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Discovery of new organoselenium compounds as antileishmanial agents

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ABSTRACT

We report new organoselenium compounds bearing the sulfonamide moiety as effective inhibitors of the β-isoform of Carbonic Anhydrase from the unicellular parasitic protozoan *L. donovani chagasi*. All derivatives were evaluated *in vitro* for their leishmanicidal activities against *Leishmania infantum* amastigotes along with their cytotoxicities in human THP-1 cells. Compounds **3e-g** showed their activity in the low micromolar range with IC_{50} values spanning from 0.72 to 0.81 μM and selectivity indexes (SI) > 8 (for **3g** SI > 30), thus much higher than those observed for the reference drugs miltefosine and edelfosine. This is the first study which reports new selenoderivatives with promising leishmanicidal properties and acting as Carbonic Anhydrase inhibitors too thus paving the way to the development of innovative agents for the treatment of neglected diseases such as leishmaniasis.

1. Introduction

The protozoan parasites *Leishmania* spp. are the causative agent of leishmaniasis, an endemic and potentially fatal disease widely distributed in the planet. According to the latest World Health Organization (WHO) report on the disease, up to 1.3 million new cases per year of leishmaniasis are estimated to occur around the globe, which in turn determine 20,000–30,000 deaths, and approximately 350 million individuals are classified as high exposed to infections [1,2].

Despite the high variability of the etiological agent, three main clinical forms of the disease are clinically recognized on the basis of the human tissues primary affected: (*i*) the cutaneous leishmaniasis (e.g. *Leishmania major*), (*ii*) the mucocutaneous (e.g. *Leishmania braziliensis*) and (*iii*) the visceral leishmaniasis, commonly known as Kala-Azar or black fever (e.g. *Leishmania donovani/infantum*). Among all, the latter is the form of leishmaniasis associated to elevated percentages of mortality of the affected patients.

Currently, there is no vaccine against leishmaniasis and the therapeutic approach is limited to few available drugs. Clinically used agents include paromomycin, amphotericin B and miltefosine, which as main drawbacks are reported to exert high toxicity to the host and thus determining administration of the drugs at sub-optimal doses, the emerging of drug resistance cases, and high cost [3,4]. In this context, different Carbonic Anhydrases (CAs, EC 4.2.1.1) from vertebrates, nematodes, fungi, protozoa and bacteria are established as promising drug targets [5–8], since interfering with their activity leads to valuable pharmacological effects. The genomes of *Leishmania* spp. encode for one α- and one β-CA. Up to now only the β-CA from *L. donovani* (LdcCA) was successfully expressed, purified and characterized [9]. *Leishmania donovani* amastigotes as well as promastigotes have an amazing ability to maintain their intracellular pH close to neutral, even when the extracellular pH is ~5.0 [10].

Several studies showed important insights towards the acid-survival strategy of *Leishmania* as the inhibition of the carbonic anhydrase activities caused intracellular acidosis, thus leading to parasite cell death by apoptosis and necrosis [11].

Since β-CAs are absent in vertebrates, the development of parasite specific β-CA inhibitors clearly paves the way to the identification of

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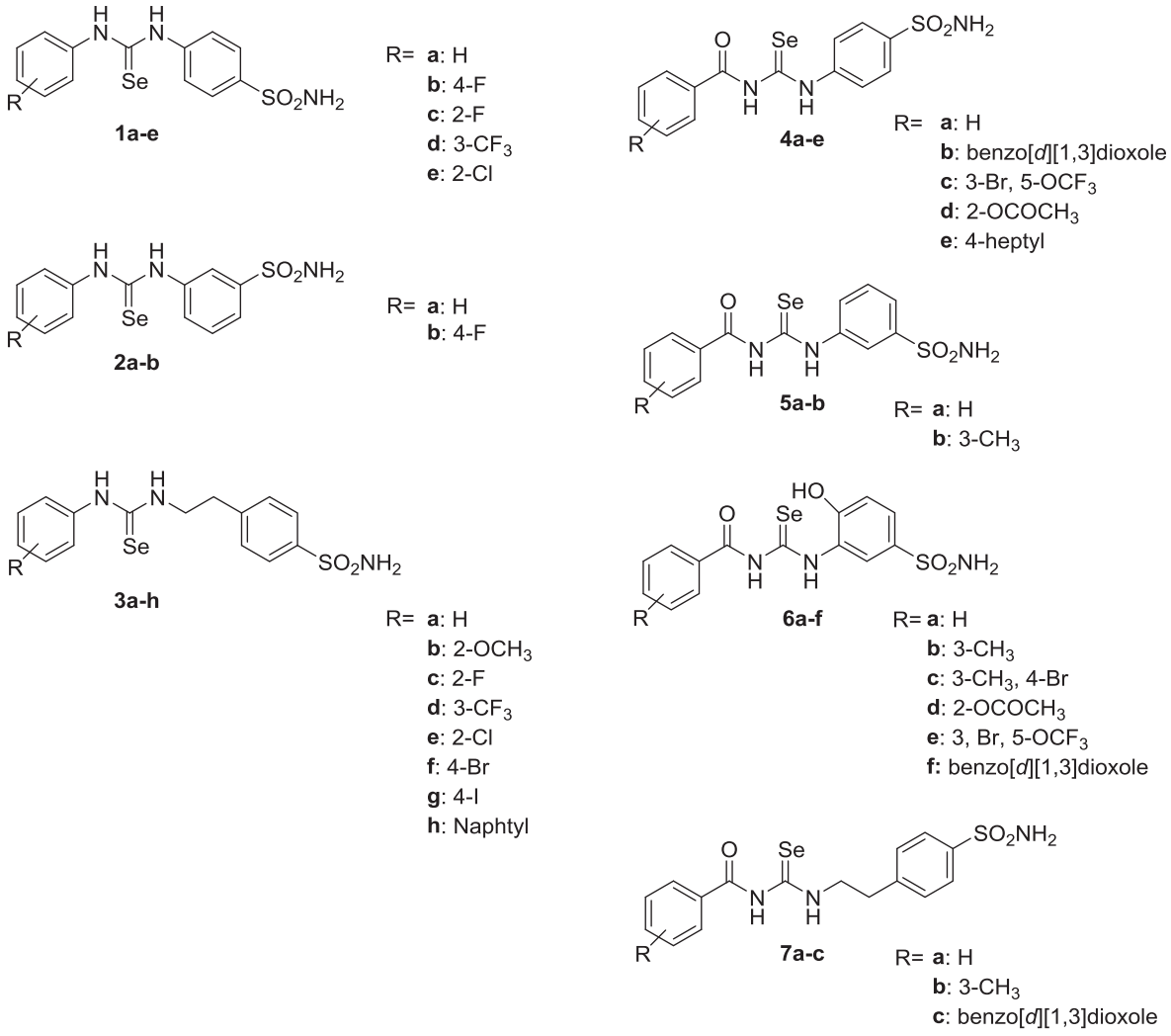


Fig. 1. Selenoureido series derivatives **1–7** as inhibitors of CAs.

highly effective and protozoan selective drug leads with the aim to properly tackle leishmaniasis.

2. Results and discussion

2.1. Chemistry

In the last years, several reports have suggested a connection between selenium and parasites and in trypanosomatids, particularly [12–15]. For this reason we became much interested in designing and synthesizing chalcogen-containing small molecules as biologically active compounds [16,17]. As result, we recently reported the synthesis of series of functionalized selenoureido containing scaffolds which were first evaluated for their inhibitory activities against the human expressed CAs (Fig. 1) [18–20]. Then, as extension of our studies we proved that compounds of this type possessed promising inhibition profiles against the bacterial CAs [21], thus giving strength to our purposes to develop selenium based organic compounds as innovative anti-infective agents [21].

In addition to the compounds above reported and with the aim to extend our screening library, we include in this study the selenocyanate **8**, the diselenide **9**, the selenides **10a-h** and **11,** the selenoamide **12**, as well as the selenazoles **13** and **14a,b** (Fig. 2). Compounds **8–11** were showed to possess *in vitro* anti-infective proprieties by inhibiting several bacterial CAs [22]. All compounds were synthesized according to literature procedures [23–26].

Some of us showed that selenocyanate and diselenide compounds have effective leishmanicidal properties *in vitro* against different classes of *Leishmania* spp. [27–29]. In agreement with such an approach we synthetized the new selenocyanate **16** and the diselenides **17**, **19a-b** bearing a primary sulfonamide as zinc binding group valuable for inhibition of the β-LdcCA. Compound **16** was obtained in excellent yield following a modified literature procedure using triselenium dicyanide and **15** [30]. Dimerization of **16** with $NaBH_4$ afforded the corresponding diselenide **17** (Scheme 1).

The synthetic approach towards **19a-b** is based on the formation of the diazonium salts of anilines **18a-b** and which were treated with Na_2Se freshly prepared by Rongalite and elemental selenium [31] (Scheme 2).

2.2. Carbonic anhydrase inhibition

All compounds **1–14**, **16**, **17** and **19a-b** were tested *in vitro* for their inhibitory activity against the β-isoform from *Leishmania donovani* species by means of the stopped-flow carbon dioxide hydration assay [32] and their activities were compared to the standard CAI acetazolamide (**AAZ**) (Table 1).

All selenoureido derivatives **1–3**, showed inhibition constant values within the micromolar range (K_i 2.4–7.8 μM). The only exception was **3a** with a K_I value sub micromolar (0.79 μM). Among these, any substitution at the aromatic tail moiety nor the positional insertion of the ZBG moiety, did not consistently influence the activity against LdcCA.

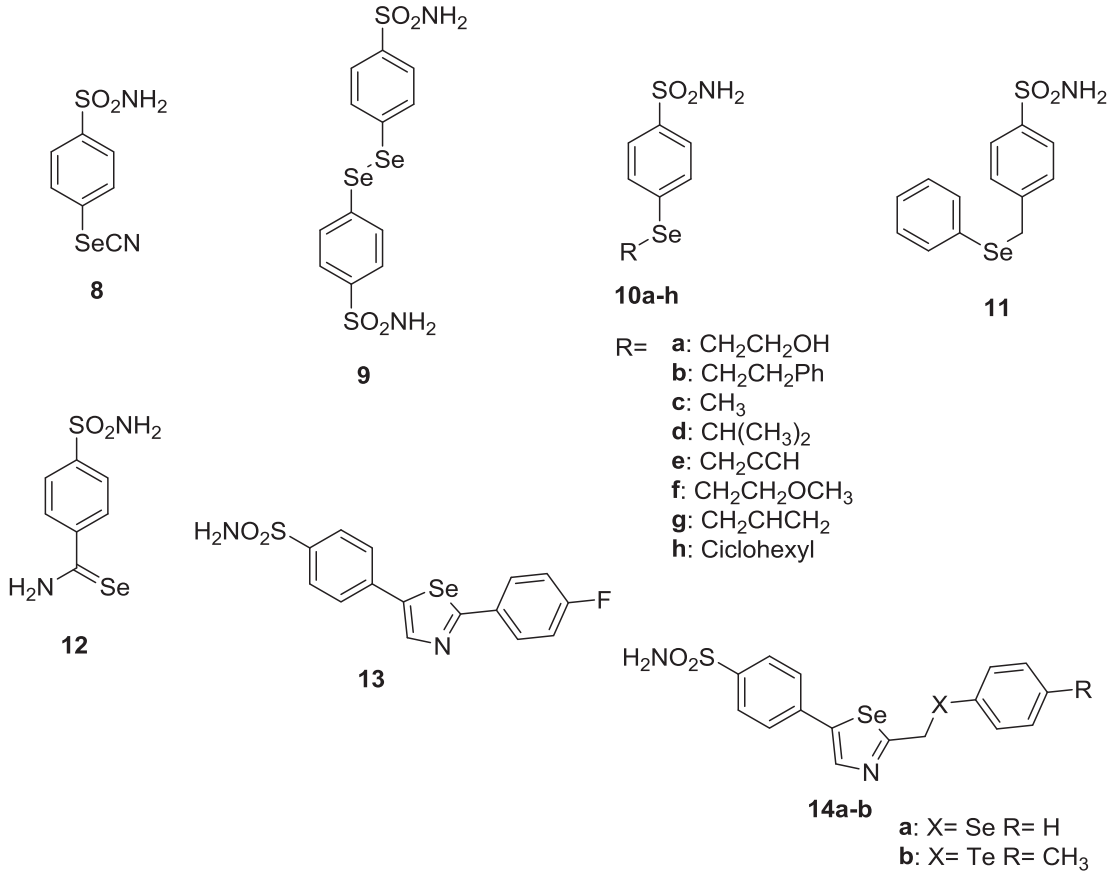
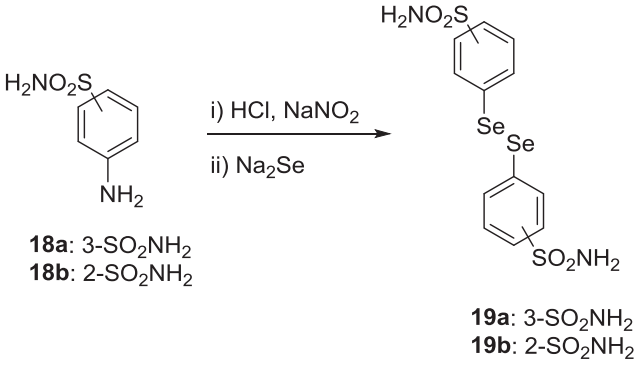
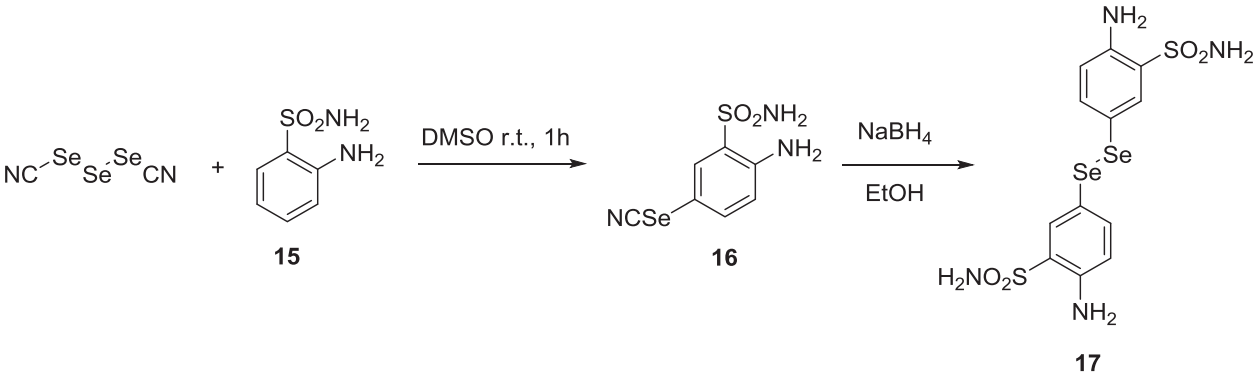


Fig. 2. Organo-selenide derivatives bearing benzenesulfonamide **8–14**.

The potency drastically increased when the phenyl selenoureido tail was replaced with the phenylacylseleno ureido moiety (series **4–7**). Compounds **4a-e** showed K_I values in ranging spanning between 0.02 and 0.90 μM. Within the 4 series the best performing were compounds **4b** and **4e** which do possess a lipophilic tail (Table 1). Insertion of the sulfonamide moiety at meta position, as in compounds **5a,b** resulted in sensible enhancement of the inhibition potency (K_Is 0.01 μM in both cases). In light of such results we explored any possible effects on the kinetic activities given from the introduction of a phenolic moiety at 2 position of the head section and keeping the sulfonamide moiety fixed at position 3 (compounds **6a-e**). Such an approach resulted in a decrease of the inhibition potencies with values comprised between 0.03 and 0.09 μM range. Furthermore, among the 6-series the various substituents in the acyl tail did not influence significantly the inhibition potencies. The introduction of an ethyl linker, such as for compounds



Scheme 2. Synthesis of diselenides **19a-b**.



Scheme 1. Synthesis of selenocyanate **16** and diselenide **17**.

Table 1
Inhibition data of LdcCAβ with compounds **1–7** and AAZ by a stopped flow CO_2 hydrase assay [22].

Compound	K_i (μM)* LdcCAβ	Compound	K_i (μM)* LdcCAβ
1a	4.7	**6e**	0.07
1b	5.8	**6f**	0.09
1c	4.3	**7a**	0.09
1d	7.0	**7b**	0.03
1e	3.3	**7c**	0.08
2a	7.8	**8**	0.007
2b	4.2	**9**	0.005
3a	0.79	**10a**	0.02
3b	4.2	**10b**	0.02
3c	4.0	**10c**	0.42
3d	4.0	**10d**	0.03
3e	4.8	**10e**	0.006
3f	3.3	**10f**	0.56
3g	2.4	**10g**	0.006
3h	4.1	**10h**	0.28
4a	0.28	**11**	0.60
4b	0.02	**12**	0.008
4c	0.49	**13**	0.01
4d	0.90	**14a**	0.24
4e	0.08	**14b**	0.029
5a	0.01	**16**	0.007
5b	0.01	**17**	0.02
6a	0.03	**19a**	0.09
6b	0.04	**19b**	0.80
6c	0.06	**AAZ**	0.09
6d	0.05		

* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5–10% of the reported values).

7a-c, afforded data analogous to the previous ones and comparable to the standard CAI **AAZ**.

Unexpectedly selenocyanates **8** and **16** showed equal and strong inhibition activity against β-LdcCA (K_i 0.007 μM). Conversely the corresponding diselenides, **9** and **17** respectively, exhibited different kinetic patterns. Compound **9** resulted slightly more potent when compared to its precursor **8** (K_i 0.005 μM) whereas the diselenide **17** was 3 fold less potent than its counterpart **16** (K_i 0.023 μM). These data gives strong support to role of the selenocyanate moiety in further development of potent LdcCA inhibitors.

The activity of diselenides **19a-b** was particularly influenced by the position of the sulfonamide moiety with the 3-substituted (**19a**) being 8 fold more potent when compared to 19b which presents the sulfonamide at position 2 (K_Is of 0.095 and 0.801 μM respectively). The different tails of selenide bearing sulfonamide moiety **10a-h** can modulate the activity against β-LdcCA. Allyl and propargyl moieties in compound **10e** and **g** showed the best inhibition activity (K_i 0.006 μM). On the other hand, little modifications in the scaffold such as for **10a** and **10f** (introduction of a OCH_3 terminal) decreased the potency up to 24 times (K_Is 0.023 and 0.566 μM respectively). Finally, the selenoamide **12** exhibited strong activity (K_i 0.008 μM) almost superimposable with the selenazole **13** (K_I 0.010 μM). Modifications of the chain at position 5 of the selenazole **13** as for compounds **14a,b** determined reduction of the inhibition potency. In particular the presence of the selenium ether (**14a**) determined a 24 fold inhibition potency decrease, whereas the tellurium analogue **14b** was only 2.9 less potent.

2.3. Biological evaluation

All compounds reported above (**1–14**, **16**, **17** and **19**) were tested for their antiprotozoal activity against amastigotes of the pathogenic *Leishmania infantum* using miltefosine and edelfosine as standard drugs [33]. The leishmanicidal assay were conducted only on the amastigote forms since the protozoan at this replication stage is responsible for all clinical manifestations in humans and is far less sensible to the therapeutic treatments when compared to the promastigote form. Even if promastigotes are significantly more susceptible to drug-induced effects, they are not fully developed forms of the parasite in vertebrate hosts and any evaluation made with them will be merely indicative of the potential leishmanicidal activity of the compounds tested. All the analyses were carried out in the amastigote form with a minimum of three independent experiments and the results were expressed as 50% of inhibition concentration (IC_{50}) values. All compounds here considered, were evaluated also for their cytotoxicity on the THP-1 cell lines in order to identify compounds with high leishmanicidal activity and low cytotoxicity. The selectivity index (SI) of the compounds are expressed by the ratio between cytotoxicity (IC_{50} value on THP-1 cells) and activity (IC_{50} value on *L. infantum* amastigotes). The data reported

Table 2
IC_{50} values for compounds **1–7** on amastigotes and cytotoxic activity in the THP-1 cell line.

Compound	IC_{50} (mean ± SEM) μM		SI[a]
	Amastigotes	THP-1	
1a	3.06 ± 0.88	1.86 ± 0.15	0.67
1b	2.335 ± 0.54	4.015 ± 0.06	1.719
1c	> 25	> 25	–
1d	2.42 ± 0.15	7.11 ± 0.14	2.94
1e	2.32 ± 0.01	5.70 ± 0.91	2.46
2a	2.22 ± 0.1	8.94 ± 0.16	4.02
2b	1.00 ± 0.29	1.78 ± 0.08	1.79
3a	> 25	> 25	–
3b	> 25	> 25	–
3c	2.70 ± 0.63	> 25	> 9.25
3d	3.54 ± 0.98	10.61 ± 0.79	3.00
3e	0.81 ± 0.06	6.84 ± 0.54	8.44
3f	0.72 ± 0.19	9.81 ± 0.88	13.56
3g	0.80 ± 0.3	25.00 ± 0.0	31.25
3h	3.35 ± 0.76	> 25	> 7.46
4a	5.94 ± 0.87	1.79 ± 0.17	0.30
4b	5.99 ± 0.48	2.04 ± 0.11	0.34
4c	5.26 ± 0.30	12.66 ± 0.43	2.41
4d	3.61 ± 0.89	1.50 ± 0.33	0.41
4e	5.09 ± 0.5	9.73 ± 0.79	1.91
5a	8.78 ± 1.51	4.26 ± 0.16	0.48
5b	16.48 ± 1.61	11.16 ± 0.64	0.68
6a	12.31 ± 1.28	> 25	> 2
6b	15.79 ± 1.54	4.06 ± 0.11	0.26
6c	6.66 ± 1.34	4.73 ± 0.44	0.71
6d	1.51 ± 0.48	4.73 ± 0.44	3.13
6e	14.27 ± 2.77	> 25	> 1.75
6f	10.31 ± 1.46	2.08 ± 0.16	0.20
7a	14.97 ± 1.6	19.80 ± 1.4	1.32
7b	10.57 ± 0.56	9.10 ± 0.78	0.86
7c	> 25	> 25	–
8	4.48 ± 1.17	> 25	> 5.58
9	8.09 ± 0.8	> 25	> 3.09
10a	> 25	> 25	–
10b	> 25	> 25	–
10c	> 25	> 25	–
10d	> 25	> 25	–
10e	6.99 ± 0.91	> 25	> 3.5
10f	> 25	> 25	–
10g	> 25	> 25	–
10h	> 25	> 25	–
11	3.77 ± 1.30	> 25	> 6.63
12	13.49 ± 1.80	4.27 ± 0.06	0.32
13	> 25	25	< 1
14a	4.11 ± 1.2	6.4 ± 0.65	1.56
14b	0.34 ± 0.06	0.68 ± 0.22	2.01
16	7.56 ± 0.15	2.28 ± 0.25	0.30
17	0.47 ± 0.14	2.6 ± 0.89	5.53
19a	14.19 ± 2.81	10.3 ± 0.72	0.73
19b	> 25	25	> 1
Miltefosine	2.84 ± 0.10	18.5 ± 0.60	6.51
Edelfosine	0.82 ± 0.13	4.96 ± 0.16	6.05

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

in Table 2 for selenoureido derivatives **1–7** showed compounds (**1c**, **3a-b** and **7c**) ineffective against the amastigotes. An interesting point from the data here reported was that the presence of halogen atom within the aromatic tail (compounds **3e-g**) showed good potency against amastigotes (IC_{50} 0.72–0.81 μM) and low cytotoxicity on THP-1 exhibiting a SI higher than the two reference drugs (with SI > 6). It is remarkable that compound **3g** was found to be 5.2 and 4.8 times more selective than edelfosine (SI > 31.4 versus SI = 6) and miltefosine (SI > 31.4 versus SI = 7) respectively. These results confirm a low toxicity for this compound. The efficacy was also modulated by the linker inserted between the selenoureido scaffold and the sulfonamide portion head as for compound **1e** and its longer derivative **3e** with the last being 2.9 fold more potent (IC_{50}s of 2.32–0.81 μM). Such differences were not present when the cytotoxicity properties were explored (Table 2).

In addition, selenocyanate compounds (**8** and **16**) showed good activity against the amastigotes with a remarkable SI > 5.58 for compound **8** (Table 2). Instead the additional presence of the amine group to afford compound **16** resulted more cytotoxic against THP-1. On the other hand, the diselenide **17**, obtained from dimerization of **16**, showed good selectivity (SI = 5.49) comparable cytotoxicity (2.6 μM) and enhanced potency against the amastigotes (0.47 μM). All selenides **10a-h**, except for **10e**, proved to be ineffective against amastigotes. Finally the only compound **11** showed good activity (3.77 μM) low cytotoxicity and high selectivity (SI > 6.63).

3. Conclusions

In the present study, we report the synthesis of new selenocyanate and diselenides bearing the sulfonamide moiety against the β-isoform of Carbonic Anhydrase from the unicellular parasitic protozoan *L. donovani chagasi*. In addition we evaluated a library of different seleno containing compounds previously obtained from us. We reported that LdcCA was efficiently inhibited in the nanomolar range by several sulfonamides reported here. In addition the leishmanicidal activities of such compounds were explored using the amastigote form of *L. infantum* along with their cytotoxicities in THP-1 cells.

Eleven compounds exhibited better potency against axenic amastigotes than the standard drug miltefosine, and five were better than edelfosine. Based on their antiparasitic activities and toxicity in THP-1 cells, compounds **3e-g** showed potent leishmanicidal activity with excellent selectivity index resulting in promising therapeutic utility.

Although no clear correlation between LdcCA and antiparasitic activity was observed for all compounds at this stage, this is the first study which reports new selenoderivatives with promising leishmanicidal properties and acting as Carbonic Anhydrase inhibitors too thus paving the way to the development of innovative agents for the treatment of neglected diseases such as leishmania.

4. Experimental part

4.1. General

All reactions were carried out in an oven-dried glassware under inert atmosphere (N_2). Ethanol was dried using a solvent purification system (Pure-Solv™). All commercial materials were used as received without further purification. Flash column chromatography purifications were performed with Silica gel 60 (230–400 mesh). Thin layer chromatography was performed with TLC plates Silica gel 60 F_{254}. NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 with Mercury 400, and Bruker 400 Ultrashield spectrometers operating at 400 MHz (for ^{1}H), 100 MHz (for ^{13}C) and 376 MHz (for ^{19}F). NMR signals were referenced to nondeuterated residual solvent signals (7.26 and 2.50 ppm for ^{1}H, 77.0 and 40.5 ppm for ^{13}C). ^{1}H NMR data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, ap d = apparent doublet, m = multiplet, dd = doublet of doublet, bs = broad singlet, bd = broad doublet, ecc.), coupling constant (J), and assignment.

The HPLC was performed by using a Waters 2690 separation module coupled with a photodiode array detector (PDA Waters 996) using a Nova-Pak C18 4 μm 3.9 mm × 150 mm (Waters) silica-based reverse phase column. The sample was dissolved in 10% acetonitrile/H_2O and an injection volume of 45 μL. The mobile phase (flow rate 1.0 mL/min) was a gradient of H2O + trifluoroacetic acid (TFA) 0.1% (A) and acetonitrile + TFA 0.1% (B), with steps as follows: (A%/B%), 0–10 min 90:10, 10–25 min gradient to 60:40, 26:28 min isocratic 20:80, 29–35 min isocratic 90:10. TFA 0.1% in water as well in acetonitrile was used as counterion. All compounds reported here were ≥95% HPLC pure.

4.1.1. Synthesis of 2-amino-5-selenocyanatobenzenesulfonamide (16)

Selenium dioxide (0.34 g, 3 mmol) was reacted with malononitrile (0.1 g, 1.5 mmol) in DMSO. Compound **15** (2.25 mmol) was added with stirring to the reaction mixture after termination of the exothermic reaction. The homogeneous solution was diluted with water (5–10 mL) after 1 h and the precipitate was filtered, dried and crystallized. The product obtained was as grey powder with a yield of 90%. **^{1}H NMR** (400 MHz, DMSO-d_6) δ (ppm): 7.89 (1H, d, J = 2.10 Hz), 7.59 (1H, dd, J = 2.12, 8.61 Hz), 7.47 (2H, s), 6.89 (1H, d, J = 8.63 Hz), 6.32 (2H, s). **^{13}C NMR** (100 MHz, DMSO-d_6) δ (ppm): 147.8, 140.2, 135.9, 125.9, 119.1, 111.4, 106.5. **^{77}Se NMR** (76 MHz, DMSO-d_6) δ (ppm): 321.1. **MS** (ESI negative) m/z: 275.8 $[M-H]^-$.

4.1.2. Synthesis of 5,5′-diselanediylbis(2-aminobenzenesulfonamide) (17)

$NaBH_4$ (4 mmol) was added in small portions with caution to a solution of 2-amino-5-selenocyanatobenzenesulfonamide **16** (1 mmol) in absolute ethanol (10 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 ethyl acetate, dried with anhydrous Na_2SO_4, and purified by crystallization from EtOH. The product obtained as yellow powder with a yield of 95%. **^{1}H NMR** (400 MHz, DMSO-d_6) δ (ppm): 7.84 (2H, d, J = 2.03), 7.43 (2H, dd, J = 2.05, 8.49), 7.27 (4H, bs, N**H$_2$**, exchange with D_2O), 6.79 (2H, d, J = 8.52), 6.23 (4H, bs, N**H$_2$**, exchange with D_2O). **^{13}C NMR** (100 MHz, DMSO-d_6) δ (ppm): 147.2, 139.8, 135.2, 125.9, 118.5, 114.9. **^{77}Se NMR** (76 MHz, DMSO-d_6) δ (ppm): 525.3. MS (ESI negative) m/z (%): 500.8 $[M-H]^-$.

4.1.3. General synthesis of diselenide 19a-b

A suspension of appropriate aminbenzenesulfonamide (**18a-b**) (1,72 g, 10 mmol) in H_2O (6 mL) with HCl (11 mL, 32%) was cooled down to −5°C. Then, an aqueous solution of $NaNO_2$ (1.2 eq) was added dropwise and the mixture was kept stirring at the same temperature until a persistent pale yellow solution was formed (5–10 min). The resulting diazonium salt, kept at −5 °C, was added by cannula in aqueous solution of Na_2Se, cooled at the same temperature, previously prepared by 4 M NaOH solution (10 mL) with elemental selenium (11 mmol) and Rongalite (22 mmol). The mixture was stirred for 6 h. The product was filtered off, washed with H_2O, dried under vacuo, and purified by flash column chromatography eluting with 1:1 mixture of hexane/ethyl acetate.

4.1.4. 3,3′-diselanediyldibenzenesulfonamide (19a)

The product **19a** was obtained as yellow powder with a yield of 40%. **^{1}H NMR** (400 MHz, DMSO-d_6) δ (ppm): 8.15 (2H, t, J = 1.6), 7.88 (2H, ddd, J = 0.98, 1.58, 7.77), 7.78 (2H, ddd, J = 0.98, 1.56, 7.80), 7.58 (4H, t, J = 7.81), 7.49 (4H, bs, N**H$_2$**, exchange with D_2O). **^{13}C NMR** (100 MHz, DMSO-d_6) δ (ppm): 146.0, 134.8, 131.7, 131.1, 128.5, 126.0. **^{77}Se NMR** (76 MHz, DMSO-d_6) δ (ppm): 459.7. MS (ESI negative) m/z (%): 470.8 $[M-H]^-$.

4.1.5. 2,2′-diselanediyldibenzenesulfonamide (19b)

The product **19b** was obtained as yellow powder with a yield of

60%. **^{1}H NMR** (400 MHz, DMSO-d_6) δ (ppm): 7.93 (2H, dd, J = 2.02, 7.14), 7.85 (2H, dd, J = 1.62, 7.44), 7.80 (4H, bs, N**H$_2$**, exchange with D_2O), 7.52–7.49 (4H, m). **^{13}C NMR** (100 MHz, DMSO-d_6) δ (ppm): 143.2, 133.7, 131.8, 129.2, 128.3, 127.9. **^{77}Se NMR** (76 MHz, DMSO-d_6) δ (ppm): 432.9. MS (ESI negative) m/z (%): 470.8 $[M-H]^-$.

4.2. Carbonic anhydrase inhibition

Production of recombinant LdcCAβ enzyme has been described in detail by Syrjänen et al. [9]. The polyhistidine-tagged protein was produced in Sf9 insect cells and purified using Protino Ni-NTA agarose (Macherey-Nagel). The correct identity of the protein was confirmed by mass spectrometry at Protein Chemistry Unit of the University of Helsinki. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity [32]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation, as reported earlier [20–24,34,35], and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in-house as reported earlier [20–24,34,35].

4.3. Biological assays

4.3.1. Culture conditions

THP-1 cells were kindly provided by Dr. Michel/Université Nice Sophia Antipolis, Nice, France) and were grown in RPMI-1640 medium (Gibco, Lieden, Netherlands) supplemented with 10% heat inactivated FBS, 5% penicillin/streptomycin, 1 nM HEPES, 2 mM glutamine and 1 mM sodium pyruvate, pH 7.2 at 37 °C and 5% CO_2 atmosphere. *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 (Gibco, Lieden, Netherlands), supplemented with 10% heat inactivated FBS, 5% penicillin/streptomycin, and 25 mM HEPES, pH 7.2 at 26 °C. *L. infantum* promastigotes were transformed in axenic amastigotes growing in M199 medium (Invitrogen, Leiden, Netherlands) supplemented with 10% heat inactivated FBS, 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 and 5% CO_2.

4.3.2. Leishmanicidal activity and cytotoxicity in vitro assays

Drug treatment of 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 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 according to the procedure described by Alzate et al. [36].

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