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 chaper 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 CO_2 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 βCAs [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 (β5) 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 $Å^2$ (Figure 1B and Figure 2A) [38].

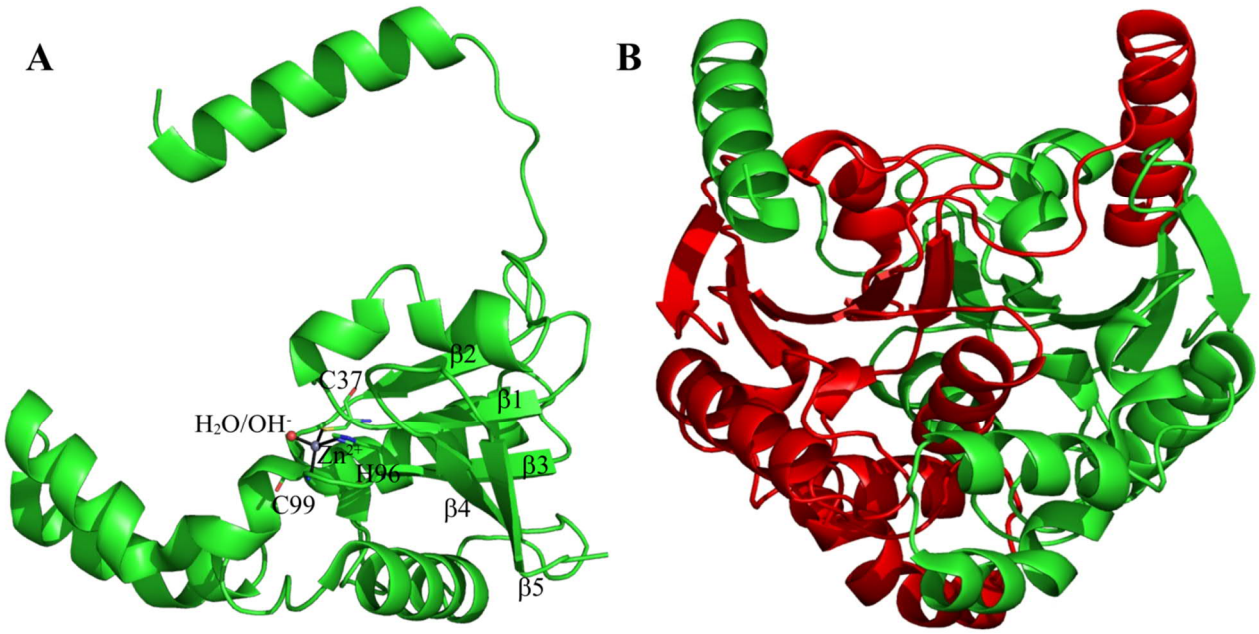

Figure1 Ribbon representation of the TvaCA1 monomer (A) and dimer (B) with one monomer colored in red and the other in green [38].

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).

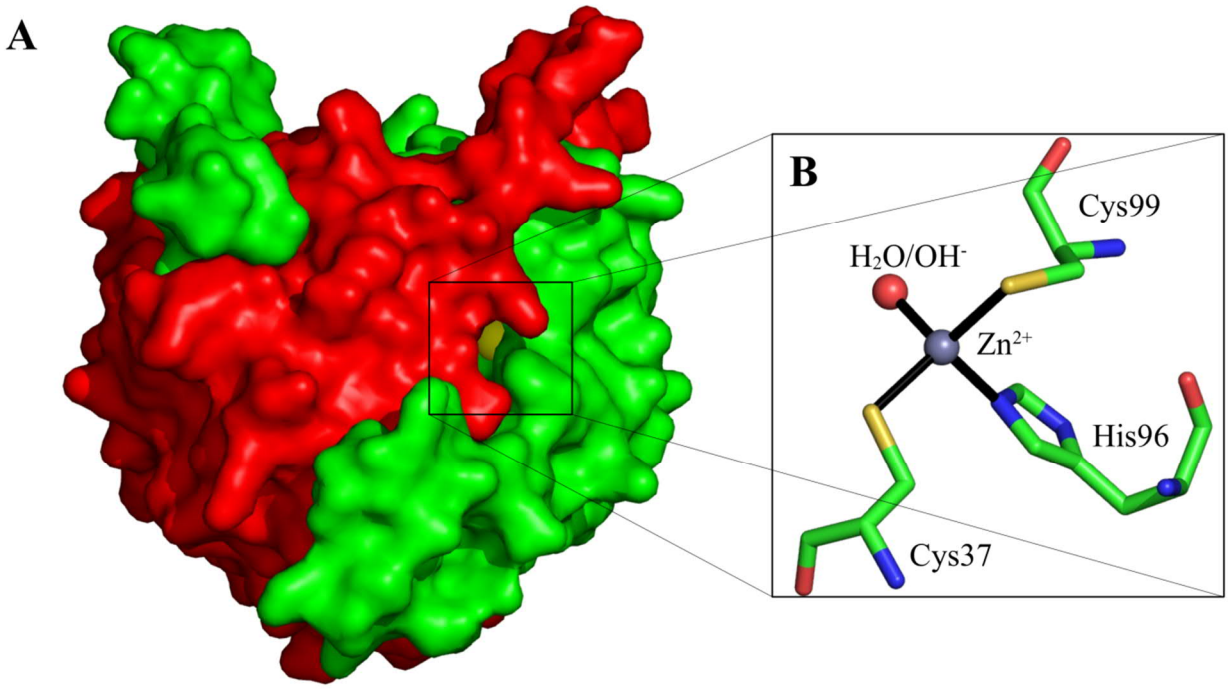

Figure 2 (A) Surface of TvaCA1 dimeric structure, with the two monomers shown in green and red. (B) Enlarged view of the active site of the enzyme, with the zinc coordinated by two Cys, one His and one water molecule/hydroxide ion [38].

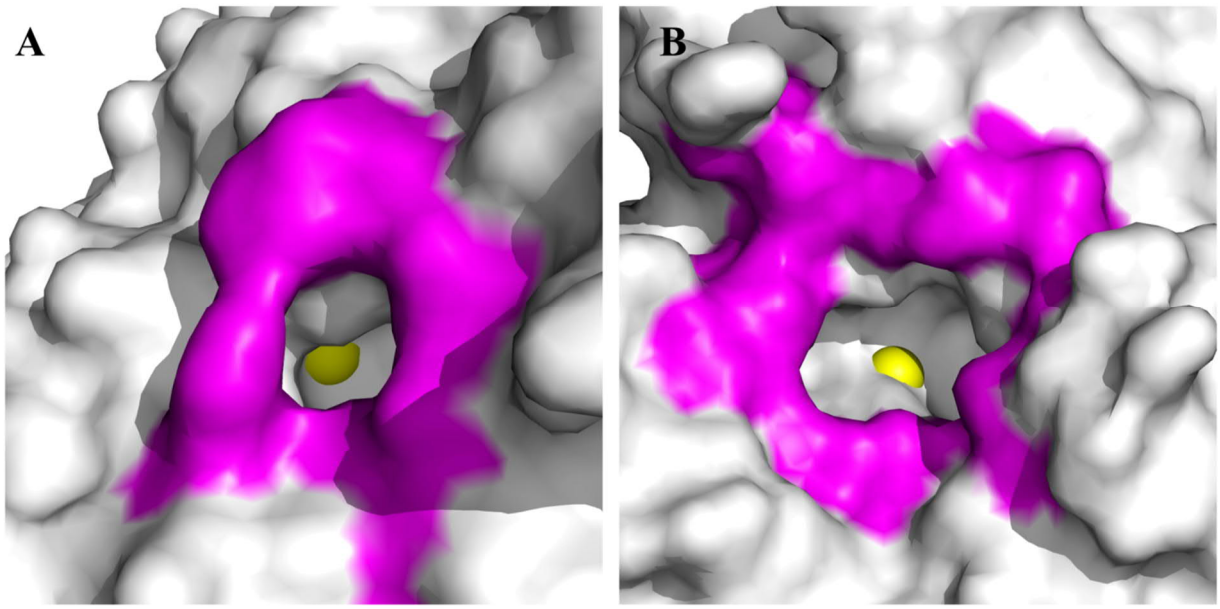

Figure 3 Surface representation of (A) TvaCA1 and (B) hCA II (chosen as a representative human isoform) showing the active site accessibility of these two enzymes [38]. Residues delimiting the rim of the active site cavity are colored in magenta, while the catalytic zinc ions are depicted as yellow spheres.

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 μM 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].

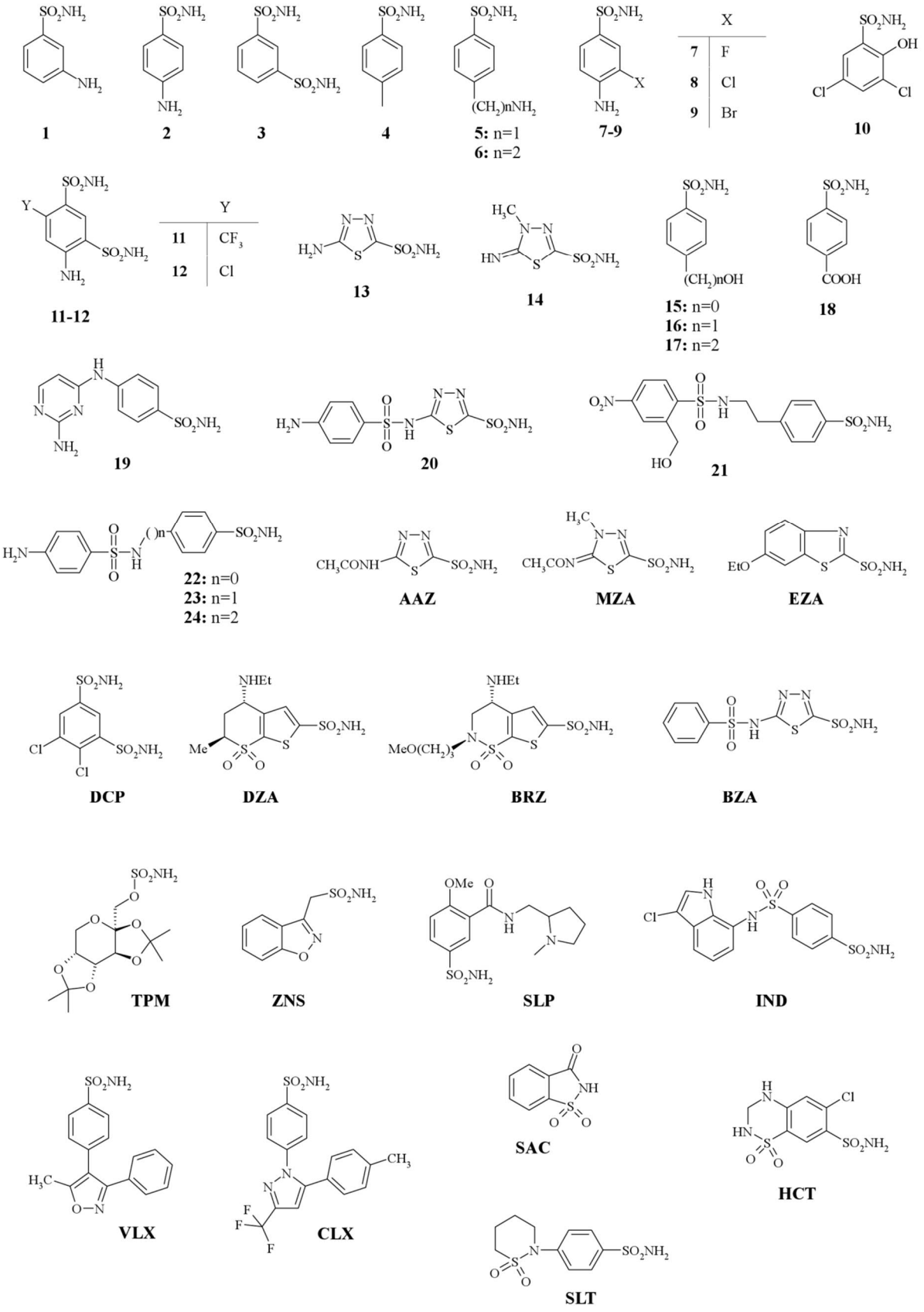

Figure 4 Schematic picture of sulfonamides **1–24** and clinically used compounds **AAZ–HCT** tested against TvaCA1 enzyme [48].

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.

References

1. Sena AC, Bachmann L, Johnston C, Wi T, Workowski K, Hook EW, 3rd, et al. Optimising treatments for sexually transmitted infections: surveillance, pharmacokinetics and pharmacodynamics, therapeutic strategies, and molecular resistance prediction. Lancet Infect Dis 2020;20(8):e181-e91.
2. de Brum Vieira P, Tasca T, Secor WE. Challenges and Persistent Questions in the Treatment of Trichomoniasis. Curr Top Med Chem 2017;17(11):1249-65.
3. Rowley J, Vander Hoorn S, Korenromp E, Low N, Unemo M, Abu-Raddad LJ, et al. Chlamydia, gonorrhoea, trichomoniasis and syphilis: global prevalence and incidence estimates, 2016. Bull World Health Organ 2019;97(8):548-62P.
4. WHO. Report on global sexually transmitted infection surveillance 2018. 2018; https://www.who.int/reproductivehealth/publications/stis-surveillance-2018/en/].
5. Sutton M, Sternberg M, Koumans EH, McQuillan G, Berman S, Markowitz L. The prevalence of Trichomonas vaginalis infection among reproductive-age women in the United States, 2001-2004. Clin Infect Dis 2007;45(10):1319-26.
6. Gardner WA, Jr., Culberson DE, Bennett BD. Trichomonas vaginalis in the prostate gland. Arch Pathol Lab Med 1986;110(5):430-2.
7. Mitteregger D, Aberle SW, Makristathis A, Walochnik J, Brozek W, Marberger M, et al. High detection rate of Trichomonas vaginalis in benign hyperplastic prostatic tissue. Med Microbiol Immunol 2012;201(1):113-6.
8. Satterwhite CL, Joesoef MR, Datta SD, Weinstock H. Estimates of Chlamydia trachomatis infections among men: United States. Sex Transm Dis 2008;35(11 Suppl):S3-7.
9. Sena AC, Miller WC, Hobbs MM, Schwebke JR, Leone PA, Swygard H, et al. Trichomonas vaginalis infection in male sexual partners: implications for diagnosis, treatment, and prevention. Clin Infect Dis 2007;44(1):13-22.
10. Lin WC, Chang WT, Chang TY, Shin JW. The Pathogenesis of Human Cervical Epithelium Cells Induced by Interacting with Trichomonas vaginalis. PLoS One 2015;10(4):e0124087.
11. Swygard H, Sena AC, Hobbs MM, Cohen MS. Trichomoniasis: clinical manifestations, diagnosis and management. Sex Transm Infect 2004;80(2):91-5.
12. Wolner-Hanssen P, Krieger JN, Stevens CE, Kiviat NB, Koutsky L, Critchlow C, et al. Clinical manifestations of vaginal trichomoniasis. Jama 1989;261(4):571-6.
13. Cotch MF, Pastorek JG, 2nd, Nugent RP, Hillier SL, Gibbs RS, Martin DH, et al. Trichomonas vaginalis associated with low birth weight and preterm delivery. The Vaginal Infections and Prematurity Study Group. Sex Transm Dis 1997;24(6):353-60.
14. Mann JR, McDermott S, Gill T. Sexually transmitted infection is associated with increased risk of preterm birth in South Carolina women insured by Medicaid. J Matern Fetal Neona 2010;23(6):563-8.
15. McClelland RS, Sangare L, Hassan WM, Lavreys L, Mandaliya K, Kiarie J, et al. Infection with Trichomonas vaginalis increases the risk of HIV-1 acquisition. J Infect Dis 2007;195(5):698-702.
16. Zhang ZF, Begg CB. Is Trichomonas vaginalis a cause of cervical neoplasia? Results from a combined analysis of 24 studies. Int J Epidemiol 1994;23(4):682-90.
17. Raffone A, Travaglino A, Angelino A, Esposito R, Orlandi G, Toscano P, et al. Gardnerella vaginalis and Trichomonas vaginalis infections as risk factors for persistence and progression of low-grade precancerous cervical lesions in HIV-1 positive women. Pathol Res Pract 2021;219:153349.
18. Twu O, Dessi D, Vu A, Mercer F, Stevens GC, de Miguel N, et al. Trichomonas vaginalis homolog of macrophage migration inhibitory factor induces prostate cell growth, invasiveness, and inflammatory responses. Proc Natl Acad Sci U S A 2014;111(22):8179-84.
19. Gardner TB, Hill DR. Treatment of giardiasis. Clin Microbiol Rev 2001;14(1):114-28.
20. Cudmore SL, Delgaty KL, Hayward-McClelland SF, Petrin DP, Garber GE. Treatment of infections caused by metronidazole-resistant Trichomonas vaginalis. Clin Microbiol Rev 2004;17(4):783-93, table of contents.
21. Ali V, Nozaki T. Current therapeutics, their problems, and sulfur-containing-amino-acid metabolism as a novel target against infections by "amitochondriate" protozoan parasites. Clin Microbiol Rev 2007;20(1):164-87.

22. Conrad MD, Gorman AW, Schillinger JA, Fiori PL, Arroyo R, Malla N, et al. Extensive genetic diversity, unique population structure and evidence of genetic exchange in the sexually transmitted parasite Trichomonas vaginalis. PLoS Negl Trop Dis 2012;6(3):e1573.
23. Alterio V, Di Fiore A, D'Ambrosio K, Supuran CT, De Simone G. Multiple binding modes of inhibitors to carbonic anhydrases: how to design specific drugs targeting 15 different isoforms? Chemical reviews 2012;112(8):4421-68.
24. D'Ambrosio K, Supuran CT, De Simone G. Are Carbonic Anhydrases Suitable Targets to Fight Protozoan Parasitic Diseases? Current medicinal chemistry 2018;25(39):5266-78.
25. Kikutani S, Nakajima K, Nagasato C, Tsuji Y, Miyatake A, Matsuda Y. Thylakoid luminal theta-carbonic anhydrase critical for growth and photosynthesis in the marine diatom Phaeodactylum tricornutum. Proceedings of the National Academy of Sciences of the United States of America 2016;113(35):9828-33.
26. Jensen EL, Clement R, Kosta A, Maberly SC, Gontero B. A new widespread subclass of carbonic anhydrase in marine phytoplankton. The ISME journal 2019;13(8):2094-106.
27. De Simone G, Di Fiore A, Capasso C, Supuran CT. The zinc coordination pattern in the eta-carbonic anhydrase from Plasmodium falciparum is different from all other carbonic anhydrase genetic families. Bioorganic & medicinal chemistry letters 2015;25(7):1385-9.
28. Carbonic Anhydrases as Biocatalysts. From Theory to Medical and Industrial Applications. 1st Edition ed. The netherlands: Elsevier; 2015.
29. Krungkrai SR, Suraveratum N, Rochanakij S, Krungkrai J. Characterisation of carbonic anhydrase in Plasmodium falciparum. International journal for parasitology 2001;31(7):661-8.
30. Krungkrai SR, Krungkrai J. Malaria parasite carbonic anhydrase: inhibition of aromatic/heterocyclic sulfonamides and its therapeutic potential. Asian Pacific journal of tropical biomedicine 2011;1(3):233-42.
31. Del Prete S, De Luca V, De Simone G, Supuran CT, Capasso C. Cloning, expression and purification of the complete domain of the eta-carbonic anhydrase from Plasmodium falciparum. Journal of enzyme inhibition and medicinal chemistry 2016;31(sup4):54-9.
32. Del Prete S, Vullo D, De Luca V, Carginale V, di Fonzo P, Osman SM, et al. Anion inhibition profiles of the complete domain of the eta-carbonic anhydrase from Plasmodium falciparum. Bioorg Med Chem 2016;24(18):4410-4.
33. Del Prete S, Vullo D, De Luca V, Carginale V, Osman SM, AlOthman Z, et al. Cloning, expression, purification and sulfonamide inhibition profile of the complete domain of the eta-carbonic anhydrase from Plasmodium falciparum. Bioorganic & medicinal chemistry letters 2016;26(17):4184-90.
34. Annunziato G, Angeli A, D'Alba F, Bruno A, Pieroni M, Vullo D, et al. Discovery of New Potential Anti-Infective Compounds Based on Carbonic Anhydrase Inhibitors by Rational Target-Focused Repurposing Approaches. ChemMedChem 2016;11(17):1904-14.
35. Vullo D, Del Prete S, Fisher GM, Andrews KT, Poulsen SA, Capasso C, et al. Sulfonamide inhibition studies of the eta-class carbonic anhydrase from the malaria pathogen Plasmodium falciparum. Bioorg Med Chem 2015;23(3):526-31.
36. Urbanski LJ, Angeli A, Hytonen VP, Di Fiore A, Parkkila S, De Simone G, et al. Inhibition of the newly discovered betacarbonic anhydrase from the protozoan pathogen Trichomonas vaginalis with inorganic anions and small molecules. Journal of Inorganic Biochemistry 2020;213:111274.
37. Di Fiore A, Supuran CT, Scaloni A, De Simone G. Human carbonic anhydrases and post-translational modifications: a hidden world possibly affecting protein properties and functions. Journal of enzyme inhibition and medicinal chemistry 2020;35(1):1450-61.
38. Urbanski LJ, Di Fiore A, Azizi L, Hytonen VP, Kuuslahti M, Buonanno M, et al. Biochemical and structural characterisation of a protozoan beta-carbonic anhydrase from Trichomonas vaginalis. Journal of enzyme inhibition and medicinal chemistry 2020;35(1):1292-9.
39. Syrjanen L, Vermelho AB, Rodrigues Ide A, Corte-Real S, Salonen T, Pan P, et al. Cloning, characterization, and inhibition studies of a beta-carbonic anhydrase from Leishmania donovani chagasi, the protozoan parasite responsible for leishmaniasis. J Med Chem 2013;56(18):7372-81.

40. Pan P, Vermelho AB, Capaci Rodrigues G, Scozzafava A, Tolvanen ME, Parkkila S, et al. Cloning, characterization, and sulfonamide and thiol inhibition studies of an alpha-carbonic anhydrase from Trypanosoma cruzi, the causative agent of Chagas disease. J Med Chem 2013;56(4):1761-71.
41. Ceruso M, Carta F, Osman SM, Alothman Z, Monti SM, Supuran CT. Inhibition studies of bacterial, fungal and protozoan beta-class carbonic anhydrases with Schiff bases incorporating sulfonamide moieties. Bioorg Med Chem 2015;23(15):4181-7.
42. Pal DS, Mondal DK, Datta R. Identification of metal dithiocarbamates as a novel class of antileishmanial agents. Antimicrobial agents and chemotherapy 2015;59(4):2144-52.
43. Pal DS, Abbasi M, Mondal DK, Varghese BA, Paul R, Singh S, et al. Interplay between a cytosolic and a cell surface carbonic anhydrase in pH homeostasis and acid tolerance of Leishmania. Journal of cell science 2017;130(4):754-66.
44. Pan P, Vermelho AB, Scozzafava A, Parkkila S, Capasso C, Supuran CT. Anion inhibition studies of the alpha-carbonic anhydrase from the protozoan pathogen Trypanosoma cruzi, the causative agent of Chagas disease. Bioorg Med Chem 2013;21(15):4472-6.
45. Guzel-Akdemir O, Akdemir A, Pan P, Vermelho AB, Parkkila S, Scozzafava A, et al. A class of sulfonamides with strong inhibitory action against the alpha-carbonic anhydrase from Trypanosoma cruzi. J Med Chem 2013;56(14):5773-81.
46. Rodrigues GC, Feijo DF, Bozza MT, Pan P, Vullo D, Parkkila S, et al. Design, synthesis, and evaluation of hydroxamic acid derivatives as promising agents for the management of Chagas disease. J Med Chem 2014;57(2):298-308.
47. Alafeefy AM, Ceruso M, Al-Jaber NA, Parkkila S, Vermelho AB, Supuran CT. A new class of quinazoline-sulfonamides acting as efficient inhibitors against the alpha-carbonic anhydrase from Trypanosoma cruzi. Journal of enzyme inhibition and medicinal chemistry 2015;30(4):581-5.
48. Urbanski LJ, Angeli A, Hytonen VP, Di Fiore A, De Simone G, Parkkila S, et al. Inhibition of the beta-carbonic anhydrase from the protozoan pathogen Trichomonas vaginalis with sulphonamides. Journal of enzyme inhibition and medicinal chemistry 2021;36(1):329-34.
49. Haapanen S, Bua S, Kuuslahti M, Parkkila S, Supuran CT. Cloning, Characterization and Anion Inhibition Studies of a beta-Carbonic Anhydrase from the Pathogenic Protozoan Entamoeba histolytica. Molecules 2018;23(12)
50. Nishimori I, Minakuchi T, Vullo D, Scozzafava A, Supuran CT. Inhibition studies of the beta-carbonic anhydrases from the bacterial pathogen Salmonella enterica serovar Typhimurium with sulfonamides and sulfamates. Bioorg Med Chem 2011;19(16):5023-30.
51. Supuran CT. Legionella pneumophila Carbonic Anhydrases: Underexplored Antibacterial Drug Targets. Pathogens 2016;5(2)
52. Kimber MS, Pai EF. The active site architecture of Pisum sativum beta-carbonic anhydrase is a mirror image of that of alpha-carbonic anhydrases. Embo J 2000;19(7):1407-18.
53. Covarrubias AS, Bergfors T, Jones TA, Hogbom M. Structural mechanics of the pH-dependent activity of beta-carbonic anhydrase from Mycobacterium tuberculosis. J Biol Chem 2006;281(8):4993-9.
54. Sawaya MR, Cannon GC, Heinhorst S, Tanaka S, Williams EB, Yeates TO, et al. The structure of beta-carbonic anhydrase from the carboxysomal shell reveals a distinct subclass with one active site for the price of two. J Biol Chem 2006;281(11):7546-55.
55. Teng YB, Jiang YL, He YX, He WW, Lian FM, Chen Y, et al. Structural insights into the substrate tunnel of Saccharomyces cerevisiae carbonic anhydrase Nce103. BMC Struct Biol 2009;9:67.
56. Huang S, Hainzl T, Grundstrom C, Forsman C, Samuelsson G, Sauer-Eriksson AE. Structural studies of beta-carbonic anhydrase from the green alga Coccomyxa: inhibitor complexes with anions and acetazolamide. PLoS One 2011;6(12):e28458.
57. Strop P, Smith KS, Iverson TM, Ferry JG, Rees DC. Crystal structure of the "cab"-type beta class carbonic anhydrase from the archaeon Methanobacterium thermoautotrophicum. J Biol Chem 2001;276(13):10299-305.
58. Mitsuhashi S, Mizushima T, Yamashita E, Yamamoto M, Kumasaka T, Moriyama H, et al. X-ray structure of beta-carbonic anhydrase from the red alga, Porphyridium purpureum, reveals a novel catalytic site for CO(2) hydration. J Biol Chem 2000;275(8):5521-6.

59. Cronk JD, Endrizzi JA, Cronk MR, O'Neill J W, Zhang KY. Crystal structure of E. coli beta-carbonic anhydrase, an enzyme with an unusual pH-dependent activity. Protein Sci 2001;10(5):911-22.
60. Cronk JD, Rowlett RS, Zhang KY, Tu C, Endrizzi JA, Lee J, et al. Identification of a novel noncatalytic bicarbonate binding site in eubacterial beta-carbonic anhydrase. Biochemistry 2006;45(14):4351-61.
61. Schlicker C, Hall RA, Vullo D, Middelhaufe S, Gertz M, Supuran CT, et al. Structure and inhibition of the CO2-sensing carbonic anhydrase Can2 from the pathogenic fungus Cryptococcus neoformans. J Mol Biol 2009;385(4):1207-20.
62. Ferraroni M, Del Prete S, Vullo D, Capasso C, Supuran CT. Crystal structure and kinetic studies of a tetrameric type II beta-carbonic anhydrase from the pathogenic bacterium Vibrio cholerae. Acta Crystallogr D Biol Crystallogr 2015;71(Pt 12):2449-56.
63. Pinard MA, Lotlikar SR, Boone CD, Vullo D, Supuran CT, Patrauchan MA, et al. Structure and inhibition studies of a type II beta-carbonic anhydrase psCA3 from Pseudomonas aeruginosa. Bioorg Med Chem 2015;23(15):4831-8.
64. McGurn LD, Moazami-Goudarzi M, White SA, Suwal T, Brar B, Tang JQ, et al. The structure, kinetics and interactions of the beta-carboxysomal beta-carbonic anhydrase, CcaA. Biochem J 2016;473(24):4559-72.
65. Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nature reviews Drug discovery 2008;7(2):168-81.
66. De Simone G, Supuran CT. (In)organic anions as carbonic anhydrase inhibitors. Journal of Inorganic Biochemistry 2012;111:117-29.

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 chagasi* 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 CO_2 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
$P_2O_7^{4-}$	25.77	48.50	>100
$V_2O_7^{4-}$	0.54	0.57	0.64
$B_4O_7^{2-}$	0.64	0.95	>100
ReO_4^-	0.110	0.75	>100
RuO_4^-	0.101	0.69	1.2
$S_2O_8^{2-}$	0.107	0.084	>100
$SeCN^-$	0.085	0.086	0.64
CS_3^{2-}	0.0087	0.0088	>100
$Et_2NCS_2^-$	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
$H_2NSO_2NH_2$	0.31	1.13	0.044
H_2NSO_3H	0.021	0.39	0.083
$Ph\text{-}B(OH)_2$	58.6	23.1	0.093
$Ph\text{-}AsO_3H_2$	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 CO_2 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].