SHORT COMMUNICATION

Antileishmanial activity of imidothiocarbamates and imidoselenocarbamates

David Moreno · Daniel Plano · Ylenia Baquedano · Antonio Jiménez-Ruiz · Juan Antonio Palop · Carmen Sanmartín

Abstract In the present study, a family of 15 imidothio- and imidoselenocarbamates (1–15) analogs have been synthesized and screened for their in vitro antileishmanial potential against *Leishmania infantum* promastigotes. The six most active ones (2, 4, 7, 13, 14, and 15) were also tested in an axenic amastigote model. In order to establish their selectivity indexes (SI) the cytotoxic effect of each compound was also assayed against Jurkat and THP-1 cell lines. Compounds 2 and 4, both with a pyridine moiety, showed a moderate antileishmanial activity with an IC_{50} value of 4.68 ± 0.46 and 3.03 ± 0.24 µM, respectively, in the amastigote model. The activity was compared with that of standard drugs, edelfosine ($IC_{50} = 0.82 \pm 0.13$ µM) and miltefosine ($IC_{50} = 2.84 \pm 0.10$ µM). Related to selectivity, the SI of both compounds are similar to those of the standard drugs when compared against the THP-1 cell line. Moreover, compound 4 was able to reduce the number of amastigote-infected THP-1 cells to 40% of that observed in untreated controls after a 96-h period of treatment. These derivatives thus represent two new leads for further studies aimed at establishing their mechanism of action.

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Introduction

In spite of the vast amount of research conducted on *Leishmania* pathogens, leishmaniasis is still one of the major worldwide public health problems, with a special incidence in the poorest populations (Lindoso and Lindoso 2009). Leishmaniasis is a spectrum of diseases ranging in symptoms from skin lesions to fatal systemic infections. These diseases are caused by several species of flagellated protozoa belonging to the genus *Leishmania* in the Trypanosomatidae family, whose members are characterized by the presence of the kinetoplast, a unique form of mitochondrial DNA (Sharma and Singh 2008). Leishmaniasis comprises three basic clinical forms: visceral, cutaneous, and mucocutaneous, among which visceral is the most severe form (David and Craft 2009). Pentavalent antimonials (Frézard et al. 2009) have been the gold standard for the treatment of these diseases, but the use of these agents is becoming limited due to the emergence of drug resistance, severe side effects, and loss of efficacy. Amphotericin B and particularly its lipid formulation (Rosenthal et al. 2009) has proved to be very effective in the treatment of leishmaniasis, but its elevated cost frequently impedes its use in poorly developed countries. Miltefosine, a phosphocholine analog (Palumbo 2008) recently introduced into clinical practice, shows a high efficacy in children. Edelfosine is another alkyl-phospholipid (Cabrera-Serra et al. 2008) that has also been tested and found to display higher in vitro activity than miltefosine (Alzate et al. 2008). Other drugs such as paromomycin, sitamaquine, azoles, and azithromycin are currently being analyzed in several clinical trials (Le Pape 2008).

In the search for new antileishmanial drugs, both synthetic and natural compounds comprising a diverse group of chemical structures have been reported. Among

those from natural sources, flavonoids, chalcones, iridoids, lignands, and taxoids have been tested (Polonio and Effert 2008). A new therapeutic option based on the use of alkaloids has been recently reported (Mishra et al. 2009). Despite all these efforts, additional rapid and cost-effective approaches for the identification and development of new lead compounds with good safety profiles and oral bioavailability are urgent. Two recent papers (Le Pape 2008; Den Boer et al. 2009) summarize available data about antileishmanial drugs, with specific information about those that are currently included in clinical trials, their status and future perspectives in the area of combinational therapy, plant product research, and novel drug targets.

During the last few years, various reports have shown the role of selenium (Se) in the modulation of the immune responses against *Trypanosoma* infections (De Souza et al. 2002; De Souza et al. 2003). It has been shown that trypanosomatids, in comparison with mammals, have a different strategy to withstand oxidative stresses because they lack classical glutathione reductase, glutathione-dependent peroxidise, and thioredoxin reductase activities. It is well known (Krauth-Siegel et al. 2003) that the glutathione system that controls the redox status in higher eukaryotes is replaced in trypanosomatids by a trypanothione system where the key enzyme is trypanothione reductase which catalyzes the reduction of trypanothione disulfide to trypanothione (Stump et al. 2007). The activity of these molecules is based in the ability of sulfur to act as a donor or an acceptor of electrons. Based on the chemical analogy of sulfur and selenium, we decided to explore the relevance of this trace element to generate new molecules with leishmanicidal activity.

In addition, computational analysis of the genomes of several species of *Trypanosoma* and *Leishmania* has revealed the presence of at least three selenoproteins: Sel T, Sel K, and the kinetoplastida-specific selenoprotein Sel Tryp. In all three proteins selenocysteine (Sec) is located within predicted redox motifs (CxxU in the case of Sel T

and Sel Tryp and CxxxU in Sel K), which is suggestive of their possible role in maintaining the redox equilibrium in these parasites (Lobanov et al. 2006; Sculaccio et al. 2008). A recent study has also implicated selenoprotein P (Sepp1) in the protection against illness caused by *Trypanosoma* due to its protective action against oxidative damage (Burk and Hill 2009). Further studies are necessary to verify parasite selenium dependence and determine the concentration of this trace element that is required for their viability.

Taking into account these considerations, and as part of our ongoing search for biologically active Se-containing compounds (Plano et al. 2007; Sanmartín et al. 2009; Plano et al. 2010), we have analyzed the biological activity of a small in-house compound collection of alkyl imidoselenocarbamates (alkyl isoselenourea) and alkyl imidothiocarbamates (alkylisothiourea) against *Leishmania* parasites. Our research group has developed these structures based on aromatic and heteroaromatic rings (Fig. 1) that have already shown remarkable anticancer activity (Plano et al. 2007).

It is interesting to point out that several of the most effective antiprotozoal agents were originally developed as anticancer drugs (Fuertes et al. 2008; Da Silva et al. 2009), which prompted us to test this class of compounds and their derivatives as antileishmanial agents. In addition, and with the aim of confirming whether the isosteric substitution of sulfur for selenium could significantly improve the leishmanicidal activity, as has already been demonstrated for their antitumor activity (Madhunapantula et al. 2008; Sharma et al. 2009; Das et al. 2009; Emmert et al. 2010), four sulfur analogs were also screened.

Material and methods

Chemistry

All the compounds were synthesized, purified, and characterized by our earlier reported method (Plano et al. 2007). The analytical

Fig. 1 General structure for alkyl imidoselenocarbamates and alkyl imidothiocarbamates
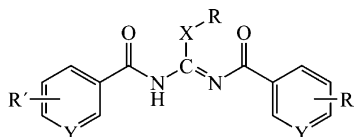
Compound	X	Y	R	R'	Compound	X	Y	R	R'
1	S	N	methyl	2-Cl	9	Se	C	methyl	3,4,5-triOCH$_3$
2	Se	N	methyl	2-Cl	10	Se	C	methyl	4-Cl
3	S	N	methyl	H	11	Se	C	isopropyl	4-Cl
4	Se	N	methyl	H	12	S	C	methyl	4-CH$_3$
5	S	C	methyl	H	13	Se	C	methyl	4-CH$_3$
6	Se	C	methyl	H	14	Se	C	methyl	4-CF$_3$
7	Se	C	methyl	3,5-diOCH$_3$	15	Se	C	methyl	4-CN
8	Se	C	isopropyl	3,5-diOCH$_3$					

Table 1 Physical constants and spectroscopic data for new compounds

Ref	Yield (%)	Mp (°C)	Molecular formula	Anal (%) calcd/found	IR (KBr; cm^{-1})	^{1}H NMR (400 MHz, CDCl$_3$, δ, J in Hz):
3	52	164–6	C$_{14}$H$_{12}$N$_4$O$_2$S	C, 56.00/55.65; H, 4.00/3.98; N, 18.67/18.83	3,448 (m, N–H); 1,698 (m, C=O)	2.68 (s, 3H, S-CH$_3$); 7.44 (dd, 1H, J_{5-4}=7.9, J_{5-6}=4.8 Hz, H$_5$); 7.52 (dd, 1H, $J_{5'-4'}$=8.0, $J_{5'-6'}$=4.8, H$_{5'}$); 8.30 (dt, 1H, $J_{4'-6'}$=$J_{4'-2'}$=1.7, H$_4$); 8.53 (dt, 1H, J_{4-6}=J_{4-2}=1, 6, H$_4$); 8.79 (dd, 1H, H$_{6'}$); 8.88 (dd, 1H, H$_6$); 9.29 (d, 1H, H$_{2'}$); 9.55 (d, 1H, H$_2$).
4	38	158–9	C$_{14}$H$_{12}$N$_4$O$_2$Se	C, 48.41/48.19; H, 3.46/3.40; N, 16.14/16.37	3,443 (m, N–H); 1,693 (m, C=O)	2.52 (s, 3H, Se-CH$_3$); 7.43 (dd, 1H, J_{5-4}=7, 9, J_{5-6}=4,8, H$_5$); 7.52 (dd, 1H, $J_{5'-4'}$=8.0, $J_{5'-6'}$=4.8, H$_{5'}$); 8.29 (dt, 1H, $J_{4'-6'}$=$J_{4'-2'}$=1, 7, H$_4$); 8.54 (dt, 1H, J_{4-6}=J_{4-2}=1, 6, H$_4$); 8.79 (dd, 1H, H$_{6'}$); 8.88 (dd, 1H, H$_6$); 9.29 (d, 1H, H$_{2'}$); 9.56 (d, 1H, H$_2$)
9	31	161–2	C$_{22}$H$_{26}$N$_2$O$_8$Se	C, 50.29/49.92; H, 4.99/5.08; N, 5.33/5.30	3,415 (m, N–H); 1,682 (m, C=O)	2.50 (s, 3H, Se-CH$_3$); 3.99 (s, 18H, 6(OCH$_3$)); 7.30 (s, 2H, H$_{2'}$+H$_{6'}$); 7.69 (s, 2H, H$_2$+H$_6$); 14.69 (s, 1H, NH)

and spectroscopic data for the new compounds described here 3 (X=, Y=N, R'=H), 4 (X=Se, Y=N, R'=H), and 9 (X=Se, Y=C, R'=3,4,5-trimethoxy) are shown in Table 1.

Biological evaluation

Cells and culture conditions

Leishmania 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 gentamicine 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$_2$.

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 exclusion method.

Eukaryotic green fluorescent protein sequence cloning and construct design

The coding sequence of eukaryotic green fluorescent protein (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) with the following primers: 5′GGGAGATC

Table 2 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
2	31.6±3.3	4.68±0.46	25.2±4.9	5	22.6±1.1	5
4	3.74±0.18	3.03±0.24	4.78±0.53	2	11.8±2.4	4
7	18.2±0.4	14.9±0.80	20.4±6.9	1	26.9±2.9	2
13	28.5±1.0	13.4±3.80	27.5±3.9	2	24.1±1.5	2
14	29.0±0.7	17.6±1.00	20.0±0.8	1	15.3±0.7	1
15	30.9±2.3	16.6±0.70	26.4±3.3	2	36.3±2.7	2
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

TATGGTGAGCAAGGGC-GAGGA3′ and 5′GGGCA
TATGTTACTTGTACAGCTCGTCCA3′. 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.

Promastigote transfection

The parasites were harvested in logarithmic growth phase
and transfected by electroporation as previously described
(Alzate et al. 2006). 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.

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
RPMI-1640 medium were added to each well. Three hours
later, all medium containing non-infecting amastigotes was
removed, plates were washed three times with 1× phosphate-
buffered saline (1× PBS), and new RPMI-1640 medium with
the corresponding treatment was added. After 48 h of
treatment, medium was removed; THP-1 cells were washed
three times with 1× PBS and detached with TrypLE™
Express (Invitrogen, Leiden, the Netherlands) according to
the manufacturer's indications. Infection quantization was
measured by flow cytometry.

Physico-chemical and absorption properties calculations

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

Results and discussion

Leishmanicidal activity

All the structures were initially tested in vitro against cultured
promastigotes of L. infantum MCAN/ES/89/IPZ229/1/89 at
25, 12.5, and 6.25 μM according to a previously described
procedure (Alzate et al. 2006). All the analyses were carried
out with a minimum of three independent experiments.

The percentages of growth inhibition obtained at these
three different concentrations are summarized as supple-
mentary data. Among the 15 evaluated compounds, six
caused more than 50% of cell growth inhibition at 25 μM
and were subsequently screened against axenic amastigotes.
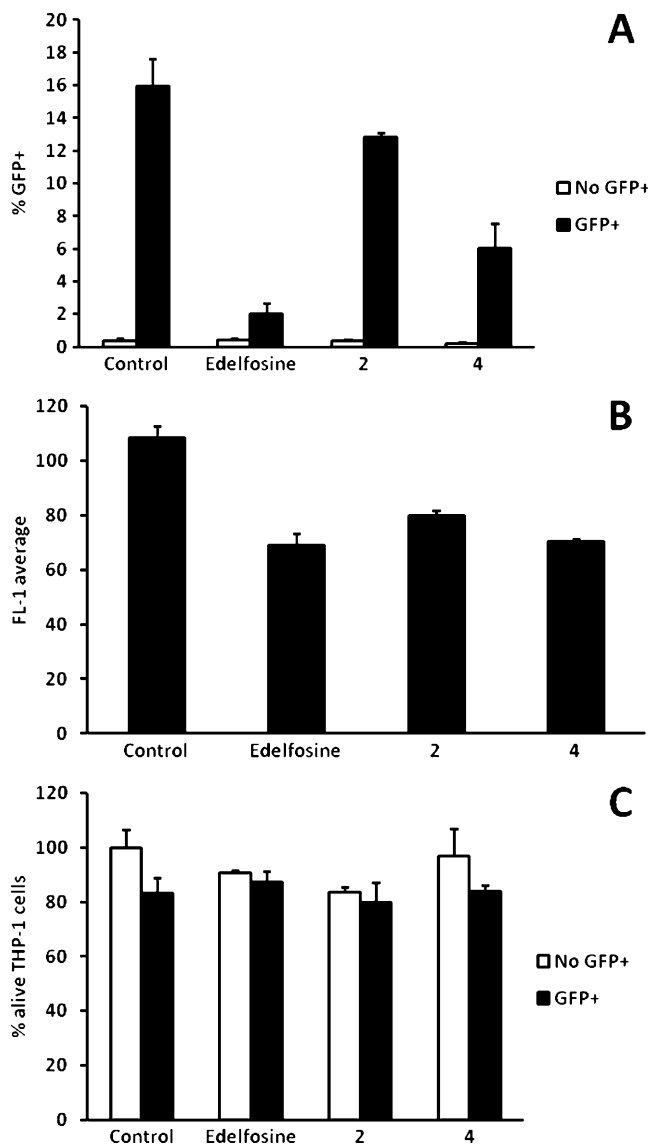The IC_{50} values for promastigotes and amastigotes are

Fig. 2 Activity of the compounds in PMA-differentiated THP-1 cells.
a Percentage of differentiated THP-1 cells showing green fluorescence
in infected (black bars) or non-infected (white bars) THP-1 cells
treated for 96 h with DMSO (control), 1 μM edelfosine, 3 μM
compound 2, and 3 μM compound 4. Green fluorescence is due to the
presence of eGFP-expressing L. infantum amastigotes inside the THP-
1 cells. b Mean fluorescence of the differentiated THP-1 cells
containing GFP-expressing amastigotes after 92 h of treatment with
DMSO (control), 1 μM edelfosine, 3 μM compound 2, and 3 μM
compound 4. The intensity of the fluorescence is proportional to the
amount of L. infantum amastigotes inside the cells. c Percentage of
infected (black bars) or non-infected (white bars) differentiated THP-
1 cells that remain alive after treatment with DMSO (control), 1 μM
edelfosine, 3 μM compound 2, and 3 μM compound 4 for 96 h

shown in Table 2. Edelfosine and miltefosine were used as reference drugs in these assays.

The results do not allow the definition of a precise structure–activity relationship, but it is still possible to point out some general tendencies:

1) Replacement of sulfur in 1, 3, 5, and 12 with selenium to obtain structures 2, 4, 6, and 13 has a positive effect on the activity: whereas the sulfur-containing compounds can be considered inactive, remarkable activity was shown by those containing selenium. This observation is in agreement with the previous studies on their antitumor activity (Madhunapantula et al. 2008; Sharma et al. 2009; Das et al. 2009; Emmert et al. 2010).

2) The heteroaromatic rings confer higher antileishmanial potency compared to the same moiety in the aromatic ring. In particular, the unsubstituted pyridine-derivative 4 is very active against the promastigote (more than 50% growth inhibition at all concentrations tested), whereas its aromatic analog 6 was not effective under the same conditions. The same conclusion can be drawn when comparing compound 2 (pyridine ring and a chloro moiety) with compound 10 (phenyl ring).

3) No correlation can be established regarding the presence of substituents with deactivating electron-withdrawing groups (e.g., trifluoromethyl, chloro) or electron-donating groups like methyl or methoxy in the phenyl ring. For example, compounds 13 with a methyl group and 14 with a trifluoromethyl moiety are active, whereas compounds 10 with a chloro and 9 with a trimethoxy substituent are inactive.

The IC_{50} values for the six most active compounds in promastigotes (2, 4, 7, 13, 14, and 15) were calculated in both the promastigote and amastigote models. Compound 4 ($IC_{50\ promastigotes}=3.74$; $IC_{50\ amastigotes}=3.03$) is four times more active than the control drug miltefosine in promastigotes

and shows a similar potency in amastigotes. Compound 2, having a chloro atom at position 2 of the pyridine ring, showed a moderate activity against the amastigote form ($IC_{50}=$ 4.68 µM). The rest of compounds have varying degrees of in vitro antileishmanial activities.

Cytotoxic activity

In order to determine their toxicity/activity index, the six 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 2. The selectivity index (SI) was defined as the ratio between the IC_{50} values obtained against either Jurkat or THP-1 cells and those obtained against *L. infantum* amastigotes. The best SI values were obtained for compounds 2 and 4, which also displayed the best inhibitory activity in the cultured amastigote model. These values are similar to those obtained for edelfosine or miltefosine when comparing with THP-1 cells but smaller when the comparison is made in Jurkat cells.

Activity in infected macrophages

The leishmanicidal activity in infected macrophages of the six most active molecules was determined by flow cytometry. Compounds 2, 4, 7, 13, 14, and 15 were assayed for 96 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. 2a). Our results indicate that whereas 16% of the cells were infected in untreated controls, only 2% of them remained infected after treatment with 1 µM edelfosine and 6% or 13% of them contained parasites after treatment with compounds 4 or 2, respectively. No significant reduction in infection was observed with any of the other compounds

Table 3 Physico-chemical and absorption properties for the most active compounds

Compound	cLogP	MW	*n*-OHNH donors	*n*-ON acceptors	Lipinski's violations	Absorption	
						HIA (%)	Caco-2 (nm/s)
2	2.71	416	1	6	0	98.87	21.61
4	3.46	346	1	6	0	97.86	22.66
7	3.04	466	1	8	0	99.32	23.92
13	4.09	374	1	4	0	97.63	23.24
14	4.91	482	1	4	0	97.58	23.42
15	3.08	396	1	6	0	98.14	20.39

cLogP logarithm of compound partition coefficient between *n*-octanol and water, *MW* molecular weight, *n-OHNH* number of hydrogen bond donors, *n-ON* number of hydrogen bond acceptors, *HIA* human intestinal absorption, *Caco-2* cells derived from human colon adenocarcinoma

(data not shown). Moreover, the mean number of parasites inside the infected cells was significantly reduced after treatment with compound 2 (26% reduction), compound 4 (35% reduction), or edelfosine (35% reduction), as revealed by the decrease in the intensity of the cellular fluorescence (Fig 2b). Viability of the differentiated THP-1 cells after drug treatment was also analyzed both for uninfected and for infected cells (Fig 2c). Edelfosine and compound 2 slightly reduced the amount of surviving cells in uninfected THP-1 cells, whereas this effect was not observed for compound 4, which showed a complete absence of toxicity in these conditions. None of the three treatments caused a significant reduction in the amount of living cells related to the untreated controls when the infected THP1 cells were analyzed. Accordingly, treatment of the cells with compound 4 at 3 μM for 96 h is able to reduce infection rates by 62% showing a negligible cytotoxic effect over the host cells.

Drug-likeness

In recent years, one of the tools for predicting drug-likeness, which discriminates between drug-like and non drug-like compounds, is the Lipinski's rule of five (Lipinski et al. 2001), which takes into consideration molecular weight, compound partition coefficient between *n*-octanol and water (cLogP), number of hydrogen bond donors, and number of hydrogen bond acceptors. According to the results obtained using the OSIRIS Property Explorer, none of the most active compounds (compounds 2, 4, 7, 13, 14, and 15) violate any of the Lipinski's criteria, an important characteristic for future drug development (Table 3).

In addition, it is well known that a lot of drug candidates have failed during clinical tests because of the problems related to absorption, distribution, metabolism, and excretion (ADME) properties. A very preliminary computational study designed to predict the absorption properties of our active and selective compounds found for each specific bioassay (2, 4, 7, 13, 14, and 15) was performed (PreADMET program), and the results are presented in Table 3. Human intestinal absorption (HIA) and Caco-2 permeability are good indicators of drug absorbance in the intestines and Caco-2 monolayer penetration, 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 (Zhao et al. 2001). 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 24 (Yamashita et al. 2000).

In summary, this study confirms the leishmanicidal activity of imidoselenocarbamates, among which compounds 2 and 4, both of them with a pyridine ring moiety, emerge as the most promising derivatives.

Compound 4 is able to reduce in vitro infection rates by 62%, showing negligible toxicity over the differentiated THP-1 cells. Since the synthesis of these compounds is straightforward, a wide variety of analogs can be prepared to optimize their in vitro and in vivo activities. All the compounds discussed in this paper can be very simply and efficiently synthesized in one-pot reactions, which may be important for subsequent studies and for scaling-up processes, the latter being an important aspect to be considered when searching for new molecules to be used in the treatment of diseases affecting poorly developed countries.

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