Inhibition of the newly discovered β-carbonic anhydrase from the protozoan pathogen *Trichomonas vaginalis* with inorganic anions and small molecules

Linda J. Urbański,[a] Andrea Angeli,[b] Vesa P. Hytönen,[a,c] Anna Di Fiore,[d] Seppo Parkkila, [a,c] Giuseppina De Simone,[d*] Claudiu T. Supuran[b]*

[a]*Faculty of Medicine and Health Technology, Tampere University, Arvo Ylpön katu 34, FI-33520 Tampere, Finland*

[b]*Neurofarba Department, Sezione di Chimica Farmaceutica e Nutraceutica, Università degli Studi di Firenze, Via U. Schiff 6, I-50019 Sesto Fiorentino (Firenze), Italy*

[c]*Fimlab Ltd., Arvo Ylpön katu 4, FI-33520 Tampere, Finland*

[d]*Institute of Biostructures and Bioimaging of the National Research Council, Via Mezzocannone 16 - 80134 Naples, Italy*

Abstract: The protozoan pathogen *Trichomonas vaginalis* encodes two carbonic anhydrases (CAs, EC 4.2.1.1) belonging to the β-class. One of these enzymes, *T. vaginalis* carbonic anhydrase 1 (TvaCA1), was recently cloned and characterized by our group, and its X-ray crystal structure reported. No inhibitors of this enzyme were reported up until now. Here we investigated the inhibition of TvaCA1 with inorganic anions and small molecules and observed that thiocyanate, cyanide, selenite, selenocyanate and divanadate are sub-millimolar inhibitors, whereas sulfamide, sulfate, phenylboronic acid and phenylarsonic acid are micromolar inhibitors. Finding effective TvaCA1 inhibitors may be useful for developing new antiprotozoan drugs.

Keywords: carbonic anhydrase; anion; beta-class enzyme; inhibitor; *Trichomonas vaginalis;* trichomoniasis

*Corresponding authors. Phone +39-081-2534579, email giuseppina.desimone@cnr.it (GDS); Phone: +39-055-4573729; e-mail: claudiu.supuran@unifi.it (CTS).

1. Introduction

Trichomonas vaginalis is a protozoan parasite provoking one of the most widespread sexually transmitted diseases worldwide, namely trichomoniasis [1, 2]. This infection accounts for almost half of the total sexually transmitted infections. In women, trichomoniasis usually affects the vagina, but it can also spread to the urethra, whereas the majority of men infected with *T. vaginalis* infections are asymptomatic but infective to their partners [1-3]. To date, the treatment of this infection is based solely on 5-nitroimidazoles, with only two available drugs, metronidazole and tinidazole, which however present relevant levels of drug resistance [2, 3].

As a consequence, the search for new drug targets to fight this infection is an ongoing task in many laboratories [4, 5]. Among the new enzymes which might be targeted in this protozoan, *T. vaginalis* carbonic anhydrase 1 (TvaCA1), belonging to the β-class of carbonic anhydrases (CAs), was recently characterized by this group [5]. Indeed, β-CAs from various pathogenic organisms, such as bacteria [6, 7], fungi [8, 9] and protozoans [10-12] were ultimately shown to be potential drug targets for the development of anti-infective agents with novel mechanisms of action compared to clinically used such drugs [13].

CAs are metallo-enzymes acting as catalysts for the CO_2 hydration reaction to bicarbonate and proton [14, 15]. They are present in most organisms, being encoded by eight genetically unrelated gene families [16-19]. Apart for being involved in pH homeostasis in many tissues/organisms due to their equilibration of CO_2/bicarbonate, these enzymes also participate in metabolic processes and the modulation of their activity by inhibitors and activators has pharmacologic applications [10, 20, 21].

There are several classes of CA inhibitors (CAIs) which act with different mechanisms [16, 20, 22]. The most investigated ones, such as the sulfonamides and their isosteres some of which are clinically used drugs [14, 15], are zinc binders which coordinate the catalytically crucial metal ion present within the enzyme active site [16, 20]. Inorganic anions also may act as zinc binders in many CAs, and constitute a second quite relevant class of CAIs [23].

We have recently reported [5] that the genome of *T. vaginalis* encodes for two CAs, one of which was quite an effective catalyst for the physiologic CO_2 hydration reaction, with the following kinetic parameters: k_{cat} of 4.9 x 10^5 s^{-1} and k_{cat}/K_M of 8.0 x 10^7 $M^{-1}s^{-1}$ [5]. The enzyme has an activity slightly higher than that of the human (h) isoform hCA I. As humans and other vertebrates encode only for α-CAs, whereas many parasites including some protozoans possess β-class enzymes, it appears of interest to explore selective inhibitors of the parasite over the human enzymes in the search of novel therapeutic candidates. In the previous work [5] we explored the kinetic properties and resolved the X-ray crystal structure of TvaCA1 but no inhibition studies were performed. Here

we report an inhibition study of this enzyme with a wide range of simple/complex inorganic anions, as well as various small molecule compounds known to target the metal ion in metallo-enzymes [23] including sulfamide, sulfamic acid, boronic and arsonic acids.

2. Materials and methods

2.1. Chemistry. All anions/small compounds used here were commercially available, highest purity reagents, from Sigma-Aldrich (Milan, Italy).

2.2. Enzymology. TvaCA1 was a recombinant enzyme obtained in-house as described earlier [5].

2.3. CA catalytic activity and inhibition assay. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity [24]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 – 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5, for α-CAs) or TRIS (pH 8.3 for β-CAs) as buffers, and 20 mM $NaClO_4$ (for maintaining constant the ionic strength). The initial rates of the CA-catalyzed CO_2 hydration reaction were followed for a period of 10-100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitors (10 mM) and dilutions up to 0.01 nM were prepared in distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 as reported earlier [25], and represent the mean from at least three different determinations.

3. Results and Discussions

We recently reported the X-ray crystal structure of TvaCA1 [5]. The enzyme, similar to many other Type I β-CAs, is dimeric (Fig. 1A) with two active sites per dimer, which are located in clefts at the dimeric interface. Each active site contains a zinc ion on the bottom, which is coordinated by three protein residues, Cys37, His96 and Cys99 and a water molecule/hydroxide ion (Fig. 1B)

Fig. 1 here

Type I β-CAs, as the most well-known α-CAs, possess a water molecule as the fourth zinc ligand, which is important both for the catalysis (as it acts as nucleophile in the catalytic cycle, in the deprotonated hydroxide form [22]) and for the inhibition of these enzymes. Indeed, in most cases the inhibitors substitute the zinc-coordinated water molecule/hydroxide ion and directly bind to the metal ion, in tetrahedral or trigonal bipyramidal geometries of Zn(II) [16, 23, 26]. This is almost always the case of small inorganic anions acting as CAIs [16, 23, 26], except one case, iodide, which was found anchored to the zinc-coordinated water in the β-CA from the algae Coccomyxa by means of X-ray crystallography [27]. Although anion inhibitors are usually not highly effective, they are relevant both for understanding in detail the inhibition mechanisms of metallo-enzymes and for drug design purposes. For example, trithiocarbonate (CS_3^{2-}) was reported by us as a rather weak inhibitor of mammalian CAs [28], but led to the discovery of dithiocarbamates and of the monothiocarbamates, which act as highly efficient, low nanomolar inhibitors and incorporate the same zinc-binding fragment of trithiocarbonate [29, 30].

Here we present the inhibition data of the protozoan enzyme TvaCA1 with a wide range of inorganic anions and small molecule compounds known to interact with the CA family of proteins [23] (Table 1). Inhibition of the two human α-CA isoforms, hCA I and II (highly abundant proteins with important physiological functions [14]), are also provided for comparison.

Table 1 here

The following should be noted regarding the inhibition data of Table 1:

(i) A group of inhibitors, among which fluoride, carbonate, hydrogensulfide, bisulfite, perosmate, diphosphate, tetraborate, perrhenate, peroxydisulfate, trithiocarbonate, perchlorate, tetrafluoroborate and fluorosulfonate did not significantly inhibit TvaCA1 up to concentrations of 100 mM in the assay system. Some of these inhibitors (perchlorate, tetrafluoroborate) are known to possess a low affinity for metal ions especially when found in the active site of metallo-enzymes [23], and in fact they also do not inhibit hCA I and II as well as many other classes of CAs on which they were assayed [25, 31-33]. However, the fact that anions with a high affinity for metal ions (in solution or within active sites of enzymes) such as hydrogen sulfide and trithiocarbonate and carbonate do not act as inhibitors against this enzyme is a surprising discovery and it is rather difficult to interpret.

(ii) Millimolar inhibitory activity was observed for the following anions: the halides (except fluoride), cyanate, azide, bicarbonate, nitrate, nitrite, stannate, tellurate, perruthenate, sulfate and iminodisulfonate. These anions had inhibition constants ranging between 1.2 and 8.7 mM. It is interesting to notice the difference between bicarbonate/carbonate (one being inhibitory while the

other one not), but also the rather effective inhibition with sulfate, which is not at all inhibitory against hCA II and it is a very weak inhibitor of hCA I (Table 1).

(iii) Submillimolar inhibition was observed with thiocyanate, cyanide, selenate, divanadate, and N,N-diethyldithiocarbamate, which had inhibition constants in the range of 0.39 – 0.91 mM (Table 1).

(iv) The most effective TvaCA1 inhibitors were sulfamide, sulfamate, phenylboronic acid and phenylarsonic acid, which acted as micromolar inhibitors, with K_Is in the range of 44 – 93 μM, thus allowing the discovery of interesting lead compounds for the development of efficient inhibitory molecules. Whereas it is rather difficult to rationalize the efficient inhibition observed with phenylboronic acid and phenylarsonic acid, due to the absence of structural information on the binding mode of these compounds to any CA, interesting hypotheses can be done on the mechanism of action of sulfamide and sulfamate. Indeed, it could be inferred that, similarly to what observed for hCA II [34] and the βCA from *Pseudomonas aeruginosa* [35], sulfamate and sulfamide bind to the TvaCA1 catalytic zinc ion in ionized form through their NH^- moiety, replacing the zinc bound solvent molecule and forming an additional hydrogen bond with an acceptor residue on the protein (Fig. 2). Support to this supposition comes from the crystal structure of the complex between the β-CA from *Coccomyxa* and the sulfonamide inhibitor acetazolamide (**AZM**), showing that the deprotonated sulfonamide replaces the zinc bound solvent molecule and donates a hydrogen bond to the carboxylate group of the Asp49 (corresponding to Asp39 in TvaCA1) (Fig. 2C) [27]. Further structural studies are currently underway to test this hypothesis.

4. Conclusions

We evaluated a series of inorganic simple/complex anions and other small molecules known to bind to metalloenzymes (sulfamide, sulfamic acid, phenylboronic/arsonic acids), for the inhibition of the protozoan β-CA TvaCA1, a recently described new potential drug target for the fight against trichomoniasis. TvaCA1 was recently shown to be a dimeric type I β-CA with significant catalytic activity for the hydration of CO_2 to bicarbonate and proton. We report here the first inhibition study of this enzyme, with inorganic anions and small molecules. Our data show that thiocyanate, cyanide, selenate, selenocyanate, divanadate and N,N-diethyldithiocarbamate were sub-millimolar inhibitors, whereas sulfamide, sulfate, phenylboronic acid and phenylarsonic acid were micromolar inhibitors. Some of the detected TvaCA1 inhibitors also showed very different inhibition profiles of

the protozoan and human major isoforms, which might be relevant for developing compounds which specifically and selectively may inhibit the parasite enzyme over the offtarget human ones. Thus, such effective TvaCA1 inhibitors may constitute the starting point for developing new antiprotozoan drugs with a different mechanism of action.

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Table 1 Inhibition constants of anion and small molecule inhibitors against hCA I, hCA II and the protozoan enzyme TvaCA1, for the CO_2 hydration reaction, at 20 °C [24].

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

(Table 1, continued)

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-$B(OH)_2$	58.6	23.1	0.093
Ph-AsO_3H_2	31.7	49.2	0.062

[§]As sodium salt; [#]Errors were in the range of 5-10 % of the reported values, from three different assays, by a CO_2 hydrase assay method

[a] From refs. [23]; [b] This work.

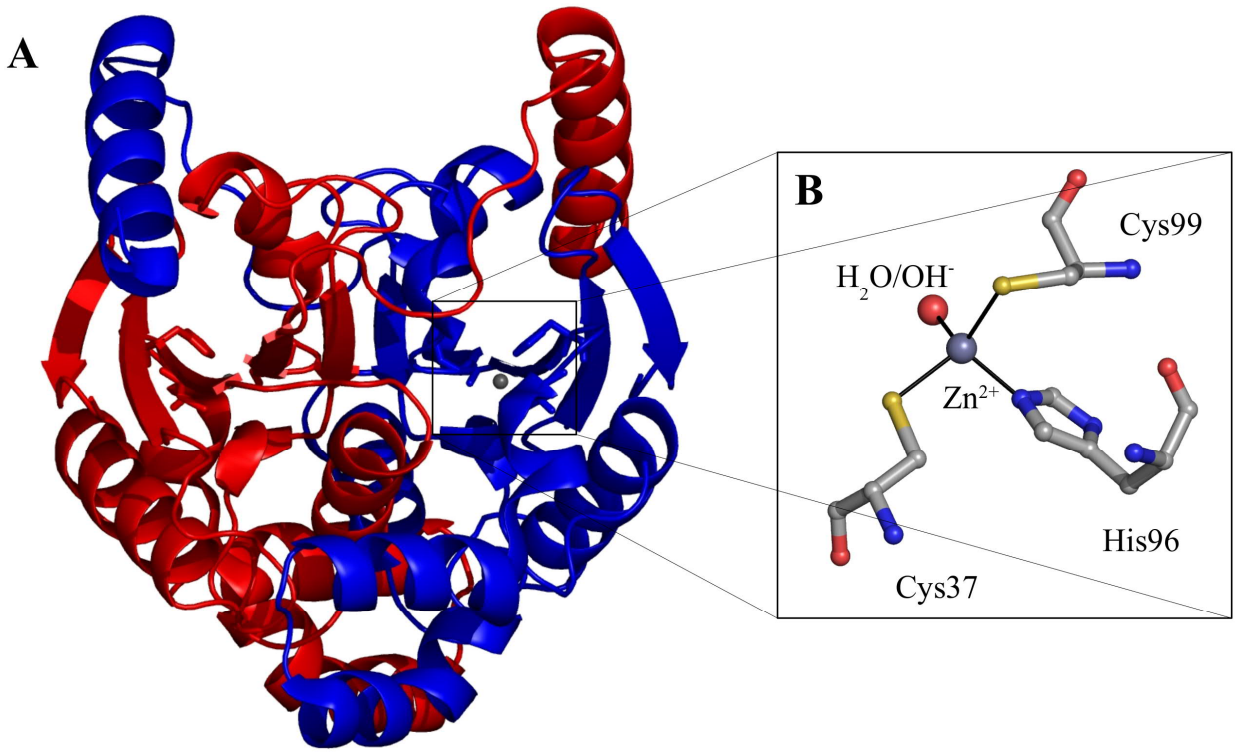

Fig. 1. **A**. Overall structure of the dimeric TvaCA1 with the two monomers colored in red and blue, respectively, and the catalytic zinc ions as gray spheres. **B.** Detailed view of the active site with the metal ion coordinated by Cys37, His96, Cys99 and a water molecule/hydroxide ion [5].

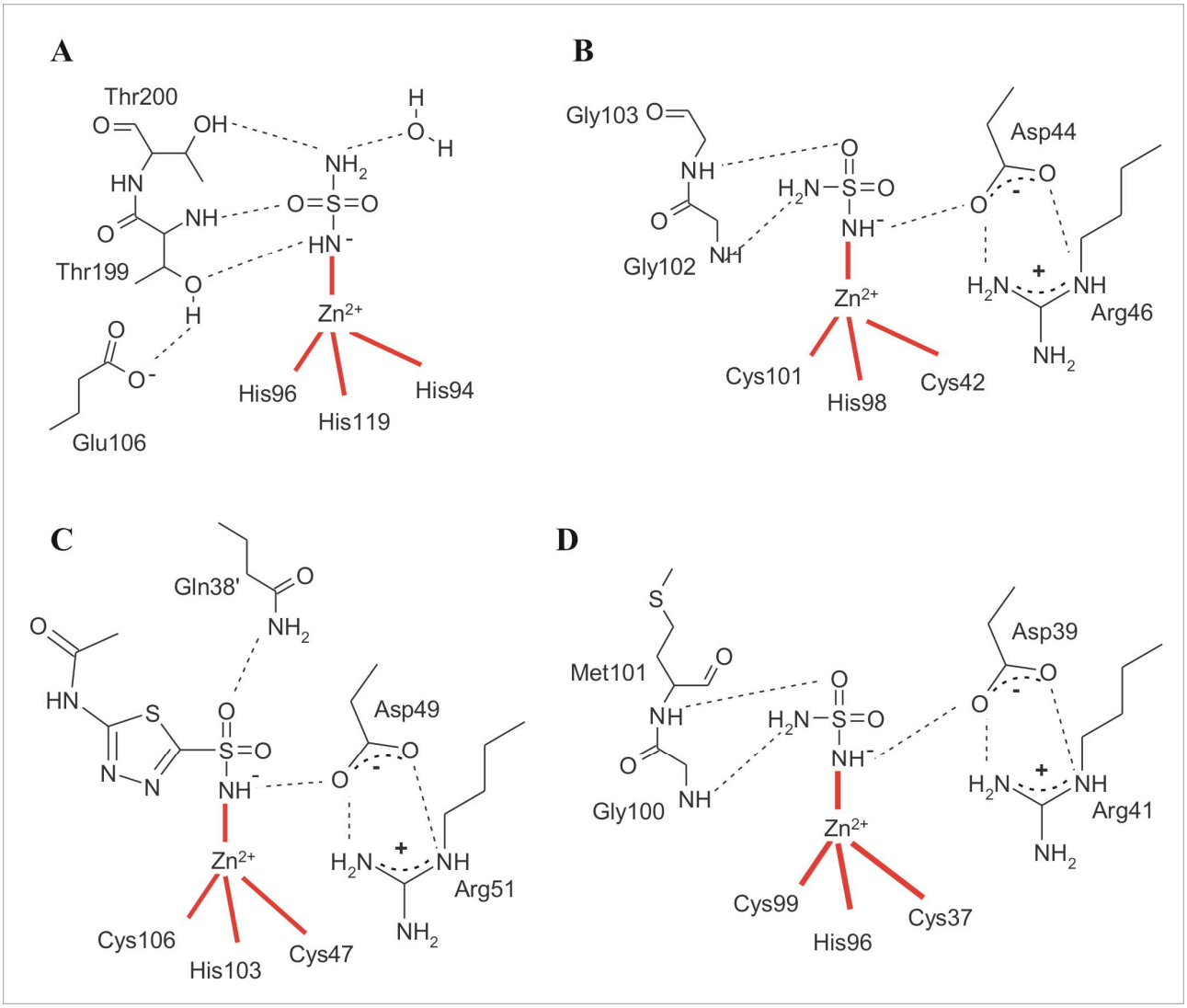

Fig. 2. Schematic representation of the binding mode of sulfamide to hCA II [34] (**A**) and to the β-CA from *Pseudomonas aeruginosa* [35] (**B**). (**C**) Schematic picture of **AZM** binding mode to β–CA from *Coccomyxa* (the residue Gln38' is from the adjacent monomer) [27]. (**D**) Hypothetical binding mode of sulfamide to TvaCA1 active site. The coordination of zinc ion is indicated by the red continuous lines, whereas hydrogen bond interactions by black dashed lines.