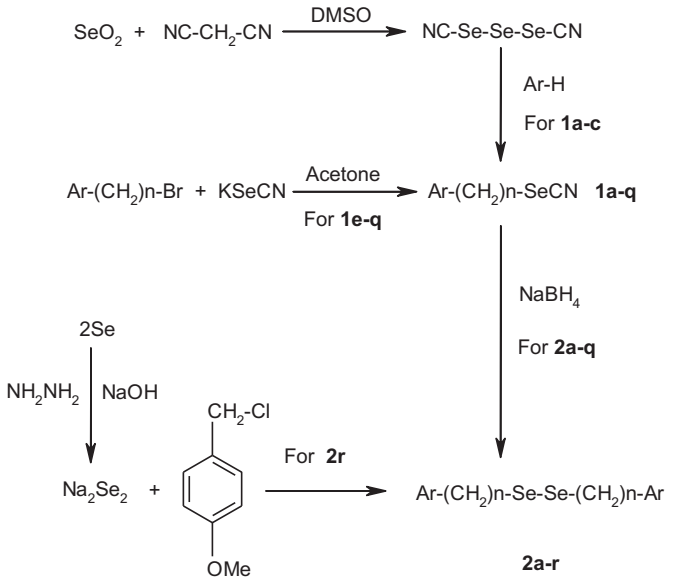
For these reasons, in the selection of the chemical groups for the new molecules described here we have considered, as a first and promising approach, the importance of possible potential interference of selenium derivatives with the redox system of the parasites. It is well known [15] that in trypanosomatids the glutathione system is replaced by a trypanothione system where the key enzyme is trypanothione reductase (TrypR) which catalyzes the reduction of trypanothione disulfide to trypanothione [16]. In contrast to its human counterpart, the enzymatic system that regulates the redox status in Trypanosomatids is not based in the activity of selenoproteins [17] so, we propose to incorporate structural moieties such as diselenide function in an attempt to find more potent and selective leishmanicidal compounds.

Taking into account all of the aforementioned and due to the urgent need for innovative drugs based on new molecular scaffolds, and as an extension of our earlier work on selenium derivatives [18–22], we decided to test the efficiency of the chemical forms selenocyanate and diselenide with different functionalities in the aryl ring as leishmanicidal agents. The substitutions of groups at various positions of the phenyl ring allowed us to explore the effect of the position on biological activity. Further studies are necessary to verify parasite selenium dependence and determine the concentration and mechanism of action of this trace element that is required for their viability.

2. Results and discussion

2.1. Chemistry

The synthetic strategy for the preparation of selenocyanate derivatives (compounds **1a**–**q**) and diselenides (compounds **2a**–**r**) is outlined in Scheme 1. Compounds **1a**–**c** were prepared by addition of selenium dioxide [23] to malononitrile in DMSO at room temperature followed by reaction with the corresponding aminoaryl derivatives. Compound **1d** was obtained from **1c** by reaction with acetic anhydride. Compounds **1e**–**q** were obtained in variable yields (15–81%) by reaction of haloarenes with potassium selenocyanate in acetone under reflux [24]. Neither of these methods gave good results in the case of derivative **1r** and nor did they allow us to carry out the synthesis in sufficient amounts to study the biological behavior. The subsequent reduction of compounds **1a**–**q** with sodium borohydride [25] in ethanol afforded derivatives **2a**–**q**. A different synthetic pathway was developed to prepare compound **2r**. The strategy [26] employed 100% hydrazine hydrate and sodium hydroxide in DMF to reduce elemental selenium and generate sodium diselenide and this was followed by reaction with *p*-methoxybenzyl chloride.



Scheme 1. Synthesis of compounds **1a**–**q** and **2a**–**r**. Compound **1d** was obtained from **1c** (see Experimental section).

All of the compounds prepared during the course of these investigations (Table 1) are stable and they were characterized by spectroscopic methods such as IR and NMR and also by elemental analysis. The spectroscopic data for the new synthesized derivatives were shown in Tables 2 and 3.

2.2. Biological evaluation

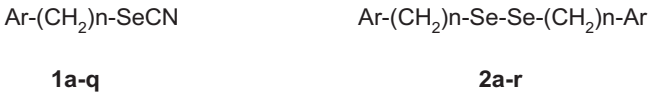
2.2.1. Activity in axenic promastigotes and amastigotes

The synthesized selenocyanates (**1a**–**q**) and diselenides (**2a**–**r**) were initially tested *in vitro* at concentrations of 25, 12.5 and 6.25 μM against cultured promastigotes of *Leishmania infantum* (MCAN/ES/89/IPZ229/1/89) according to a previously described procedure [27]. All the analyses were carried out with a minimum of three independent experiments.

The percentages of growth inhibition obtained at three different concentrations are summarized in Table 4. This procedure allowed us to select the compounds that caused more than 50% cell growth inhibition at 25 μM. These compounds were subsequently screened against amastigotes. Edelfosine and miltefosine were used as the reference drugs in these assays.

Of the thirty five compounds evaluated against promastigotes, twenty of them caused more than 50% cell growth inhibition at 25 μM; thirteen retained inhibition over 50% at 12.5 μM and nine at 6.25 μM. The IC_{50} values for promastigotes and amastigotes obtained with the fifteen most active compounds are shown in Table 5.

Table 1
Structures of compounds **1a**–**q** and **2a**–**r**.



1a-q **2a-r**

Compounds	Aryl ring (Ar)	n
1a/**2a**	4-Aminophenyl	0
1b/**2b**	4-(*N*,*N*-dimethylamino)phenyl	0
1c/**2c**	4-Amino-3-carboxyphenyl	0
1d/**2d**	4-Acetamido-3-carboxyphenyl	0
1e/**2e**	4-Nitrophenyl	1
1f/**2f**	3-Nitrophenyl	1
1g/**2g**	2-Nitrophenyl	1
1h/**2h**	4-Bromophenyl	1
1i/**2i**	4-Trifluoromethylphenyl	1
1j/**2j**	4-Methylthiophenyl	1
1k/**2k**	4-Methylphenyl	1
1l/**2l**	4-Cyanophenyl	1
1m/**2m**	3-Cyanophenyl	1
1n/**2n**	2-Cyanophenyl	1
1o/**2o**	Phenyl	1
1p/**2p**	Naphthyl	1
1q/**2q**	4-Nitrophenyl	2
2r	4-Methoxyphenyl	1

Table 2
Physical constants of the new synthesized compounds.

Ar-(CH$_2$)n-SeCN **1a-q**

Ar-(CH$_2$)n-Se-Se-(CH$_2$)n-Ar **2a-r**

Comp.	Aryl ring (Ar)	n	Yield (%)	Mp (°C)	PM[a]	Molecular formula	Anal (%) calcd/found
1d	4-Acetamino-3-carboxyphenyl	0	89	172–173	C	$C_{10}H_8N_2O_3Se$	C, 42.40/42.20; H, 2.83/3.10; N, 9.89/9.55.
1f	3-Nitrophenyl	1	30	84–86	B	$C_8H_6O_2N_2Se$	C, 39.83/39.95; H, 2.49/2.60; N, 11.62/11.24.
1g	2-Nitrophenyl	1	83	72–74	B	$C_8H_6O_2N_2Se$	C, 39.83/39.56; H, 2.49/2.54; N, 11.62/11.40.
1i	4-Trifluoromethylphenyl	1	67	54–55	B	$C_9H_6F_3NSe$	C, 40.90/41.10; H, 2.27/2.27; N, 5.30/4.94.
1j	4-Methylthiophenyl	1	41	81–83	C	C_9H_9NSSe	C, 44.62/44.63; H, 3.71/3.80; N, 5.78/5.55.
1l	4-Cyanophenyl	1	80	129–131	B	$C_9H_6N_2Se$	C, 49.05/48.95; H, 2.76/2.69; N, 12.47/12.33.
1m	3-Cyanophenyl	1	80	60–62	B	$C_9H_6N_2Se$	C, 49.05/48.83; H, 2.76/2.68; N, 12.47/12.26.
1n	2-Cyanophenyl	1	63	120–121	B	$C_9H_6N_2Se$	C, 49.05/48.83; H, 2.76/2.80; N, 12.47/12.40.
1p	Naphthyl	1	43	119–120	C	$C_{12}H_9NSe$	C, 58.54/58.31; H, 3.66/3.90; N, 5.69/5.42.
1q	4-Nitrophenyl	2	70	67–68	C	$C_9H_8N_2O_2Se$	C, 42.35/42.44; H, 3.14/3.31; N, 10.98/11.08.
2c	4-Amino-3-carboxyphenyl	0	63	231–232	B	$C_{14}H_{12}N_2O_4Se_2$	C, 39.07/38.73; H, 2.79/2.78; N, 6.51/6.14.
2d	4-Acetamino-3-carboxyphenyl	0	84	142–143	B	$C_{18}H_{16}N_2O_6Se_2$	C, 42.02/41.69; H, 3.11/3.45; N, 5.45/5.03.
2e	4-Nitrophenyl	1	35	99–100	B	$C_{14}H_{12}O_4N_2Se_2$	C, 39.07/39.09; H, 2.79/2.78; N, 6.51/6.37.
2f	3-Nitrophenyl	1	22	86–88	B	$C_{14}H_{12}O_4N_2Se_2$	C, 39.07/39.25; H, 2.79/2.73; N, 6.51/6.46.
2g	2-Nitrophenyl	1	65	99–100	B	$C_{14}H_{12}O_4N_2Se_2$	C, 39.07/39.08; H, 2.79/2.76; N, 6.51/6.56.
2h	4-Bromophenyl	1	67	102–103	B	$C_{14}H_{12}Br_2Se_2$	C, 33.73/33.37; H, 2.41/2.31.
2i	4-Trifluoromethylphenyl	1	38	64–66	B	$C_{16}H_{12}F_6Se_2$	C, 40.34/40.84; H, 2.52/2.53.
2j	4-Methylthiophenyl	1	15	99–100	B	$C_{16}H_{18}S_2Se_2$	C, 44.44/44.12; H, 4.16/4.20.
2l	4-Cyanophenyl	1	30	136–137	B	$C_{16}H_{12}N_2Se_2$	C, 49.23/49.04; H, 3.07/3.11; N, 7.18/7.07.
2m	3-Cyanophenyl	1	33	118–120	B	$C_{16}H_{12}N_2Se_2$	C, 49.23/48.88; H, 3.07/3.27; N, 7.18/7.29.
2n	2-Cyanophenyl	1	11	103–105	B	$C_{16}H_{12}N_2Se_2$	C, 49.23/49.63; H, 3.07/3.11; N, 7.18/7.07.
2q	4-Nitrophenyl	2	83	86–88	B	$C_{16}H_{16}N_2O_4Se_2$	C, 41.92/41.77; H, 3.49/3.52; N, 6.11/5.97.

A = Recrystallized from a mixture of MeOH:H_2O (1:1); B = Recrystallized from EtOH; C = Washed with H_2O; D = Recrystallized from 1,4-dioxane; E = Washed with hot EtOH:*N*,*N*-DMF (1:1); F = Recrystallized from *n*-hexane.
[a] PM = Purification method.

The results presented in Table 4 indicate that most of the aryl rings confer higher activity against *Leishmania* promastigotes when present in the selenocyanate derivatives compared to the same moiety in the diselenide scaffold. However, a detailed analysis of the IC_{50} values for amastigotes (see Table 5) reveals that the rings **a**, **b**, **e** and **o**, with the substituent group in *para* position generate more active compounds when present in the diselenide forms. In fact, all of the IC_{50} values obtained for compounds **2a**, **2b**, **2e** and **2o** are below 1 μM when tested against amastigotes. The cytotoxic activity of these four compounds was further confirmed in an additional assay by direct counting of living cells in a neubauer chamber after staining of the dead cells with trypan blue (Supplementary data).

Comparison of the IC_{50} values of our compounds with those obtained for miltefosine in the promastigote model reveals that nineteen of them are more potent than miltefosine, with IC_{50} values in the range 0.96–11.9 μM. When considering the amastigote form, thirteen of the compounds tested (**1b**, **1e**, **1f**, **1g**, **1i**, **1l**, **1m**, **1o**, **2a**, **2b**, **2e, 2g** and **2o**) show IC_{50} values (IC_{50} = 0.29–2.79 μM) better than that obtained for miltefosine (IC_{50} = 2.84 μM). Moreover, ten of them (**1e**, **1f**, **1g**, **1l**, **1m**, **2a**, **2b**, **2e, 2g** and **2o**), with IC_{50} values in the range 0.29–0.77 μM, show stronger *in vitro* antileishmanial activity than edelfosine (IC_{50} = 0.82 μM). It is remarkable that **2g**, **1g** and **2e** are 9.8, 8.3 and 7.5 times more effective respectively than miltefosine and 2.8, 2.4 and 2.2 times more potent than edelfosine.

Given the relatively small number of derivatives tested, a detailed SAR analysis is not feasible but some observations and comparisons on the effect of the size of the linker on activity can be made. For example, analysis of the nitro derivatives (compounds **1e**, **1q**, **2e** and **2q**) that contain chains with one or two carbons reveals that an increase in chain length has a detrimental effect on the activity. The results for compounds **1e** and **2e** (aryl ring = 4-nitrophenyl; n = 1, $IC_{50\ amastigotes}$ = 0.68 and 0.38 μM, respectively) and **1q** and **2q** (aryl ring = 4-nitrophenyl; n = 2, $IC_{50\ amastigotes}$ = 3.80 μM and inactive, respectively) support this conclusion, with the effect being more evident for the diselenide function. Finally, for diselenide compounds, when n = 1, the activity is improved by the presence in the aromatic moiety of strongly electron-withdrawing substituents (NO_2 in **2e**) compared to electron-donating ones (for example, CH_3 in **2k**, OCH_3 in **2r**). However, for selenocyanate compounds the activity does not seem to correlate with modulation of the electronic distribution through the aromatic ring. In order to evaluate the role in the biological activity of the position of substituent group in the aryl ring we synthesized various *ortho* and *meta* derivatives of some of the most active *para* compounds. The selection of the new compounds to be synthesized was made according to the commercial availability so as the synthetic accessibility. So, the functionalization of positions 2 and 3, in order to obtain the corresponding *ortho* and *meta* derivatives from **1a**, **2a**, **1b** and **2b**, that requires an electrophilic substitution on a deactivated position, does not occur [23]. This difficulty has not been overcome, using other synthetic methods [24–26]. In general, the structure–activity relationship (SAR) information indicates that in the promastigote model the highest activity among selenocyanate compounds is obtained with the cyano group in the *ortho* position whereas the nitro group located at the *para* position in the aryl ring results in the best activity among diselenides. However, a very slight increase in the activity for *ortho* nitro and *meta* cyano related to their corresponding *para* is obtained in the amastigote model.

2.2.2. Cytotoxic activity in human cells

In order to determine the toxicity/activity index, the most active compounds against *Leishmania* promastigotes were also tested against two leukemia cell lines derived from either lymphoblasts (Jurkat) or monocytes (THP-1) at 0.08, 0.4, 2.0, 10.0 and 50.0 μM. The IC_{50} values obtained are gathered in Table 5. The selectivity index (SI) was defined as the ratio of the IC_{50} values of compounds against either Jurkat or THP-1 cells relative to those obtained

Table 3
Spectroscopic data for the new synthesized compounds.

Comp.	IR (KBr; cm^{-1})	^1H NMR (400 MHz, DMSO-d_6, δ, J in Hz)	^{13}C NMR (400 MHz, DMSO-d_6, δ)
1d	3245 (m, N–H), 2154 (m, C≡N), 1694 (s, C=O(OH)), 1654 (C O)	2.16 (s, 3H, CH_3); 7.91 (dd, 1H, $J_{4-3}=8.8$, $J_{4-6}=2.2$, H_4); 8.25 (d, 1H, H_6); 8.51 (d, 1H, H_3); 11.09 (s, 1H, NH)	25.8 (1C, CH_3); 106.3 (1C, CN); 117.4 (1C, $C_{3\text{-phenyl}}$); 118.7 (1C, $C_{1\text{-phenyl}}$); 122.2 (1C, $C_{5\text{-phenyl}}$); 137.2 (1C, $C_{2\text{-phenyl}}$); 140.0 (1C, $C_{6\text{-phenyl}}$); 142.4 (1C, $C_{4\text{-phenyl}}$); 169.2 (1C, CONH); 169.7 (1C, COOH)
1f	2147 (m, C≡N), 1520 (s, C–NO_2)	4.44 (s, 2H, CH_2); 7.67–7.71 (m, 1H, H_5); 7.83 (d, 1H, $J_{6-5}=7.7$, H_6); 8.18 (d, 1H, $J_{4-5}=8.1$, H_4); 8.27 (s, 1H, H_2)	31.9 (1C, CH_2); 105.6 (1C, CN); 123.5 (1C, $C_{4\text{-benzyl}}$); 124.3 (1C, $C_{2\text{-benzyl}}$); 131.1 (1C, $C_{5\text{-benzyl}}$); 136.3 (1C, $C_{6\text{-benzyl}}$); 141.8 (1C, $C_{1\text{-benzyl}}$); 148.6 (1C, $C_{3\text{-benzyl}}$)
1g	2146 (m, C≡N), 1515 (s, C–NO_2)	4.60 (s, 2H, CH_2); 7.60–7.64 (m, 1H, H_4); 7.67 (d, 1H, $J_{6-5}=7.6$, H_6); 7.77–7.81 (m, 1H, H_5); 8.12 (d, 1H, $J_{3-4}=8.2$, H_3)	29.6 (1C, CH_2); 104.9 (1C, CN); 126.3 (1C, $C_{3\text{-benzyl}}$); 130.5 (1C, $C_{4\text{-benzyl}}$); 133.5 (1C, $C_{6\text{-benzyl}}$); 133.9 (1C, $C_{1\text{-benzyl}}$); 135.1 (1C, $C_{5\text{-benzyl}}$); 148.0 (1C, $C_{2\text{-benzyl}}$)
1i	2155 (s, C≡N), 1326 (s, C–F)	4.37 (s, 2H, CH_2); 7.59 (d, 2H, $J_{2-3}=J_{6-5}=8.1$, H_2+H_6); 7.75 (d, 2H, H_3+H_5)	32.3 (1C, CH_2); 105.7 (1C, CN); 124.1 (1C, CF_3); 126.4 (2C, C-3, $C_{5\text{-benzyl}}$); 129.2 (1C, $C_{4\text{-benzyl}}$); 130.5 (2C, C-2, $C_{6\text{-benzyl}}$); 144.2 (1C, $C_{1\text{-benzyl}}$)
1j	2143 (s, C≡N), 1490 (s, S–CH_3)	2.47 (s, 3H, CH_3); 4.29 (s, 2H, CH_2); 7.24, (d, 2H, $J_{2-3}=J_{6-5}=8.2$, H_2+H_6); 7.31 (d, 2H, H_3+H_5)	15.4 (1C, SCH_3); 33.4 (1C, CH_2); 105.8 (1C, CN); 126.7 (2C, C_3, $C_{5\text{-benzyl}}$); 130.3 (2C, C_2, $C_{6\text{-benzyl}}$); 135.6 (1C, $C_{1\text{-benzyl}}$); 138.8 (1C, $C_{4\text{-benzyl}}$)
1l	2229 (s, C≡N), 2144 (s, C≡N)	4.36 (s, 2H, CH_2); 7.56 (d, 2H, $J_{2-3}=J_{6-5}=8.3$, H_2+H_6); 7.85 (d, 2H, H_3+H_5)	32.5 (1C, CH_2); 105.6 (1C, SeCN); 111.3 (1C, $C_{4\text{-benzyl}}$); 119.5 (1C, CN); 130.7 (2C, C_2, $C_{6\text{-benzyl}}$); 133.4 (2C, C_3, $C_{5\text{-benzyl}}$); 145.2 (1C, $C_{1\text{-benzyl}}$)
1m	2226 (s, C≡N), 2150 (s, C≡N)	4.35 (s, 2H, CH_2); 7.59–7.62 (m, 1H, H_5); 7.72 (d, 1H, $J_{6-5}=8.2$, H_6); 7.78 (d, 1H, $J_{4-5}=7.7$, H_4); 7.80 (s, 1H, H_2)	32.0 (1C, CH_2); 105.6 (1C, SeCN); 112.3 (1C, $C_{3\text{-benzyl}}$); 119.4 (1C, CN); 130.8 (1C, $C_{5\text{-benzyl}}$); 132.4 (1C, $C_{4\text{-benzyl}}$); 133.1 (1C, $C_{2\text{-benzyl}}$); 134.7 (1C, $C_{6\text{-benzyl}}$); 141.2 (1C, $C_{1\text{-benzyl}}$)
1n	2230 (s, C≡N), 2151 (s, C≡N)	4.47 (s, 1H, CH_2); 7.49–7.53, (m, 1H, H_4); 7.63 (d, 1H, $J_{6-5}=7.7$, H_6); 7.71–7.75, (m, 1H, H_5); 7.86 (d, 1H, $J_{3-4}=7.6$, H_3)	30.5 (1C, CH_2); 104.9 (1C, SeCN); 112.3 (1C, $C_{2\text{-benzyl}}$); 117.9 (1C, CN); 129.6 (1C, $C_{4\text{-benzyl}}$); 131.7 (1C, $C_{6\text{-benzyl}}$); 134.1 (1C, $C_{3\text{-benzyl}}$); 134.3 (1C, $C_{5\text{-benzyl}}$); 142.2 (1C, $C_{1\text{-benzyl}}$)
1p	2148 (s, C≡N)	($CDCl_3$): 4.63 (s, 2H, CH_2); 7.54–7.56 (m, 2H, H_6+H_7); 7.62 (dd, 1H, $J_{3-4}=8.4$, $J_{3-1}=1.9$, H_3); 7.91–7.95 (m, 4H, $H_1+H_4+H_5+H_8$)	34.0 (1C, CH_2); 105.9 (1C, CN); 127.2 (1C, C_6); 127.3 (1C, C_7); 127.8 (1C, C_1); 128.3 (1C, C_4); 128.5 (1C, C_8); 128.6 (1C, C_5); 129.3 (1C, C_3); 133.2 (1C, C_{10}); 133.5 (1C, C_9); 136.6 (1C, C_2)
1q	2145 (m, C≡N), 1508, 1343 (s, NO_2)	3.42 (t, 2H, $J_{CH_2-CH_2}$ = 7.5, CH_2–SeCN); 3.54 (t, 1H, Ph–CH_2); 7.66 (d, 2H, $J_{3-2}=J_{5-6}=8.7$, H_3+H_5); 8.24 (d, 2H, H_2+H_6)	30.4 (1C, CH_2Se); 36.9 (1C, CH_2); 105.2 (1C, CN); 124.4 (2C, C_3, $C_{5\text{-benzyl}}$); 130.9 (2C, C_2, $C_{6\text{-benzyl}}$); 147.2 (1C, $C_{4\text{-benzyl}}$); 148.4 (1C, $C_{1\text{-benzyl}}$)
2c	3490, 3406, 3379, 3275 (m, NH_2), 1682 (s, C=O), 824 (m, Se–Se)	6.63 (d, 2H, $J_{3-4}=J_{3'-4'}=8.5$, $H_3+H_{3'}$); 7.29 (dd, 2H, $J_{4-6}=J_{4'-6'}=2.2$, $H_4+H_{4'}$); 7.98 (d, 2H, $H_6+H_{6'}$)	114.3 (2C, C_3, $C_{3'\text{-phenyl}}$); 114.6 (2C, C_5, $C_{5'\text{-phenyl}}$); 117.8 (2C, C_1, $C_{1'\text{-phenyl}}$); 139.2 (2C, C_2, $C_{2'\text{-phenyl}}$); 139.6 (2C, C_6, $C_{6'\text{-phenyl}}$); 152.5 (2C, C_4, $C_{4'\text{-phenyl}}$); 170.7 (2C, COOH)
2d	1690 [s, C=O(OH)], 1664 (s, C=O), 836 (w, Se–Se)	2.14 (s, 6H, $2CH_3$); 7.80 (dd, 2H, $J_{4-3}=J_{4'-3'}=8.8$, $J_{4-6}=J_{4'-6'}=2.2$, $H_4+H_{4'}$); 8.13 (d, 2H, $H_6+H_{6'}$); 8.42 (d, 2H, $H_3+H_{3'}$); 11.07 (s, 2H, 2NH)	25.9 (2C, CH_3); 118.3 (2C, C_3, $C_{3'\text{-phenyl}}$); 121.6 (2C, C_1, $C_{1'\text{-phenyl}}$); 123.7 (2C, C_5, $C_{5'\text{-phenyl}}$); 135.9 (2C, C_2, $C_{2'\text{-phenyl}}$); 138.7 (2C, C_6, $C_{6'\text{-phenyl}}$); 141.7 (2C, C_4, $C_{4'\text{-phenyl}}$); 169.2 (2C, CONH); 169.5 (2C, COOH)
2e	1516 (s, NO_2), 741 (m, Se–Se)	4.12 (s, 4H, $2CH_2$); 7.47 (d, 4H, $J_{2-3}=J_{6-5}=8.5$, $H_2+H_6+H_{2'}+H_{6'}$); 8.17 (d, 4H, $H_3+H_5+H_{3'}+H_{5'}$)	30.9 (2C, CH_2); 124.3 (4C, C_3, C_5, $C_{3'}$, $C_{5'\text{-benzyl}}$); 130.9 (4C, C_2, C_6, $C_{2'}$, $C_{6'\text{-benzyl}}$); 147.1 (2C, C_1, $C_{1'\text{-benzyl}}$); 148.5 (2C, C_4, $C_{4'\text{-benzyl}}$)
2f	1521 (s, C–NO_2), 737 (m, Se–Se)	4.13 (s, 4H, $2CH_2$); 7.58–7.62 (m, 2H, $H_5+H_{5'}$); 7.67 (d, 2H, $J_{6-5}=J_{6'-5'}=7.7$, $H_6+H_{6'}$); 8.05 (s, 2H, $H_2+H_{2'}$); 8.09 (d, 2H, $J_{4-5}=J_{4'-5'}=8.2$, $H_4+H_{4'}$)	30.5 (2C, CH_2); 122.6 (2C, C_4, $C_{4'\text{-benzyl}}$); 124.1 (2C, C_2, $C_{2'\text{-benzyl}}$); 130.6 (2C, C_5, $C_{5'\text{-benzyl}}$); 136.3 (2C, C_6, $C_{6'\text{-benzyl}}$); 142.7 (2C, C_1, $C_{1'\text{-benzyl}}$); 148.4 (2C, C_3, $C_{3'\text{-benzyl}}$)
2g	1511 (s, C–NO_2), 741 (m, Se–Se)	4.27, (s, 4H, $2CH_2$); 7.36 (d, 2H, $J_{6-5}=J_{6'-5'}=7.3$, $H_6+H_{6'}$); 7.51–7.55 (m, 2H, $H_4+H_{4'}$); 7.66–7.69 (m, 2H, $H_5+H_{5'}$); 8.05 (d, 2H, $J_{3-4}=J_{3'-4'}=8.0$, $H_3+H_{3'}$)	30.4 (2C, CH_2); 126.2 (2C, C_3, $C_{3'\text{-benzyl}}$); 129.6 (2C, C_4, $C_{4'\text{-benzyl}}$); 133.1 (2C, C_6, $C_{6'\text{-benzyl}}$); 134.6 (2C, C_1, $C_{1'\text{-benzyl}}$); 135.5 (2C, C_5, $C_{5'\text{-benzyl}}$); 148.0 (2C, C_2, $C_{2'\text{-benzyl}}$)
2h	1008 (s, C–Br), 710 (m, Se–Se)	3.93 (s, 4H, $2CH_2$); 7.19 (d, 4H, $J_{2-3}=J_{6-5}=8.4$, $H_2+H_6+H_{2'}+H_{6'}$); 7.51 (d, 4H, $H_3+H_5+H_{3'}+H_{5'}$)	31.4 (2C, CH_2); 120.9 (2C, C_4, $C_{4'\text{-benzyl}}$); 131.9 (4C, C_2, C_6, $C_{2'}$, $C_{6'\text{-benzyl}}$); 132.1 (4C, C_3, C_5, $C_{3'}$, $C_{5'\text{-benzyl}}$); 139.6 (2C, C_1, $C_{1'\text{-benzyl}}$)
2i	1325 (s, C–F), 746 (d, Se–Se)	4.04 (s, 4H, $2CH_2$); 7.43 (d, 4H, $J_{2-3}=J_{6-5}=7.9$, $H_2+H_6+H_{2'}+H_{6'}$); 7.67 (d, 4H, $H_3+H_5+H_{3'}+H_{5'}$)	31.2 (2C, CH_2); 123.8 (2C, CF_3); 126.0 (4C, C_3, C_5, $C_{3'}$, $C_{5'\text{-benzyl}}$); 128.2 (2C, C_4, $C_{4'\text{-benzyl}}$); 130.5 (4C, C_2, C_6, $C_{2'}$, $C_{6'\text{-benzyl}}$); 145.1 (2C, C_1, $C_{1'\text{-benzyl}}$)
2j	1491 (s, S–CH_3), 718 (w, Se–Se)	2.45 (s, 6H, $2CH_3$); 3.90 (s, 4H, $2CH_2$); 7.19 (s, 8H, $H_2+H_{2'}+H_3+H_{3'}+H_5+H_{5'}+H_6+H_{6'}$)	15.6 (2C, SCH_3); 32.1 (2C, CH_2); 126.7 (4C, C_3, C_5, $C_{3'}$, $C_{5'\text{-benzyl}}$); 130.4 (4C, C_2, C_6, $C_{2'}$, $C_{6'\text{-benzyl}}$); 136.5 (2C, C_1, $C_{1'\text{-benzyl}}$); 137.6 (2C, C_4, $C_{4'\text{-benzyl}}$)
2l	2229 (s, C≡N), 729 (w, Se–Se)	4.02 (s, 4H, $2CH_2$); 7.40 (d, 4H, $J_{2-3}=J_{6-5}=8.2$, $H_2+H_6+H_{2'}+H_{6'}$); 7.77 (d, 4H, $H_3+H_5+H_{3'}+H_{5'}$)	31.3 (2C, CH_2); 110.3 (2C, C_4, $C_{4'\text{-benzyl}}$); 119.8 (2C, CN); 130.7 (4C, C_2, C_6, $C_{2'}$, $C_{6'\text{-benzyl}}$); 133.1 (4C, C_3, C_5, $C_{3'}$, $C_{5'\text{-benzyl}}$); 146.2 (2C, C_1, $C_{1'\text{-benzyl}}$)
2m	2223 (s, C≡N); 687 (w, Se–Se)	4.01 (s, 4H, $2CH_2$); 7.51–7.55 (m, 2H, $H_5+H_{5'}$); 7.57 (d, 2H, $J_{6-5}=J_{6'-5'}=8.0$, $H_6+H_{6'}$,); 7.64 (s, 2H, $H_2+H_{2'}$); 7.72 (d, 2H, $J_{4-5}=J_{4'-5'}=7.3$, $H_4+H_{4'}$)	30.6 (2C, CH_2); 112.0 (2C, C_3, $C_{3'\text{-benzyl}}$); 119.5 (2C, CN); 130.5 (2C, C_5, $C_{5'\text{-benzyl}}$); 131.0 (2C, C_4, $C_{4'\text{-benzyl}}$); 133.0 (2C, C_2, $C_{2'\text{-benzyl}}$); 134.6 (2C, C_6, $C_{6'\text{-benzyl}}$); 141.9 (2C, C_1, $C_{1'\text{-benzyl}}$)
2n	2224 (s, C≡N); 762 (w, Se–Se)	4.18 (s, 4H, $2CH_2$); 7.43–7.48 (m, 4H, $H_4+H_6+H_{4'}+H_{6'}$); 7.65–7.69 (m, 2H, $H_5+H_{5'}$); 7.82 (d, 2H, $J_{3-4}=J_{3'-4'}=7.9$, $H_3+H_{3'}$)	30.0 (2C, CH_2); 112.2 (2C, C_2, $C_{2'\text{-benzyl}}$); 118.5 (2C, CN); 128.8 (2C, C_4, $C_{4'\text{-benzyl}}$); 131.2 (2C, C_6, $C_{6'\text{-benzyl}}$); 133.9 (2C, C_3, $C_{3'\text{-benzyl}}$); 134.1 (2C, C_5, $C_{5'\text{-benzyl}}$); 143.4 (2C, C_1, $C_{1'\text{-benzyl}}$)
2q	1508, 1340 (s, NO_2), 747 (m, Se–Se)	($CDCl_3$): 3.16–3.19 (m, 8H, $4CH_2$); 7.38 (d, 4H, $J_{2-3}=J_{6-5}=J_{2'-3'}=J_{6'-5'}=8.8$, $H_2+H_6+H_{2'}+H_{6'}$); 8.19 (d, 4H, $H_3+H_5+H_{3'}+H_{5'}$)	29.8 (2C, Se–CH_2); 37.0 (2C, CH_2–Ph); 124.3 (4C, C_3, C_5, $C_{3'}$, $C_{5'\text{-phenyl}}$); 130.7 (4C, C_2, C_6, $C_{2'}$, $C_{6'\text{-phenyl}}$); 146.9 (2C, C_1, $C_{1'\text{-phenyl}}$); 149.7 (2C, C_4, $C_{4'\text{-phenyl}}$)

Table 4
In vitro activities of compounds against *L. infantum* promastigotes expressed as growth inhibition (%).

Ar-(CH_2)n-SeCN **1a-q**

Ar-(CH_2)n-Se-Se-(CH_2)n-Ar **2a-r**

Compound	Aryl ring	n	25 μM	12.50 μM	6.25 μM
1a	4-Aminophenyl	0	76.3	38.2	14.4
1b	4-(*N*,*N*-dimethylamino)phenyl	0	97.3	86.7	54.4
1c	4-Amino-3-carboxyphenyl	0	NE[a]	–	–
1d	4-Acetamino-3-carboxyphenyl	0	NE	–	–
1e	4-Nitrophenyl	1	98.0	95.0	82.3
1f	3-Nitrophenyl	1	97.0	80.5	47.2
1g	2-Nitrophenyl	1	98.6	89.8	74.0
1h	4-Bromophenyl	1	97.1	80.8	49.2
1i	4-Trifluoromethylphenyl	1	98.8	97.2	79.4
1j	4-Methylthiophenyl	1	97.2	92.9	77.3
1k	4-Methylphenyl	1	98.6	82.8	52.8
1l	4-Cyanophenyl	1	97.0	80.4	51.5
1m	3-Cyanophenyl	1	93.0	71.2	42.2
1n	2-Cyanophenyl	1	90.2	62.4	31.4
1o	Phenyl	1	99.4	64.1	46.5
1p	Naphthyl	1	91.5	81.1	37.1
1q	4-Nitrophenyl	2	88.9	81.8	46.6
2a	4-Aminophenyl	0	98.0	97.6	95.8
2b	4-(*N*,*N*-dimethylamino)phenyl	0	77.6	67.7	51.5
2c	4-Amino-3-carboxyphenyl	0	NE	–	–
2d	4-Acetamino-3-carboxyphenyl	0	NE	–	–
2e	4-Nitrophenyl	1	98.7	96.3	81.8
2f	3-Nitrophenyl	1	41.2	29.4	18.1
2g	2-Nitrophenyl	1	77.2	58.9	50.0
2h	4-Bromophenyl	1	12.2	1.9	NE
2i	4-Trifluoromethylphenyl	1	12.2	6.0	11.0
2j	4-Methylthiophenyl	1	17.5	NE	NE
2k	4-Methylphenyl	1	2.5	NE	NE
2l	4-Cyanophenyl	1	32.8	21.9	10.9
2m	3-Cyanophenyl	1	44.3	35.4	22.6
2n	2-Cyanophenyl	1	31.5	16.5	7.3
2o	Phenyl	1	60.4	43.6	33.2
2p	Naphthyl	1	NE	–	–
2q	4-Nitrophenyl	2	28.4	3.2	1.2
2r	4-Methoxyphenyl	1	31.7	9.9	0.5

[a] NE = no effect.

against *L. infantum* amastigotes. It can be deduced that selenocyanate analogs displayed a strong inhibitory activity in the cultured amastigote model but the SI values of all the compounds were below those obtained for the diselenides with the exception of **2g**. The selectivity of the diselenide compounds for amastigotes increased in the order **2a** < **2o** < **2b** < **2e** with SI > 32 for Jurkat and SI > 24 for THP-1 in all of them. Among selenocyanates, the most selective was **1g** (SI = 38 and 21 when compared with Jurkat and THP-1 respectively) followed by the derivatives **1f** (SI = 21 and 16) and **1l** (SI = 24 and 15). Taken all the results together, the more promising candidates were, in this order, **2e**, **2b** and **2o**. Comparison with the SI values obtained for edelfosine (SI = 15 for Jurkat and SI = 6 for THP-1) and miltefosine (SI = 17 for Jurkat and SI = 7 for THP-1) reveals that compound **2e** is 4.7 and 12.7 times more selective than edelfosine for Jurkat and THP-1 cells, respectively, and 4 and 11 times more selective than miltefosine for the same cell lines. The selectivity index for **2b** is 4.3 and 4.8 higher than edelfosine and 3.8 and 4.1higher than miltefosine, whereas the indexes for **2o** are 2.7 and 8.5 better than edelfosine and 2.4 and 7.3 superior to those obtained for miltefosine.

2.2.3. Leishmanicidal activity in infected macrophages

Leishmanicidal activity was assayed in amastigote-infected THP-1 cells. The four molecules that showed the best SI in these cells were selected and their activity was determined by flow cytometry. Accordingly, compounds **2a**, **2b**, **2e** and **2o** were assayed for 48 h in differentiated THP-1 cells infected with *L. infantum* amastigotes expressing the green fluorescent protein (eGFP). Infected cells were identified by the green fluorescence emitted by their intracellular amastigotes (Fig. 1) [28]. Our results indicate that whereas 35% of the cells were infected in untreated controls, only 5% of them remained infected after treatment with 3 μM edelfosine and 9%, 23%, 17% or 24% of them contained parasites after treatment with compounds **2a**, **2b**, **2e** or **2o** respectively.

It is important to emphasize that treatment of the cells with compound **2a** at 3 μM for 48 h is able to reduce infection rates by 74% while showing a negligible cytotoxic effect over the host cells. Cytotoxic activity of this compound was also tested by microscopic

Table 5
IC_{50} ± SEM (μM) values for the most active compounds on promastigotes, amastigotes and cytotoxic activity in Jurkat and THP-1 cell lines.

Compound	Promastigote	Amastigote	Jurkat	SI[a]	THP-1	SI
1a	11.2 ± 1.1	9.29 ± 1.16	43.8 ± 6.2	5	> 50	>5
1b	9.24 ± 0.33	2.79 ± 0.17	29.3 ± 2.2	10	44.7 ± 3.4	16
1e	4.30 ± 0.74	0.68 ± 0.08	6.86 ± 1.3	10	16.8 ± 1.8	25
1f	4.18 ± 1.06	0.57 ± 0.08	12.2 ± 0.7	21	9.32 ± 0.1	16
1g	3.32 ± 0.16	0.34 ± 0.19	12.9 ± 1.4	38	7.10 ± 0.2	21
1h	11.6 ± 0.6	7.95 ± 1.02	12.7 ± 2.1	2	28.9 ± 3.9	4
1i	11.3 ± 0.4	2.14 ± 1.05	7.31 ± 0.4	3	19.8 ± 2.7	9
1j	11.6 ± 0.7	3.02 ± 0.59	5.07 ± 1.0	2	17.0 ± 1.8	6
1k	11.8 ± 0.3	3.33 ± 0.79	14.4 ± 1.7	4	24.0 ± 2.2	7
1l	4.14 ± 0.23	0.55 ± 0.21	13.4 ± 2.5	24	7.97 ± 1.0	15
1m	6.06 ± 1.7	0.53 ± 0.11	8.71 ± 2.6	16	7.60 ± 2.0	14
1n	7.75 ± 0.2	3.16 ± 0.02	17.1 ± 1.9	5	17.1 ± 1.1	5
1o	7.95 ± 0.34	2.58 ± 0.21	10.7 ± 1.7	4	25.0 ± 2.9	10
1p	11.2 ± 1.2	3.72 ± 1.08	14.0 ± 1.4	4	16.8 ± 3.1	5
1q	11.9 ± 0.9	3.80 ± 0.27	2.40 ± 1.2	1	17.2 ± 2.2	5
2a	0.96 ± 0.07	0.65 ± 0.02	20.4 ± 2.8	32	15.3 ± 0.8	24
2b	3.18 ± 0.23	0.77 ± 0.03	> 50	>65	22.1 ± 1.4	29
2e	4.04 ± 0.38	0.38 ± 0.03	26.8 ± 3.3	71	29.1 ± 2.3	77
2g	12.5 ± 2.8	0.29 ± 0.10	2.62 ± 0.5	9	2.42 ± 0.1	8
2o	17.8 ± 1.9	0.63 ± 0.05	26.1 ± 5.9	41	32.2 ± 2.5	51
Edelfosine	3.65 ± 0.19	0.82 ± 0.13	12.2 ± 2.4	15	4.96 ± 0.16	6
Miltefosine	15.0 ± 0.8	2.84 ± 0.10	48.2 ± 4.8	17	18.5 ± 0.6	7

[a] Selectivity index (SI) is the ratio of IC_{50} values of compounds against either Jurkat or THP-1 cells relative to those against *L. infantum* amastigotes.

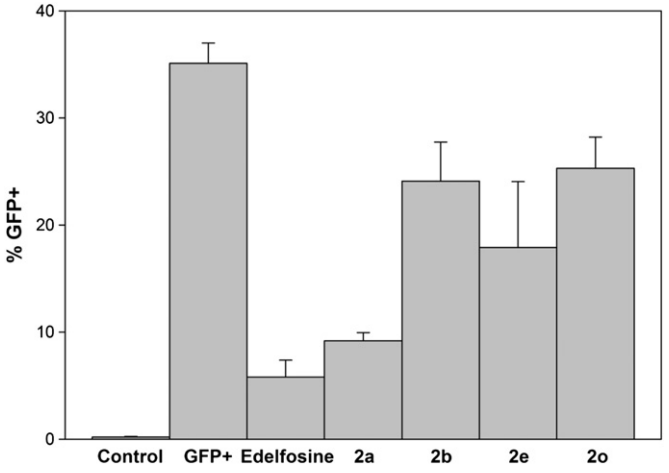


Fig. 1. The percentage of infected THP-1 cells after 48 h of drug-treatment was evaluated by flow cytometry. THP-1 cells were infected for 24 h with amastigotes expressing the green fluorescent protein (eGFP) and then treated with the different compounds for 48 additional hours. Infected macrophages were detected by the fluorescence emitted by the intracellular GFP-expressing amastigotes. Results are presented as the percentage of GFP positive cells in the THP-1 population. **Control**: non-infected/non-treated THP-1 cells. **GFP+**: infected/non-treated THP-1 cells. **Edelfosine, 2a, 2b, 2e and 2o**: infected THP-1 cells treated with the indicated drugs.

visualization of promastigotes, amastigotes, Jurkat and Thp-1 cells. The results presented in Fig. 2 confirm the absence of cytotoxicity of **2a** at 1 μM and 3 μM in the human cell lines tested. Remarkably, this compound is able to kill 100% of the axenic amastigotes at 3 μM without almost any deleterious effect in the Jurkat or Thp-1 cell lines.

2.3. Physico-chemical and absorption properties calculations

Analysis of the Lipinski descriptors for bioavailability estimation using the freely accessible program OSIRIS Explorer Properties (http://www.chemexper.com/tools/propertyExplorer/main.html) was also carried out (Table 6). These parameters describe molecular properties important for drug pharmacokinetics in the human body, especially for their oral absorption. According to "Lipinski rules", an orally active drug must not violate more than one of the following criteria: ≤5 hydrogen donors (nOHNH), ≤10 hydrogen acceptors (nON), MW ≤ 500 and clogP ≤ 5. Compounds **2a** (clogP = 0.36, MW = 344, nOHNH = 4, nON = 2), **2b** (clogP = 1.08, MW = 400, nOHNH = 0, nON = 2), **2e** (clogP = 1.85, MW = 432, nOHNH = 0, nON = 6), and **2o** (clogP = 2.11, MW = 342, nOHNH = 0, nON = 0) meet these claims. However, one of the reference drugs for pharmacological testing, miltefosine (clogP = 5.67, MW = 509, nOHNH = 0, nON = 7), which was also subjected to OSIRIS Explorer Properties does not fulfil two out of four Lipinski descriptors. In addition, it is well known that a lot of drug candidates have failed during clinical tests because of the problems related to ADME (absorption, distribution, metabolism and excretion) properties. So, we utilized a web-based application called PreADMET (http://preadmet.bmdrc.org/preadmet/index.php) which has been developed for rapid prediction of ADME/Tox data. We present the results

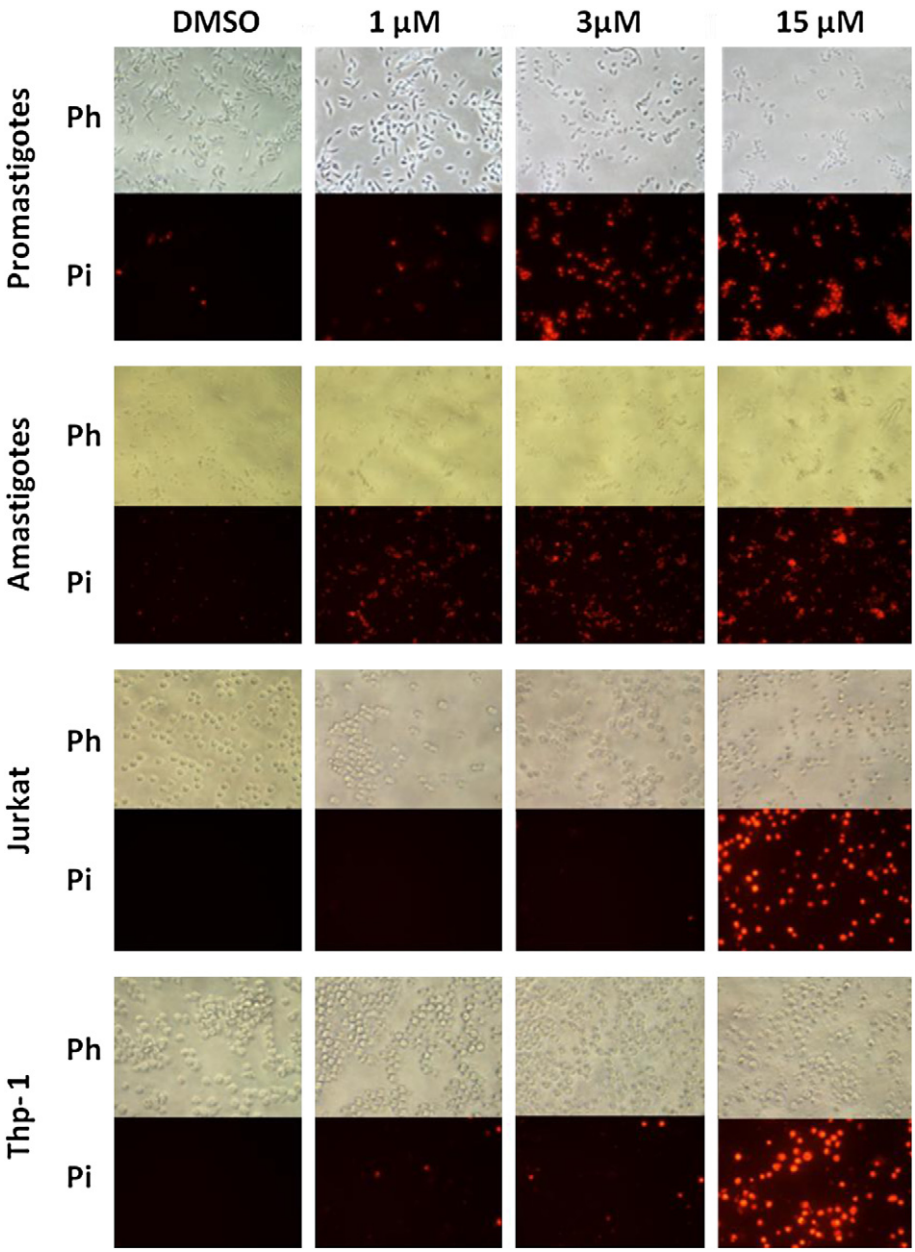


Fig. 2. Microscopic visualization of *L. infantum* axenic promastigotes, *L. infantum* axenic amastigotes, Jurkat and Thp-1 human cell lines. Cells were treated with compound **2a** at 1 μM, 3 μM and 15 μM. Non-treated control cells exposed to the maximum DMSO concentration of the assay are also shown. Propidium iodide was added to a final concentration of 5 μg/mL and the cells were immediately analyzed by phase contrast (Ph) and fluorescence microscopy (PI). Propidium iodide is excluded from viable cells but stains the nucleic acids from dead cells.

Table 6
Physico-chemical and absorption properties for the most active compounds.

Compound	cLogP[a]	MW[b]	*n*-OHNH[c] donors	*n*-ON[d] acceptors	Lipinski's violations	Absorption	
						HIA[e] (%)	Caco-2[f] (nm/s)
2a	0.36	344	4	2	0	97.64	22.89
2b	1.80	400	0	2	0	100.00	56.07
2e	1.85	432	0	6	0	98.33	20.14
2o	2.11	342	0	0	0	100.00	58.33
Edelfosine	4.34	407	1	5	0	98.43	39.38
Miltefosine	5.67	509	0	7	2	98.96	21.74

[a] cLogP: logarithm of compound partition coefficient between *n*-octanol and water.
[b] MW: molecular weight.
[c] *n*-OHNH: number of hydrogen bond donors.
[d] *n*-ON: number of hydrogen bond acceptors.
[e] HIA: Human Intestinal Absorption.
[f] Caco-2, cells derived from human colon adenocarcinoma.

of some properties of our active and selective compounds, (**2a**, **2b**, **2e**, and **2o**) in Table 6. Human Intestinal Absorption (HIA) and Caco-2 permeability are good indicators of drug absorbance in the intestines and Caco-2 monolayers respectively. Human Intestinal Absorption data are the sum of bioavailability and absorption evaluated from the ratio of excretion or cumulative excretion in urine, bile and feces [29]. The predicted percentages of intestinal absorption are excellent for all the compounds tested, with values above 97% in all cases. The compounds present middle permeability values in Caco-2 cells ranging from 20 to 58 [30].

3. Conclusions

In summary, we have presented the synthesis of thirty five compounds that contain either a selenocyanate (**1a**–**q**) or a diselenide (**2a**–**r**) moiety. These compounds were evaluated as a potential novel class of antileishmanial agents in the promastigote and amastigote models and their cytotoxic activity against the cancer cell lines Jurkat and THP-1 was investigated. Twenty analogs exhibit promising leishmanicidal activity in both *Leishmania* models, nineteen and thirteen of them being more active than miltefosine in promastigotes and amastigotes respectively. Moreover, ten compounds (**1e**, **1f**, **1g**, **1l**, **1m**, **2a**, **2b**, **2e, 2g** and **2o**) are more potent than edelfosine in amastigotes. In particular, based on IC_{50} and SI values, seven compounds (**1f**, **1g**, **1l**, **2a**, **2b**, **2e** and **2o**) combined high potency and low cytotoxicity with SI values higher than those obtained for the reference drugs.

Taken together, our results and the predicted values for the Lipinski parameters indicate that the diselenide compounds **2a** (with amino group), **2e** (with a 4-NO_2 aryl moiety), **2b** (with an *N*,*N*-dimethylamino group) and **2o** (with an unsubstituted aromatic ring) have the best profiles, with IC_{50} values ranging from 0.4 to 0.8 μM against *L. infantum* amastigotes. These compounds show SI values that are two- to four-times and four- to twelve-times higher than those obtained for the reference drugs miltefosine and edelfosine respectively. Furthermore, compound **2a** emerges as the most promising derivative, taking into account that it is able to reduce *in vitro* infection rates by 74% showing a negligible cytotoxic effect over the host cells.

Despite this encouraging advance, there is still a need to develop safe, efficient and affordable new treatments for the different clinical forms of leishmaniasis. In this respect, extensive mechanism-based studies are required to gain a better understanding of how these compounds exert their cytotoxicity. The series of compounds described here not only show promising drug-like properties but they are also easy and economical to prepare. These are important points that support further *in vivo* studies and an assessment of their possible use in developing countries, where the cost of drug therapies is a major factor.

4. Experimental protocols

4.1. Chemistry

Melting points were determined with a Mettler FP82 + FP80 apparatus (Greifensee, Switzerland) and are not corrected. The ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 Ultrashield™ spectrometer (Rheinstetten, Germany) using TMS as the internal standard. The IR spectra were obtained on a Thermo Nicolet FT-IR Nexus spectrophotometer with KBr pellets. Elemental microanalyses were carried out on vacuum-dried samples using a LECO CHN-900 Elemental Analyzer. Silica gel 60 (0.040–0.063 mm) 1.09385.2500 (Merck KGaA, 64271 Darmstadt, Germany) was used for Column Chromatography and Alugram® SIL G/UV_{254} (Layer: 0.2 mm) (Macherey-Nagel GmbH & Co. KG. Postfach 101352, D-52313 Düren, Germany) was used for Thin Layer Chromatography. Chemicals were purchased from E. Merck (Darmstadt, Germany), Scharlau (F.E.R.O.S.A., Barcelona, Spain), Panreac Química S.A. (Montcada i Reixac, Barcelona, Spain), Sigma–Aldrich Química, S.A. (Alcobendas, Madrid, Spain), Acros Organics (Janssen Pharmaceuticalaan 3a, 2440 Geel, België) and Lancaster (Bischheim-Strasbourg, France).

4.1.1. General procedure for the synthesis of compounds *1a–c*

Selenium dioxide (6 mmol) was added with stirring to solution of malononitrile (3 mmol) in DMSO (15 mL). The mixture was stirred at room temperature for 15 min in order to obtain triselenium dicyanide. When the exothermic reaction had finished the appropriate aromatic amine (5 mmol) was added. The mixture was stirred for 30–60 min. Water (150 mL) was added to the reaction mixture and the resulting precipitate was filtered off, dried and crystallized according to the method shown in Table 2.

*4-Aminophenylselenocyanate (**1a**)*: From aniline. The synthesis of this compound has been previously reported by Kachanov et al. [23].

*4-(N,N-dimethylamino)phenylselenocyanate (**1b**)*: From *N,N*-dimethylaniline. The synthesis of this compound has been previously reported by Kachanov et al. [23].

*4-Amino-3-carboxyphenylselenocyanate (**1c**)*: From *o*-aminobenzoic acid. The synthesis of this compound has been previously reported by Kachanov et al. [23].

*4-Acetamido-3-carboxyphenylselenocyanate (**1d**)*: From compound **1c** by reaction with acetic anhydride.

*4.1.2. General procedure for the synthesis of compounds **1e**–**q***

KSeCN (4 mmol) was added to a solution of the appropriate benzyl bromide derivative (4 mmol) in acetone (50 mL) and the mixture was heated under reflux for 2–4 h. The resulting precipitate (KBr) was filtered off. The filtrate was evaporated under vacuum and the residue was treated with water (50 mL), filtered and purified according to Table 2.

*4-Nitrobenzylselenocyanate (**1e**)*: From 4-nitrobenzyl bromide. The synthesis of this compound has been previously reported by Maartmann-Moe et al. [31].

*3-Nitrobenzylselenocyanate (**1f**)*: From 3-nitrobenzyl bromide. The synthesis of this compound has been previously reported by Grung et al. [32].

*2-Nitrobenzylselenocyanate (**1g**)*: From 2-nitrobenzyl bromide. The synthesis of this compound has been previously reported by Grung et al. [32]

*4-Bromobenzylselenocyanate (**1h**)*: From 4-bromobenzyl bromide. The synthesis of this compound has been previously reported by Jacob et al. [33].

*4-Trifluoromethylbenzylselenocyanate (**1i**)*: From 4-trifluoromethylbenzyl bromide.

*4-Methylthiobenzylselenocyanate (**1j**)*: From 4-methylthiobenzyl bromide.

*4-Methylbenzylselenocyanate (**1k**)*: From 4-methylbenzyl bromide. The synthesis of this compound has been previously reported by Jacob et al. [34].

*4-Cyanobenzylselenocyanate (**1l**)*: From 4-bromomethylbenzonitrile. The synthesis of this compound has been previously reported by Prabhu et al. [35]

*3-Cyanobenzylselenocyanate (**1m**)*: From 3-bromomethylbenzonitrile.

*2-Cyanobenzylselenocyanate (**1n**)*: From 2-bromomethylbenzonitrile.

*Benzylselenocyanate (**1o**)*: From benzyl bromide. The synthesis of this compound has been previously reported by Krief et al. [25].

*2-Selenocyanatemethylnaphthalene (**1p**)*: From 2-bromomethylnaphthalene.

*2-(4-Nitrophenyl)ethylselenocyanate (**1q**)*: From 4-nitrophenethyl bromide.

*4.1.3. General procedure for the synthesis of compounds **2a**–**q***

$NaBH_4$ (3 mmol) was added in small portions with caution to a solution of the appropriate selenocyanate derivative **1a**–**q** (3 mmol) in absolute ethanol (40 mL). The mixture was stirred at room temperature for 2 h. The solvents were removed under vacuum by rotary evaporation and the residue was treated with water. The mixture was extracted with dichloromethane (3 × 50 mL). The organic phase was washed with water (3 × 50 mL) and dried with anhydrous Na_2SO_4. The dichloromethane was removed under vacuum and the residue was purified according to the method shown in Table 2.

*Bis(4-aminophenyl)diselenide (**2a**)*: From **1a**. The synthesis of this compound has been previously reported by Banks et al. [36].

*Bis[4-(N,N-dimethylamino)phenyl]diselenide (**2b**)*: From **1b**. The synthesis of this compound has been previously reported by Pinto et al. [37].

*Bis(4-amino-3-carboxyphenyl)diselenide (**2c**)*: From **1c**.

*Bis(4-acetamido-3-carboxyphenyl)diselenide (**2d**)*: From **1d**.

*Bis(4-nitrobenzyl)diselenide (**2e**)*: From **1e**. The synthesis of this compound has been previously reported by Prabhu et al. [35] and Degrand et al. [38].

*Bis(3-nitrobenzyl)diselenide (**2f**)*: From **1f**.

*Bis(2-nitrobenzyl)diselenide (**2g**)*: From **1g**.

*Bis(4-bromobenzyl)diselenide (**2h**)*: From **1h**. The synthesis of this compound has been previously reported by Wang et al. [39].

*Bis(4-trifluoromethylbenzyl)diselenide (**2i**)*: From **1i**.

*Bis(4-methylthiobenzyl)diselenide (**2j**)*: From **1j**.

*Bis(4-methylbenzyl)diselenide (**2k**)*: From **1k**. The synthesis of this compound has been previously reported by Tian et al. [40].

*Bis(4-cyanobenzyl)diselenide (**2l**)*: From **1l**. The synthesis of this compound has been previously reported by Prabhu et al. [35]

*Bis(3-cyanobenzyl)diselenide (**2m**)*: From **1m**.

*Bis(2-cyanobenzyl)diselenide (**2n**)*: From **1n**.

*Bisbenzyldiselenide (**2o**)*: From **1o**. The synthesis of this compound has been previously reported by Tian et al. [40].

*Bis(2-methylnaphthyl)diselenide (**2p**)*: From **1p**. The synthesis of this compound has been previously reported by Tian et al. [40].

*Bis[2-(4-nitrophenyl)ethyl]diselenide (**2q**)*: From **1q**.

*4.1.4. Procedure for preparation of bis(4-methoxybenzyl)diselenide (**2r**)*

Hydrazine hydrate (11 mmol) was added to a mixture of NaOH (15 mmol), gray selenium (10 mmol) and DMF (5 mL) under a nitrogen atmosphere and the mixture was stirred for 6 h. A solution of 1-chloromethyl-4-methoxybenzene (5 mmol) in DMF (3 mL) was added dropwise to the reaction mixture and this was stirred for 45 min. Water (150 mL) was added and the mixture was extracted with dichloromethane (3 × 50 mL). The organic phase was washed with a 6 N HCl (20 mL) and water (2 × 50 mL). The organic phase was dried with anhydrous Na_2SO_4, filtered and the dichloromethane was removed under vacuum. The residue was purified by recrystallization from *n*-hexane. The synthesis of this compound has been previously reported by Tian et al. [36].

4.2. Biological evaluation

4.2.1. Cells and culture conditions

L. infantum promastigotes (MCAN/ES/89/IPZ229/1/89) were kindly provided by Dr. Colmenares (Centro de Investigaciones Biológicas, CIB, Madrid, Spain) and were grown in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat inactivated fetal calf serum (FCS), antibiotics and 25 mM HEPES, pH 7.2 at 26 °C. *L. infantum* axenic amastigotes were grown in M199 (Invitrogen, Leiden, The Netherlands) medium supplemented with 10% heat inactivated FCS, 1 g/L β-alanine, 100 mg/L L-asparagine, 200 mg/L sacarose, 50 mg/L sodium pyruvate, 320 mg/L malic acid, 40 mg/L fumaric acid, 70 mg/L succinic acid, 200 mg/L α-ketoglutaric acid, 300 mg/L citric acid, 1.1 g/L sodium bicarbonate, 5 g/L MES, 0.4 mg/L hemin, 10 mg/L gentamicin pH 5.4 at 37 °C. Jurkat cells were kindly provided by Dr. Mollinedo [Centro de Investigación del Cáncer, Instituto de Biología Molecular y Celular del Cáncer, Consejo Superior de Investigaciones Científicas (C.S.I.C.) – Universidad de Salamanca, Salamanca, Spain] and were grown in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat inactivated FCS, antibiotics and 10 mM HEPES, pH 7.2 at 37 °C and 5% CO_2. THP-1 cells were kindly provided by Dr. Michel (Université Nice Sophia Antipolis, Nice, France) and were grown in RPMI-1640 medium (Gibco, Leiden, The Netherlands) supplemented with 10% heat inactivated FCS, antibiotics, 1 mM HEPES, 2 mM glutamine and 1 mM sodium pyruvate, pH 7.2 at 37 °C and 5% CO_2.

4.2.2. Leishmanicidal activity and cytotoxicity assays

Drug treatment of promastigotes and amastigotes was performed during the logarithmic growth phase at a concentration of 2×10^6 parasites/mL at 26 °C or 1×10^6 parasites/mL at 37 °C for 24 h, respectively. Drug treatment of Jurkat and THP-1 cells was performed during the logarithmic growth phase at a concentration of 4×10^5 cells/mL at 37 °C and 5% CO_2 for 24 h. The percentage of living cells was evaluated by flow cytometry by the propidium iodide (PI) exclusion method [J.F. Alzate, A.A. Arias, D. Moreno-Mateos,

A. Alvarez-Barrientos, A. Jiménez-Ruiz. Mitochondrial superoxide mediates heat-induced apoptotic-like death in *L. infantum*. Mol. Biochem. Parasitol. 152 (2007) 192–202]. Microscopic evaluation of the cells was performed by direct observation of the parasites in 96 well plates with a Nikon eclipse Ti–S microscope either by phase contrast or by fluorescence microscopy.

4.2.3. Eukaryotic green fluorescent protein (eGFP) sequence cloning and construct design

The coding sequence of eGFP was PCR-amplified from the p6.5-eGFP construct kindly provided by Dr. K.P. Chang (Chicago Medical School – Rosalind Franklin University, North Chicago, IL, USA) with the following primers: 5′GGGAGATCTATGGTGAGCAAGGGC-GAG-GA3′ and 5′GGGCATATGTTACTTGTACAGCTCGTCCA3′. The PCR product was purified, doubly digested with the restriction endonucleases Nde I/Bgl II and cloned into the vector pIRmcs3(–) digested with the same endonucleases.

4.2.4. Promastigote transfection

The parasites were harvested in logarithmic growth phase and transfected by electroporation as previously described [27]. Stable transfected strains were selected at 100 μg/mL nourseothricin (Axxora, San Diego, CA, USA) in RPMI/20%FCS. The *pIRmcs3-eGFP* construct was linearized with the enzyme Swa I and gel purified (Illustra GFX gel purification kit, General Electric, UK) prior to transfection.

4.2.5. Leishmania infection assay

THP-1 cells were seeded at 120,000 cells/mL in 24 multidishes plates (Nunc, Roskilde, Denmark) and differentiated to macrophages for 24 h in 1 mL of RPMI-1640 medium containing 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma–Aldrich, St. Louis, MO, USA). Medium culture was removed and 1.2×10^6 *Leishmania* amastigotes in 1 mL of THP-1 medium were added to each well. 3 h later all medium with non-infecting amastigotes was removed, washed 3 times with 1X phosphate buffered saline (1X PBS) and replaced with new THP-1 medium and corresponding treatment. After 48 h treatment, medium was removed; THP-1 cells were washed 3 times with 1X PBS and detached with TrypLE™ Express (Invitrogen, Leiden, The Netherlands) according to the manufacturer's indications. Infection quantization was measured by flow cytometry.

4.2.6. Physico-chemical and absorption properties calculations

The physico-chemical properties of the most active compounds were calculated using the OSIRIS Property Explorer (http://www.chemexper.com/tools/propertyExplorer/main.html). The absorption properties were calculated using PreADMET program (http://preadmet.bmdrc.org/preadmet/index.php).

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.04.054.

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