Activation studies with amines and amino acids of the \( \beta \)-carbonic anhydrase encoded by the \( \text{Rv3273} \) gene from the pathogenic bacterium \( \text{Mycobacterium tuberculosis} \)

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ABSTRACT

The activation of a \( \beta \)-class carbonic anhydrase (CAs, EC 4.2.1.1) from \( \text{Mycobacterium tuberculosis} \), encoded by the gene \( \text{Rv3273} \) (mtCA 3), was investigated using a panel of natural and non-natural amino acids and amines. mtCA 3 was effectively activated by D-DOPA, L-Trp, dopamine and serotonin, with \( K_a \) ranging between 8.98 and 12.1 \( \mu \)M. L-His and D-Tyr showed medium potency activating effects, with \( K_a \) in the range of \( 17.6-18.2 \mu \)M, whereas other amines and amino acids were relatively ineffective activators, with \( K_a \) in the range of \( 28.9-52.2 \mu \)M. As the physiological roles of the three mtCAs present in this pathogen are currently poorly understood and considering that inhibition of these enzymes has strong antibacterial effects, discovering molecules that modulate their enzymatic activity may lead to a better understanding of the factors related to the invasion and colonisation of the host during \( \text{Mycobacterium tuberculosis} \) infection.

1. Introduction

Among the bacterial infections which create huge medical problems worldwide, the \( \text{Mycobacterium tuberculosis} \) one is among the most threatening due to a number of causes: (i) it is estimated that one in each three people is latently infected with this pathogen, and although clinical manifestations emerge only in ill, old-aged or immunosuppressed patients, the ease of transmission of this infection creates serious medical challenges\(^1,2\); (ii) a large number of \( \text{M. tuberculosis} \) strains became drug resistant or extensively drug resistant to most of almost all clinically used antimycobacterial\(^3,4\); (iii) no new such drugs were launched for the last 30 years, which coupled to the general antibiotic resistance of many other pathogenic bacteria, of which \( \text{M. tuberculosis} \) is the tip of the iceberg, may lead to the resurgence of fatal bacterial infections worldwide.

In fact, since the 1950s, it has been considered that the fight against infective diseases caused by bacteria has been a success\(^5,6\). However, this does not seem to be the case any longer and new medical problems worldwide. The first step involves the nucleophilic attack of a zinc-bound hydroxide species of the enzyme on the \( \text{CO}_2 \) substrate, bound in a
hydropic pocket nearby and optimally orientated for the hydration reaction (Equation (1))7,8. Bicarbonate formed in the hydration reaction is then replaced by an incoming water molecule, with the generation of the catalytically acid form of the enzyme, EZN2+–OH2 (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:

\[
EZN^2+ – OH_2 + A \rightleftharpoons [EZN^2+ – OH_2 – A] \rightleftharpoons [EZN^2+ – HO^- – AH^+] \rightleftharpoons EZN^2+ – HO^- + AH^+ + activator complexes
\]

In the presence of activators (A in Equation (3)), the formation of enzyme–activator complexes occurs, in which the proton transfer reaction became intramolecular, being thus more efficient than the corresponding intermolecular process7,8. This mechanism of CA activation was demonstrated by kinetic and crystallographic studies for the human isoforms hCA I and II9. The activator-binding site was shown to be situated at the entrance of the active site cavity. Most of the activators belong to the amino and/or amino acid chemotypes, and possess moieties with an appropriate pKa (generally in the range of 6–8) for efficient proton shuttling processes between the active site and the environment7,9.

CAAs were extensively investigated in the last period for their interaction with all human CAs24–30, but their effects on bacterial enzymes were poorly studied up until now31–33. The same situation is in fact valid for the investigation of bacterial CA inhibitors, as mentioned above31–33.

Considering the fact that few CA activity studies of bacterial enzymes are available, and none of them for the mycobacterial enzymes, here we report the first such study in which we evaluated the activation of mtCA 3 (encoded by the gene Rv3273) with a panel of amines and amino acid derivatives.

2. Materials and methods

2.1. Materials

Amino acids and amines 1–19 were commercially available, highest purity reagents from Sigma-Aldrich, Milan, Italy. Rv3273 was a recombinant protein produced as reported earlier by our group4,5.

2.2. 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 isoforms for CO2 hydration reaction3. 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 Na2SO4 (for maintaining constant ionic strength), following the catalysed CO2 hydration reaction for a period of 10 s at 25°C. Activity of the x-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 closed7. The CO2 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 uncatalysed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activators (10 mM) were prepared in distilled-deionised 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 (KA), defined similarly with the inhibition constant KI, 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:

\[
v = \frac{v_{\text{max}}/\left(1 + [A]/[K_A]\right)}{1 + [A]/[K_A]}
\]

where [A] is the free concentration of activator.

3. Results and discussion

Natural and non-natural amino acids and amines 1–19 were included among the investigated compounds (Figure 1). These activators were included in this study, as they were employed for investigations as CAAs against many classes of CAs, including the few bacterial ones investigated so far3,30.

Data of Table 1 show that L-Trp (at 10 μM concentration), which is a medium potency activator for all enzymes considered here, i.e. hCA I, II and mtCA 3, enhanced kcat values for all of them, whereas KM remained unchanged, a situation observed for all CAAs investigated so far, both for those belonging to vertebrates (x-class enzymes) and micro-organism (enzymes belonging to various CA genetic families23–30,33). L-Trp was a micromolar activator for all these enzymes with KA in the range of 27–44 μM for hCA I and II, and with a KA of 8.98 μM against mtCA 3 (Table 1).

L-Trp induced an increase of the kinetic constant of hCA I and II compared with the uncatalysed rate (of 1.7–3.5 times, which for such efficient enzymes is highly significant). For the mtCA 3, a similar kinetic effect was observed, with an increase of kcat of 3.2 times in the presence of 10 μM L-Trp (compared with the rate in the absence of activator). We stress again, KM remained the same in the presence and absence of activator, which proves that the substrate and activator binding sites are different (this has been confirmed by X-ray crystallography for several x-CAs complexed with activators23–30).

Amino acids and amines 1–19 (Figure 1) previously investigated as CAAs of human (x-class) CAs25 and for the activation of few bacterial enzymes33 showed significant activating effects against mtCA 3, as observed from data of Table 2, in which the activation constants (KS) of these compounds against three CAs are presented (hCA I and II data are included for comparison reasons23). The following structure-activity relationship (SAR) can be evidenced from the data of Table 2:

i. The most effective mtCA 3 activators were D-DOPA, L-Trp, dopamine and serotonin, with KA ranging between 8.98 and 12.1 μM. Thus, both amino acid and amine types of activators show efficient activating effects on mtCA 3.

ii. L-His and D-Tyr showed medium potency activating effects, with KA in the range of 17.6–18.2 μM.
iii. The remaining derivatives showed a weaker mtCA 3 activation potency, with $K_a$ in the range of 28.9–52.2 μM. The SAR is thus rather well defined. For example, with few exceptions the L-amino acids were more effective mtCA 3 activators compared with the corresponding D-enantiomer. The exceptions are D-DOPA and D-Tyr which were more effective mtCA 3 activators compared with the corresponding L-enantiomer. Amines (with the exception of dopamine and serotonin) were generally less effective mtCA 3 activators compared with structurally related amino acid derivatives (compare histamine and L/D-His; L-adrenaline and L/D-DOPA, etc.), but the differences were not very important. In fact, no submicromolar mtCA 3 activators were detected in this study.

iv. There were important differences of activity for these CAAs against the human isoforms hCA I and II compared to the mycobacterial enzyme mtCA 3. Only L-Trp and serotonin were better activators of the bacterial versus the human isoforms, whereas all other compounds were more effective (sometimes in the nanomolar range) for activating the human CAs (Table 2).

### 4. Conclusions

The first activation study of a mycobacterial CA is reported here. mtCA 3 was effectively activated by D-DOPA, L-Trp, dopamine and...
serotonin, with $K_a$ ranging between 8.98 and 12.1 $\mu$M. l-His and $\delta$-Tyr showed medium potency activating effects, with $K_a$ in the range of 17.6–18.2 $\mu$M, whereas other amines and amino acids were weakly effective activators, with $K_a$ in the range of 28.9–52.2 $\mu$M. As the physiological role of the three mTCAs is poorly understood at this moment and the inhibition of such enzymes was shown to lead to strong antibacterial effects, modulating the activity of these CAs may lead to a better understanding of factors connected to the invasion and colonisation of the host during *Mycobacterium tuberculosis* infection.

**Disclosure statement**

The authors do not declare any conflict of interest.

**Funding**

This research was financed in part by a Distinguished Scientist Fellowship Programme (DSFP) of King Saud University, Saudi Arabia to CTS. The Australian Research Council is also acknowledged for support [DP160102681].

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