

The carbonic anhydrase enzymes as new targets for the management of neglected tropical diseases

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Abstract

Diseases caused by protozoan parasites represent a huge challenge to global health care, due to the lack of selective and efficient treatments for the management and spreading of such complex pathologies. The protozoans *Trypanosoma cruzi* (*T. cruzi*) and *Leishmania* spp. are the etiological agents of the so-called neglected tropical diseases (NTDs), that is, Chagas disease (CD) and leishmaniasis, respectively. In such a context, the metalloenzymes carbonic anhydrases (CAs; EC 4.2.1.1) emerged as potential protozoan druggable enzymes, being involved in the parasites' life cycle. Several studies suggested the relevance of the protozoan-expressed CAs as future candidates for the management of NTDs.

KEYWORDS

carbonic anhydrases, Chagas disease, *Leishmania chagasi donovani*, leishmaniasis, neglected tropical diseases, *Trypanosoma cruzi* (*T. cruzi*)

1 | INTRODUCTION

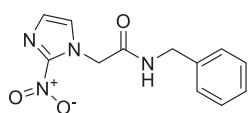
Neglected tropical diseases (NTDs) constitute a huge family of infectious disorders linked together by protozoan parasites, which does make use of human host for their replication cycle and further spreading. Among NTDs, almost 20 diverse conditions are included, which are predominantly widespread as endemic within the tropical areas of the globe. Main feature associated with such diseases is the primary impact on individual health, followed by significant effects at economic and social levels. Overall, NTDs epidemiology proved to be complex due to their multistep life cycle features and, moreover, being often strictly related to environmental circumstances. Within NTDs, our focus regard the main widespread diseases, that is, American trypanosomiasis, also known as Chagas disease (CD) and leishmaniasis.^[1–3]

1.1 | Chagas disease

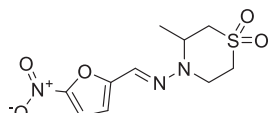
Chagas disease was named after the Brazilian physician and researcher Carlos Ribeiro Justiniano Chagas, who discovered the disease in 1909, identifying the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*) as its causative agent. Approximately 7 million people worldwide are estimated to be infected with *T. cruzi*, with transmission occurring through triatomine bug bites, blood transfusions, or ingestion of contaminated food. Historically endemic to Central and South America, globalization has led to its spread into new regions, making it a global health concern.^[4–8] Infection with *T. cruzi* follows a biphasic pattern: an acute phase lasting 40–60 days marked by mild symptoms (e.g., fever, skin lesions, edema palpebrae/periocular tissues (*Romana sign*) and inflammation of nodules called *Chagoma*) and an asymptomatic chronic phase that can last decades, thus complicating clinical diagnosis.^[9–11]

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Benznidazole



Nifurtimox

FIGURE 1 Chemical structures of benznidazole and nifurtimox belonging to nitroheterocyclic compounds developed for Chagas disease.

About 30% of patients progress to severe cardiac or digestive complications, which can be life-threatening, if untreated. The chronic phase often shows low treatment response, leading to irreversible health issues.^[4]

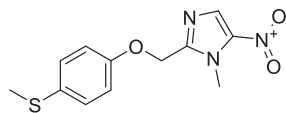
1.2 | Current approved drugs for CD

Up to now, there are no vaccines for CD, thus prevention still remains the best strategy for avoiding health outcomes. Primary prevention focuses on vector control and transmission interruption, blood donor screening, and insecticide application in infested areas. Few drugs are developed for such diseases since they are considered neglected, and there is limited industry interest in discovering new effective treatments.^[12,13]

Barriers to development include the lack of biomarkers for disease stages and the genetic diversity of *T. cruzi* strains. Current treatment relies on small molecules like nifurtimox and benznidazole (Figure 1), with the latter being preferred for its better tissue penetration and remarkable safety, although its treatment is endowed with adverse reactions, including allergic dermatitis and gastrointestinal issues. Nifurtimox is effective when administered early but has a high incidence of side effects (43%–97.5%).^[14–21] Both drugs generate free radicals that target *T. cruzi*, but their effectiveness is limited to early infection stages, and resistance is emerging.^[22,23] Therefore, novel therapeutic alternatives are urgently needed. Therefore, research is ongoing to develop new therapies, including optimizing existing treatments through dosage adjustments and exploring drug repurposing strategies, alongside combination therapies to enhance efficacy while minimizing toxicity and resistance risks. New strategies involve repurposing existing drugs like allopurinol and triazole derivatives, which have shown promising results in reducing parasite load and inflammation. The ongoing globalization of CD necessitates the rapid development of new pharmacological treatments to address its spread beyond traditional tropical regions.^[6,8]

1.3 | New Chagas treatments

Since the introduction of treatments for CD, researchers have sought new methods to enhance disease management due to the increasing resistance of *T. cruzi* to both benznidazole and nifurtimox, as well as the presence of multiple parasite strains in individual patients. Efforts to optimize benznidazole therapy include dose reductions, intermittent



Fexinidazole

FIGURE 2 Structure of fexinidazole.

dosing, and combination therapies, which have shown improved outcomes but remain unimplemented in clinical practice.^[24–28]

Repurposed drugs such as allopurinol and triazole derivatives have been explored in clinical trials.^[29] Administering allopurinol after benznidazole treatment has demonstrated reduced parasite load and inflammation.^[30] Monotherapy with triazole antifungals like posaconazole^[31] and ravuconazole^[32,33] has also shown effectiveness against chronic infections.^[32,34–40]

Fexinidazole (Figure 2), currently in Phase II/III trials for late-stage CD, shares a similar action mechanism with azole derivatives but has a unique toxicity profile due to its metabolites.^[41–43] Experimental compounds like D0870 have shown promise in acute and chronic models but faced development halts due to cardiac events.^[44–47] In contrast, TAK187 has demonstrated better efficacy against cardiac damage in both infection stages.^[48,49]

New therapeutic strategies include synergistic therapies combining benznidazole with azoles like itraconazole or nitric oxide, which can reduce parasitemia and inflammation. This combination approach aims to enhance treatment efficacy while minimizing toxicity and resistance risks.^[50] However, the lack of validated biomarkers for assessing treatment response remains a significant challenge in managing chronic cases.^[51]

1.4 | Leishmaniasis

Leishmaniasis is a neglected vector-borne disease caused by protozoan parasites of the genus *Leishmania*, primarily affecting impoverished populations in tropical and subtropical regions. Transmitted by infected female phlebotomine sandflies, over 1.5 billion people live in endemic areas, facing a significant disease burden that results in over 30,000 deaths annually.^[52,53]

The *Leishmania* parasites exist in two forms: promastigotes and amastigotes.^[54] Infection begins when sandflies inject promastigotes into the host, which are then phagocytosed by macrophages and transform into amastigotes, causing disease.^[55–57] Leishmaniasis can manifest as cutaneous, mucocutaneous, or visceral forms. Cutaneous leishmaniasis presents as skin ulcers, while the visceral form can be fatal if untreated, leading to symptoms like fever, weight loss, and organ enlargement.^[58,59]

1.5 | Leishmaniasis treatment

Leishmaniasis is described as the condition in which the patient becomes ill due to *Leishmania* infection, described as more than just

FIGURE 3 Structure of pentavalent antimonials used as intravenous treatment.

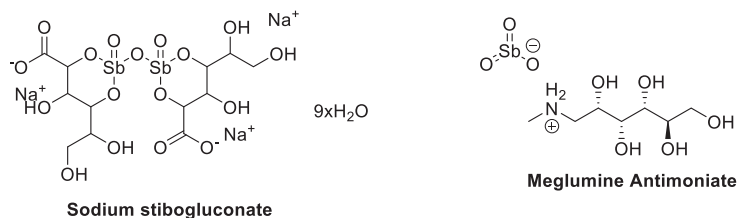
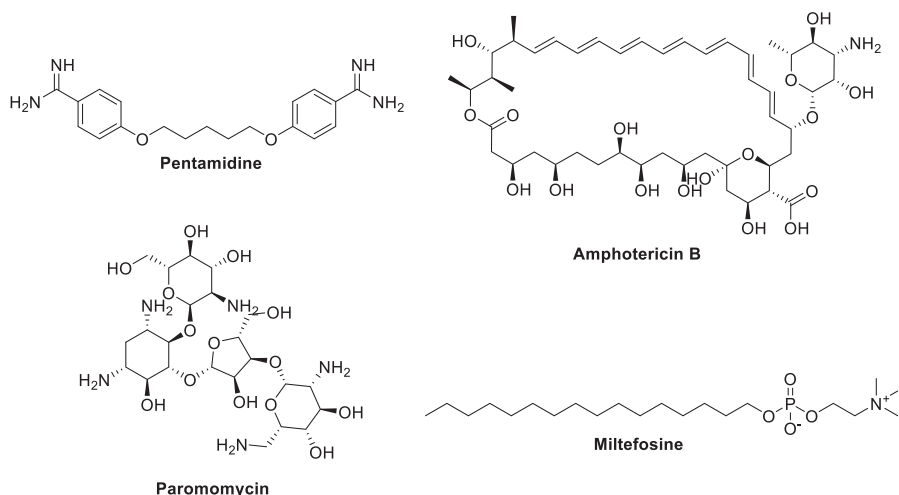


FIGURE 4 Alternative medications for leishmaniasis treatment.



carrying the parasite, although most infected individuals remain asymptomatic, making early diagnosis critical. Treatment options are limited due to high costs and potential adverse reactions, with drug resistance complicating management. Moreover, the ongoing lack of an approved vaccine highlights the need for continued research into effective therapies.

Currently, pentavalent antimonials such as sodium stibogluconate or meglumine antimoniate (Figure 3) are the primary treatment despite emerging resistance and required hospitalization for administration over approximately 30 days, since they are administered through the intravenous route,^[60,61] and with several mechanisms of action which have been proposed to explain their antileishmanial activity.^[62–65] Nevertheless, the strong antileishmanial activity exhibited by pentavalent antimonials is surpassed by their cardiotoxic, nephrotoxic, and hepatotoxic effects.^[66,67]

Alternative treatments include amphotericin B, pentamidine, paromomycin, and miltefosine (Figure 4), each with specific efficacy depending on the *Leishmania* species involved. However, these drugs can have severe side effects and potential for resistance for leishmaniasis.^[68–75]

2 | CARBONIC ANHYDRASES (CAs): A NEW PROMISING TARGET

Moreover, no progress in new drug approval was done since 1960s, based on a lack of interest from pharmaceutical companies along with inadequate methodologies for findings translation from in vitro and in vivo studies into new drugs discovery, thus making disease control

even more difficult. For such reasons, therapeutic strategies have shifted toward the exploration of new targets endowed with relevant role in disease-advanced treatments: In this regard, in recent years, many efforts were directed leading through the discovery of an enzyme involved in protozoan survival, CA (EC 4.2.1.1).^[76] Such enzyme represents an innovative and high promising druggable target for the design, synthesis, and development of new compounds for the management of diseases progression. Furthermore, it is essential for proper cellular functioning and plays an important role in the infection process operated by the protozoan to the host organism.^[76]

CAs are widespread metalloenzymes all over the phylogenetic tree, present ubiquitously both in prokaryotes and eukaryotes. Classified as a superfamily of enzymes, up to date eight distinct families were discovered, phylogenetically and evolutionarily distinct from each other, indicated with the Greek letters α , β , γ , δ , ζ , η , θ e ι .^[77] Such enzymes do catalyze a very simple physiological reaction, the reversible hydration of carbon dioxide to bicarbonate and a proton, and are thus involved in crucial physiological processes connected with respiration and transport of CO₂/bicarbonate between metabolizing tissues and lungs, pH and CO₂ homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes.^[78–84] As mentioned above, CAs are defined as metalloenzymes, hence their catalytic sites contain a metal ion cofactor. The metal ion, typically Zn (II) in all known α -CAs, is essential for catalysis.^[78–81,85,86] X-ray crystallographic studies proved that this ion is situated at the bottom of a 15 Å deep active site cleft, thus coordinated by three histidine residues (His94, His96, and His119) and a water molecule/hydroxide

ion.^[78–81,84–87] Additionally, the zinc-bound water molecule engages hydrogen bond interactions with the hydroxyl group of Thr199, which, in turn, interacts with the carboxylate group of Glu106, which is essential for the enzyme mechanism of action.^[76]

Up to now, only one α -class carbonic anhydrase (α -CA) was identified, cloned, and characterized by our groups in the genome of *T. cruzi*, denominated TcCA, several years ago by Pan et al.,^[88] leading to the purified enzyme which showed high catalytic activity for the conversion CO₂ into bicarbonate and protons.^[89–93]

As well as TcCA, *Leishmania donovani chagasi* carbonic anhydrase (LdcCA), a β -CA isoform was identified, cloned, and characterized in 2013.^[88,94]

Such metalloenzyme is deeply implicated in the growth and virulence mechanisms of the pathogen, playing key roles in respiration, CO₂ and bicarbonate transport, pH regulation, electrolyte secretions, and various biosynthetic reactions. Inhibition of TcCA may be critical for eliminating the parasite, though the role of the enzyme within the pathogen organism remains still poorly understood. Such inhibition results in alterations on ion exchange mechanisms, strongly reducing the parasite's metacyclogenesis capacity and causing its growth inhibition. Consequently, these biological disruptions led the parasite to a large amount of suffered stress, eventually resulting in its death.^[95,96]

Several in vitro studies in literature focus on establishing comprehensive structure–activity relationships (SAR) for TcCA or LdcCA-directed inhibitors, involving screening of primary sulfonamides, sulfamates, thiols, hydroxamates, and phenols, which will be individually discussed further.^[88,97–106] Nevertheless, the interesting in vitro and ex vivo results obtained with numerous classes of CAs prompted us to further investigate novel inhibitors targeting such pathogenic enzymes.

Recently findings showed that bacterial CA's can act as a drug that targets the development of antibiotics.^[107] and as an efficient way to overcome drug resistance^[108] were subjected to in vivo validation of some of them by means of the development of accurate animal models of such bacterial diseases. In contrast, concerning protozoan CAs, although there are plenty of effective and fairly selective in vitro inhibitors, the lack of animal models for most of these infections poses a significant challenge. Such issues are related to the intricate life cycles of these pathogens.^[109]

The complexity is particularly evident in CD: *T. cruzi* has two distinct forms, with the former being the trypomastigotes, the circulating infective but nonreplicative form, and the latter being the amastigotes, the replicative intracellular form, both of which were endowed with infection characteristics. Consequently, the inability to evaluate the efficacy of newly developed enzyme inhibitors, such as those being investigated here, on both forms of *T. cruzi* makes it challenging to accurately assess the actual contribution of protozoan CAs to the pathogenicity and infectivity of these protozoa.^[109]

Thus, as long as it will be impossible to test the efficacy of newly designed enzyme inhibitors, as those investigated here, on both evolutive forms of *T. cruzi*, it is difficult to estimate the

real contribution of protozoan CAs on their pathogenicity and infectiveness.^[101]

2.1 | *T. cruzi* TcCA and *Leishmania donovani chagasi* LdcCA inhibition

2.1.1 | Sulfonamide inhibitors

Sulfonamides represent the main class of zinc-binding CA inhibitors, and they were the first compounds endowed with antimicrobial features, discovered by Domagk in 1935. Beyond their antimicrobial utility, sulfonamides are also used with versatile applications as diuretics and in clinical pathologies such as glaucoma and epilepsy.^[83,110] Moreover, they are under investigation for potential roles in obesity treatment and have recently entered into advanced clinical trials for cancer therapy.^[111–113]

For *T. cruzi*, several aromatic and heterocyclic sulfonamides were explored as TcCA inhibitors, with structures displayed in Figure 5. Among them, classically and sistemically CA inhibitors were tested and evaluated, such as acetazolamide (AAZ), methazolamide (MZA), ethoxzolamide (EZA), and dichlorophenamide (DCP). Furthermore, other sulfonamides were used, such as dorzolamide (DZA) and brinzolamide (BRZ), anti-glaucoma agents with topical action, benzolamide (BZA), orphan sulfonamide drug, zonisamide (ZNS), topiramate (TPM) and sulthiame (SLT), widely used for antiepileptic treatment. Moreover, valdecoxib (VLX), indisulam (IND), saccharin (SAC), celecoxib (CLX), sulpiride (SLP), and hydrochlorothiazide (HCT) were evaluated, displaying inhibition constants in the range of 61.6–93.6 nM for some of such classes against constitutive human isoforms hCA I/II, as off-target enzymes, and the protozoan enzyme TcCA, showing on this isoform greater inhibition and a positive selectivity. Nevertheless, some sulfonamide derivatives, consisting of aromatic derivatives **1–10** and **15–18**, did not show inhibition against such isoform; conversely, sulfonamides **11–14**, **19**, **21–24**, DCP, ZNS, and HCT exhibited greater potency, with *K_i* values within the range of 128–867 nM (Table 1).

However, elucidating a structure–activity relationship (SAR) for such compounds is difficult due to the diversity of scaffolds, for instance, disulfamoylbenzene derivatives (**11–12** and HCT), or heterocyclic sulfonamides (**13**, **14**, and ZNS), and so on. Nevertheless, some sulfonamide derivatives have emerged as potent inhibitors of TcCA, with *K_i* values below 100 nM, such as AAZ, EZA, DZA, TPM, SLP, SLT, and compound **20**.

Simultaneously, their inhibitor efficacy against LdcCA was assessed using both aromatic/heterocyclic sulfonamides and 5-mercapto-1,3,4-thiadiazoles, by testing the same inhibitors used for *T. cruzi*. However, several sulfonamides, including compounds **1–17**, **19**, and clinically used drugs such as DCP, DZA, BRZ, BZA, TPM, ZNS, SLP, IND, VLX, CLX, SLT, and SAC, showed to be ineffective inhibitors. Conversely, derivatives **18**, **20–24**, AAZ, MZA, EZA, and HCT exhibited enhanced effectiveness, showing *K_i* values ranging from 50.2 to 95.1 nM, with the most potent inhibitors revealed once again to be the thiols.

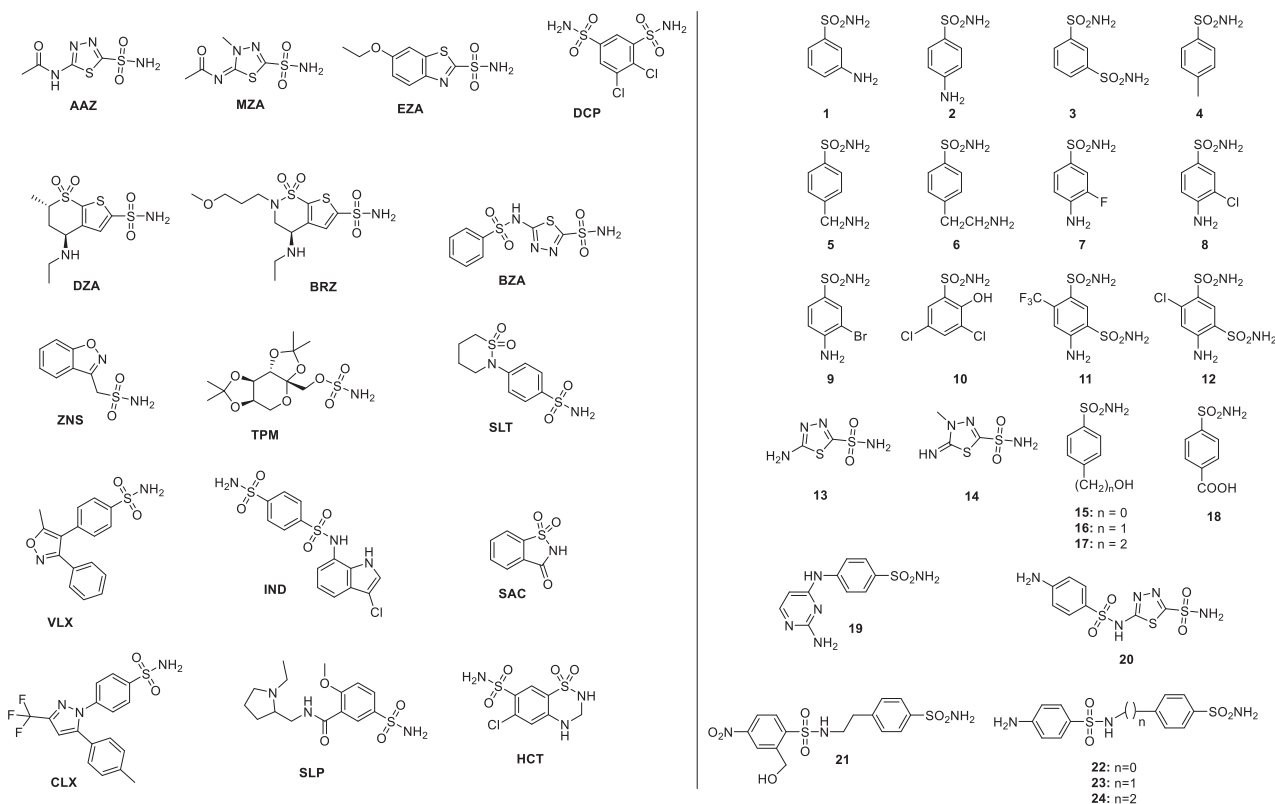


FIGURE 5 Structures of acetazolamide AAZ-HCT and 1-24, representing available CA inhibitors. AAZ, hydrochlorothiazide; CA, carbonic anhydrase; HCT, hydrochlorothiazide.

Despite their efficacy as TcCA or LdcCA inhibitors, sulfonamides were associated with difficulties in their in vitro testing due to their low lipophilicity, limiting their efficacy against the parasite enzyme. In contrast, thiol-based inhibitors have shown enhanced in vivo inhibitory activity, probably attributed to their increased lipophilicity, which facilitates penetration into parasite membranes.

Interestingly, while certain aromatic and heterocyclic sulfonamides incorporating halogen or methoxyphenyl acetamide tails have shown remarkable TcCA inhibition, with K_i values in the range of 0.5–12.5 nM, their highly polar nature makes them ineffective as anti-trypanosomal agents, due to their inability to cross biological membranes effectively.^[104]

2.1.2 | Different substituted sulfonamides

After the revealed high inhibition activity for sulfonamide compounds, many strategies were pursued for the development of new compounds endowed with selectivity toward protozoans CAs. Among these strategies, nitroaromatic benzenesulfonamides were discovered to possess nanomolar-to-micromolar inhibition constant toward the inhibition of both protozoan parasite, *T. cruzi*, and *Leishmania* spp. Design of N-nitrosulfonamides was driven by the presence of the nitro group observed in various anti-protozoan agents, such as the nitroimidazoles, displayed in Figure 6.

This structural feature is essential for the mechanism of action of these drugs, confirming its potential role as a strong protozoan inhibitor. Inhibition data presented in Table 2 provide valuable insights for constructing a structure–activity relationship (SAR).^[100]

The investigated 3-nitrobenzenesulfonamides displayed effective inhibition of TcCA, with K_i s ranging from medium nanomolar to low micromolar values from 0.08 to 4.79 μ M. Notably, compound 2a emerged as the most potent TcCA inhibitor with a K_i of 80 nM, endowed with minimal steric hindrance. However, introducing some groups, also small ones such as nitro, amino, or hydroxy groups, resulted in decreased inhibitory efficacy. This indicates that increasing steric hindrance at position 4 adversely affects the binding affinity for the compound to TcCA. The same trend was obtained for ureas compound, in which the unsubstituted phenyl derivative 8a and the pentafluorinated derivative 12a exhibited the most effective inhibition, with K_i values of 0.32 and 0.28 μ M, respectively. Conversely, other substitutions on the ureido-aromatic ring impacted inhibition activity.

The inhibition profiles for LdcCA share similarities with those observed for TcCA, with the simplest derivatives—namely 2a, 3a, 4a, and 5a—exhibit the most potent inhibition of LdcCA, with K_i s between 0.21 and 0.46 μ M, respectively. Increasing steric hindrance is also associated with reduced LdcCA inhibitory properties, resulting in micromolar range K_i s. In general, most compounds exhibit greater selectivity against TcCA and LdcCA than CA I, while only few

TABLE 1 Inhibition of human isoforms hCA I and hCA II, and protozoan-associated isoforms, *Trypanosoma cruzi* (TcCA) and *Leishmania chagasi donovani* (LdcCA), by a CO₂ hydrase, stopped-flow assay with sulfonamides 1–24, and the clinically used agents AAZ-HCT.

Inhibitor	K _i (nM) ^a			
	hCA I	hCA II	TcCA	LdcCA
1	28,000	300	25,460	5960
2	25,000	240	57,300	9251
3	79	8	63,800	8910
4	78,500	320	44,200	>100,000
5	25,000	170	7231	>100,000
6	21,000	160	9238	>100,000
7	8300	60	8130	15,600
8	9800	110	6925	9058
9	6500	40	8520	8420
10	7300	54	9433	9135
11	5800	63	842	9083
12	8400	75	820	4819
13	8600	60	534	584
14	9300	19	652	433
15	5500	80	73,880	927
16	9500	94	71,850	389
17	21,000	125	66750	227
18	164	46	84,000	59.6
19	109	33	810	>100,000
20	6	2	88.5	95.1
21	69	11	134	50.2
22	164	46	365	136
23	109	33	243	87.1
24	95	30	192	73.4
AAZ	250	12	61.6	91.7
MZA	50	14	74.9	87.1
EZA	25	8	88.2	51.5
DCP	1200	38	128	189
DZA	50,000	9	92.9	806
BRZ	45,000	3	87.3	764
BZA	15	9	93.6	236
ZNS	56	35	867	>100,000
TPM	250	10	85.5	>100,000
SLT	374	9	71.9	834
VLX	54,000	43	82.7	338

TABLE 1 (Continued)

Inhibitor	K _i (nM) ^a			
	hCA I	hCA II	TcCA	LdcCA
IND	31	15	84.5	316
SAC	18,540	5959	8210	>100,000
CLX	50,000	21	91.1	705
SLP	1200	40	87.9	>100,000
HCT	328	290	134	50.2

Abbreviations: AAZ, hydrochlorothiazide; hCA, human carbonic anhydrase; HCT, hydrochlorothiazide.

^aMean from three different assays, by a stopped-flow technique (errors were in the range of ±5%–10% of the reported values).

derivatives inhibit both protozoan isoforms more efficiently than CA II. However, all compounds demonstrate lower inhibition of the screened isoforms compared with the standard AAZ, albeit lacking selectivity for the target TcCA and LdcCA when compared to the ubiquitous hCAs.

Additionally, Bonardi et al.^[99] proved N-nitrosulfonamides (Figure 7) as a newly scaffold capable of inhibition toward protozoan isoforms, providing strong inhibition of the targeted CA isoform, with data displayed in Table 3.

It was shown that all N-nitro aromatic sulfonamides displayed limited effectiveness in inhibiting CA I and II, with K_i values ranging from low to medium micromolar concentrations (2.2–80.6 μM). Notably, among such compounds, heteroaromatic derivatives **9b** and **10b** emerged as the most potent inhibitors against such isoforms, while a significant selectivity was obtained toward protozoan CA isoforms with submicromolar K_i values (0.32–0.52). Furthermore, TcCA was found to be the most affected α-CA among those studied, with most derivatives inhibiting TcCA in the medium nanomolar range (0.10–0.85 μM), showing a quite good selectivity for protozoan isoform rather than human enzymes.

Incorporating primary amino groups at the *meta*- or *para*-position of the phenyl ring conferred the greatest TcCA inhibitory properties to compounds **2b** and **3b**. According to previously reported inhibition data,^[114] β-CAs were generally more efficiently inhibited by N-nitrosulfonamides than α-CAs. Thus, the selectivity profiles against target and off-target CAs varied significantly among N-nitrosulfonamide derivatives, with some compounds displaying striking selectivity toward TcCA and LdcCA over ubiquitous human isoforms CA I and II by up to two orders of magnitude. For instance, derivative **3b** exhibited inhibition ratios of 540 for TcCA/CA II and 230 for LdcCA/CA II. Such promising in vitro inhibition results against TcCA and LdcCA prompted further investigation into the inhibitory activity of selected inhibitors against various forms of *T. cruzi* and *Leishmania*, for instance, for compounds **2b** and **3b** which demonstrated potent and selective inhibition against the target CAs.

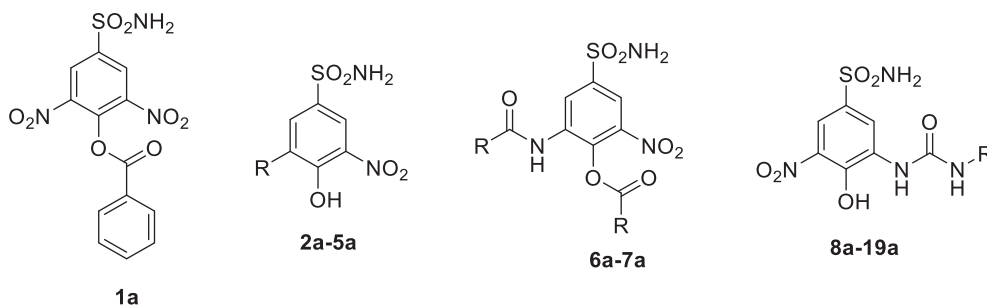


FIGURE 6 Structures of 3-nitrobenzenesulfonamides synthesized 1a-19a.

TABLE 2 Inhibition of human isoforms hCA I and hCA II, and protozoan-associated isoforms, *Trypanosoma cruzi* (TcCA) and *Leishmania chagasi donovani* (LdcCA), by a CO₂ hydrase, stopped-flow assay with 3-nitrobenzenesulfonamides compounds 1a-19a using AAZ as a reference drug.

Compound	K _i (μM) ^a			
	hCA I	hCA II	TcCA	LdcCA
1a	4.79	0.84	2.52	4.68
2a	0.91	0.24	0.08	0.21
3a	4.35	0.18	0.16	0.34
4a	6.18	0.61	0.24	0.46
5a	6.21	0.64	0.11	0.39
6a	1.38	0.39	3.54	3.87
7a	2.92	0.46	4.79	8.49
8a	>50	2.78	0.32	1.06
9a	5.39	0.53	0.46	0.98
10a	5.20	0.24	0.51	2.34
11a	7.58	0.21	0.38	2.96
12a	0.69	0.27	0.28	1.36
13a	8.21	5.15	0.91	0.95
14a	>50	4.33	1.02	2.03
15a	8.33	0.45	0.69	1.86
16a	5.99	1.72	1.35	3.65
17a	9.29	3.08	0.74	0.86
18a	>50	2.53	0.41	1.02
19a	5.67	1.89	2.47	3.64
AAZ	0.25	0.012	0.06	0.09

Note: The different substituent groups R are available in the reference article.^[100]

Abbreviations: AAZ, hydrochlorothiazide; hCA, human carbonic anhydrase.

^aMean from three different assays, by a stopped-flow technique (errors were in the range of ±5%–10% of the reported values).

2.1.3 | Anionic inhibitors

Anionic inhibitors, along with other small molecules like sulfamide, sulfamic acid, and phenylboronic/arsonic acids, endowed with the ability to interact with zinc proteins were tested against TcCA, with data reported in Table 4. Among them, diethyldithiocarbamate emerged as the most potent inhibitor ($K_i = 5 \mu\text{M}$). However, iodide, cyanate, thiocyanate, hydrogen sulfide, and trithiocarbonate also exhibited inhibitory effects on the enzyme, albeit activity in the low micromolar range (K_i s ranging from 44 to 93 μM).^[88]

2.1.4 | Hydroxamates

Hydroxamic acid derivatives (in Figure 8) have emerged as versatile tools endowed with several applications in design for therapeutics targeting a spectrum of diseases, spanning from cancer and cardiovascular disorders to HIV, Alzheimer's, malaria, allergies, tuberculosis, and antimicrobial interventions.^[115] Such compounds do inhibit the enzyme through their metal ion chelating properties,^[116] coordinating to the metal site particularly in metalloenzymes such as matrix metalloproteinases (MMPs) and CAs.^[117,118]

Although their binding modalities may vary, the prevailing mode involves bidentate coordination, where the deprotonated oxygens of the hydroxamate or hydroximate moiety (Figure 8) form a very stable 5-membered ring with the metal. Monodentate binding mode, by means of the deprotonated atom of nitrogen or oxygen, was also reported, although it requires specially designed coordination environments for providing additional stability.^[119]

Several experiments showed the inhibitory potential of hydroxamic acids against α -CA from *T. cruzi* and its associated peptidases, including metallo- and cysteine peptidases.^[103,120,121] To explore further, a series of 4,5-dihydroisoxazoles bearing the hydroxamate moieties (Figure 9) such as compounds 1c-12c underwent synthesis and evaluation against both human isoform hCA I and hCA II, and protozoan isoform expressed in *T. cruzi* (Table 5).

Among these compounds which showed mid-high inhibition features, compound 7c displayed potent inhibitory activity against

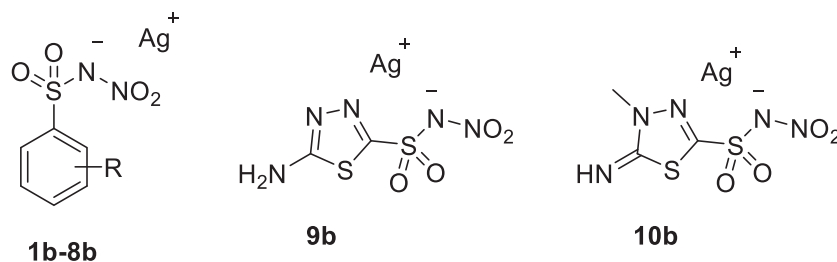


FIGURE 7 Structures of N-nitrosulfonamides synthesized **1b–10b**.

TABLE 3 Inhibition of human isoforms hCA I and hCA II, and protozoan-associated isoforms, *Trypanosoma cruzi* (TcCA) and *Leishmania chagasi donovani* (LdcCA), by a CO₂ hydrase, stopped-flow assay with N-nitrosulfonamides compounds **1b–10b** using AAZ as a reference drug.

Compound	K_i (μM) ^a			
	hCA I	hCA II	TcCA	LdcCA
1b	29.0	60.9	3.2	4.7
2b	54.7	7.7	0.15	0.49
3b	67.4	53.4	0.10	0.23
4b	80.6	6.2	5.0	4.8
5b	45.9	18.1	1.4	0.50
6b	58.3	64.2	0.43	0.65
7b	39.6	55.8	0.47	0.71
8b	19.8	45.0	0.85	1.0
9b	7.3	2.9	0.35	0.52
10b	4.9	2.2	0.32	0.44
AAZ	0.25	0.012	0.06	0.09

Note: The different substituent groups R are available in the reference article.^[99]

Abbreviations: AAZ, hydrochlorothiazide; hCA, human carbonic anhydrase.

^aMean from three different assays, by a stopped-flow technique (errors were in the range of $\pm 5\%$ – 10% of the reported values).

TcCA in vitro, thus determining inhibition of parasite growth across various developmental stages at very low concentrations. Importantly, compound **7c** demonstrated selective action against parasite cells without eliciting cytotoxic effects on macrophages, through eliminating the parasite from infected cells with a selectivity index (SI) of 6.7. Moreover, such selectivity was afforded by detection through an in vitro macrophage cytotoxicity assay. In vivo preliminary studies in *T. cruzi*-infected mice confirmed its efficacy, showing a greater reduction of parasites in the bloodstream than the standard drug, benznidazole.^[103]

Such compound showed the most promising inhibition against TcCA ($K_i = 39.8$ nM) and only moderate inhibition toward human isoforms CA I and CA II (K_i hCA I = 641 nM, K_i hCA II = 815 nM).

The crucial role of the hydroxamic acid moiety in anti-*T. cruzi* activity was evident from a comparative analysis between compounds bearing or not such scaffold, as its absence resulted in the

loss of both antiparasitic and enzyme inhibitory activities. Furthermore, the inclusion of a p-benzyloxy moiety in compound **7c** enhanced its efficacy against trypomastigote forms without affecting its action against replicative forms, amastigotes. These findings suggest that such compounds do inhibit whether parasite peptidases and CA, both essential for the parasite's life cycle and dependent on effective chelation.

In this compound series, the hydroxamate moiety was indispensable for anti-*T. cruzi* activity, as its replacement in compound **11c** by an acyl hydrazine moiety in compound **12c** led to a complete loss of activity in enzyme inhibition against TcCA and hCA I/II (Table 5).^[103,122]

2.1.5 | Thiols

Similar to previous zinc-binding groups, such as sulfonamide and hydroxamates moieties, thiols were investigated to afford their useful role in CA inhibition as possible TcCA inhibitors, assuming the mercapto moiety as an efficient and well-documented promising zinc-binding group (in ionized, anionic form, similar to the SO_2NH^- one) for obtaining effective CAIs (Figure 10).^[88,123,124]

Previously reported by our group, a 1,3,4- thiadiazole-5-mercapto derivatives **1d–10d** series showed medium potency inhibition on hCA I and hCA II, further investigated for their interaction with TcCA and LdcCA, with in vivo evaluations of their effect on the promastigote phase of *Leishmania chagasi* (Table 6).^[101,125]

Data displayed in Table 6 showed relevant TcCA inhibitory properties, with inhibition constant in the nanomolar range between 21.1 and 125 nM, suggesting strong inhibitory properties of Schiff bases among other derivatives. Therefore, it is evident how slight modifications to compound scaffold, whether being thiol or sulfonamide moiety, can lead to dramatic changes in its affinity for the protozoan enzyme TcCA. Thiols showed better inhibition profiles toward TcCA (nanomolar range K_i values) than human CA I and CA II (micromolar range K_i values), which underscores the huge significance to achieve selective inhibition of the parasite over the host enzymes, thus limiting the risk of side effects due to off-target inhibition.^[126] Moreover, among thiol-based inhibitors, Schiff bases **2d** and **4d** incorporating phenylazomethine and 3-methoxyphenylazomethine moieties have displayed particularly potent inhibition (K_i values of 21.1–34.5 nM).^[88,89,101]

Toward LdcCA conversely with TcCA inhibition, the semi-carbazide derivative **1d** effectively inhibited LdcCA (K_i of 74.1 nM),

TABLE 4 Inhibition of human isoforms hCA I and hCA II, and protozoan-associated isoform of *Trypanosoma cruzi* (TcCA) by a CO₂ hydrase, stopped-flow assay by different anion inhibitors using AAZ as a reference drug.

Inhibitor ^b	K _i (mM) ^a		
	hCA I	hCA II	TcCA
F ⁻	>300	>300	0.90
Cl ⁻	6	200	0.81
Br ⁻	4	63	0.73
I ⁻	0.3	26	0.044
CNO ⁻	0.0007	0.03	0.080
CN ⁻	0.0005	0.02	1.01
N ₃ ⁻	0.0012	1.51	1.12
HCO ₃ ⁻	12	85	0.58
CO ₃ ²⁻	15	73	0.69
NO ₃ ⁻	7	35	0.77
NO ₂ ⁻	8.4	63	0.70
HS ⁻	0.0006	0.04	0.078
HSO ₃ ⁻	18	89	0.91
SnO ₃ ²⁻	0.57	0.83	0.72
SeO ₄ ²⁻	118	112	0.66
TeO ₄ ²⁻	0.66	0.92	0.71
OsO ₅ ²⁻	0.92	0.95	0.80
S ₂ O ₇ ²⁻	0.99	0.97	0.76
P ₂ O ₇ ⁴⁻	25.77	48.5	16.9
V ₂ O ₇ ⁴⁻	0.54	0.57	0.68
B ₄ O ₇ ²⁻	0.64	0.95	24.3
ReO ₄ ⁻	0.11	0.75	0.65
RuO ₄ ⁻	0.101	0.69	0.65
S ₂ O ₈ ²⁻	0.107	0.084	0.84
SeCN ⁻	0.085	0.086	0.78
CS ₃ ²⁻	0.0087	0.0088	0.093
Et ₂ NCS ₂ ⁻	0.00079	0.0031	0.005
SO ₄ ²⁻	63	>200	6.9
ClO ₄ ⁻	>200	>200	>100
BF ₄ ⁻	>200	>200	>100
FSO ₃ ⁻	0.79	0.46	15.8
NH(SO ₃) ₂ ²⁻	0.31	0.76	7.1
H ₂ NSO ₂ NH ₂	0.31	1.13	0.12
H ₂ NSO ₃ H	0.021	0.39	10.6
Ph-B(OH) ₂	58.6	23.1	0.86

TABLE 4 (Continued)

Inhibitor ^b	K _i (mM) ^a		
	hCA I	hCA II	TcCA
Ph-AsO ₃ H ₂	31.7	49.2	0.62
AAZ	0.00025	1.2 × 10 ⁻⁵	6.2 × 10 ⁻⁵

Abbreviations: AAZ, hydrochlorothiazide; hCA, human carbonic anhydrase.

^aMean from three different assays, by a stopped-flow technique (errors were in the range of ±5%–10% of the reported values).

^bAs sodium salt.

while compounds **2d–6d** and **8d** showed good inhibition of *Leishmania* enzyme, comparable to the one obtained toward TcCA with K_i values ranging from 13.4 to 40.1 nM

For in vivo experiments, promastigote forms of *L. chagasi* and *Leishmania amazonensis* (Raimundo strains) were treated with inhibitors **1d–8d** at a concentration of 256 μM. Among these, inhibitor **5d** exhibited the highest inhibition activity, inhibiting promastigote growth by 100% and 97%, respectively.^[88,89,101]

2.1.6 | Phenols

In addition to previously discussed scaffolds, CAs are targeted by several classes of inhibitors that interact with the enzyme in intricate ways.^[127–129] Traditional inhibitors like inorganic/organic anions, sulfonamides, and their isosteres (sulfamides and sulfamates) displace the coordinated nucleophilic water molecule or hydroxide ion, crucial for catalysis, from catalytic metal ion.^[130,131]

However, newer classes of CA inhibitors recently identified, such as phenols, exhibit different ways of interaction. The inhibition process concerns anchoring to the metal-ion coordinated water molecule, blocking the entrance to the active site cavity, or binding outside the active site.^[132–136] Phenols, in particular, have been observed through X-ray crystallography^[137] to form hydrogen bonds with the zinc-coordinated water molecule by using their OH moiety, often interacting strongly with amino acid residues near the catalytic core in various CA isoforms.^[129]

Phenols-based compounds, although well-known human CAs inhibitors (displayed in Figure 11), have recently reported for the first time as innovative moieties for the inhibition of protozoan CAs, that is, TcCA, with a comparison of such inhibition with constitutive human CA isoforms, CA I and CA II: Such results were demonstrated by Bonardi et al.^[97] and reported in Table 7.^[138–140]

All phenolic compounds investigated **1e–22e**, exhibited inhibition of TcCA within the micromolar range, ranging from 1.8 to 47.9 μM, while the most effective inhibitors of TcCA were identified as compounds **1e**, **2e**, **10e**, **16e**, **18e**, and **20e–22e**, with K_i between

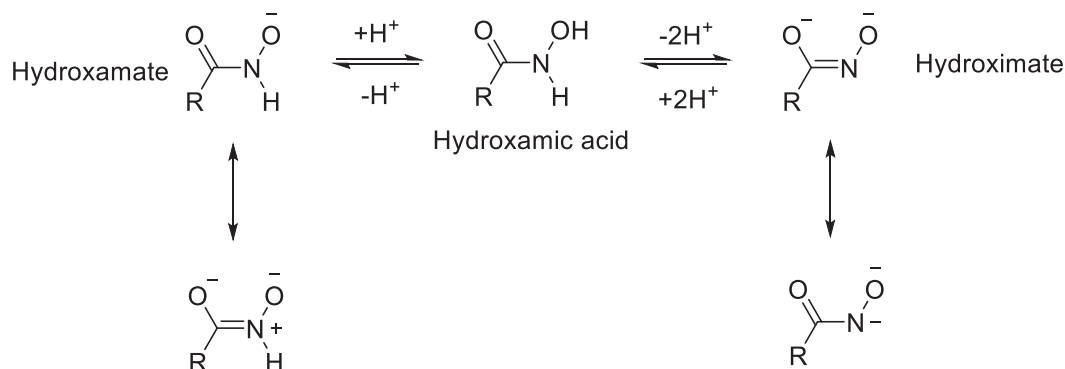


FIGURE 8 Hydroxamic acids, hydroxamates, and hydroximates structures and their resonance hybrids.

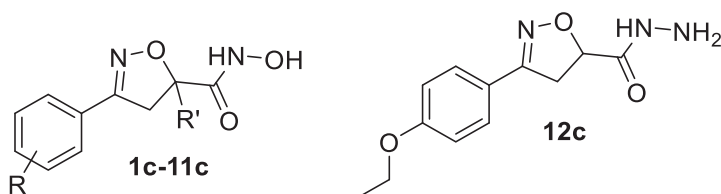


FIGURE 9 Chemical structures of different hydroxamates synthesized 1c-12c.

TABLE 5 Inhibition of human isoforms hCA I and hCA II, and protozoan-associated isoform of *Trypanosoma cruzi* (TcCA) by a CO₂ hydratase, stopped-flow assay with hydroxamates compounds 1c-12c using AAZ as a reference drug.

Compound	<i>K_i</i> (nM) ^a		
	hCA I	hCA II	TcCA
1c	612	802	428
2c	516	27,900	263
3c	133	47,300	267
4c	3240	94,500	189
5c	72,200	297	94.1
6c	47,000	808	71.3
7c	641	815	39.8
8c	28,000	847	365
9c	598	733	615
10c	47,600	257	182
11c	64,000	3810	141
12c	>100,000	>100,000	>100,000
AAZ	250.0	12.1	61.6

Note: The different substituent groups R are available in the reference article.^[103]

Abbreviations: AAZ, hydrochlorothiazide; hCA, human carbonic anhydrase.

^aMean from three different assays, by a stopped-flow technique (errors were in the range of ±5%–10% of the reported values).

1.8 and 7.3 μM. Notably, while caffeic acid contains the pyrocatechol fragment found in compound 18e, which is the most effective inhibitor, its inhibitory activity was not consistently the same. For instance, compound 5e, despite incorporating this fragment, only exhibited moderate inhibition with a *K_i* of 13.2 μM. The structural diversity among such derivatives makes discussions on structure–activity relationships (SAR) complicated. However, observations suggest that the presence of amino groups positioned *meta* or *para* to the phenolic functionality (as observed in compounds 6e and 14e) was associated with diminished TcCA inhibitory potency. Nevertheless, it is important to note that all phenolic compounds are significantly less potent CAIs compared with the sulfonamide acetazolamide, which effectively inhibits all three enzymes at nanomolar concentrations.

2.1.7 | Organoselenium compounds as antileishmanial agents

Al-Tamimi et al.^[141] reported a small library of organoselenium compounds bearing the sulfonamide moiety as effective inhibitors of the β-CA in the protozoan *L. donovani chagasi* LdcCA. In particular, some in vitro evaluations were made by different types of assays with the aim to confirm their inhibition efficacy against the protozoan parasite and their safety profile through the host cells. Thus, for such purposes, a series of selenoureido derivatives were synthesized and tested among *L. donovani chagasi* isoform, with structures reported in Figure 12.

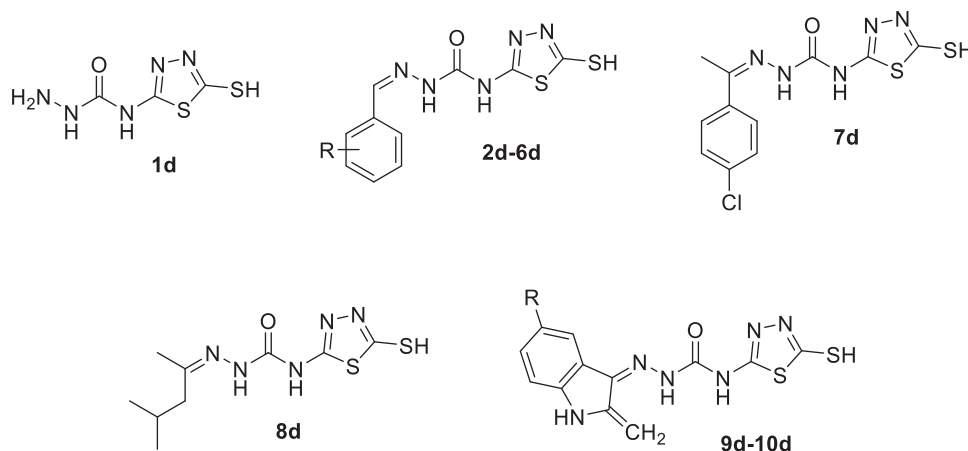


FIGURE 10 Chemical structures of different thiadiazole-2-thiols synthesized and tested **1d–10d**.

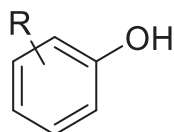
TABLE 6 Inhibition of human isoforms hCA I and hCA II, and protozoan-associated isoforms, *Trypanosoma cruzi* (TcCA) and *Leishmania chagasi donovani* (LdcCA), by a CO₂ hydrase, stopped-flow assay with 1,3,4-thiadiazole-2-thiol compounds **1d–10d** using AAZ as a reference drug.

Compound	K _i (nM) ^a			
	hCA I	hCA II	TcCA	LdcCA
1d	7100	9200	125	74.1
2d	3000	>100,000	21.1	27.9
3d	30,770	3830	28.6	33.1
4d	18,740	13,460	64.3	18.4
5d	71,600	>100,000	43.1	13.4
6d	8540	2670	34.5	40.1
7d	>100,000	3890	94.7	95.3
8d	8530	8850	52.4	19.5
9d	7890	8360	79.0	144
10d	3710	7970	72.5	152
AAZ	250.0	12.1	61.6	91.7

Note: The different substituent groups R are available in the reference article.^[88]

Abbreviations: AAZ, hydrochlorothiazide; hCA, human carbonic anhydrase.

^aMean from three different assays, by a stopped-flow technique (errors were in the range of ±5%–10% of the reported values).



1e–22e

FIGURE 11 Chemical structures of different tested phenol-based compounds **1e–22e**.

All selenoureido derivatives, except for a few, which showed inhibition constant values within the micromolar range, showed huge inhibition of LdcCA isoform (K_i 2.4–7.8 μM), as displayed in Table 8. Among these, any substitution at the aromatic tail moiety nor the positional insertion of the sulfonamide moiety did not consistently influence the activity against LdcCA. The potency drastically increased when the phenyl selenoureido tail was replaced with the phenylacetylselenoureido moiety (compounds **1f–31f**) with K_i values within a submicromolar range for all derivatives of the series.

This represents the first study that reports new selenoderivatives with promising leishmanicidal properties acting as CA inhibitors, thus paving the way to the development of innovative agents for the treatment of neglected diseases such as leishmaniasis.

2.1.8 | Benzoxaborole compounds as antiprotozoan agents

Considerable interest has been aroused in recent years by boron-containing derivatives, which has driven research toward the discovery of new compounds that can act selectively against infections caused by protozoa. Acoziborole (SCYX-7158), in Figure 13, is one of the very few compounds currently undergoing Phase III clinical trials for treating *Trypanosoma brucei* infection. It belongs to the benzoxaborole derivative class, a totally new chemotype to the array of antiprotozoal drugs.^[142,143]

Beyond their antiprotozoal effects, benzoxaboroles are endowed with a range of pharmacological activities,^[144] including antiviral, antibacterial, antifungal, and CA inhibitory actions.^[105,145,146]

Such findings prompted research on potential inhibition of this scaffold toward protozoan CA, paving the way to a new moiety capable of CAs inhibition. Previously, a novel class of CA inhibitors were introduced by Alterio et al.^[147] based on the benzoxaborole scaffold. Such inhibitors exhibited a novel binding mode and demonstrated effectiveness against both human α-CA and β-CA found in pathogenic fungi.^[148]

TABLE 7 Inhibition of human isoforms hCA I and hCA II, and protozoan-associated isoform of *Trypanosoma cruzi* (TcCA) by a CO₂ hydrazide, stopped-flow assay with different substituted phenols compounds 1e–22e using AAZ as a reference drug.

Compound	K_i (μ M) ^a		
	hCA I	hCA II	TcCA
1e	10.2	5.5	3.4
2e	>100	5.5	2.1
3e	>100	9.4	32.7
4e	10.7	0.1	18.5
5e	>100	>100	13.2
6e	4.9	4.7	41.7
7e	>100	>100	25.3
8e	10.0	6.2	19.8
9e	>100	0.1	15.1
10e	9.9	7.1	4.5
11e	9.8	10.6	28.4
12e	68.9	95.3	17.8
13e	6.3	4.9	13.6
14e	57.8	57.5	47.9
15e	>100	>100	21.1
16e	38.8	33.9	7.3
17e	>100	>100	26.9
18e	1.1	0.5	2.4
19e	5.7	5.2	15.9
20e	4.2	4.1	7.1
21e	1.1	1.3	4.8
22e	2.4	1.6	1.8
AAZ	0.25	0.012	0.06

Note: The different substituent groups R are available in the reference article.^[97]

Abbreviations: AAZ, hydrochlorothiazide; hCA, human carbonic anhydrase.

^aMean from three different assays, by a stopped-flow technique (errors were in the range of $\pm 5\%$ – 10% of the reported values).

Thus, according to Nocentini et al.^[105] herein are discussed the inhibitory activities of a series of 6-substituted urea/thiourea benzoxaboroles against CAs derived from the pathogenic protozoans TcCA and LdcCA, with structures reported in Figure 14. Tavaborole, a benzoxaborole compound widely utilized as topical antifungal medication, was utilized as standard. The inhibitory activities were assessed by comparing them with those against two physiologically relevant but off-target human isoforms, hCA I and hCA II (as shown in Table 9). Analyzing such presented data, the following structure–activity relationship for compounds **1g–23g** are identified:

Benzoxaboroles **1g–23g**, bearing ureido and thioureido moieties, exhibited inhibitory activities in micromolar concentrations, reaching low micromolar for some of them, against the protozoans TcCA and LdcCA. Notably, they exhibited pronounced selectivity toward the β -isoform LdcCA, with inhibition constants ranging from 3 to 0.65 μ M.

Compound **13g** demonstrated the highest selectivity for TcCA compared with LdcCA. Notably, the para-methoxy derivative **17g** emerged as the most potent inhibitor against the TcCA isoform, while all tested derivatives were endowed with K_i values ranging from 12.6 to 75.1 μ M.

Among the simple benzoxaboroles tested, such as **1g**, 6-nitro- (**2g**), and 6-amino benzoxaboroles (**3g**), they exhibited activity in the low micromolar range against LdcCA, comparable to their effects on hCA I, with K_i s ranging from 2.37 to 4.01 μ M, thus showing a good activity in inhibition terms, although such compounds did not show significant activity against TcCA ($K_i > 100 \mu$ M). On the other hand, tavaborole, used as a reference standard, showed inhibition potency similar to compounds **2g** and **3g** against LdcCA, with a slight selectivity against human isoform hCA II, indicated by a selectivity ratio of 5.5 ($K_i(\text{LdcCA})/K_i(\text{hCA II})$).

Compounds bearing para substitution on the phenyl ring (i.e., 4-F (**20g**) and 4-CF₃ (**22g**)) also displayed selectivity against LdcCA over CA II in the thioureido series. Conversely, a reversal of selectivity was observed for such compounds in the ureido series, where compounds **9g** (4-F derivative) and **10g** (4-CF₃) exhibited higher selectivity against hCA II over LdcCA, with selectivity ratios of 1.6 and 5.6, respectively ($K_i(\text{LdcCA})/K_i(\text{hCA II})$).

The most potent inhibitor was the para-nitrophenyl thioureido derivative **18g**, demonstrating a high selectivity ratio against the pathogenic isoform LdcCA over hCA II, around 110-fold higher inhibition activity. This compound could potentially offer an improved solution compared with the clinically used standard acetazolamide, as it displayed selectivity for hCA II over LdcCA and TcCA with selectivity ratios of 6 and 9, respectively.

3 | FUTURE PERSPECTIVES: STRUCTURE-BASED AND COMPUTATIONAL TECHNIQUES APPLIED TO NEW INHIBITORS DESIGN

Based on these studies, TcCA and LdcCA are emerging as a new and promising therapeutic target. Llanos et al.^[98] utilized a structure-based approach to identify new compounds with the potential to inhibit *T. cruzi* carbonic anhydrase, TcCA. A virtual screening protocol was used to guide the development of a zinc-binding function-sharing set of compounds (i.e. sulfonamides, sulfamates, and sulfamides). A combination of computational methodologies, in association with docking-based virtual screening, allowed the authors to easily identify potent compounds endowed with K_i values in the nanomolar range. Moreover, the crucial finding concerned selectivity toward inhibition of TcCA, thus exhibiting trypanocidal effects against *T. cruzi* epimastigotes and trypomastigotes. Hence, they

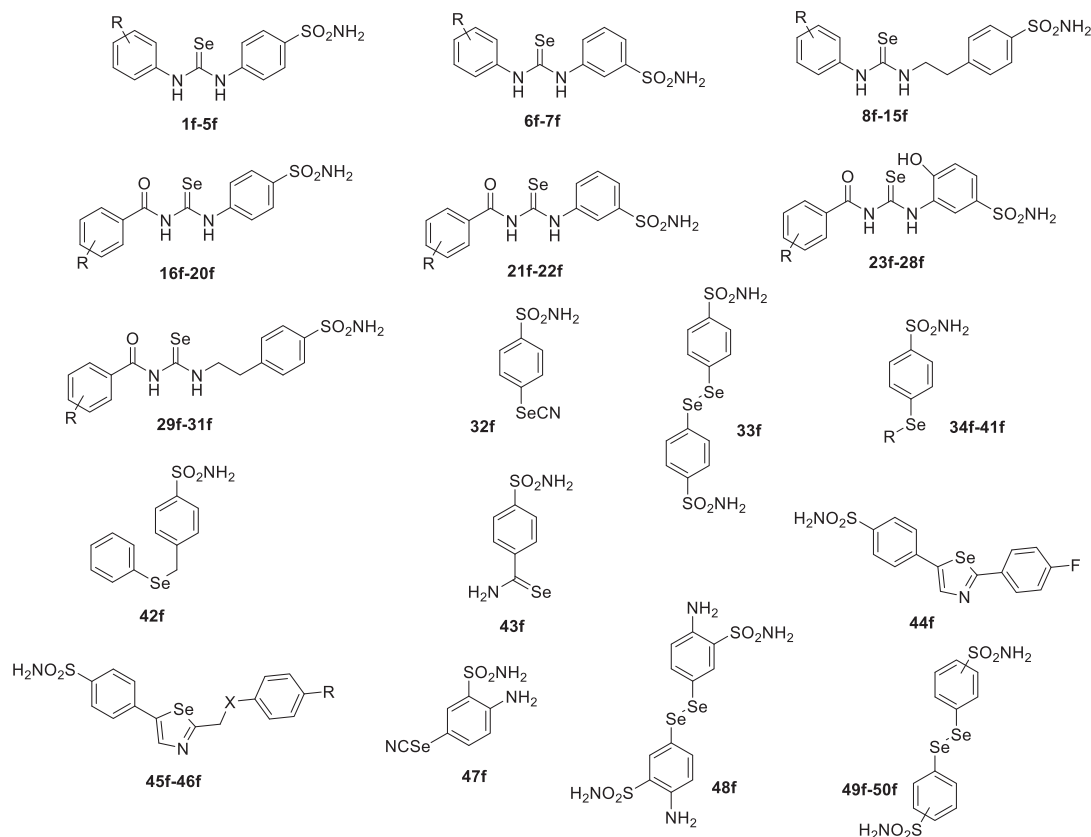


FIGURE 12 Chemical structures of different sulfonamide derivatives synthesized **1f–50f**.

reported the discovery of novel TcCA inhibitors with low interaction with human carbonic anhydrase (hCA), two of which are commercially available artificial sweeteners endowed with extensive toxicological data.^[96]

Table 10 shows inhibition of human isoform hCA II and protozoan-associated isoform of *T. cruzi* (TcCA) by a CO₂ hydrase, stopped-flow assay with compounds **1h–8h** using acesulphame K and sodium cyclamate as reference drugs (Figure 15).

Data obtained and reported in Table 10 exhibit a remarkable inhibitory potency of selected compounds (showed in the same table) against the protozoan isoform TcCA, showing a strong activity with high nanomolar range, with *K_i* values ranging from 261.4 to 951.8 nM for most of the tested compounds. Only compound **7** and acesulphame reported inhibition activity toward such isoform within the micromolar range with *K_i* values spanning from 2.24 to 7.25 μM.

Nevertheless, the most interesting results were achieved concerning selectivity of such compounds against the protozoan isoform compared with the human hCA II isoform: Most of compounds exhibited ineffective properties against the human CA II isoform, with only compounds **3h**, **5h**, and **7h** showing inhibitory activity within micromolar range (*K_i* from 4.96 to 8.88 μM) thus demonstrating high selectivity ratio for protozoan isoform.

Interestingly, the reduction of the tail bulkiness with the introduction of a *n*-hexyl tail in **8h** instead of a phenyl group in **7h** allowed

it to increase inhibition activity up to 448.8 nM, that is, 16-fold higher potency than **7h**.

Furthermore, sulphamide **5h** (which was reported as the most potent inhibitor with a *K_i* value of 0.26 μM) and sodium cyclamate both showed efficacy against the protozoan target to be about 30 times higher than human CA II, representing an important feature to be considered for further development.

3.1 | Nanoemulsions (NEs)

Sulfonamide CAs proved to be the more efficient inhibitors for CAs, employed for several pharmacological purposes, as detailed previously. Despite discovering potent in vitro TcCA inhibitors within low nanomolar concentrations, initial findings failed to demonstrate their efficacy in vivo, particularly in disrupting the pathogen's life cycle. Thus, it was hypothesized that this lack of efficacy could be attributed to difficulties in sulfonamide permeability across the biological membrane of the protozoan. To evaluate further, and eventually confirm, such issue, *T. cruzi* CA inhibitors formulated as NEs in clove oil were explored. This strategic approach yielded promising results, with several potent sulfonamide TcCA inhibitors exhibiting significant anti-*T. cruzi* effects against two distinct strains of the pathogen. Observed effects are ascribed to an enhanced permeation of the enzyme inhibitor through the innovative NE formulation, thereby

TABLE 8 Inhibition of the protozoan-associated isoform of *Leishmania chagasi donovani* (LdcCA) by a CO₂ hydratase, stopped-flow assay with different substituted sulfonamide compounds 1f–50f using AAZ as a reference drug.

Cpd	K _i (μM) ^a LdcCA
1f	4.7
2f	5.8
3f	4.3
4f	7.0
5f	3.3
6f	7.8
7f	4.2
8f	0.79
9f	4.2
10f	4.0
11f	4.0
12f	4.8
13f	3.3
14f	2.4
15f	4.1
16f	0.28
17f	0.02
18f	0.49
19f	0.90
20f	0.08
21f	0.01
22f	0.01
23f	0.03
24f	0.04
25f	0.06
26f	0.05
27f	0.07
28f	0.09
29f	0.09
30f	0.03
31f	0.08
32f	0.007
33f	0.005
34f	0.02
35f	0.02
36f	0.42
37f	0.03

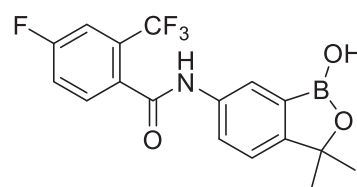
TABLE 8 (Continued)

Cpd	K _i (μM) ^a LdcCA
38f	0.006
39f	0.56
40f	0.006
41f	0.28
42f	0.60
43f	0.008
44f	0.01
45f	0.24
46f	0.029
47f	0.007
48f	0.02
49f	0.09
50f	0.80
AAZ	0.09

Note: The different substituent groups R are available in the reference article.^[141]

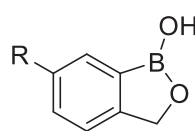
Abbreviations: AAZ, hydrochlorothiazide; hCA, human carbonic anhydrase.

^aMean from three different assays, by a stopped-flow technique (errors were in the range of ±5%–10% of the reported values).

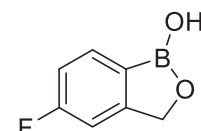


Acoziborole

FIGURE 13 Structure of acoziborole (SCYX-7158).



1g–23g



Tavaborole

FIGURE 14 Chemical structures of different benzoxaboroles synthesized **1g–23g** and tavaborole.

disrupting the pathogen's life cycle, potentially by interfering with pH regulation or carboxylating reactions involving bicarbonate/CO₂, representing a promising new way for the development of novel therapeutic strategies against this neglected disease.^[106]

TABLE 9 Inhibition of human isoforms hCA I and hCA II, and protozoan-associated isoforms, *Trypanosoma cruzi* (TcCA) and *Leishmania chagasi donovani* (LdcCA), by a CO₂ hydrase, stopped-flow assay with benzoxaboroles compounds 1g–23 g using AAZ as a reference drug.

Compound	K_i (μM) ^a			
	hCA I	hCA II	TcCA	LdcCA
1g	5.69	8.18	>100	3.79
2g	6.35	0.50	>100	4.01
3g	9.43	0.60	>100	2.37
4g	0.56	0.44	75.1	2.04
5g	0.56	0.28	87.6	4.30
6g	0.65	0.73	32.3	0.74
7g	3.46	0.71	37.8	0.62
8g	0.61	0.84	67.6	3.54
9g	0.23	0.48	24.0	0.78
10g	0.49	0.46	60.7	2.59
11g	0.45	0.27	69.5	3.85
12g	0.10	0.09	38.6	0.67
13g	0.29	0.80	33.6	0.48
14g	0.64	1.55	72.8	3.20
15g	0.32	1.25	32.5	0.67
16g	0.55	1.15	58.8	3.06
17g	0.51	1.25	12.6	0.59
18g	0.38	>100	46.8	0.91
19g	0.38	1.30	59.6	4.37
20g	0.35	1.50	19.7	0.66
21g	0.26	2.23	42.1	4.36
22g	0.42	1.84	16.6	0.85
23g	0.53	1.62	23.4	0.65
Tavaborole	2.01	0.46	60.5	2.54
AAZ	0.25	0.012	0.06	0.09

Note: The different substituent groups R are available in the reference article.^[105]

Abbreviations: AAZ, hydrochlorothiazide; hCA, human carbonic anhydrase.

^aMean from three different assays, by a stopped-flow technique (errors were in the range of ±5%–10% of the reported values).

Discovery of such inhibitors has led to multiple ways to increase the bioavailability and cellular intake of these molecules, aiming to act as therapeutic agent from the inside. Some of these inhibitors, particularly sulfonamides and hydroxamates (displayed in Figure 16) formulated as NEs, were shown to gain promising antiprotozoal effects in ex vivo cell cultures. However, the definitive classification of TcCA as a potential drug target for Chagas disease remains uncertain due to the lack of available animal model to investigate antiprotozoan effects.^[109]

TABLE 10 Inhibition of human isoform hCA II and protozoan-associated isoform *Trypanosoma cruzi* (TcCA), by a CO₂ hydrase, stopped-flow assay with secondary sulfonamides derivatives 1h–8 h using acesulphame K and sodium cyclamate as reference drugs.

Compound	K_i (nM) ^a		Selectivity ratio >16.8
	hCA II	TcCA	
1h	>10,000	594.4	>16.5
2h	>10,000	604.6	>6.6
3h	4957	752.5	>10.5
4h	>10,000	950.5	>32.6
5h	8528	261.4	>10.5
6h	>10,000	951.8	1.22
7h	8884	7250	8
8h	>10,000	448.8	>22.3
Acesulphame	>20,000	2242	>8.9
Sodium cyclamate	>10,000	348.1	>28.7

Abbreviation: hCA, human carbonic anhydrase.

^aMean from three different assays, by a stopped-flow technique (errors were in the range of ±5%–10% of the reported values).

4 | CONCLUSIONS

Protozoan diseases Chagas disease and leishmaniasis, caused by *T. cruzi* and *Leishmania spp.*, respectively, have traditionally impacted tropical regions but are now spreading to North America and Europe due to factors such as immigration and climate change. Despite significant efforts by various institutions and research groups, NTDs still represent challenges without effective solutions and limited treatment alternatives. However, recent drug discovery initiatives have unveiled promising new targets and potential drug candidates aimed at improving treatment options for these conditions.

Furthermore, many currently used drugs have led to significant drug resistance, complicating treatment efforts. Thus, the metalloenzyme CA, recently cloned and characterized in protozoan genomes, represents an innovative and promising target for therapy, holding the potentiality to direct research toward identifying new drug targets specifically for leishmaniasis and Chagas disease.

To date, extensive studies have led to the discovery of several potent and selective in vitro inhibitors, including sulfonamides, thiols, dithiocarbamates, and hydroxamates. Ex vivo experiments with some of these inhibitors have demonstrated significant inhibition of parasite growth, suggesting their potential to disrupt parasite viability. Although these results are promising, further research is necessary to fully elucidate the role of CAs in pathogens' life cycle, crucial for designing inhibitors that exhibit improved efficacy.

The inhibition of TcCA has emerged as a novel mechanism of action and a relatively unexplored target for developing anti-Chagas disease agents.^[89] However, a major challenge remains: ensuring selectivity in inhibiting pathogenic enzymes over host enzymes, particularly since TcCA has not yet been crystallized,^[149] making this

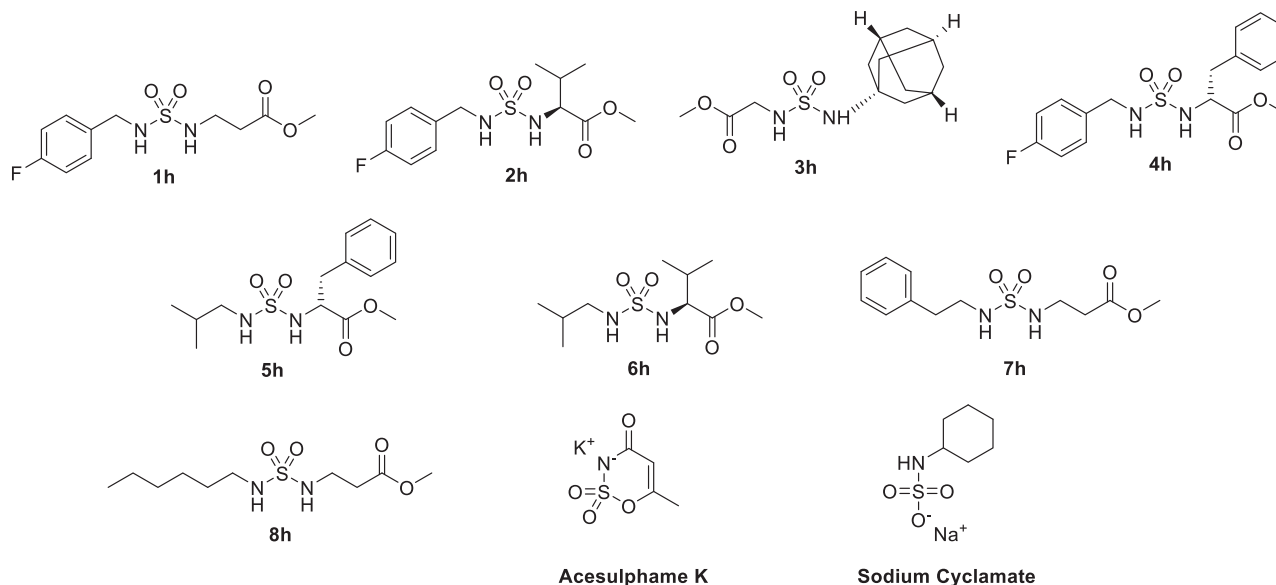


FIGURE 15 Chemical structures of different secondary sulfonamide derivatives synthesized by structure-based computational techniques 1h–8h, acesulphame K, and sodium cyclamate.

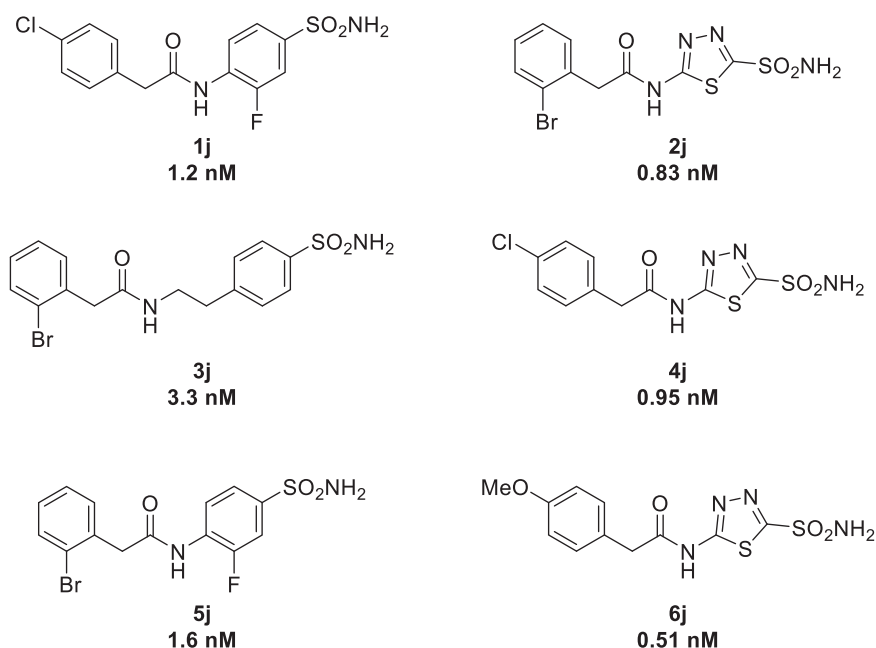


FIGURE 16 Sulfonamides 1j–6j and their TcCA inhibition activity (expressed as K_i) involved in the study.

strategy important for advancing therapeutic strategies against such diseases.

5 | FUTURE PERSPECTIVES

Among the CA inhibitors tested toward protozoan isoforms, several classes of sulfonamides and thiols have emerged as potent low nanomolar inhibitors *in vitro*. Although detailed investigations remain limited, it is clear that these enzymes do play critical roles in the life cycles of parasites, as they represent one of the most promising strategies for selectively counteracting pathogens without harming the host. Inhibition

of CA activity has been shown to impair pathogen growth, highlighting their potential as targets for developing anti-infective drugs with novel mechanisms of action, representing a promising strategy for NTDs treatment. Research has identified pathogen-specific CA isoforms and demonstrated successful inhibition using various classes of CA inhibitors, including phenols, sulfonamides, thiols, and hydroxamates, with some of them showing promising results in preclinical studies. Although certain classes exhibit lower efficacy, they demonstrate remarkable selectivity toward protozoan isoforms, paving the way for new anti-infective treatments with different mechanisms of action. Additionally, the risk of resistance development poses a challenge that must be considered, as well as potential toxicity to human enzymes.

Detailed structural studies, high-throughput screening methods, and enzyme-inhibitor simulations will be crucial in this effort. Among such techniques, docking studies stand out as a promising method for predicting ligand–enzyme interactions reliably. Furthermore, artificial intelligence can be harnessed to optimize the design of compounds endowed with improved selectivity and activity toward protozoan isoforms, while reducing interactions with human host enzymes.

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CONFLICTS OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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