

**Beta-carbonic anhydrase 1 from *Trichomonas vaginalis*
as new antiprotozoan drug target**

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Abstract

Trichomonas vaginalis is a unicellular parasite responsible for trichomoniasis, which is one of the world's leading sexually transmitted infections (STIs). The diagnosis and effective treatment of trichomoniasis has become an extremely important goal for global health, due to the increasing experimental evidences showing the relationship between trichomoniasis and other critical pathologies and the appearance of resistance to the existing pharmacological treatments. Consequently, in recent years research of novel drug targets for fighting this STI has seen an increased interest. In this scenario and considering recent experimental evidences which indicate Carbonic Anhydrases (CAs) as potential targets for the treatment of protozoan parasitic diseases, our group focused the attention on TvaCA1, a β -CA from *T. vaginalis*, carrying out a complete biochemical, structural and kinetic characterization of this enzyme. In this chapter we will summarize these studies, showing that this enzyme is a druggable target and that its selective inhibition is feasible with the aim to obtain new anti-trichomoniasis drugs.

Key words: *Trichomonas vaginalis*, β -carbonic anhydrase, drug design, inhibition, drug target

Introduction

Globally, more than 1 million sexually transmitted infections (STIs) occur each day which are associated with significant morbidity and mortality worldwide. In this scenario, a very important issue is the identification of a strategy to manage STI epidemic potential, changing patterns of the diseases, preventability of the diseases, and their social and economic effects. Critical points for the prevention and control of STIs include rapid diagnosis and early therapeutic treatment of infections in order to interrupt the transmission and reduce the untreated cases [1].

The human-infective parasite *Trichomonas vaginalis* is the causative agent of the most widespread non-viral STI worldwide, namely the trichomoniasis [2]. Based on the “Report on global sexually transmitted infection surveillance”, there were roughly 156 million new infections every year attributable to *T. vaginalis* pathogen [3, 4]. In particular, *T. vaginalis* is a flagellate protozoan that affects lower female genital tract, with a prevalence of 2.1 % in reproductive age-women [5], and the prostate epithelium [6, 7]. Clinically, symptoms of *T. vaginalis* infection can appear weeks, months or years after an initial infection [8, 9] and include mild to moderate inflammation of the cervix, vagina and urethra [10-12]. However, since many cases are asymptomatic, millions of *T. vaginalis* infections remain undiagnosed and therefore untreated [3], suggesting that asymptomatic individuals represent an infection risk to their sexual partners.

The recent increased interest for the treatment of trichomoniasis infection depends on the observation that this STI can cause serious damage in some physiological or pathological conditions. In fact, it has been observed that during pregnancy trichomoniasis could be responsible of premature rupture of the amniotic sac, preterm labor and delivery of a low birth weight [13, 14], while infected individuals could show increased susceptibility to human immunodeficiency virus (HIV) acquisition and/or transmission [15]. In addition, previous investigations suggested that *T. vaginalis* infection might determine an increased risk of cervical neoplasia [16]. Recently, the effect of this pathogen as risk factor for persistence and/or progression of low-grade cervical precancerous lesions has also been evaluated in HIV-1 positive women, showing that *T. vaginalis* infection can negatively modulate this pathological condition [17]. On the other side, infections in men that occur mainly in colonization of the prostate can increase the risk of aggressive prostate cancer [18]. In particular, the pathogen expresses a protein involved into cellular pathways linked to inflammation and cell proliferation, thus contributing to the initiation and progression of cancer.

In this scenario, enabling an early diagnosis and an effective pharmacological treatment of *T. vaginalis* infection represent very important goals for global health protection. At present, trichomoniasis management involves the use of only one type of drug, the 5-nitroimidazoles, which could be associated to several side effects [19-21]. However, the major disadvantage of this therapy

is the rapid appearance of resistance and a natural drug tolerance among a certain population of *T. vaginalis* isolates [22]. Since no alternative treatments were so far developed, a large number of research studies has been focused on the identification of new and more effective compounds acting as anti-infective drugs. In particular, different classes of molecules have been tested *in vitro* for their anti-parasitic action, as an example 5-nitroimidazole derivatives, benzo[f]cinnoline N-oxide and metronidazole containing dual active chemical group, while other potential therapeutic agents have been identified by screening natural compounds [2].

An alternative therapeutic strategy to counteract STIs caused by non-viral microorganisms involves the identification of new molecular targets. The cloning of the genomes of many pathogenic microorganisms offered the possibility of exploring alternative pathways for inhibiting virulence factors or proteins essential for their life cycle.

Carbonic Anhydrases (CAs, EC 4.2.1.1), ubiquitous metallo-enzymes which catalyze the reversible hydration of carbon dioxide to bicarbonate and proton [23], have been recently proposed as new potential targets for the treatment of protozoan parasitic diseases. Indeed, convincing data in the literature strongly indicate that the inhibition of CA activity in various parasites leads to a damage of parasite growth and virulence, causing a significant anti-infective effect [24]. Eight evolutionarily unrelated CA families have been so far identified (α -, β -, γ -, δ -, ζ -, η -, θ -, and ι -CAs), which do not show significant structural homology with each other [23, 25-27]; therefore, the possibility to develop specific inhibitors of one family, which do not interact with the other ones, is highly feasible [28]. Interestingly, only α -CAs are present in humans, whereas many parasites contain β -, γ - and η -CAs; thus suggesting that the latter enzymes could represent excellent target molecules for development of drugs free of potential side effects [24]. For this reason in recent years many studies describing the production, the characterization and the inhibition of parasite β -, γ and/or η -CAs have been carried out [29-48].

In this context we recently reported the expression in *E. coli* and the structural, biochemical and kinetic characterization of a new β -CA from *T. vaginalis* (TvaCA1) [36, 38, 48]. In this chapter, we will summarize these studies, showing that this enzyme is a druggable target and that its selective inhibition is feasible with the aim to obtain new anti-trichomoniasis drugs.

TvaCA1 biochemical, kinetic and structural characterization

TvaCA1 was expressed in *E. coli* and purified with high yield, in order to carry out a complete biochemical, kinetic and structural characterization [38]. In the same paper size exclusion chromatography and light scattering experiments were described showing a dimeric quaternary structure for the recombinant protein. These data were in agreement with the observation that β -

CAs show always a dimeric structure that in some cases can arrange in higher oligomers such as tetramers, hexamers or octamers. The CO₂ hydration activity was also measured by means of a stopped flow instrument revealing a rather high catalytic efficiency comparable to that of human (h) CA I, protozoan β -CAs from *Entamoeba histolytica* [49] and *Leishmania donovani chagasi* [39] and some prokaryotic β -CAs, such as *Salmonella enterica* [50] and *Legionella pneumophila* [51] (Table 1), but lower than that of the highly efficient isoform hCA II.

Subsequently, the crystallographic structure of the enzyme was determined [38], showing the typical α/β -fold previously observed for other β CAs [52-64], consisting of a central five-stranded β -sheet core, formed by four parallel (with strand order 2-1-3-4) and one antiparallel β -strands (β 5) and flanking helices (Figure 1A). Two monomers associated to form the biologically relevant dimer, originating an extended β -sheet core of ten β -strands and generating a total buried surface area at the dimeric interface of 4366 Å² (Figure 1B and Figure 2A) [38].

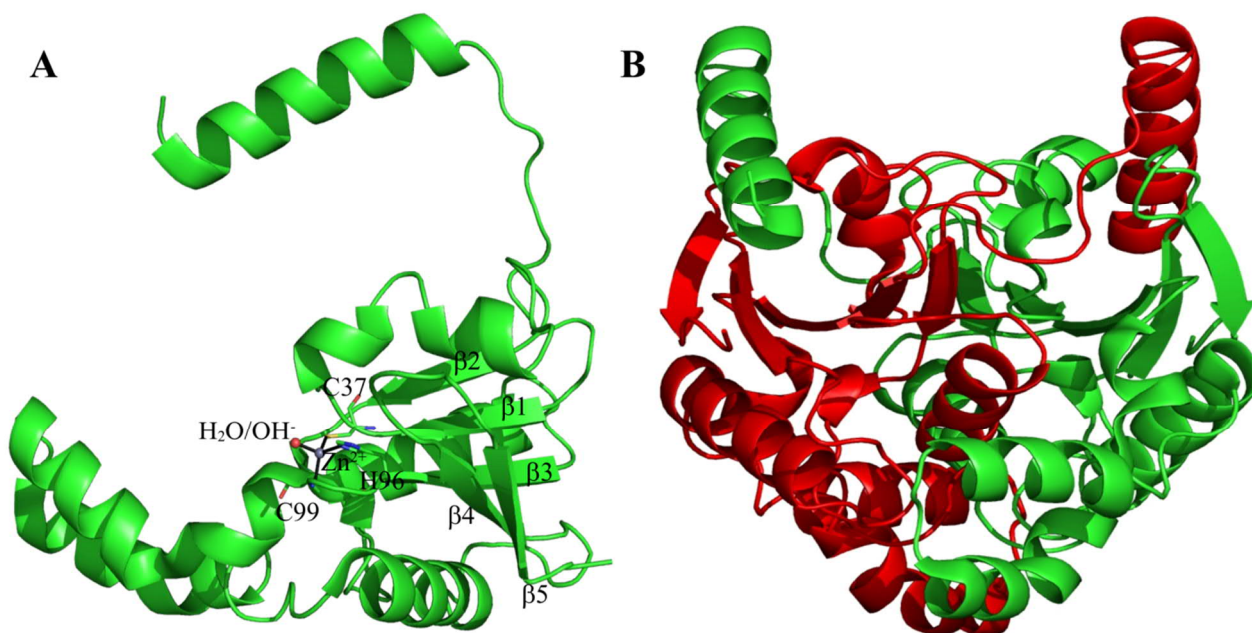


Figure1 Ribbon representation of the TvaCA1 monomer (A) and dimer (B) with one monomer colored in red and the other in green [38].

In this dimer, the N-terminal helix of each monomer extends away from the rest of the molecule, making extensive contacts with the other monomer. The dimeric structure contained two active sites, which were located in clefts at the dimeric interface, each one containing a zinc ion on the bottom coordinated by three protein residues, Cys37, His96 and Cys99 and a solvent molecule/hydroxide ion (Figure 2). Interestingly, these active sites were scarcely accessible when compared to the active sites of hCAs, whose active site is situated in a large and deep conical cavity (Figure 3).

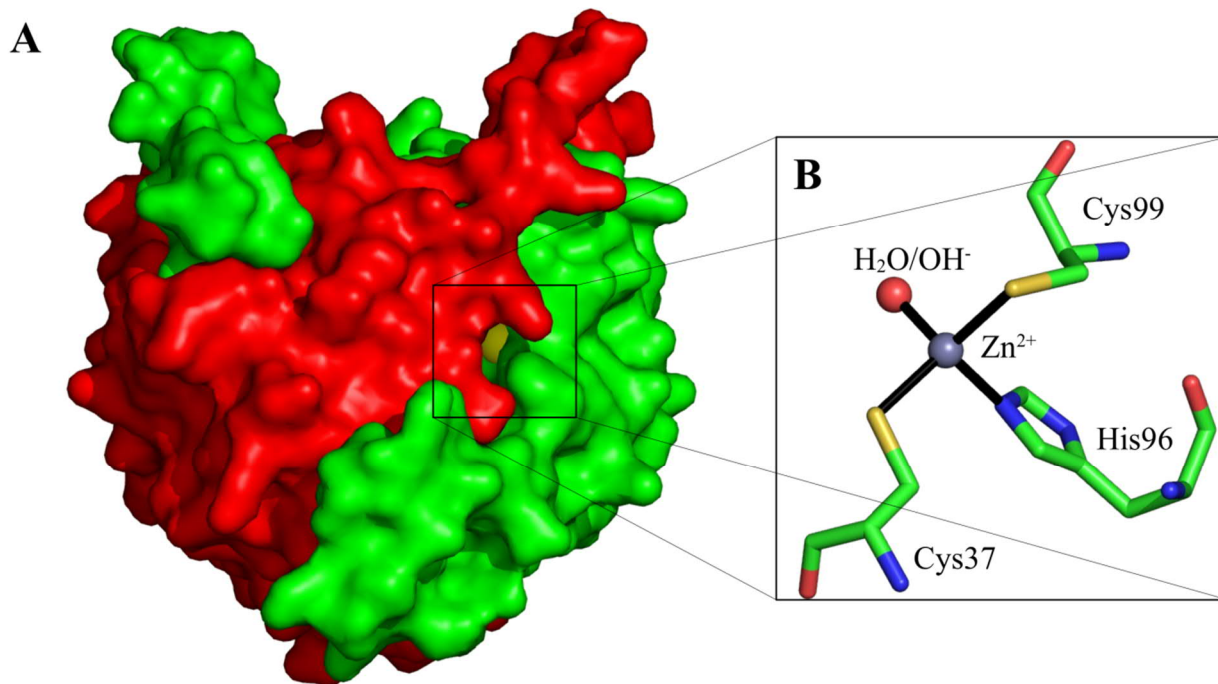


Figure 2 (A) Surface of TvaCA1 dimeric structure, with the two monomers shown in green and red. (B) Enlarged view of the active site of the enzyme, with the zinc coordinated by two Cys, one His and one water molecule/hydroxide ion [38].

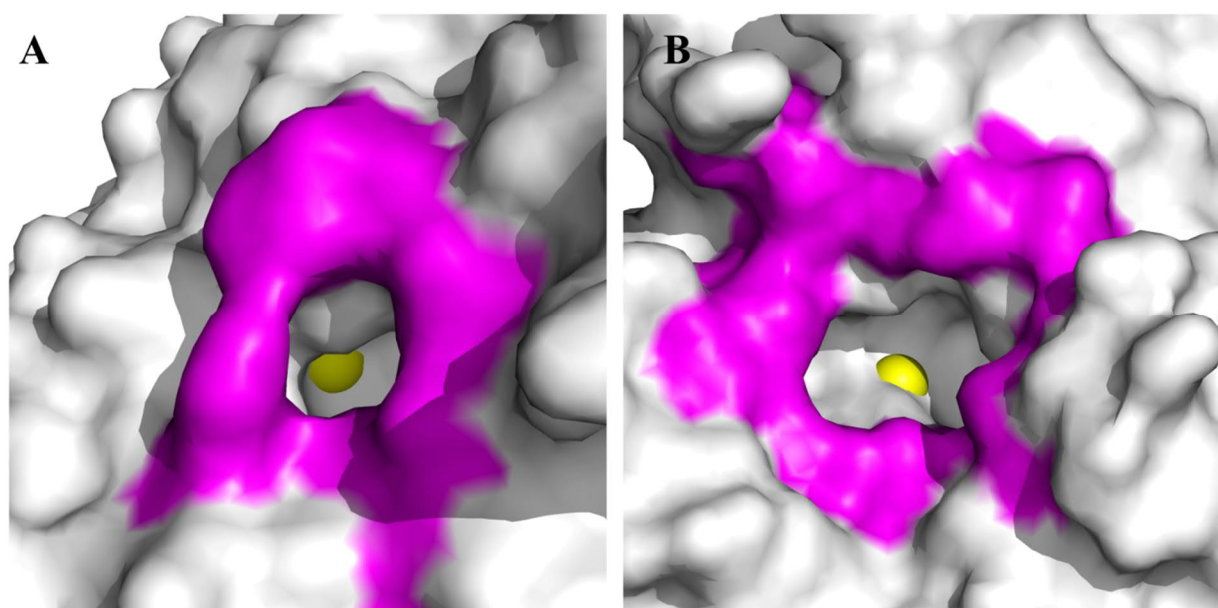


Figure 3 Surface representation of (A) TvaCA1 and (B) hCA II (chosen as a representative human isoform) showing the active site accessibility of these two enzymes [38]. Residues delimiting the rim of the active site cavity are colored in magenta, while the catalytic zinc ions are depicted as yellow spheres.

This is an important difference that can be exploited for the design of inhibitors selective for the protozoan enzyme with respect to the human CAs, which represent an off-target for the development of antiparasitic drugs.

Inhibition with anions and sulfonamides

In order to gain information on the molecules which could be used for the development of TvaCA1 selective inhibitors, a wide range of inorganic anions and small molecule compounds were investigated for their inhibition properties against the parasitic enzyme and results were compared those obtained for hCA I and hCA II with the same set of molecules (Table 2) [36]. These studies identified thiocyanate, cyanide, selenate, selenocyanate, divanadate and N,N-diethyldithiocarbamate as sub-millimolar inhibitors, and sulfamide, sulfate, phenylboronic acid and phenylarsonic acid as micromolar inhibitors. The latter two compounds were the most interesting ones, since they were rather selective for TvaCA1 with respect to hCA I and hCA II (see Table 2), thus emerging as lead compounds for the development of new antiprotozoan drugs with a different mechanism of action [36].

Subsequently a series of simple aromatic/heterocyclic primary sulfonamides as well as several clinically approved/investigational such drugs for a range of pathologies were also investigated (Figure 4) (Table 3) [48] and compared with results previously obtained for the off-target hCA II. Interestingly out of the 40 tested derivatives only 14 were able to inhibit TvaCA1, the remaining 26 being ineffective up to 50 μ M concentration in the assay system. Among the inactive compounds were the clinically used agents **DCP, DZA, BRZ, BZA, TPM, ZNS, SLP, IND, VLX, CLX, SLT, SAC** and **HCT**. Most of them possessed rather bulky scaffolds, thus explaining why they could not enter the scarcely accessible TvaCA1 active site and on the contrary were very good inhibitor of hCA II, possessing a very large and well accessible active site. Similar considerations can be done for compounds of the series **1-24**; among these, molecules with very bulky scaffold were completely ineffective as CA inhibitors, whereas those incorporating more compact, simple benzenesulfonamide/thiadiazole sulfonamide scaffolds with few compact substituents (such as **1-4, 7, 13-15** and **MZA**) were able to better accommodate within the enzyme active site producing micromolar inhibition. However, also in this case the human enzyme was significantly better inhibited. These studies clearly indicate that sulfonamide molecules generally behave as better inhibitors of human isoforms with respect to TvaCA1 and do not represent ideal lead compounds for the development of selective TvaCA1 inhibitors [48].

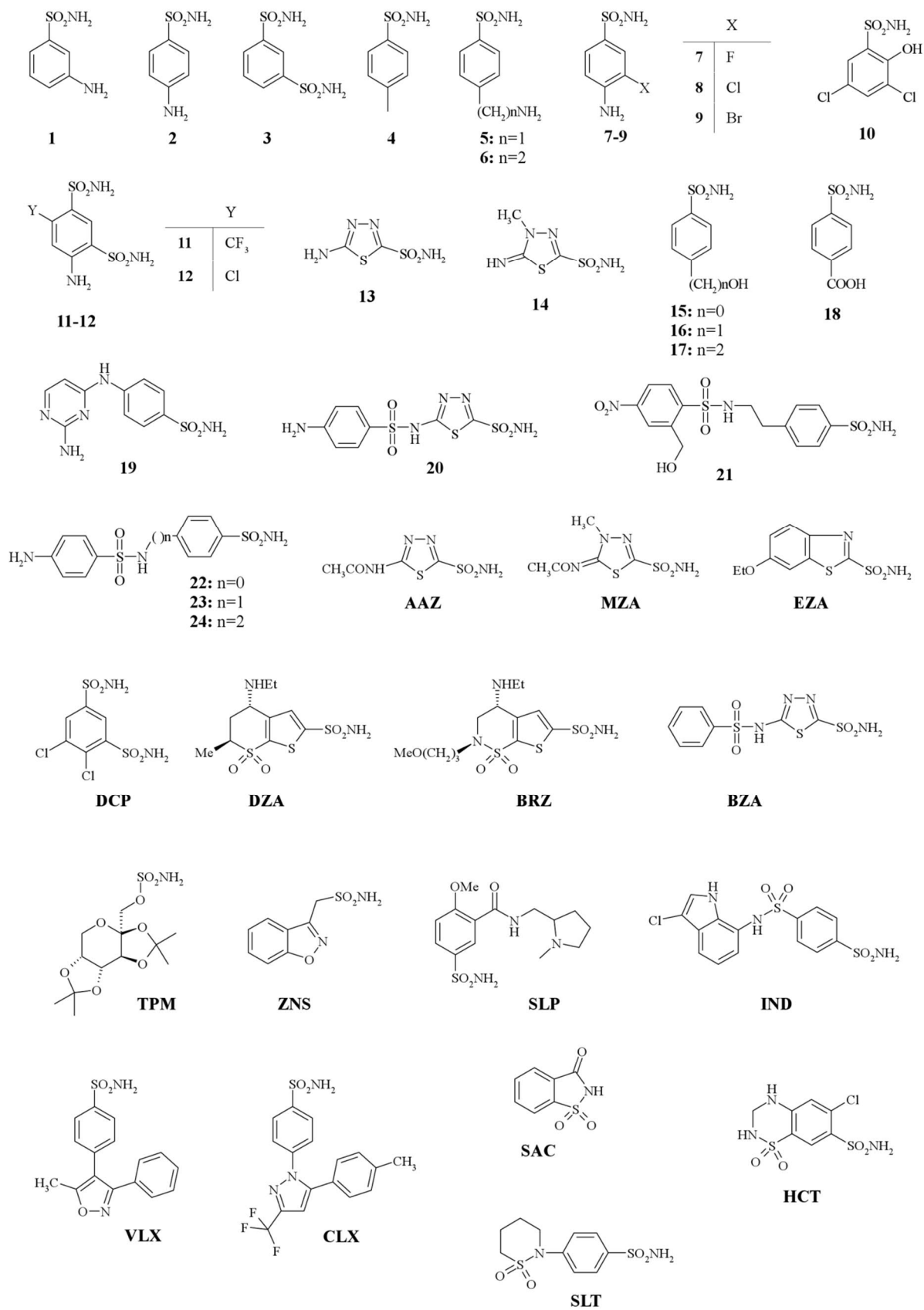


Figure 4 Schematic picture of sulfonamides **1–24** and clinically used compounds **AAZ–HCT** tested against TvaCA1 enzyme [48].

Conclusions and future perspectives

The diagnosis and effective treatment of *T. vaginalis* infection has become an extremely important goal for global health in both women and men, due to the increasing experimental evidences showing the relationship between trichomoniasis and other critical pathologies and the appearance of resistance to the existing pharmacological treatments. Consequently, in recent years research of novel drug targets for fighting trichomoniasis has seen an increased interest. In this scenario and considering recent experimental evidences, which indicate CAs as potential targets for the treatment of protozoan parasitic diseases, our group focused the attention on TvaCA1, a β -CA from *T. vaginalis*, carrying out a complete biochemical, structural and kinetic characterization of this enzyme. The enzyme was demonstrated to possess a rather high catalytic efficiency and to behave as a non-covalent dimer in solution. The crystal structure determination highlighted significant differences between the active site of TvaCA1 and that of human CAs. Moreover, the parasitic enzyme could be inhibited both by sulfonamides in the nanomolar range and by other small molecules such as phenylboronic and phenylarsonic acid in the micromolar range. The latter two compounds, although being less efficient than sulfonamides, emerged as ideal lead compounds for the development of anti-trichomoniasis drugs, since they were rather selective for TvaCA1 with respect to hCA I and hCA II.

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Table 1 Kinetic parameters of TvaCA1. For comparison, kinetic parameters of hCA I, hCA II, and other representative protozoan and bacterial β -CAs are reported.

Enzyme	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1} s^{-1}$)	K_I (AAZ) (nM)
TvaCA1 [38]	4.9×10^5	8.0×10^7	391
hCA I [65]	2.0×10^5	5.0×10^7	250
hCA II [65]	1.4×10^6	1.5×10^8	12
EhiCA [49]	6.7×10^5	8.9×10^7	509
LdcCA [39]	9.4×10^5	5.9×10^7	92
SenCA1 [50]	1.0×10^6	8.3×10^6	59
SenCA2 [50]	7.9×10^5	5.2×10^7	84
LpnCA1 [51]	3.4×10^5	4.7×10^7	76
LpnCA2 [51]	8.3×10^5	8.5×10^7	72

AAZ = acetazolamide, EhiCA = *Entamoeba histolytica* β -CA, LdcCA = *Leishmania donovani* β -CA, SenCA = *Salmonella enterica* β -CA, LpnCA = *Legionella pneumophila* β -CA.

Table 2 Inhibition constants of anion and small molecule inhibitors against hCA I, hCA II and the protozoan enzyme TvaCA1 measured by CO₂ hydrase assay method.

Inhibitor	K _i (mM)		
	hCA I ^a	hCA II ^a	TvaCA1 ^b
F ⁻	>300	>300	>100
Cl ⁻	6	200	8.7
Br ⁻	4	63	7.7
I ⁻	0.3	26	2.1
CNO ⁻	0.0007	0.03	2.2
SCN ⁻	0.2	1.60	0.71
CN ⁻	0.0005	0.02	0.91
N ₃ ⁻	0.0012	1.51	3.3
HCO ₃ ⁻	12	85	7.1
CO ₃ ²⁻	15	73	>100
NO ₃ ⁻	7	35	3.7
NO ₂ ⁻	8.4	63	1.8
HS ⁻	0.0006	0.04	>100
HSO ₃ ⁻	18	89	>100
SnO ₃ ²⁻	0.57	0.83	3.9
SeO ₄ ²⁻	118	112	0.39
TeO ₄ ²⁻	0.66	0.92	8.5
OsO ₅ ²⁻	0.92	0.95	>100
P ₂ O ₇ ⁴⁻	25.77	48.50	>100
V ₂ O ₇ ⁴⁻	0.54	0.57	0.64
B ₄ O ₇ ²⁻	0.64	0.95	>100
ReO ₄ ⁻	0.110	0.75	>100
RuO ₄ ⁻	0.101	0.69	1.2
S ₂ O ₈ ²⁻	0.107	0.084	>100
SeCN ⁻	0.085	0.086	0.64
CS ₃ ²⁻	0.0087	0.0088	>100
Et ₂ NCS ₂ ⁻	0.00079	0.0031	0.49
SO ₄ ²⁻	63	>200	2.8
ClO ₄ ⁻	>200	>200	>100
BF ₄ ⁻	>200	>200	>100
FSO ₃ ⁻	0.79	0.46	>100
NH(SO ₃) ₂ ²⁻	0.31	0.76	2.2
H ₂ NSO ₂ NH ₂	0.31	1.13	0.044
H ₂ NSO ₃ H	0.021	0.39	0.083
Ph-B(OH) ₂	58.6	23.1	0.093
Ph-AsO ₃ H ₂	31.7	49.2	0.062

^a From reference De Simone & Supuran 2012 [66]; ^b From reference Urbanski et al. 2020 [36].

Table 3 Inhibition constants of hCA II and TvaCA1 with sulphonamides **1–24** and the clinically used drugs **AAZ–HCT**, measured by a CO₂ hydrase, stopped-flow assay^a.

Inhibitor	K _i (nM)	
	hCA II	TvaCA1
1	300	3246
2	240	4742
3	8	3559
4	320	3599
5	170	>50,000
6	160	>50,000
7	60	4282
8	110	>50,000
9	40	>50,000
10	54	4536
11	63	>50,000
12	75	>50,000
13	60	1889
14	19	3987
15	80	2027
16	94	>50,000
17	125	>50,000
18	46	>50,000
19	33	4528
20	2	>50,000
21	11	3450
22	46	>50,000
23	33	>50,000
24	30	>50,000
AAZ	12	391
MZA	14	3827
EZA	8	283
DCP	38	>50,000
DZA	9	>50,000
BRZ	3	>50,000
BZA	9	>50,000
TPM	10	>50,000
ZNS	35	>50,000
SLP	40	>50,000
IND	15	>50,000
VLX	43	>50,000
CLX	21	>50,000
SLT	9	>50,000
SAC	5959	>50,000
HCT	290	>50,000

^a From reference Urbanski et al. 2021 [48].