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N-Nitrosulfonamides as Carbonic Anhydrase Inhibitors: A Promising Chemotype for Targeting Chagas Disease and Leishmaniasis

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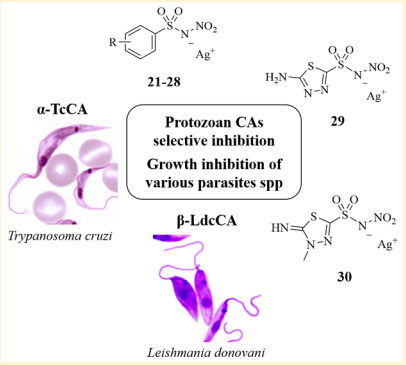
Supporting Information

ABSTRACT: *Trypanosoma cruzi* and Leishmania spp. are protozoa of the Trypanosomatidae family, respectively, responsible of the neglected tropical disorders (NTDs) Chagas disease and leishmaniasis. The present pharmacotherapy is often ineffective and exhibits serious side effects. The metalloenzyme carbonic anhydrases (CAs, EC 4.2.1.1) recently identified in these protozoans (α-TcCA and β-LdcCA) are novel promising targets for chemotherapeutic interventions. Herein, we report a series of *N*-nitrosulfonamides, as a novel chemotype to yield the target CA isoform selective inhibition over ubiquitous human isozymes. Two derivatives selected among the most active and selective ones for TcCA/LdcCA over off-target CAs were progressed as silver salts to in vitro studies with various developmental forms and spp of *Trypanosoma cruzi* and leishmania. Excellent values of parasites growth inhibition (IC_{50}) were observed, with some selectivity index (over cytotoxicity for macrophages and Vero cells) being comparable or better than reference drugs. These findings make *N*-nitrosulfonamides and their salts promising lead compounds for a rational optimization of innovative agents for the treatment of Chagas disease and leishmaniasis based on CA inhibition.



KEYWORDS: *Chagas disease, Trypanosoma cruzi, leishmania, carbonic anhydrase, zinc-binding group, inhibition, silver, antiparasitic*

World Health Organization (WHO) included Chagas disease (American trypanosomiasis) and leishmaniasis in the list of neglected tropical diseases (NTDs). Parasites of the kinetoplastidae family are responsible for these infections, both belonging to the vector-borne diseases affecting 20 million people and killing more than 50,000 every year.[1]

Trypanosoma cruzi is naturally transmitted by kissing bugs (mainly belonging to the genera *Triatoma* and *Rhodnius*), which primarily diffuse in Latin America. The disease evolves producing potentially fatal lesions to organs in the cardiac, digestive, or neurological systems.[1]

Leishmaniasis is transmitted by the bite of an infected phlebotomine and works out skin or visceral aches that could turn out to be fatal if untreated. Among the NTDs, leishmaniasis is the first-in-class in terms of mortality and morbidity.[1]

Available pharmacological treatments for the majority of NTDs are limited in terms of cost and toxicity and ineffective, and resistance phenomena constantly increase throughout the world.[2−4] Pharmaceutical industry shows poor interest in searching new effective drugs for the treatment of NTDs due to high costs and expected low financial return. It is urgent to find new therapeutic targets for these parasitosis, which WHO classifies as priority infections.[2,5] Novel targets have been identified driven by large-scale analysis on the completely known genome sequence of both protozoans. Indeed, endeavors to enrich the therapeutic arsenal against Chagas disease and leishmaniasis based on enzymatic inhibition have been starting in many laboratories with synthetic drugs representing a valuable source for new treatments.[6,7]

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The metalloenzyme carbonic anhydrases (CAs, EC 4.2.1.1) recently identified in these protozoans are novel promising targets for chemotherapeutic interventions.[6,8,9] CAs catalyze the reversible hydration of CO_2 to bicarbonate and proton, a pivotal reaction for all cells and complex organisms, which is also basic in the growth and virulence of pathogenic microorganisms.[9] CAs from *Trypanosoma cruzi* (TcCA) and *Leishmania donovani chagasi* (LdcCA) were cloned and characterized in 2013,[10−12] resulting in the design of novel antiprotozoal agents that act by a totally new mechanism of action and lack cross-resistance to existing drugs. The α-CA TcCA is endowed with a very high catalytic activity for the CO_2 hydration reaction and was shown to be inhibited in the nanomolar range by many types of CA inhibitors (CAIs) such as aromatic/heterocyclic sulfonamides,[10,13,14] sulfamates,[10] thiols,[10] anions,[15] dithiocarbamates,[15] hydroxamates,[16] and benzoxaboroles.[17] Thiols and hydroxamates exhibited *in vitro* antitrypanosomal activity, inhibiting the three phases of the pathogen's life cycle.[10,16] The β-CA LdcCA also features an effective catalytic activity and was shown to be efficiently inhibited by sulfonamides and heterocyclic thiols with nanomolar inhibition constants.[12,18] Some such thiol derivatives displayed *in vitro* antileishmania activity in preliminary assays being able to reduce parasites' growth and causing their death.[12] Identification of new protozoans CAIs with effective antitrypasonomal or antileishmania activities is more than ever worth the endeavor due to such targets' remarkable druggability.

Nifurtimox and benznidazole have been the first effective drugs for treating acute-phase human Chagas infection, with the first being no longer available on the market because of undesirable side effects.[19] They feature heteroaromatic nitro moieties that are pivotal for the antiprotozoa mechanism of action. Parasite resistance arisen with benznidazole drove the development of alternative therapies. Indeed, combined treatment of benznidazole with drugs with different mechanisms of action such as azoles, nitric oxide, or clomipramine could be a strategy to improve the pharmacotherapy efficacy.[6]

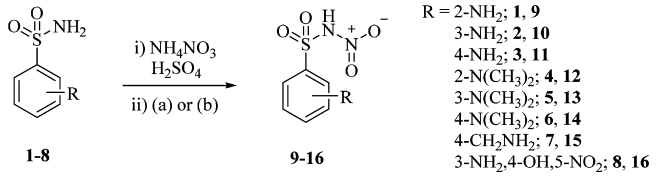
Noteworthy, a new chemotype able to afford α- and β-CAs inhibition was reported by us in 2016, namely, *N*-nitrosulfonamides.[20] Interestingly, these latter were shown to inhibit ubiquitous, off-targets isoforms, such as CA II, feebler than lead sulfonamides though holding remarkable submicromolar inhibition of the human (h) tumor-associated CA IX (α-CA) and the β-CA from the pathogen fungus *Malassezia Globosa*.

Considering the above, we have herein extended the set of *N*-nitrosulfonamides and screened them on a wider pattern of human and pathogen (from protozoa and fungi) CAs, among which are the targets α-TcCA and β-LdcCA.

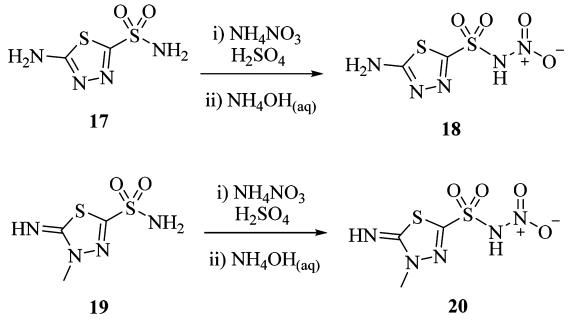
Furthermore, we produced silver salts of all such derivatives based on their marked effects against viruses, bacteria, fungi, and protozoa.[20] The antimicrobial behaviors of silver, silver ions, and silver-containing compounds have long been investigated with various antimicrobial mechanisms of action having been proposed to date.[21−24] The biologically active silver ion (Ag^+) irreversibly damages key enzyme systems in the cell membranes of pathogens. Conversely, silver exhibits low toxicity in the human body and minimal risk is expected due to clinical exposure.[21] Recently, silver nanoparticles (Ag-NPs) were demonstrated to produce reactive oxygen species to which *Leishmania* parasites are very sensitive.[24] Moreover, the commercially available antibiotic silver sulfadiazine shares a wealth of features with silver *N*-nitrosulfonamides. These latter derivatives are thus endowed with multiple potential antiprotozoa entities to be synergistically exploited to overcome resistance issues displayed by single-targeted therapy.

The general synthetic strategy proposed by Minksztym[24] for the chemoselective mononitration of aminosulfonamides was applied to a set of ten starting compounds being commercially available (**1**−**3**, **7**, **8**) or yielded by methylation (**4**−**6**) or deacetylation (**17**, **19**) reactions (Schemes 1 and 2).

Scheme 1. Synthesis of Aromatic *N*-Nitrosulfonamides: (a) H_2O; (b) NH_4OH(aq)



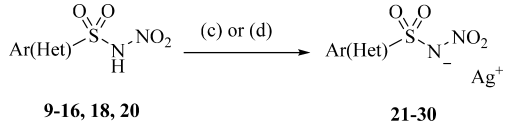
Scheme 2. Synthesis of Heteroaromatic *N*-Nitrosulfonamides



Quenching and workup of the NH_4NO_3/H_2SO_4 based nitration reaction was switched for the most unstable derivatives **16**, **18**, and **20** from water to NH_4OH(aq) to generate the stable ammonium salts instead of the zwitterion forms of *N*-nitrosulfonamides (Schemes 1 and 2).[25,26]

The production of the silver salts of the derivatives was achieved by different methods depending on the nature of the compound or the form it was produced as in the previous step (Scheme 3). Silver carbonate was used as the base (to remove

Scheme 3. Synthesis of Silver Salts of *N*-Nitrosulfonamides: (c) Ag_2CO_3, H_2O; (d) NaOH, $AgNO_3$, H_2O



the proton) and the source of silver ion in an aqueous phase in case of zwitterion of amino aromatic compounds. NaOH/$AgNO_3$ was used for the zwitterion of the amino aliphatic compound **15** and for ammonium salts **16**, **18**, and **20**. All compounds silver salts precipitated in aqueous phases and were therefore recrystallized by the same solvent. All the obtained derivatives were properly characterized by means of ^{1}H NMR, ^{13}C NMR, and MS (see Supporting Information).

The inhibition profiles of the *N*-nitrosulfonamide derivatives were evaluated against six α-CAs and three β-CA isoforms in addition to acetazolamide (**AAZ**) as standard inhibitor, by a stopped flow CO_2 hydrase assay.[27]

Five human CA isoforms, among which the ubiquitous CA I and II (involved in many physiopathological processes), the

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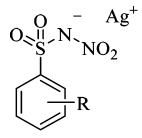
membrane-associated CA IV (involved in ocular aches, stroke and arthritis), and IX and XII (overexpressed in hypoxic tumors) were included in the kinetic study to work out thorough structure−activity relationships (SARs) and selectivity profiles.[28] Along with the target TcCA and LdcCA, the activity of two additional β-CA isoforms from pathogenic fungi was studied with the reported inhibitors, namely, MgCA from *Malassezia globosa* (responsible of the production of dandruff)[29] and Can2 from *Cryptococcus neoformans* (that can cause fungal meningitis and encephalitis).[30] The inhibitory efficacy against nine such isoforms was also assessed with the silver salts of all derivatives to verify whether the monovalent metal ion affects the enzymatic activities. The inhibition constants (K_Is) of these latter do not show significant variations out of the error ranges, witnessing no significant action of the Ag^+ ion to each CA activity. Tables 1 (hCAs) and 2 (pathogens CAs) gather the inhibition data of silver salts **21**−**30** (inhibition data of **9**−**20** are not shown; comparisons can be made with data previously reported).[20]

Table 1. Inhibition Data of CA I, II, IV, IX, and XII with N-Nitrosulfonamides 21−30 and the Standard Sulfonamide Inhibitor Acetazolamide (AAZ) by a Stopped Flow CO_2 Hydrase Assay[27]



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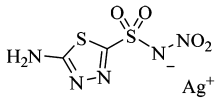

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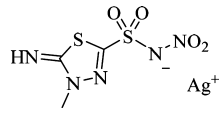

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		K_I (μM)[a]				
compd	R	CA I	CA II	CA IV	CA IX	CA XII
21	2-NH_2	29.0	60.9	39.2	0.52	0.75
22	3-NH_2	54.7	7.7	4.3	5.4	2.6
23	4-NH_2	67.4	53.4	23.6	9.4	2.0
24	2-$N(CH_3)_2$	80.6	6.2	32.2	8.0	3.6
25	3-$N(CH_3)_2$	45.9	18.1	4.5	6.8	5.7
26	4-$N(CH_3)_2$	58.3	64.2	11.0	4.5	3.9
27	CH_2NH_2	39.6	55.8	3.1	5.4	0.65
28	3-NH_2, 4-OH, 5-NO_2	19.8	45.0	1.9	5.2	0.55
29		7.3	2.9	1.4	0.84	0.92
30		4.9	2.2	4.8	0.23	0.76
AAZ		0.25	0.012	0.075	0.025	0.006

[a]Mean from three different assays, by a stopped flow technique (errors were in the range of ±5−10% of the reported values).

The following structure−activity relationships (SAR) can be drawn from the inhibition data reported in Table 1 and 2.

According to preliminary data previously reported,[20] *N*-nitro aromatic sulfonamides exhibited low CA I and II inhibitory effectiveness, with K_Is spanning in a low to medium micromolar range (2.2−80.6 μM). Heteroaromatic derivatives **29** and **30** turned out as the most potent inhibitors against these ubiquitous hCAs. Whereas CA IV was targeted by all derivatives in a low micromolar range (1.4−39.2 μM), a wealth of submicromolar K_I values against CA IX and XII (0.23−9.4 μM) confirmed the favorite efficacy of *N*-nitrosulfonamides against the tumor-associated isoforms. CA XII was the most affected isozyme among the considered cluster, though the greatest inhibition was measured with the thiadiazole derivative **30** with CA IX (K_I of 0.23 μM). It should be noted that CA XII features more Thr and

Table 2. Inhibition Data of TcCA, LdcCA, MgCA, and Can2 with *N*-Nitrosulfonamides and AAZ

		K_I (μM)[a]			
compd	R	TcCA	LdcCA	MgCA	Can2
21	2-NH_2	3.2	4.7	0.52	7.4
22	3-NH_2	0.15	0.49	1.7	0.25
23	4-NH_2	0.10	0.23	0.76	0.40
24	2-$N(CH_3)_2$	5.0	4.8	32.2	4.3
25	3-$N(CH_3)_2$	1.4	0.50	4.5	1.1
26	4-$N(CH_3)_2$	0.43	0.65	0.30	0.42
27	CH_2NH_2	0.47	0.71	7.1	1.0
28	3-NH_2, 4-OH, 5-NO_2	0.85	1.0	0.57	0.35
29		0.35	0.52	4.1	0.76
30		0.32	0.44	2.7	2.3
AAZ		0.06	0.09	0.076	0.01

[a]Mean from three different assays, by a stopped flow technique (errors were in the range of ±5−10% of the reported values).

Ser residues in the active site than other hCAs. Likely extended H-bond networks between *N*-nitrosulfonamide moieties and such hydrophilic residues could justify the reported low K_I values. Noteworthy, TcCA turned out to be the most affected α-CA among those herein studied (Table 2). Most derivatives inhibited TcCA in a medium nanomolar range (0.10−0.85 μM), except for compounds bearing 2-NH_2, 2-$N(CH_3)_2$, or 3-$N(CH_3)_2$ moieties at the phenyl ring (K_Is in the range 1.4−5.0 μM). The incorporation of primary amino groups at the *meta* or *para* position of the phenyl ring confers to **22** and **23**, the greatest TcCA inhibitory efficacies as well as the strongest CA inhibition properties of the study. In agreement with the inhibition data previously reported,[20] β-CAs were generally more efficiently inhibited by *N*-nitrosulfonamides than α-CAs. Indeed, most K_Is shown in Table 2 for LdcCA, MgCA, and Can2 lie into a submicromolar range. While the first isozyme is undoubtedly the most affected one among the three (K_Is in the range 0.23−4.8 μM), equally efficient inhibitions were measured against the fungal MgCA and Can2. The 4-NH_2-phenyl derivative **23** arose again as the most potent one against the target LdcCA (K_I of 0.23 μM). Unlike against TcCA, **22** inhibited the isozyme comparably with the heterocyclic derivatives **29** and **30** (K_Is of 0.49, 0.52, and 0.44 μM, respectively).

Striking target/off-target CAs selectivity profiles can be ascribed to many *N*-nitrosulfonamide derivatives. As a general trend, the designed compounds acted one to more than two orders of magnitude more potently against TcCA and LdcCA than ubiquitous h-isoforms CA I and II; e.g., derivative **23** showed TcCA/CA II and LdcCA/CA II inhibition ratios of 540 and 230, respectively.

The noticeable *in vitro* inhibition results of *N*-nitrosulfonamides against TcCA and LdcCA isoforms led us to study the inhibitory activity of some such inhibitors against various *Trypanosoma cruzi* and *Leishmania* forms. Compounds **22** and **23** demonstrated the most potent and selective inhibition against the target CAs and were progressed in the study in the silver salt forms.

The percentages of inhibition of the *Trypanosoma cruzi* epimastigote forms at different concentrations of synthetic compounds are shown in Table 3. The experiments showed that compounds **22** and **23** possess better activity than the reference drug benznidazole (Bnz) against the epimastigotes forms of *Trypanosoma cruzi* in both Dm28c clone and Y strain. At the

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Table 3. Minimum Inhibitory Concentration (MIC) and Concentration That Reduced the Proliferation of Epimastigotes by 50% (IC_{50}) Values Derived from Growth Inhibition Assays of *T. cruzi* Dm28c Clone and Y Strain[a]

	MIC (μM)		IC_{50} (μM)			selectivity index (SI)	
compd	epimastigotes forms *T. cruzi* Dm28c	epimastigotes forms *T. cruzi Y*	epimastigotes forms *T. cruzi* Dm28c	epimastigotes forms *T. cruzi Y*	raw 267.4 cells toxicity CC_{50} (μM)	epimastigotes forms *T. cruzi* Dm28c	epimastigotes forms *T. cruzi Y*
benznidazole	32	32	29.12 ± 3.03	17.00 ± 0.64	137.54 ± 12.05	4.77 ± 0.91	8.09 ± 0.40
22	16	32	5.03 ± 0.95	12.00 ± 1.06	29.28 ± 0.38	5.87 ± 1.14	2.47 ± 0.41
23	32	8	11.99 ± 0.14	2.51 ± 0.40	34.89 ± 3.47	2.32 ± 0.79	11.58 ± 2.72

[a]Determination of cytotoxicity (CC_{50}) and the selectivity index (SI_{50}) of **22** and **23** was done using RAW 264.7 macrophages. Average values of three independent experiments ± standard deviations. SI^3 = IC_{50} Raw 267.4 cells/IC_{50} epimastigote forms of *T. cruzi* Dm28c and *T. cruzi* Y.

Table 4. Analysis of Cytotoxicity and Trypanocidal Effect of Compounds[a]

	IC_{50} (μM)			selectivity index (SI)	
compd	trypomastigotes	intracellular amastigotes	Vero cells toxicity CC_{50} (μM)	trypomastigotes	intracellular amastigotes
benznidazole	15.6 ± 1.9	1.7 ± 0.3	>500	>32	>294.1
22	0.8 ± 0.3	5.2 ± 1.1	21.1 ± 2.8	26.4	4.1
23	3.9 ± 1.1	8.3 ± 1.5	24.1 ± 3.6	6.2	2.9

[a]Average values of three independent experiments ± standard deviations. SI = IC_{50} Vero cells/IC_{50} trypomastigote and intracellular amastigote forms of *T. cruzi*, Dm28c-Luc clone.

concentration of 5.03 ± 0.95 μM compound **22** inhibited by 50% (IC_{50}) the proliferation of *T. cruzi* Dm28c. For *T. cruzi* Y, IC_{50} values were reached at the concentrations of 12.00 ± 1.06 and 2.51 ± 0.40 μM for **22** and **23**, respectively. Anyhow, the two derivatives possess higher toxicity than Bnz for Raw 267.4 macrophages cells (Table 3). As a result, only **22** shows a better SI (5.87 ± 1.14) than benznidazole (4.77 ± 0.91) for *T. cruzi* Dm28c, whereas uniquely **23** display a higher SI for the Y strain of the parasite 11.58 ± 2.72 with respect to the standard (8.09 ± 0.40). Compounds **22** and **23** were also screened against both *T. cruzi* forms relevant to human infection. Table 4 summarizes the trypanocidal activity against the nonreplicative (trypomastigotes) and replicative (amastigotes) stages of *T. cruzi*, Dm28c-Luc clone. Both inhibitors **22** and **23** showed a potent activity against trypomastigotes, reaching IC_{50} values of 4- to 19.5-fold better than Bnz, respectively. For intracellular amastigotes, **22** (IC_{50} = 5.2 ± 1.1) and **23** (IC_{50} = 8.3 ± 1.5) showed lower efficacy than Bnz (IC_{50} = 1.7 ± 0.3). The higher toxicity than Bnz against Vero cells found for compounds **22** and **23** resulted in reduced selectivity index with respect to the reference drug (Table 4).

Minimum inhibitory concentrations (MIC) of **22** and **23** against *Leishamnia infantum* and *L. amazonensis* are shown in Table 5, whereas IC_{50}, CC_{50}, and SI values are represented in Figure 1. While compound **22** shows MIC values of 25 against both *Leishmania* spp, **23** exhibits a 2-fold greater activity against *L.amazonensis* than *L. infantum* (Table 5). These values are higher than the reference drug amphotericin B (AMP).

Table 5. Minimum Inhibitory Concentration (MIC) Assays of *L. amazonensis* and *L. infantum*[a]

	MIC (μM)	
compd	promastigotes forms *L. amazonensis*	promastigotes forms *L. infantum*
amphotericin B	8	8
22	25	25
23	12.5	25

[a]Average values of three independent experiments ± standard deviations.

Derivatives **22** and **23**, respectively, display IC_{50} of 16.61 and 8.43 μM against the promastigote forms of *L.amazonensis* and 16.64 and 17.67 μM for *L. infantum*. The standard AMP is more effective with IC_{50} values of 1.65 and 1.77 μM against the two *Leishmania* sp. Nonetheless, the concentration of AMP, which reduced 50% of RAW 267.4 macrophage cells viability (CC_{50}), is very low (1 μM), whereas inhibitors **22** and **23** possess lower toxicity (CC_{50} values of 29.28 and 34.89 μM, respectively; Figure 1B). As a result of the latter, whereas the SI of the reference compound AMP was more than 2-fold higher than **22** against both Leishmanias, **23** showed a better selectivity profile than AMP against *L. amazonensis* (Figure 1C).

We proposed herein an innovative approach to afford agents for the treatment of Chagas disease and leishmaniasis based on a new CA inhibitory chemotype and on the antimicrobial properties of silver. A set of *N*-nitrosulfonamides and silver salts thereof were screened against a panel of nine human and pathogens isoforms, among which the targets are TcCA and LdcCA. Most such derivatives showed selective (nanomolar) inhibition of the parasite CAs over human ubiquitous ones. The best inhibitors **22** and **23** showed potent inhibition activity against various developmental forms and spp of *Trypanosoma cruzi* and *Leishmania*. The two compounds showed to be more effective than the reference drug benznidazole in inhibiting epimastigote proliferation of both *T. cruzi* stocks belonging to TcI (Dm28c clone) and TcII (Y strain) lineage. Anyhow, their higher toxicity than Bnz against macrophage cells led to SI comparable to the reference drug. Moreover, both tested compounds displayed 4- to 19.5-fold greater efficacy against *T. cruzi* forms relevant to human infection, but rather low SI values were calculated owing to low micromolar inhibition of Vero cells in comparison to Bnz.

Both *L. amazonensis* and *L. infantum* were inhibited by **22** and **23** in a low micromolar range, but less efficiently than the reference drug amphotericin B. Nevertheless, comparable SI values with the standard were calculated based on a rather less toxic effect of **22** and **23** against RAW 264.7 macrophages. The SI of **23** against *L.infantum* is even better than amphotericin B.

The present results make *N*-nitrosulfonamides innovative chemotypes to yield the selective inhibition of the target pathogens CAs over human isoforms. The reported *in vitro*

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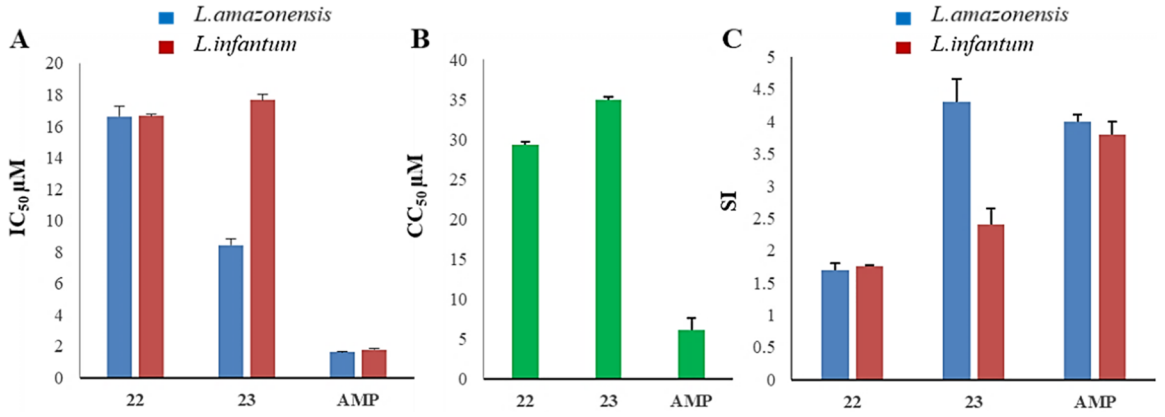

Figure 1. (A) Concentration that reduced the proliferation of promastigotes by 50% (IC_{50} μM). (B) Cytotoxic concentration that reduced 50% of RAW 267.4 cells (CC_{50} μM). (C) Selectivity index: RAW 267.4 cells (CC_{50})/IC_{50} against *L. amazonensis* and *L. infantum*.

assays against various strains of *T. cruzi* and *L. donovani* are of remarkable interest in the field of NTDs and represent a new interesting starting point for the rational CAI optimization for the treatment of Chagas disease and leishmaniasis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00430.

Synthetic procedures, characterization of compounds, *in vitro* kinetic procedure, and antiparasitic and cytotoxicity assays (PDF)

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■ ABBREVIATIONS

CA, carbonic anhydrase; CAI, carbonic anhydrase inhibitor; K_I, inhibition constant; Bzn, benznidazole; AMP, amphotericin B

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