Novel Heteroaryl Selenocyanates and Diselenides as Potent Antileishmanial Agents

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A series of new selenocyanates and diselenides bearing interesting bioactive scaffolds (quinoline, quinoxaline, acridine, chromene, furane, isosazole, etc.) was synthesized, and their in vitro leishmanicidal activities against *Leishmania infantum* amastigotes along with their cytotoxicities in human THP-1 cells were determined. Interestingly, most tested compounds were active in the low micromolar range and led us to identify four lead compounds (1h, 2d, 2e, and 2f) with 50% effective dose (ED50) values ranging from 0.45 to 1.27 μM and selectivity indexes of >25 for all of them, much higher than those observed for the reference drugs. These active derivatives were evaluated against infected macrophages, and in order to gain preliminary knowledge about their possible mechanism of action, the inhibition of trypanothione reductase (TryR) was measured. Among these novel structures, compounds 1h (3,5-dimethyl-4-isoxazolyl selenocyanate) and 2d [3,3′-(diselenodiyldimethanediyl)bis(2-bromothiophene)] exhibited good association between TryR inhibitory activity and antileishmanial potency, pointing to 1h, for its excellent theoretical ADME (absorption, distribution, metabolism, and excretion) properties, as the most promising lead molecule for leishmanicidal drug design.

Leishmaniasis is an infectious poverty-associated disease caused by protozoan parasites of the genus *Leishmania*. In fact, this term includes a wide spectrum of vector-borne diseases with great epidemiological and clinical diversity. Even though exact statistical data are lacking (1, 2), within the 350 million people that live in areas where leishmaniasis is endemic, approximately 12 million people get infected per year. There are three major clinical types: cutaneous (CL), mucocutaneous (MCL), and visceral leishmaniasis (VL; also known as kala-azar), which differ in their immunopathologies and degrees of morbidity and mortality (3). Among the different manifestations, VL is the most severe form, with nearly 200,000 to 400,000 new cases, causing more than 20,000 deaths, per year. Left untreated, it is usually fatal within 2 years.

The efficacy of the different drugs available seems to vary according to the *Leishmania* species, and the current chemotherapy is far from being satisfactory. Furthermore, they present several problems, including toxicity, many adverse side effects, and high costs. The most relevant problem is related to the fact that many of these drugs were developed many years ago, and currently, there are resistant strains (4).

Since their discovery in the 1940s, the toxic pentavalent antimony [Sb(V)] compounds have been the mainstay of treatment against all forms of leishmaniasis through parenteral administration, and their efficacy is progressively decreasing owing to the development of resistance (5). For this reason, in the last decades several drugs, such as amphotericin B and miltefosine (6), paromomycin and pentamidine (7), sitamaquine (8), and edelfosine (9), have been used in the treatment of leishmaniasis. Nevertheless, their high cost and therapeutic complications limit their use. Nowadays, several other drugs based on natural products show promising antileishmanial activity, but despite significant progress, an ideal drug is still awaited (10).

The development of new antiparasitic drugs has not been much of a priority for the pharmaceutical industry because many of the parasitic diseases occur in poor countries where the populations cannot afford to pay a high price for the drugs. Thus, although important initiatives, such as the Drugs for Neglected Diseases Initiative (DNDi), are attracting more interest in these neglected pathologies, an investment in drug development against parasitic diseases is needed.

The incorporation of different functionalities bearing the Se atom (i.e., methylseleno, selenocyanate, and diselenide) onto organic scaffolds can be considered a promising rational design to achieve potent and selective cytotoxic compounds (11). Several reports have shown vast and miscellaneous types of structures applying this approach, resulting in very promising antitumoral compounds in preclinical models (12, 13). Recently, our research group has been using this rational design in order to obtain new derivatives with potent and selective antileishmanial activity. Continuing with these efforts, herein we have designed novel Se compounds which gather two different chemical entities: the selenium entity on its selenocyanate and diselenide forms; and different carbo- and heterocyclic entities with proven leishmanicidal activity. Below in this section, a brief description with several reported data that supports the selection for each of these subunits can be found.

During the last years, various reports have shown recognition of an increase in plasma selenium levels as a new defensive strategy...
against *Leishmania* infection (14, 15). The choice of the chemical form for the selenium derivatives can modulate the level of this element on the basis of several metabolic routes (16). The mechanism of action for selenium is unknown, though some enzymatic pathways, such as mitochondrial peroxiredoxins (17), selenophosphate synthetases (18), or ascorbate peroxidases (19), could be implicated. On the other hand, the incorporation of selenium into novel nanomaterials has demonstrated effectiveness in the treatment of leishmaniasis (20). We have reported (21–24) new selenium compounds with potent *in vitro* antiparasitic activity against *Leishmania infantum* and *Leishmania major*, and selectivity indexes higher than those observed for the reference drugs miltefosine, edelfosine, and paromomycin. Additionally, some of them induced nitric oxide production and alterations in gene expression profiling related to proliferation (PCNA), treatment resistance (ABC-transporter and α-tubulin), and virulence (QDPR) (23). Among the various antileishmanial scaffolds containing selenium earlier reported by us, selenocyanate and diselenide showed promising activity against *Leishmania* parasites (24).

We have paid special attention to quinoline, which constitutes the central nucleus of sitamaquine (25, 26), acridine (27, 28), quinoxaline (29–31), and coumarins (32, 33). On the other hand, nitrofuran compounds (34, 35), the most relevant registered as nifurtimox, and derivatives of the benzodioxol core (36) have been selected. In addition, substituted five-membered heterocyclic rings such as isoxazol (37) and thiophenyl (38) or pirrol (39) have been tested as leishmanicidal agents. Furthermore, related to heterocycles derivatives, some fused aryl azo and triazo molecules have been described (34, 40). Finally, some carbocycles, such as ones with an adamantane ring (41) or anthraquinone structure (42, 43), have been described.

Among the potential molecular targets for the treatment of leishmaniasis, trypanothione reductase (TryR) is considered an ideal enzyme, since it is involved in the unique thiol-based metabolism observed in the *Trypanosomatidae* family and is a validated target in the search for drugs against members of this family. TryR catalyzes the reduction of trypanothione disulfide to trypanothione (44). Therefore, during recent years a great number of inhibitors of this key enzyme have been reported (45–47). Based on the chemical analogy of sulfur and selenium, we decided to explore the relevance of this trace element to generate new TryR inhibitors.

In summary, as a continuation of an ongoing program aiming to find new structural leaders with potential leishmanicidal activity, we have constructed a new class of seleno derivatives. They were designed by incorporating selenocyanate or diselenide moieties onto other bioactive carbo- or heterocycles selected on the basis of the above-mentioned findings. In this work, we present the synthesis of 23 new Se compounds (Fig. 1) and their leishmanicidal activities against the amastigote form of *L. infantum*. In parallel, the cytotoxicities of these newly synthesized molecules were assessed. Moreover, the leishmanicidal activities of the most active compounds were evaluated in *L. infantum*-infected macrophages. Finally, in order to elucidate a preliminary mechanism of action, their inhibitory activities against trypanothione reductase were determined.

**MATERIALS AND METHODS**

**Chemistry.** Melting points (mp) were determined with a Mettler FP82+FP80 apparatus (Greifense, Switzerland) and are not corrected. The $^1$H nuclear magnetic resonance (NMR) and $^{13}$C NMR spectra were recorded on a Bruker 400 Ultrashield spectrometer (Rheinstetten, Germany) using TMS (tetramethylsilane) as the internal standard. The infrared (IR) spectra were obtained on a Thermo Nicolet FT-IR Nexus spec-


(6,7-Dimethoxy-2-oxo-2H-chromen-4-yl)methyl selenocyanate (1c).

Compound 1c was from 6,7-dimethoxy-4-bromomethyl-2H-chromen-2-one and potassium selenocyanate. The compound was washed with ethyl ether (2 × 50 ml). Yield: 28.1%, mp: 197 to 199°C. IR (KBr) cm⁻¹: 2,150 (m, C=O); 1,709 (s, C=O). ¹H NMR (400 MHz, DMSO-d₆): δ: 3.83 (3H, OCH₃); 3.87 (3H, OCH₃); 4.42 (2H, CH₂-Se); 6.33 (1H, CH-CHO); 7.10 (1H, H₇); 7.43 (1H, H₈). ¹³C NMR (100 MHz, DMSO-d₆): δ: 29.5 (CH₂-Se); 39.1 (C₄); 72.7 (1H, H₇, J₄₋₅ = 11.0 Hz); 7.79 (2H, H₈, J₅₋₆ = 9.4 Hz); 7.98 (dd, 1H, H₅, J₅₋₆ = 7.7 Hz, J₄₋₅ = 7.4 Hz); 8.41 (d, 1H, H₆, J₆₋₇ = 9.4 Hz). ¹⁵N NMR (100 MHz, DMSO-d₆): δ: 126.8 (C₂₈); 127.3 (C₂₉); 131.7 (C₃₀); 136.0 (C₃₁); 154.1 (C₃₁). MS (m/z [percent abundance]): 228 (58); 158 (35); 142 (100); 130 (13); 115 (18); 89 (8); 63 (6). Elemental analysis for C₁₅H₁₀N₂Se, calculated/found (percent): C, 48.64/48.60; H, 3.40/3.40; N, 4.33/4.20.

(5-Nitro-2-yl) methyl selenocyanate (1f).

Compound 1f was from 5-nitro-2-bromomethylfenuramine and potassium selenocyanate. The compound was washed with ethyl ether (2 × 50 ml). Yield: 42%, mp: 87 to 88°C. IR (KBr) cm⁻¹: 2,152 (m, C=O). ¹H NMR (400 MHz, DMSO-d₆): δ: 4.43 (2H, CH₂-Se); 6.80 (d, 1H, H₆, J₆₋₇ = 7.8 Hz); 7.70 (1H, H₇). ¹³C NMR (100 MHz, DMSO-d₆): 23.0 (CH₃-Se); 101.1 (CN); 109.4 (C₄); 111.2 (C₅); 150.0 (C₆); 156.3 (C₇). MS (m/z [abundance]): 126 (100); 113 (85). Elemental analysis for C₉H₉N₂O₄Se, calculated/found (percent): C, 31.71/31.78; H, 1.72/2.02; N, 12.12/11.72.

(6-Bromo-1,3-benzodioxol-5-yl)methyl selenocyanate (1g).

Compound 1g was from 5-bromo-6-(bromomethyl)-1,3-benzodioxole and potassium selenocyanate. The compound was washed with ethyl ether (2 × 50 ml). White solid. Yield: 78.6%, mp: 110 to 111°C. IR (KBr) cm⁻¹: 2,150 (s, C=O); 1033 (s, C-Br). ¹H NMR (400 MHz, DMSO-d₆): δ: 4.36 (3H, CH₃-Se); 6.10 (2H, CH₂-Se); 7.11 (s, 1H, H₇); 7.27 (s, 1H, H₈). ¹³C NMR (100 MHz, DMSO-d₆): δ: 153.3 (C₄); 113.3 (C₅); 113.8 (C₆); 130.6 (C₇); 148.2 (C₈); 149.4 (C₉). MS (m/z [abundance]): 213 (100); 157 (7); 75 (19); 50 (15). Elemental analysis for C₁₅H₁₁N₂O₄Se, calculated/found (percent): C, 33.86/34.02; H, 1.88/2.05; N, 4.39/4.13.

(3,5-Dimethyl-4-isoxazolyl) methyl selenocyanate (1h).

Compound 1h was from 4-chloromethyl-3,5-dimethylisoxazole and potassium selenocyanate. The brown oil obtained after washed with water was extracted with dichloromethane (3 × 50 ml). The organic layer was dried with Na₂SO₄. The dichloromethane was removed under vacuum, and the residue was treated with ethyl ether (3 × 25 ml); a clear brown powder was obtained. Yield: 21.3%. mp: 69 to 70°C. IR (KBr) cm⁻¹: 2,143 (m, C=O); 1,628 (s, C=O). ¹H NMR (400 MHz, DMSO-d₆): δ: 2.23 (2H, CH₂-Se); 7.11 (s, 1H, H₇); 7.27 (s, 1H, H₈). ¹³C NMR (100 MHz, DMSO-d₆): δ: 153.3 (C₄); 113.3 (C₅); 113.8 (C₆); 130.6 (C₇); 148.2 (C₈); 149.4 (C₉). MS (m/z [abundance]): 213 (100); 157 (7); 75 (19); 50 (15). Elemental analysis for C₁₅H₁₃BrN₂O₄Se, calculated/found (percent): C, 33.86/34.02; H, 1.88/2.05; N, 4.39/4.13.
analysis for C_{10}H_{11}NOSe, calculated (found): C, 45.29/44.93; H, 3.62/4.00; N, 6.33/6.10.

1H-Benzotriazol-1-ylmethyl selenocyanate (11). Compound 11 was from 1-chloromethyl-1H-benzotriazole and potassium selenocyanate. The orange oil obtained after washed with water was extracted with di-chloromethane (3 × 30 ml) and dried over Na_2SO_4. The di-chloromethane was removed under vacuum, and the residue was recrystallized from ethanol to give a yellow solid. Yield: 77%. mp: 108 to 110°C. IR (KBr) cm^(-1): 1751 (C=O). MS (m/z [abundance]): 163 (4); 135 (100); 107 (8); 93 (17); 79 (1); 67 (17). Elemental analysis for C_{10}H_{11}NOSe, calculated (found): C, 53.51/53.58; H, 6.03/6.19; N, 4.96/4.70.

2-Adamant-1-yl-2-oxoethyl selenocyanate (1m). Compound 1m was from 1-adamant-1-yl-2-bromomethanone and potassium selenocyanate. The yellow oil was extracted with ethyl ether (2 × 30 ml). Yellow solid. Yield: 77.3%. mp: 158 to 160°C. IR (KBr) cm^(-1): 1751 (C=O). MS (m/z [abundance]): 163 (4); 135 (100); 107 (8); 93 (17); 79 (1); 67 (17). Elemental analysis for C_{12}H_{16}NSe, calculated (found): C, 51.88/51.39; H, 3.86/4.01; N, 10.73/10.07.

2-(Diselenodiyldimethanediyl)diacridine (2b). Compound 2b was from (quinolin-8-yl)methyl selenocyanate and sodium borohydride. The resultant solid was washed with ethyl ether (3 × 25 ml) and dried from Na_2SO_4. The ethyl ether was removed under vacuum, and the residue was recrystallized from ethanol to give a yellow solid. Yield: 60.3%. mp: 103 to 104°C. IR (KBr) cm^(-1): 1751 (C=O); 790 (s, Se-Se). MS (m/z [abundance]): 442 (M^+; 5); 222 (75); 142 (100); 130 (11); 115 (79); 87 (63); 5 (m/z [percent abundance]). Elemental analysis for C_{10}H_{11}NSe_2, calculated (found): C, 54.30/54.80; H, 3.62/4.00; N, 6.33/6.10.

2-(Diselenodiyldimethanediyl)diquinoline (2a). Compound 2a was from (quinoxin-8-yl)methyl selenocyanate (1a) and sodium borohydride. The resultant solid was washed with ethyl ether (3 × 30 ml) and recrystallized from ethanol to give a yellow solid. Yield: 60.3%. mp: 103 to 104°C. IR (KBr) cm^(-1): 1751 (C=O); 790 (s, Se-Se). MS (m/z [abundance]): 442 (M^+; 5); 222 (75); 142 (100); 130 (11); 115 (79); 87 (63); 5 (m/z [percent abundance]). Elemental analysis for C_{10}H_{11}NSe_2, calculated (found): C, 54.30/54.80; H, 3.62/4.00; N, 6.33/6.10.
promastigotes (MCAN/ES/89/IPZ229/1/89) were grown in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) 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 obtained by incubation of 4.5 × 10^5 late-logarithmic-phase promastigotes in 5 ml of M199 medium (In-vitrogen, Leiden, The Netherlands) supplemented with 10% heat-inactivated FCS, 1 g liter⁻¹ of β-alanine, 100 mg liter⁻¹ of l-asparagine, 200 mg liter⁻¹ of sucrose, 50 mg liter⁻¹ of sodium pyruvate, 320 mg liter⁻¹ of malic acid, 40 mg liter⁻¹ of uramic acid, 70 mg liter⁻¹ of succinic acid, 200 mg liter⁻¹ of α-ketoglutaric acid, 300 mg liter⁻¹ of citric acid, 1.1 g liter⁻¹ of sodium bicarbonate, 5 g liter⁻¹ of morpholinoethanesulfonic acid (MES), 0.4 mg liter⁻¹ of hemin, and 10 mg liter⁻¹ of gentamicin, pH 5.4, at 37°C. After 48 h of incubation, all parasites was rounded morphologically and a flagellum and divided during several weeks under the described conditions.

THP-1 cells 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% CO₂.

(ii) Leishmanial activity and cytotoxicity assays. Drug treatment of amastigotes was performed during the logarithmic growth phase at a concentration of 2 × 10⁶ parasites/ml at 26°C or 1 × 10⁵ parasites/ml at 37°C for 24 h, respectively. Drug treatment of THP-1 cells was performed during the logarithmic phase growth at a concentration of 4 × 10⁵ cells/ml at 37°C and 5% CO₂ for 24 h. The percentage of living cells was evaluated by flow cytometry using the propidium iodide (PI) exclusion method (51).

Drug concentrations ranged from 0.2 μM to 25 μM.

(iii) Leishmania infection assay. Human THP-1 mononuclear cells were seeded at 1.2 × 10⁶ cells/ml in 24-multidish plates (Nunc, Roskilde, Denmark) and differentiated to macrophages for 24 h in 1 ml of RPMI 1640 medium containing 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO). Medium culture was removed, and 1.2 × 10⁶ Leishmania amastigotes in 1 ml of THP-1 medium were added to each well. Four hours later, all medium with noninfective amastigotes was removed, washed 3 times with 1× phosphate-buffered saline (PBS), and replaced with new THP-1 medium and corresponding treatment. After 48 h of treatment, medium was removed; THP-1 cells were washed 3 times with 1× PBS and detached with TrypLE Express (Invitrogen, Leiden, The Netherlands) according to the manufacturer’s indications. Infection quantification was measured by flow cytometry. Drug concentrations ranged from 0.2 μM to 25 μM.

(iv) Trypanothione reductase assay. Oxidoreductase activity was determined according to the method described by Toro et al. (52). Briefly, reactions were carried out at 26°C in 250 μl of 40 mM HEPES buffer (pH 8.0) containing 1 mM EDTA, 150 μM NADPH, 30 μM NAPD⁺, 25 μM DTNB [5,5'-dithiobis-(2-nitrobenzoic acid) or Ellman’s reagent], 1 μM T[S]₂, 0.02% glycerol, 1.5% dimethyl sulfoxide (DMSO), and 7 nM recombinant Li-tryR. Enzyme activity was monitored by the increase in absorbance at 412 nm for 1 h at 26°C in a VERSAmax microplate reader ( Molecular Devices, CA). All the assays were conducted in triplicate in at least three independent experiments. Data were analyzed using a nonlinear regression model with Grafit software (Erithacus, Horley, Surrey, United Kingdom).

Drug likeness parameters. The drug likeness and drug score values along with the topological polar surface area (TPSA) values and the properties described in the Lipinski’s rule of five (molecular mass ≤ 500 Da, log P ≤ 5, H-bond donors [HBD] ≤ 5, and H-bond acceptors [HBA] ≤ 10) were calculated using Osiris (53) and Molinspiration property calculation (54), respectively. Topological polar surface area was used to calculate the percentage of absorption (ABS) according to the equation 109 − (0.345 × TPSA) (55).

RESULTS

Chemistry. The synthetic approaches adopted to obtain the target compounds are depicted in Fig. 2. The selenolate derivatives

3,3'-([Diselenodylithio]methylidinyl)bis(2-bromophene) (2d). Compound 2d was from (2-bromophene-3-yl)methyl selenocyanate (1i) and sodium borohydride. The mixture was extracted with ether (3 × 50 ml). The organic phase was washed with water (3 × 50 ml) and dried with anhydrous Na₂SO₄. The ethyl ether was removed under vacuum, and a yellow powder was obtained. Yield: 92%; mp: 49 to 50°C. IR (KBr) cm⁻¹: 3,099 (w, C-H); 722 (s, Se-Se). ¹H NMR (400 MHz, DMSO-d₆): δ = 4.04 (s, 4H, CH₂-Se, CH₂-Se); 7.00 (d, 2H, H₁ + H₃, J₃-4 = 5.6 Hz); 7.58 (d, 2H, H₁ + H₃, J₃-4 = 5.6 Hz). ³¹C NMR (100 MHz, DMSO-d₆): δ = 26.5 (CH₂-Se); 111.1 (C₁₁, C₁₂); 128.4 (C₁₂, C₁₃); 130.6 (C₁₁, C₁₂); 140.0 (C₁₁, C₁₂). MS (m/z [percent abundance]): 510 (M⁺, 3); 175 (100); 169 (9); 45 (8). Elemental analysis for C₁₄H₁₂N₆Se₂·H₂O, calculated/found (percent): C, 38.40/38.40; H, 1.58/1.52.

1,1'-([Diselenodylithio]methylidinyl)bis(1H-benzotriazole) (2e). Compound 2e was from 1H-benzotriiazol-1-ylmethylantheneselenolate (1k) and sodium borohydride. The mixture was extracted with dichloromethane (3 × 50 ml). The organic phase was washed with water (3 × 50 ml) and dried with anhydrous Na₂SO₄. The dichloromethane was removed under vacuum, and a white powder was obtained. Yield: 25%; mp: 197 to 199°C. IR (KBr) cm⁻¹: 744 (s, Se-Se, CH₂-Se); 3,12/3.44.

The drug likeness and drug score values along with the topological polar surface area (TPSA) values and the properties described in the Lipinski’s rule of five (molecular mass ≤ 500 Da, log P ≤ 5, H-bond donors [HBD] ≤ 5, and H-bond acceptors [HBA] ≤ 10) were calculated using Osiris (53) and Molinspiration property calculation (54), respectively. Topological polar surface area was used to calculate the percentage of absorption (ABS) according to the equation 109 − (0.345 × TPSA) (55).

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(compounds 1a to 1o) were obtained in variable yields (27 to 92\%) by reaction between the commercially available haloalkyl carbo or heterocyclic reactives with potassium selenocyanate in the molar ratio 1:1 in acetone under reflux over 3 to 4 h (24). The subsequent reduction of compounds 1a to 1o with sodium borohydride or sodium cyanoborohydride in ethanol afforded derivatives 2a to f and 2g (24) in yields ranging from 25 to 70\%. Unfortunately, for some selenocyanates (1a, 1d, 1e, 1f, 1 h, 1i, 1k, and 1m) several difficulties were found, and this procedure failed to afford the expected products. This prompted us to seek alternative routes to prepare the corresponding diselenides. Surprisingly, modification of the reaction conditions (temperature, solvents, or molar ratio) resulted in decomposition of the starting materials by rupture of the bond between the heterocycle and methylene. This hypothesis was confirmed by the disappearance of the signal corresponding to the methylene group in $^1$H NMR. Additionally, an undesired mixture of side compounds was identified in TLC. The alternative strategy employing 100\% hydrazine hydrate and sodium hydroxide resulted in decomposition of the starting materials by rupture of the bond between the heterocycle and methylene. This hypothesis was confirmed by the disappearance of the signal corresponding to the methylene group in $^1$H NMR. Additionally, an undesired mixture of side compounds was identified in TLC.

Finally, and contrary to our expectations, the reduction of 1o with NaBH$_4$ in ethanol yielded compound 3, an unexpected compound instead of the corresponding diselenide that was obtained by reaction with NaBH$_2$CN.

All of the compounds prepared during the course of these investigations are stable. Their purity was assessed by TLC and elemental analyses, and their structures were identified from spectroscopic data. IR, $^1$H NMR, $^{13}$C NMR, mass spectrometry, and elemental analysis methods were used for structure elucidation (Fig. 2).

The IR spectra of compounds 1a to 1o illustrate sharp peaks around 2,138 to 2,155 cm$^{-1}$ due to a CN group. Derivatives 2a to 2g showed multiple bands in the range of 710 to 790 cm$^{-1}$ attributable to the Se-Se group and lacked the CN band, confirming the reduction. In NMR all the signals were fully consistent with proposed structures. Copies of the registered $^1$H and $^{13}$C NMR spectra for the selected compounds (1h, 2d, and 2e) can be found in the supplemental material.

Biological evaluation. (i) Activity in amastigotes and cytotoxic activity in human cells. Compounds 1a to 1o, 2a to 2g, and 3 were tested for their antiprotozoal activity against amastigotes of the pathogenic Leishmania infantum using miltefosine and edelfosine as standard drugs according to a previously described procedure (57). Although most of the studies on the in vitro biological activity of new compounds against Leishmania spp. are performed on promastigote forms, this assay must be considered preliminary because this stage of parasite is significantly more susceptible to drug-induced effects than the amastigote form. Moreover, promastigotes are not the developed forms of the parasite in vertebrate hosts so the evaluations made with them are merely indicative of the potential leishmanicidal activity of the compounds tested. Accordingly, because amastigotes are responsible for all clinical manifestations in humans, the intracellular amastigote model has been cited as the “gold standard” for in vitro Leishmania drug discovery research. Taking this into account, all the analyses were carried out in the amastigote form with a minimum of three independent experiments and the results are expressed as 50\% effective dose (ED$_{50}$) values. In addition, for a compound to be a candidate for antileishmanial drug, it is required to have both high leishmanicidal activity and low cytotoxicity. Cytotoxicity for the THP-1 cell line was evaluated for all compounds in order to identify drugs with low toxicity in human cells and as a prelude to selecting drugs for in vitro assay on the relevant clinical Leishmania amastigote stage. The selectivity index (SI) of the compounds is expressed by the ratio between cytotoxicity (ED$_{50}$ value on THP-1 cells) and activity (ED$_{50}$ value on L. infantum amastigotes).

Table 1 shows the ED$_{50}$ values obtained after 24 h of exposure to the compounds in L. infantum axenic amastigotes. Values for the reference drugs miltefosine and edelfosine are included in all cases for comparison. Biological data evidenced that most of the screened compounds (15 out of 23) showed high bioactivity (ED$_{50}$ $\leq$ 2.53 $\mu$M) against L. infantum, being more potent than miltefosine (ED$_{50}$ = 2.84 $\mu$M). In addition, under our experimental conditions, seven compounds (1d, 1e, 1h, 1m, 1n, 2e, and 2f) displayed in vitro potency comparable to or higher than that of edelfosine (ED$_{50}$ = 0.82 $\mu$M).

Different authors have claimed that compounds having SI values greater than 20 can be considered ideal candidates for further development as leishmanicidal drugs (58). However, in this study and with rigorous criteria, we have considered the SI threshold of >25 for further analysis of their activity in amastigote-infected THP-1 cells. This requirement is satisfied by compounds 1h, 2d, 2e, and 2f considering them as the lead ones in this series due to their excellent biological behavior.

(ii) Leishmanicidal activity in infected macrophages. According to their activity and selectivity, four compounds (1h, 2d,
2e, and 2f) were advanced for testing leishmanicidal activity in amastigote-infected THP-1 cells. Compound 2f (ED$_{50} = 0.68$ μM; SI = 36.8) was not further tested due to the reproducibility issues showed by this derivative pertaining to its lack of solubility under the assay conditions. The ED$_{50}$ values for the other selected derivatives (1h, 2d, and 2e) were calculated and summarized in Table 2. The potency of the analogues was compared with that of edelfosine, a current antileishmanial agent (ED$_{50}$ = 0.82 μM), excellent SI (29.9 and 25.8), and a marked inhibitory activity against TryR inhibition. Compound 1h, as well as compound 2d, can be considered promising antileishmanial lead candidates because they show a strong inhibitory activity against axenic amastigotes (IC$_{50}$ = 0.73 and 6.85 μM), excellent SI (29.9 and 25.8), and a marked inhibitory activity against TryR.

### Drug likeness properties.
Employing the Molinspiration (54) and Osiris (53) software, the selected compounds (1h, 2d, and 2e) were subjected to Lipinski’s rule of five analyses (drug likeness), which helps to predict and explain the biological behavior of small molecules. This preliminary analysis allows prediction of the physicochemical properties related to their absorption and bioavailability. We have found that the derivatives 1h and 2e show no violations of Lipinski’s rule of five (Table 4). Among leishmanicidal drugs available on the market, only miltefosine does not violate Lipinski’s rule of five; all other drugs have at least 1 violation (edelfosine). It has been well established that optimal lipophili-

<p>| TABLE 1 | ED$_{50}$ values for compounds on amastigotes and cytotoxic activity in the THP-1 cell line |
|------------------------|------------------------|------------------------|</p>
<table>
<thead>
<tr>
<th><strong>Compound</strong></th>
<th><strong>R (substituted group)</strong></th>
<th><strong>ED$_{50}$ (mean ± SEM), μM</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Quinol-8-yl</td>
<td>4.49 ± 0.21 14.48 ± 0.37</td>
</tr>
<tr>
<td>1b</td>
<td>Quinol-2-yl</td>
<td>1.76 ± 0.04 14.91 ± 0.92</td>
</tr>
<tr>
<td>1c</td>
<td>Acridin-9-yl</td>
<td>7.40 ± 0.60 15.01 ± 0.68</td>
</tr>
<tr>
<td>1d</td>
<td>Quinoxaline-2,3-dimethanediyl</td>
<td>0.69 ± 0.06 13.83 ± 1.59</td>
</tr>
<tr>
<td>1e</td>
<td>6,7-Dimethoxy-2-oxo-2H-chromen-4-yl</td>
<td>0.82 ± 0.07 15.95 ± 1.54</td>
</tr>
<tr>
<td>1f</td>
<td>5-Nitrofur-2-yl</td>
<td>1.99 ± 0.23 3.95 ± 0.29</td>
</tr>
<tr>
<td>1g</td>
<td>6-Bromo-1,3-benzodioxol-5-yl</td>
<td>10.10 ± 1.81 15.26 ± 1.19</td>
</tr>
<tr>
<td>1h</td>
<td>3,3-Dimethylisoxazol-4-yl</td>
<td>0.73 ± 0.10 21.82 ± 2.40</td>
</tr>
<tr>
<td>1i</td>
<td>2-Benzothien-3-yl</td>
<td>2.86 ± 0.29 19.54 ± 0.52</td>
</tr>
<tr>
<td>1j</td>
<td>5-Chlorothien-2-yl</td>
<td>1.85 ± 0.33 21.02 ± 0.52</td>
</tr>
<tr>
<td>1k</td>
<td>N-Phenylpyrrol-4-yl</td>
<td>8.87 ± 1.32 23.74 ± 0.48</td>
</tr>
<tr>
<td>1l</td>
<td>Benzotriazol-1-yl</td>
<td>1.11 ± 0.21 22.00 ± 1.20</td>
</tr>
<tr>
<td>1m</td>
<td>2-Adamant-1-yl-2-oxoethyl</td>
<td>0.83 ± 0.03 19.68 ± 1.98</td>
</tr>
<tr>
<td>1n</td>
<td>9,10-Dioxy-9,10-dihydroanthracen-2-yl</td>
<td>0.74 ± 0.18 6.05 ± 1.19</td>
</tr>
<tr>
<td>1o</td>
<td>Phthalimidyl</td>
<td>2.53 ± 0.32 22.50 ± 1.63</td>
</tr>
<tr>
<td>2a</td>
<td>Quinol-8-yl</td>
<td>2.05 ± 0.24 8.60 ± 1.10</td>
</tr>
<tr>
<td>2b</td>
<td>Acridin-9-yl</td>
<td>5.46 ± 0.01 3.76 ± 0.07</td>
</tr>
<tr>
<td>2c</td>
<td>6-Bromo-1,3-benzodioxol-5-yl</td>
<td>3.99 ± 0.62 &gt;25</td>
</tr>
<tr>
<td>2d</td>
<td>2-Benzothien-3-yl</td>
<td>1.20 ± 0.03 30.9 ± 0.02</td>
</tr>
<tr>
<td>2e</td>
<td>Benzotriazol-1-yl-2-oxoethyl</td>
<td>0.68 ± 0.30 &gt;25</td>
</tr>
<tr>
<td>2f</td>
<td>9,10-Dioxy-9,10-dihydroanthracen-2-yl</td>
<td>1.35 ± 0.17 9.26 ± 0.31</td>
</tr>
<tr>
<td>2g</td>
<td>Phthalimidyl</td>
<td>&gt;25</td>
</tr>
<tr>
<td>3</td>
<td>Phthalimidyl</td>
<td>&gt;25</td>
</tr>
<tr>
<td>Edelfosine</td>
<td>0.82 ± 0.13 4.96 ± 0.16</td>
<td>6.0</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>2.84 ± 0.10 18.50 ± 0.60</td>
<td>7.0</td>
</tr>
</tbody>
</table>

| TABLE 2 | ED$_{50}$ values for compounds in amastigote-infected THP-1 cells |
|------------------------|------------------------|------------------------|
| **Compound** | **ED$_{50}$ (mean ± SEM), μM** |
|------------------------|------------------------|------------------------|
| 1h | 23.2 ± 4.3 |
| 2d | 14.0 ± 2.1 |
| 2e | 14.4 ± 2.6 |
| Edelfosine | 3.1 ± 0.1 |

| TABLE 3 | IC$_{50}$ for the selected compounds against TryR inhibition |
|------------------------|------------------------|------------------------|
| **Compound** | **IC$_{50}$ (mean ± SEM), μM** |
|------------------------|------------------------|------------------------|
| 1h | 0.46 ± 0.01 |
| 2d | 6.85 ± 0.49 |
| 2e | >75 |
| Mepacrine | 16.99 ± 1.18 |
bioavailability (60). According to the theoretical study carried out by de Toledo et al. (61), the TPSA of most leishmanicidal drugs currently on the market is higher than this limit, which probably restricts their absorption and bioavailability. The log P and TPSA values for compounds 1h, 2d, and 2e range from 0.75 to 4.68 and 0 to 61.44, respectively, suggesting that these compounds are potentially able to cross cell membranes in a permeation process, which could explain the ability to reach the amastigotes inside the phagolysosome.

The drug score uses and relates other molecular parameters, such as drug likeness, log P, molecular weight, and toxicity risks, and can be considered a convenient value that may be used to judge the overall potential of a compound to become a drug. A value of 0.5 or more is indicative of a promising lead for future development of a safe and efficient drug (62). Compound 1h possesses the maximum drug score (0.5) for the selected compounds, whereas edelfosine presented a drug score of 0.3.

DISCUSSION

There are many available antileishmanial agents, but the drug of choice is still awaited because of several limitations of current drugs, such as high cost, poor compliance, drug resistance, low efficacy, and poor safety. The high prevalence and severity of this illness justify the urgency for the discovery of new drugs. In the last 2 decades, several interesting drug targets have been proposed, including many proteins and enzymes that differ from mammalian counterparts which can interfere with the redox system. Among the promising targets that the scientific community considers for the design of useful therapies, enzymes (trypanothione reductase, proteinases, superoxide dismutase, dihydrofolate reductase, metacaspase, topoisomerase, kinases, sirtuins, etc.) are one of the classes with the most representation. In this context, we notice that selenium plays an important role in medicinal chemistry, particularly in antioxidant, antitumoral, chemopreventive, or antiparasitic agents. Thus, we have described here the synthesis and leishmanicidal activity of novel selenocyanate and diselenide compounds.

For the novel seleno derivatives presented in this work, there seems to exist a tendency suggesting that analogues with the diselenide unit were more active than those with the selenocyanate moiety (1a versus 2a, 1c versus 2b, 1g versus 2c, 1i versus 2d, 1l versus 2e, and 1o versus 2g) against L. infantum amastigotes. Regarding the selectivity index, the addition of the diselenide scaffold clearly improved the selectivity, for example, in compounds 1g versus 2c, 1i versus 2d, 1l versus 2e, and 1n versus 2f. In general, it was observed that tricyclic nitrogenated rings, such as acridine (1c and 2b), are detrimental to the biological activity and selectivity compared with bicyclic nitrogenated rings (1b, 1d, 1I, 1o, 2e, and 2g). Furthermore, no regular order of decrease or increase in the activity among the rest derivatives can be concluded.

Taking into account the results related to activity and selectivity and considering our exigent criteria for both parameters (ED50 of <2.5 μM and SI of >25), four derivatives, one selenocyanate, 1h, and two diselenides (2d and 2e) were selected for further studies. Despite the fact that compound 2f fulfilled these criteria, it could not be tested due to solubility problems. When we performed intracellular form tests, these derivatives did not improve the activity compared to that of edelfosine. However, their lack of toxicity against THP-1 cells (Table 1) represents a remarkable advantage over the reference drug.

We hypothesized that TryR inhibition may be related with the leishmanicidal activity observed for seleno derivatives. The results for in vitro assays revealed that compounds 1h and 2d exhibited a good correlation between leishmanicidal activity and TryR inhibition, confirming our previous proposal. On the other hand, 2e did not show inhibitory activity, suggesting that not only is this enzyme involved in its potent leishmanicidal activity but also other mechanisms can be implicated.

Finally, in silico prediction studies were performed in order to determine the drug like properties for the lead compounds. Considering these properties, derivatives 1h and 2e were shown to meet the Lipinski’s rule of five, indicating favorable properties for drug development. The in silico toxicity profile, drug likeness, and drug score (0.5) data for compound 1h make it a promising leader for future development of safer and more efficient leishmanicidal drugs.

In conclusion, the present study reports the synthesis of new selenocyanates and diselenides bearing interesting bioactive scaffolds (quinoline, quinoxaline, acridine, chromene, furane, and isozazole) and their in vitro leishmanicidal activities against L. infantum amastigotes along with their cytotoxicities in THP-1 cells. Fifteen such compounds exhibited better potency against axenic amastigotes than the standard drug miltefosine. Based on their antiparasitic activities and low toxicity in THP-1 cells, compounds 1h, 2d, 2e, and 2f were identified as the best candidates for further studies with infected macrophages. Although their potency against intracellular amastigotes is lower than that observed for the reference drug, these compounds combined a potent leishmanicidal activity with excellent selectivity index (>25), resulting in promising therapeutic utility. In order to get further insight into their putative mechanism of action, their activity against L. infantum TryR was determined. A clear correlation between enzyme inhibition and antiparasitic activity was observed for compounds 1h and 2d, which may be considered evidence for one of their many possible mechanisms of action. No correlation was detected for 2e, which suggest the existence of different targets in this family of compounds. The ADME (absorption, distribution, metabolism, and excretion) parameters calculated for derivatives 1h and 2e predict a good bioavailability. In silico ADME profiling and drug score results along with in vitro leishmanicidal activity, cytotoxicity, and TryR inhibitory activity make 1h a promising candidate.
lead compound for the development of more potent antiparasitic drugs. A graphical summary of the conclusions drawn from this work is depicted in Fig. 3.

Moreover, to the best of our knowledge, this is the first study that reports new seleno-derivatives as leishmanicidal and TryR inhibitors and opens new possibilities in the field of neglected diseases.

ACKNOWLEDGMENTS

We express our gratitude to the Foundation for Applied Medical Investigation (FIMA), University of Navarra. We also acknowledge financial support from the Ministerio de Educación y Ciencia, Spain (grant SAF2012-39760-C02-02). We have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in this article apart from those disclosed.

FUNDING INFORMATION

This work, including the efforts of Verónica Alcolea, was funded by Foundation for Applied Medical Investigation (FIMA), University of Navarra. The authors also acknowledge financial support from the Ministerio de Educación y Ciencia, Spain (grant SAF2012-39760-C02-02). The authors express their gratitude to the Foundation for Applied Medical Investigation (FIMA), University of Navarra. The authors also acknowledge financial support from the Ministerio de Educación y Ciencia, Spain (grant SAF2012-39760-C02-02). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in this article apart from those disclosed.

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