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# 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 ($K_A$s 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*.

## 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 Eqs. (1) and (2) (where 'E' denotes enzyme):

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

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

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 (Eq. (1)) [14,15]. Bicarbonate formed in the hydration reaction

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replaced by an incoming water molecule to generate the catalytic acid form of the enzyme, $EZn^{2+}—OH_2$ (Eq. (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 (Eq. (2)), which is the rate-determining step of the entire catalytic cycle.

$$EZn^{2+}--OH_2 + A \Leftrightarrow [EZn^{2+}--OH_2{-}A] \underset{\text{enzyme-activator complexes}}{\Longleftrightarrow} [EZn^{2+}{-}OH^{-}{-}AH^{+}] \Leftrightarrow EZn^{2+}{-}OH^{-} + AH^{+} \quad (3)$$

In the presence of activators (A in Eq. (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 p$K_a$ (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.

**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] | $2.0 \times 10^5$ | 4.0 | $3.4 \times 10^5$ | 44.0 |
| hCA II[a] | $1.4 \times 10^6$ | 9.3 | $4.9 \times 10^6$ | 27.0 |
| LdcCA[b] | $9.35 \times 10^5$ | 15.8 | $28.6 \times 10^5$ | 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.

## 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

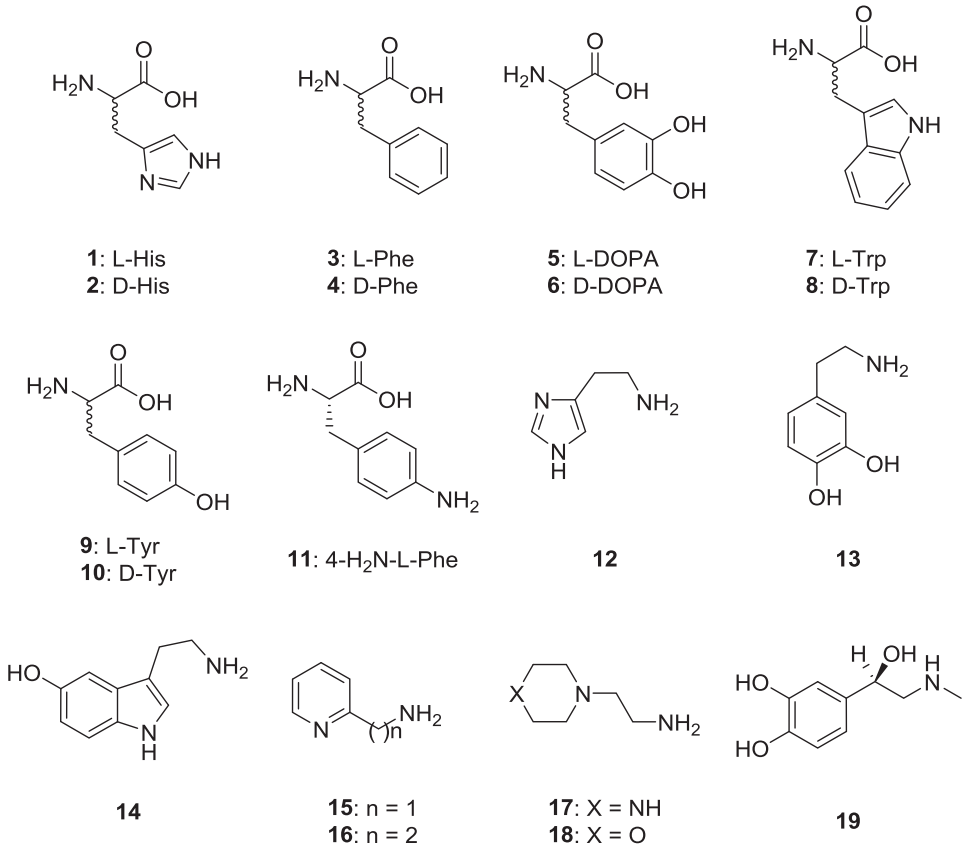


**Fig. 1.** Amino acids **1–11** and amines **12–19** investigated as LdcCA activators.