Activation studies with amines and amino acids of the β-carbonic anhydrase from the pathogenic protozoan *Leishmania donovani chagasi*

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Abstract. The activation of a β-class carbonic anhydrase (CAs, EC 4.2.1.1) from *Leishmania donovani chagasi* (LdcCA) was investigated using a panel of natural and non-natural amino acids and amines. The most effective activators belonged to the amine class, with histamine, dopamine, serotonin, 2-pyridyl-methylamine and 4-(2-aminoethyl)-morpholine with activation constants in the range of 0.23 – 0.94 μM. In addition, 2-(2-aminoethyl)pyridine and 1-(aminoethyl)-piperazine were even more effective activators (K_As of 9-12 nM). Amino acids such as L-/D-His, L-/D-Phe, L-/D-DOPA, L-/D-Trp and L-/D-Tyr were slightly less effective activators compared to the amines, but showed activation constants in the low micromolar range (1.27 – 9.16 μM). Many of the investigated activators are autacoids that are present in rather high concentrations in different tissues of the host mammals infected by these parasites. As CA activators have not yet been investigated for protozoan CAs, this study may be relevant for an improved understanding of the role of this enzyme in the life cycle of *Leishmania*.

Keywords: carbonic anhydrase; metalloenzymes, pathogens; activators; *Leishmania donovani chagasi*

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1. Introduction

Leishmaniasis is a rather diffuse sub-tropical disease provoked by protozoan belonging to *Leishmania* spp.[1,2] There are multiple forms of this disease, among which the visceral (VL), caused by *L. infantum* and *L. donovani*, as well as the tegumentary forms of the disease, which may include the cutaneous (CL), diffuse (DCL), and muco-cutaneous (MCL) leishmaniases.[1,2] The disease is transmitted by sand flies, and the life cycle of the pathogen is rather complex, as one of its developmental forms, the amastigote, dwells within immunological cells of the host, making its targeting by the immune system or by drugs rather challenging.[1,2] There are limited available drugs to treat this condition, and many strains of the parasite are increasingly resistant to drug treatment. Pentavalent antimonium salts (such as sodium stibogluconate, used via the parenteral route), the orally available miltefosine, or parenterally used paromomycin, and amphotericin B, show various degrees of resistance worldwide.[1,2] Thus, there is a strong need to design alternative therapies and to understand in more detail the life cycle of the parasite and its interactions with the mammalian host. Recently, we have proposed protozoan carbonic anhydrases (CAs, EC 4.2.1.1) as potential targets for dealing with this problem, showing that in *L. donovani chagasi*,[3,4] *Trypanosoma cruzi*, [5-7] or *Plasmodium falciparum*,[8-11] members of this family of enzymes are present, and that their inhibition interferes with the growth of the parasites *in vitro* and *in vivo*.[2-11] *L. donovani chagasi* encodes for a β-class CA (denominated LdcCA),[3] *T. cruzi* for an α-CA (TcCA),[5] and in *P. falciparum*, a new genetic family of these enzymes, the η-CA class has been discovered (PfaCA).[8] These three protozoans thus encode CAs belonging to three different genetic families, which suggests that their detailed investigation may lead to the discovery of potential drug targets with activities that can be selectively modulated for the management of the diseases they provoke. However, in contrast to the CA inhibitors (CAIs), were shown to inhibit the growth of these protozoan species, investigation of the CA activators (CAAs) have been limited.[12,13] Such compounds participate to the CA catalytic cycle, which is shown schematically in Equations 1 and 2 (where 'E' denotes enzyme):

$$EZn^{2+}\text{—}OH^- + CO_2 \Leftrightarrow EZn^{2+}\text{—}HCO_3^- \overset{H_2O}{\Leftrightarrow} EZn^{2+}\text{—}OH_2 + HCO_3^- \quad (1)$$

$$EZn^{2+}\text{—}OH_2 \Leftrightarrow EZn^{2+}\text{—}OH^- + H^+ \quad (2)$$

The first step involves a zinc-bound hydroxide species of the enzyme nucleophilically attacking a CO_2 substrate that is bound in a hydrophobic pocket nearby in an optimal orientation for the hydration reaction (Equation 1).[14,15] Bicarbonate formed in the hydration reaction replaced by an incoming water molecule to generate the catalytic acid form of the enzyme, EZn^{2+}—OH_2 (Equation 1). For the regeneration of the zinc hydroxide species, a proton transfer reaction occurs from the

Zn(II)-bound water molecule to the external medium (Equation 2), which is the rate-determining step of the entire catalytic cycle.

$EZn^{2+}—OH_2 + A \Leftrightarrow [EZn^{2+}—OH_2 - A] \Leftrightarrow [EZn^{2+}—OH^- - AH^+] \Leftrightarrow EZn^{2+}—OH^- + AH^+$ (*3*)

enzyme - activator complexes

In the presence of activators (A in Equation 3), the formation of enzyme-activator complexes occurs, in which the proton transfer reaction becomes intramolecular and thus, more efficient than the corresponding intermolecular process.[14,15] This mechanism of CA activation was demonstrated by kinetic and crystallographic studies for the human isoforms hCA I and II.[16] Based on crystal structures, the activator was bound at the entrance to the active site cavity. Most of the activators belong to the amino and/or amino acid chemotypes, and possess moieties with an appropriate pK_a (generally in the range of 6-8) for efficient proton shuttling processes between the active site and the environment.[14-16]

CAAs belonging to various classes were extensively investigated for the activation of all mammalian (human) CA isoforms known to date, hCA I – XIV.[17-21] Several drug design studies for CAAs belonging to the amine and amino acid classes have also been reported, and led to the discovery of the activation profile of the different isoforms with several classes of activators.[17-21] Recently, the potential of this class of pharmacological agents for the therapy of memory disorder and cognition impairment has also been demonstrated.[22] However, unlike CAIs, which are clinically used as diuretics,[23] antiglaucoma drugs,[24] antiobesity,[25] antitumor,[26] anti-neuropathic pain,[27] or anti-arthritis agents,[28] there are no clinically approved CAAs. Natural and non-natural amino acids and amines of type **1-19** are among the most investigated simple CAAs, and they were evaluated in the present study (Fig. 1). These compounds were investigated for their potential as CAAs against many classes of CAs, including a limited number of bacterial CAs.[29] However, no protozoan CA enzymes have been investigated for their activation to date. Here we report the first activation study of LdcCA, the β-class enzyme from the protozoan, *L. donovani chagasi*, one of the causative agents of visceral leishmaniasis.

2. Results and Discussion

To confirm that the activator binds to a different site than the substrate, the effect of the presence of L-Trp on the enzyme kinetics was investigated (Table 1). We have chosen this amino acid derivatives for the detailed kinetic studies due to the fact that it is a rather effective activator of many CAs, and its X-ray crystal structure in adduct with hCA II has also been reported.[13] Furthermore, this amino acid is present in rather high concentrations in many tissues in vertebrates, including humans, being the precursor of important autacoids/neurotransmitters such as serotonin.[13]

The K_m value does not depend on the presence of L-Trp. In contrast, k_{cat} depends strongly on the presence of L-Trp. For example, the use of 10 μM L-Trp resulted in the k_{cat} of LdcCA increasing by 3.05 times than without the activator (Table 1). These data are as expected, and confirm that the activator is not binding to the same site as the substrate, which has been documented by X-ray crystallography for the human enzymes.[13,16a] Moreover, L-Trp is a more effective towards LdcCA than the human enzymes used for comparison purposes.

Table 1: Activation of human carbonic anhydrase (hCA) isozymes I, II, and LdcCA with L-Trp, at 25°C, for the CO_2 hydration reaction.[30]

Isozyme	k_{cat}* (s^{-1})	K_M* (mM)	$(k_{cat})_{L\text{-}Trp}$** (s^{-1})	K_A*** (μM) L-Trp
hCA I[a]	$2.0x10^5$	4.0	$3.4x10^5$	44.0
hCA II[a]	$1.4x10^6$	9.3	$4.9x10^6$	27.0
LdcCA[b]	9.35×10^5	15.8	28.6×10^5	4.02

* Observed catalytic rate without activator. K_M values in the presence and the absence of activators were the same for the various CAs (data not shown).

** Observed catalytic rate in the presence of 10 μM activator.

*** The activation constant (K_A) for each enzyme was obtained by fitting the observed catalytic enhancements as a function of the activator concentration.[13] Mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[30] Standard errors were in the range of 5-10 % of the reported values (data not shown).

[a]Human recombinant isozymes, from ref.[13]; [b] Protozoan recombinant enzyme, this work.

The structure-activity relationship (SAR) for the activation of LdcCA with compounds **1-19**, can be delineated considering the data shown in Table 2, where the activation data of the human isoforms hCA I and II are also presented for comparison.

All amino acids and amines investigated act as efficient LdcCA activators, with activation constants that range from the low nanomolar (9 nM for **17**) to the micromolar (15.9 μM for **11**). Amines are generally more effective activators compared to the amino acids, except for L-adrenaline **19** (the only secondary amine investigated here) which has the same potency as most of the amino acids (K_A of 4.89 μM). The most effective LdcA activators were aminopyridine **16** and piperazine **17** (K_As of 9 – 12 nM), which incorporate a heterocyclic ring and the aminoethyl moiety (both of which can participate in proton transfer reactions between the zinc coordinated water and the external milieu). Amines **12-15** and **18**, structurally related to the most effective activators discussed above,

also show an interesting and efficient activating profile, with K_As ranging between 0.23 and 0.94 μM. Thus, the SAR here is rather well defined, with all these compounds possessing the aminoethyl- or aminomethyl tails appended to an aromatic or heterocyclic moiety. X-ray crystallography for adducts of some of these derivatives with hCA I or II showed that both these fragments of the activator are participating to the stabilization of the enzyme-activator complex, by forming hydrogen bonds and hydrophobic interaction with amino acid residues at the entrance of the active site cavity.[13,16]

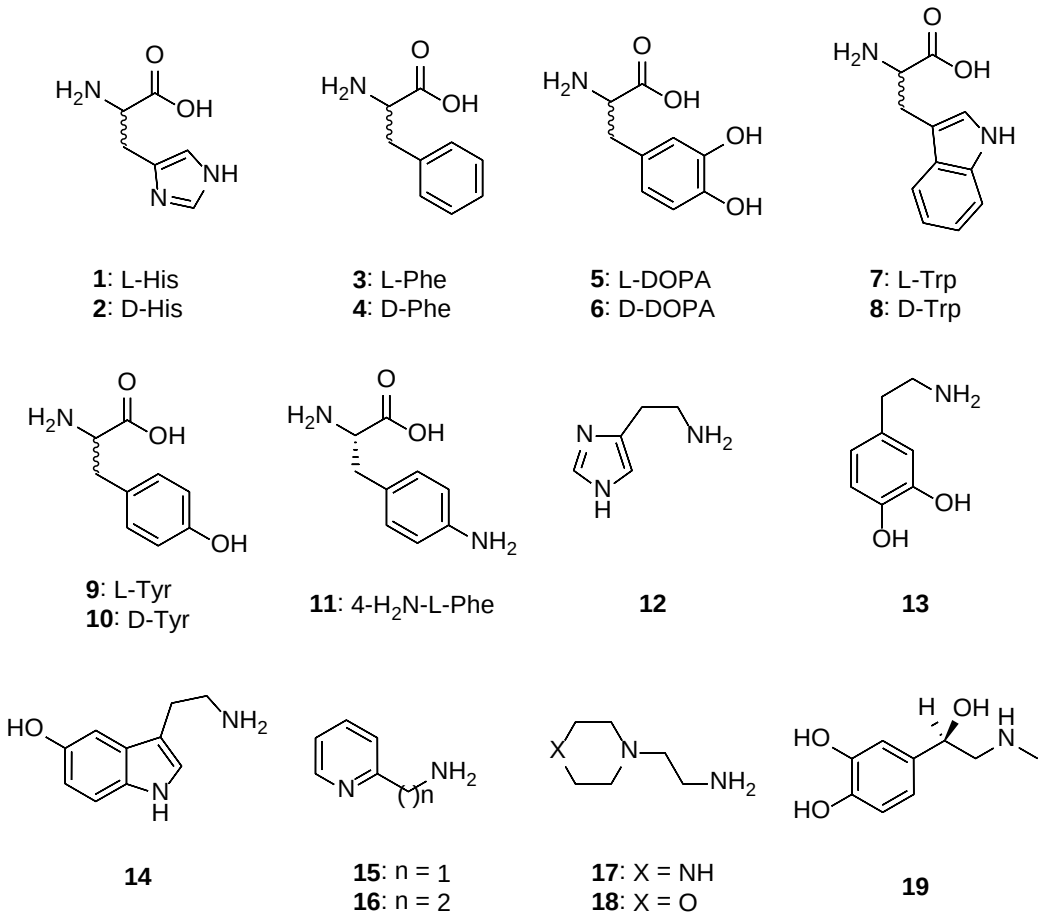

Fig. 1: Amino acids **1-11** and amines **12-19** investigated as LdcCA activators.

Table 2: Activation constants of hCA I, hCA II and the protozoan enzyme LdcCA with amino acids and amines **1 – 19**. Data for hCA I and II are from ref.[13]

No.	Compound	K_A (μM)*		
		hCA I[a]	hCA II[a]	LdcCA[b]
1	L-His	0.03	10.9	8.21
2	D-His	0.09	43	4.13
3	L-Phe	0.07	0.013	9.16
4	D-Phe	86	0.035	3.95

5	L-DOPA	3.1	11.4	1.64
6	D-DOPA	4.9	7.8	5.47
7	L-Trp	44	27	4.02
8	D-Trp	41	12	6.18
9	L-Tyr	0.02	0.011	8.05
10	D-Tyr	0.04	0.013	1.27
11	4-H_2N-L-Phe	0.24	0.15	15.9
12	Histamine	2.1	125	0.74
13	Dopamine	13.5	9.2	0.81
14	Serotonin	45	50	0.62
15	2-Pyridyl-methylamine	26	34	0.23
16	2-(2-Aminoethyl)pyridine	13	15	0.012
17	1-(2-Aminoethyl)-piperazine	7.4	2.3	0.009
18	4-(2-Aminoethyl)-morpholine	0.14	0.19	0.94
19	L-Adrenaline	0.09	96	4.89

* Mean from three determinations by a stopped-flow, CO_2 hydrase method.[30] Standard errors were in the range of 5-10 % of the reported values (data not shown).

[a] Human recombinant isozymes, stopped flow CO_2 hydrase assay method;[13,30]

[b]This work.

The amino acids **1-10** were slightly less effective as LdcCA activators compared to the amines discussed above, with K_As ranging between 1.27 and 15.9 μM. In several cases (D-His, D-Phe, D-Tyr), the D-enantiomer was more efficient as LdcCA activator compared to the corresponding L-enantiomer, whereas for the DOPA and Trp, the L-enantiomers were the more effective activators compared to the corresponding D-amino acids (Table 1). Small changes in the scaffold of the activator lead to important differences of activity: replacement of the phenolic OH from L-Tyr (**9**) by an amine moiety (as in **11**) lead to an almost 2-fold loss of activating properties.

The activation of the protozoan enzyme was rather different from that of the α-class enzymes hCA I and II. For example, L-/D-Tyr were highly effective, nanomolar hCA I and II activators, whereas their effects on LdcCA are seen only at micromolar concentrations. On the contrary, amine **16** may be considered as a LdcCA – selective activator, with a K_A of 12 nM for the protozoan enzyme and of 13-15 μM for the human CAs. Thus, this compound may be used as a pharmacologic tool to explore the role that LdcCA might play in the life cycle of this protozoan and whether CA activation

is important for the infection or host colonization by *Leishmania*, in diverse phases of the pathogen's life cycle.

3. Conclusions

The first activation study of a protozoan CA is reported here. The β-class enzyme from *Leishmania donovani chagasi* (LdcCA) was investigated for its interaction with a panel of natural and non-natural amino acids and amines acting as CAAs of mammalian and bacterial enzymes. Nanomolar activators were identified that belong to the amine class, with histamine, dopamine, serotonin, 2-pyridyl-methylamine and 4-(2-aminoethyl)-morpholine (activation constants of 0.23 to 0.94 μM). The most effective activators were 2-(2-aminoethyl)pyridine and 1-(aminoethyl)-piperazine which had K_As of 9-12 nM. Amino acids such as L-/D-His, L-/D-Phe, L-/D-DOPA, L-/D-Trp and L-/D-Tyr were less effective activators compared to the amines, but showed activation constants in the low micromolar range (1.27 – 9.16 μM). Because activators have not been identified for protozoan CAs, this study should be important for understanding the role that this enzyme has in the life cycle of *Leishmania*, particularly considering the fact that many of the activators identified are autacoids present in rather high concentrations in different tissues of the host mammals that are infected by these parasites.

4. Experimental

4.1. Chemistry. Amino acids and amines **1-19** were commercially available, highest purity reagents from Sigma-Aldrich, Milan, Italy. LdcCA was a recombinant protein produced as reported earlier by our group.[3]

4.2. CA enzyme activation assay

An Sx.18Mv-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic activity of various CA isozymes for CO_2 hydration reaction.[30] Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) or TRIS (pH 8.3) as buffers, 0.1 M Na_2SO_4 (for maintaining constant ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s at 25 ◦C. Activity of the α-CAs was measured at pH 7.5 whereas that of the β-class enzymes at pH 8.3 in order to avoid the possibility that their active site is closed.[3,12] The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation constants. For each activator 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 activators (10 mM) were prepared in distilled-deionized water and dilutions up to 1 nM were done thereafter with the assay buffer. Activator and enzyme solutions were pre-incubated together for 15 min (standard assay at room temperature) prior to assay, in order to allow for the formation of the E–A complex. The activation constant (K_A), defined similarly with the inhibition constant K_I, can be obtained by considering the classical Michaelis–Menten equation (equation 4), which has been fitted by non-linear least squares by using PRISM 3:

$$v = v_{max}/\{1+K_M/[S](1+[A]_f/K_A)\} \quad (4)$$

where $[A]_f$ is the free concentration of activator.

Working at substrate concentrations considerably lower than K_M ($[S] << K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$)and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by equation 5:

$$v=v_0.K_A/\{K_A+([A]_t-0.5\{([A]_t+[E]_t+K_A)-([A]_t+[E]_t+K_A)^2-4[A]_t.[E]_t)^{1/2}\}\} \quad (5)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[12,13,16]

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