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Activation of β- and γ-carbonic anhydrases from pathogenic bacteria with tripeptides

Azzurra Stefanucci[a]*, Andrea Angeli[b]*, Marilisa Pia Dimmito[a], Grazia Luisi[a], Sonia Del Prete[b,c], Clemente Capasso[c], William A. Donald[d], Adriano Mollica[a] and Claudiu T. Supuran[b,d]

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ABSTRACT

Six tripeptides incorporating acidic amino acid residues were prepared for investigation as activators of β- and γ-carbonic anhydrases (CAs, EC 4.2.1.1) from the pathogenic bacteria *Vibrio cholerae, Mycobacterium tuberculosis,* and *Burkholderia pseudomallei*. The primary amino acid residues that are involved in the catalytic mechanisms of these CA classes are poorly understood, although glutamic acid residues near the active site appear to be involved. The tripeptides that contain Glu or Asp residues can effectively activate VchCAβ and VchCAγ (enzymes from *V. cholerae*), Rv3273 CA (mtCA3, a β-CA from *M. tuberculosis*) and BpsCAγ (γ-CA from *B. pseudomallei*) at 0.21–18.1 μM levels. The position of the acidic residues in the peptide sequences can significantly affect bioactivity. For three of the enzymes, tripeptides were identified that are more effective activators than both L-Glu and L-Asp. The tripeptides are also relatively selective because they do not activate prototypical α-CAs (human carbonic anhydrases I and II). Because the role of CA activators in the pathogenicity and life cycles of these infectious bacteria are poorly understood, this study provides new molecular probes to explore such processes.

KEYWORDS

Carbonic anhydrase; tripeptide; activator; proton transfer; pathogenic bacteria

Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous enzymes that interconvert carbon dioxide and bicarbonate[1–9]. There are seven genetically distinct CA families known to date in organisms across the phylogenetic tree. The α-CAs are widespread in vertebrates (in the form of a multitude of isoforms, including 15 in humans)[1,2], prokaryotes, and simpler eukaryotes (such as protozoa, fungi and some bacteria)[3–6]. The β- and γ-class enzymes are widespread in bacteria and archaea, but are not found in eukaryotic organisms[7–9]. CA inhibitors (CAIs) show pharmacologic applications in pathologies in which the activity of these enzymes is dysregulated (in humans), such as edema[10], glaucoma[11], neurologic diseases (epilepsy, etc.)[12], obesity[13], and some tumors[14], and many sulphonamide or sulphamate CAIs have been in clinical use for decades[15]. In contrast, investigation of activators of these enzymes (CAAs) have been relatively limited[2]. Recently, the potential to use of CAAs as pharmacological agents for pathologies related to cognitive impairment has been demonstrated, which may result in innovative memory therapies[16].

The CA catalytic mechanism is represented by Equations (1) and (2), where "E" corresponds to enzyme.

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

$$\mathrm{EZn^{2+} - OH_2 \Longleftrightarrow EZn^{2+}{-}HO^- + H^+} \quad (2)$$

First, a reactive metal hydroxide species nucleophilically attacks a CO_2 molecule that is bound in a hydrophobic pocket within the active site of the enzyme to form a metal-bound bicarbonate[17]. Typically, zinc is the metal ion in the active sites of most CA classes, although Cd(II) and Fe(II) may also work for some CAs[1]. The bicarbonate can be readily replaced by an incoming water molecule to generate an acidic metal-bound water molecule. In the rate-determining step, a proton is transferred from the metal-coordinated water molecule to the reaction medium to reform the metal-hydroxide species[18,19]. In all CA classes that have been investigated in detail to date, the rate-determining step is assisted by amino acid residues that are positioned in the active site pocket to favor the proton-transfer process[1–4,18–21]. For α-CAs that have been the most extensively studied CAs, the proton shuttling residue is a His placed in the middle of the active site pocket (His64, CA I numbering system)[18]. However, this proton-transfer process is less well understood in all other CA classes. For β-CAs, His and Tyr residues (His92 and Tyr88, *Coccomyxa* CA numbering)[20] may act as proton shuttle residues. For γ-CAs, Ferry's group[21] reported that one or two Glu residues (Glu84 and Glu62, Cam numbering system; Cam is the enzyme from *Methanosarcina thermophila*)[21] act as proton shuttles in the catalytic cycle. In the presence of activators (A in Equation (3), enzyme-activator complexes can be formed[19], and the proton transfer reaction is intramolecular and more efficient than intermolecular transfer to

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buffer molecules, which conventionally occurs in the absence of activators (Equation (3)).

$$EZn^{2+} - OH_2 + A \Longleftrightarrow [EZn^{2+} - OH_2 - A] \Longleftrightarrow [EZn^{2+} - HO^- - AH^+] \Longleftrightarrow EZn^{2+} - HO^- + AH^+ \quad (3)$$

enzyme–activator complexes

CAAs have been investigated in detail for human (h) α-CAs, by means of X-ray crystallography, kinetic and spectroscopic methods[19,22–24], and several drug design studies have also been reported[24–27]. However, no drug-design CAA studies are available for bacterial, β- and γ-CAs. These enzymes have only recently been started to be investigated for their activation with amines and amino acids[28–31]. The design of bioactive molecules that modulate these enzymes may be useful for controlling the intra- and extracellular pH of microorganisms, which can play crucial roles in the life cycles of pathogenic microorganisms. Here, we report such a study for investigating whether tripeptides incorporating acidic amino acid residues do show activating effects against β- and γ-class CAs from pathogenic bacteria such as *Vibrio cholerae* (the enzymes included in the study were VchCAβ and VchCAγ), *Mycobacterium tuberculosis* (Rv3273, also called mtCA3, one of the three β-CAs from this bacterium was considered here) and *Burkholderia pseudomallei* (BpsCAγ, a γ-CA from this pathogenic organism was used for our investigations)[28–31]. The amino acids used for obtaining these tripeptides, apart for the acidic ones (Glu and Asp) included aromatic (His, Phe and Tyr), hydroxy (Ser and Thr) as well as aliphatic (Ile) residues, in order to investigate the role that such structural elements may induce to the CA activating effects.

Materials and methods

Chemistry

All solvents and coupling reagents were purchased from VWR (Radnor, PN, USA). Fmoc amino acids and Fmoc-Rink-amide MBHA resin (0.68 mmol/g) were purchased from Chem-Impex (Wood Dale, IL, USA) and IRIS Biotech GmbH (Marktredwitz, DH, Germany) respectively. O*t*Bu was chosen as orthogonal protection on Tyr, Thr, Ser, Asp and Glu side chains, Boc protecting group for His side chain and Trt for Asn and Gln side chains. The peptides were synthesized by Fmoc-SPPS (standard solid phase peptide synthesis) using TBTU/HOBt for coupling reactions and piperidine 20% solution in DMF for Fmoc group deprotection as previously described[32].

Purification of compounds was carried out by RP-HPLC using a Waters XBridge Prep BEH130 C18, 5.0 μm, 250 × 10 mm column at a flow rate of 4.0 ml/min on a Waters Binary pump 1525, and a linear gradient of H_2O/acetonitrile 0.1% TFA ranging from 5% acetonitrile to 95% acetonitrile for 35 min. The purity of all final TFA salts was confirmed by NMR analysis, ESI-LRMS, and analytical RP-HPLC (C18-bonded 4.6 × 150 mm) at a flow rate of 1 ml/min, using as eluent a gradient of H_2O/acetonitrile 0.1% TFA ranging from 5% acetonitrile to 95% acetonitrile in 30 min and was found to be ≥95% (R_t reported in Table 1). Nuclear magnetic resonance (NMR) spectra of the final compounds were recorded on a Varian Inova 300 MHz spectrometer using DMSO-d6 as solvent. The mass spectrometry (MS) system used consisted of an LCQ (Thermo Finnigan) ion trap mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The capillary temperature was set at 300 °C and the spray voltage at 4.00 kV. The fluid was nebulized using nitrogen (N_2) as the sheath and auxiliary.

Table 1. Characterization data for the new peptides NH_2-Xaa_1-Xaa_2-Xaa_3-NH_2 **1**–**6** (TFA salts).

Compounds	Xaa_1	Xaa_2	Xaa_3	R_t (HPLC)[a] min.	MS calcd.	MS found
1	Tyr	Phe	Asp	12.44	442.19	443.31
2	His	Phe	Glu	11.57	430.21	431.41
3	Glu	Ile	Thr	11.78	360.41	361.56
4	Gln	Asp	Ser	11.11	347.14	348.25
5	Asn	Asp	Ser	11.08	333.13	334.18
6	Glu	Phe	Glu	11.70	422.43	423.51

[a]Analytical RP HPLC: C18 linear gradient of H_2O/acetonitrile 0.1% TFA starting from 5% acetonitrile to 95% acetonitrile in 30 min (solvent ramp: from 0 to 5 min: 5% ACN; from 5 to 20 min: 80% ACN; from 20 min to 25 min: 20% ACN; from 25 min to 30 min: 5% ACN).

General procedures for the tripeptide synthesis

Loading of the first amino acid

The resin was treated with a 20% piperidine solution in DMF (2*15 min) and then washed with DMF/MeOH/DCM. Then, the Fmoc protected amino acid (3 equiv) was dissolved in DMF (3 ml). TBTU (3 equiv) and DIPEA (6 equiv) were added and the resulting mixture was added to the resin. The Kaiser test was used to check the reaction. When complete, the resin was washed with DMF/MeOH/DCM.

Amino acids couplings

3 Equiv. of amino acid was dissolved in DMF (3 ml) together with TBTU (3 equiv.) and DIPEA (6 equiv.). Then the resulting mixture was added to the resin. The Kaiser test was used to check the reaction. When complete, the resin was washed with DMF/MeOH/DCM.

Cleavage and purification

The resin was treated with TFA/H_2O/TIPS 95:2.5:2.5 (5 ml for 1 h) and filtered. The solution was concentrated to 1 ml and precipitated in 10 ml of cold Et_2O. The suspension was centrifuged and washed three times with fresh Et_2O. The crude solid was dried in high vacuum and purified on RP-HPLC.

Characterization data for new compounds.

TFA·NH_2-Tyr-Phe-Asp-NH_2 (**1**): 81% yield; R_t (HPLC) = 12.44 min. ^{1}H NMR (DMSO-d_6) δ: 8.78 (1H, d, NH Phe), 8.44 (1H, d, NH Asp), 7.92 (3H, s, NH_3^+), 7.26–7.16 (7H, m, NH_2 Asp + 5H Phe aromatics), 7.14 (2H, s, NH_2 Asp amide), 7.03 (2H, dd, Tyr aromatics), 6.66 (2H, dd, Tyr aromatics), 4.58–4.52 (2H, m, CH^{α} Phe + CH^{α} Tyr), 3.85 (1H, m, CH^{α} Asp), 3.04 (1H, dd, CH^{β} Phe), 2.98–2.52 (1H, dd, CH^{β} Phe + 2H, m, CH_2^{β} Tyr); MS calcd: 442.19, found: 443.31.

TFA·NH_2-His-Phe-Glu-NH_2 (**2**): 57% yield; R_t (HPLC) = 11.57 min. ^{1}H NMR (DMSO-d_6) δ: 8.80 (3H, s, NH_3^+), 8.64 (1H, d, NH Phe), 7.32 (1H, d, NH Glu), 7.24 (1H, s, CH^{τ} His), 7.15 (2H, s, NH_2 Glu amide), 7.07 (1H, d, NH His), 6.90 (1H, d, CH^{π} His), 4.59 (1H, m, CH^{α} Phe), 4.20 (1H, m, CH^{α} His), 3.96 (1H, m, CH^{α} Glu), 3.08–2.83 (2H, m, CH_2^{β} Phe), 2.73–2.52 (2H, m, CH_2^{β} His), 2.41–2.20 (2H, m, CH_2^{β} Glu), 1.81–1.68 (2H, m, CH_2^{γ} Glu). MS calcd: 430.21, found: 431.41.

TFA·NH_2-Glu-Ile-Thr-NH_2 (**3**): 83% yield; R_t (HPLC) = 11.78 min. ^{1}H NMR (DMSO-d_6) δ: 8.54 (1H, d, NH Thr), 8.12 (3H, s, NH_3^+), 7.92 (2H, s, NH Ile), 7.11 (2H, s, NH_2 Thr amide), 4.15 (1H, d, CH^{α} Ile), 4.11 (1H, d, CH^{α} Thr), 3.98 (1H, quint, CH^{β} Thr), 3.85 (1H, m, CH^{α} Glu), 2.27 (2H, t, CH_2^{β} Glu), 1.87 (2H, m, CH_2^{γ} Glu), 1.72 (1H, m, CH^{β} Ile), 1.43 (1H, m, CH^{γ} Ile), 1.07 (1H, m, CH^{γ} Ile), 0.96 (3H, d,

CH_3 Thr), 0.94 (3H, d, CH_3 Ile), 0.84 (3H, t, CH_3^{δ} Ile). MS calcd: 360.41, found: 461.56.

TFA·NH_2-Gln-Asp-Ser-NH_2 (**4**): 87% yield; R_t (HPLC) = 11.11 min. ^{1}H NMR (DMSO-d_6) δ: 8.79 (1H, d, NH Asp), 8.15 (3H, s, NH_3^+), 7.98 (1H, d, NH Ser), 7.17 (2H, s, NH_2 Gln), 7.11 (2H, s, NH_2 Ser amide), 4.64 (1H, m, CH^{α} Asp), 4.13 (1H, m, CH^{α} Ser), 3.52 (1H, m, CH^{α} Gln), 2.75–2.56 (2H, m, CH_2^{β} Asp and CH_2^{β} Ser), 2.21 (2H, t, CH_2^{β} Gln), 1.91 (2H, m, CH_2^{γ} Gln). MS calcd: 347.14, found: 348.25.

TFA·NH_2-Asn-Asp-Ser-NH_2 (**5**): 84% yield; R_t (HPLC) = 11.08 min. ^{1}H NMR (DMSO-d_6) δ: 8.76 (1H, d, NH Asp), 8.08 (3H, s, NH_3^+), 7.94 (1H, d, NH Ser), 7.71 (1H, s, NH_2 Asn), 7.31 (1H, s, NH_2 Asn), 7.15 (2H, s, NH_2 Ser amide), 4.61 (1H, m, CH^{α} Asp), 4.14–3.62 (4H, m, CH^{α} Ser, CH^{α} Asn, CH^{α} Ser and OH Ser broad singlet at 3.863), 2.73 (2H, m, CH_2^{β} Asp), 2.69–2.48 (4H, m, CH_2^{β} Asn, CH_2^{β} Ser). MS calcd: 333.13, found: 334.18.

TFA·NH_2-Glu-Phe-Glu-NH_2 (**6**): 77% yield; R_t (HPLC) = 11.70 min. ^{1}H NMR (DMSO-d_6) δ: 8.61 (1H, d, NH Phe), 8.32 (1H, d, NH Glu), 8.05 (3H, s, NH_3^+ Glu), 7.31–7.19 (5H, m, Phe aromatics), 7.08 (2H, s, NH_2 Glu amide), 4.58 (1H, m, CH^{α} Phe), 4.19 (1H, m, CH^{α} Glu), 3.73 (1H, m, CH^{α} Glu), 3.07 (1H, dd, CH^{β} Phe), 2.75 (1H, dd, CH^{β} Phe), 2.31–2.23 (4H, m, $2CH_2^{\beta}$ Glu), 1.93–1.76 (4H, m, $2CH_2^{\gamma}$ Glu). MS calcd: 422.43, found: 423.51.

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[33]. 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-catalysed 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[34]. 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 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-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 ($[S] \ll K_M$), 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):

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

where v_0 represents the initial velocity of the enzyme-catalysed reaction in the absence of activator[35–38].

Results and discussion

Chemistry

Here, we designed the tripeptides **1**–**6** with amidated C-termini that incorporate at least one acidic amino acid (Asp and Glu) residue at various positions in the sequence (Table 1). In the sequence assemblage, we focused on our previous observation that single acidic amino acids like Asp and Glu act as powerful activators of selected bacterial CAs, in view of the fact that both possess the $-COO^-$ functionality that can participate in the proton transfer process[28–31]. Aromatic amino acids such as Phe and Tyr were also found to have significant activating abilities on the CAs belonging to pathogenic bacteria[28–31]. Thus, we considered three groups of compounds: (i) a pair of tripeptides that share the terminal dipeptide motif Asp-Ser, i.e. H-Gln-Asp-Ser-NH_2 (**4**) and H-Asn-Asp-Ser-NH_2 (**5**); (ii) H-Tyr-Phe-Asp-NH_2 (**1**), H-His-Phe-Glu-NH_2 (**2**), and H-Glu-Phe-Glu-NH_2 (**6**), all containing a central Phe, and characterized by Tyr, His, and Glu, respectively, as amino-terminals, and Glu or Asp at the carboxy-end; and (iii) H-Glu-Ile-Thr-NH_2 (**3**), which features a Glu residue at the N-terminal position. Peptides **1**–**6** were efficiently synthesized by following routine SPPS procedures[32], and obtained in the amidated form as TFA salts. Their main analytical data (HPLC and MS) are reported in Table 1. The complete characterization is shown in section 2.

CA activation studies

The six peptides activated the enzymes from pathogenic bacteria investigated here (Table 2), i.e. the β- and γ-CAs from *V. cholerae* (VchCAβ and VchCAγ), the Rv3273 CA (also called mtCA3, a β-CA from *M. tuberculosis*) and BpsCAγ (a γ-CA from *B. pseudomallei*). These four pathogens produce serious diseases in humans, and understanding factors connected to their invasion, colonization and virulence, and how these factors are influenced by modulators of CA activity, may be relevant to developing new therapeutic strategies devoid of the extensive drug resistance that has ultimately emerged for most clinically used anti-infective drugs[28–31].

In Table 2, the activation constants of tripeptides **1**–**6**, and some amino acids for four bacterial enzymes and the ubiquitous isoforms hCA I and II are shown. The six amino acids were included in this study for comparative reasons. The activation hCA I and II by these six amino acids were measured previously[23,24]. The activation constants of L-Phe and L-His for the four enzymes from pathogenic bacteria were also recently reported[28–31].

The following structure–activity relationship (SAR) can be obtained from the data in Table 2:

(i) VchCAβ was effectively activated by tripeptides **1**–**6** with activation constants ranging between 0.21 and 7.16 μM. The most effective activator was **4** (GlnAspSer), whereas the least effective one was **5** (AsnAspSer). Thus, the extra methylene group in Gln compared to Asn resulted in tripeptide **4** more effectively activating this enzyme by 34 times compared to **5** (Table 2). Other effective activators against this enzyme include tripeptides **2** and **3** that incorporate a Glu residue in the sequence. However, the tripeptide with two Glu residues (**6**) was less effective as an activator compared to **2** and **3**. It is interesting that L-Glu is a very effective VchCAβ activator (K_A of 0.69 μM), whereas L-Gln, L-His and L-Phe are much less effective activators (Table 2). L-Asp is moderately potent as an activator (K_A of 9.87 μM), but L-Asn is not.

(ii) The other β-CA investigated here, Rv3273, was less sensitive to these activators compared to that from *V. cholerae* enzyme; i.e.

Table 2. Activation of hCA I, hCA II, VchCAβ, Rv3273, VchCAγ and BpsCAγ with tripeptides **1**–**6** and simple amino acid derivatives, by a stopped-flow, CO_2 hydrase assay, at 25 °C and pH 8.4[33].

	K_A (μM)*					
Activator	hCA I[a]	hCA II[a]	VchCAβ	Rv3273	VchCAγ	BpsCAγ
1	>50	>50	3.52 ± 0.18	8.45 ± 0.11	14.7 ± 0.21	10.1 ± 0.09
2	>50	>50	1.16 ± 0.05	6.29 ± 0.14	5.84 ± 0.15	1.63 ± 0.12
3	>50	>50	1.15 ± 0.10	4.32 ± 0.08	11.9 ± 0.42	3.75 ± 0.15
4	>50	>50	0.21 ± 0.07	15.8 ± 0.76	12.9 ± 0.61	6.18 ± 0.30
5	>50	>50	7.16 ± 0.34	18.1 ± 1.02	10.6 ± 0.74	0.95 ± 0.09
6	>50	>50	4.18 ± 0.23	9.40 ± 0.62	2.74 ± 0.16	5.24 ± 0.30
L-Asp	5.20	>50	9.87 ± 0.41	10.1 ± 0.84	8.95 ± 0.46	10.7 ± 0.85
L-Asn	11.3	>50	>50	10.0 ± 0.71	6.37 ± 0.29	0.98 ± 0.08
L-Glu	6.43	>50	0.69 ± 0.05	>50	6.48 ± 0.50	3.25 ± 0.17
L-Gln	>50	>50	18.1 ± 0.94	21.6 ± 1.1	9.21 ± 0.82	6.15 ± 0.30
L-His[a]	0.03	10.9	20.3	18.2	1.01	24.7
L-Phe[a]	0.07	0.013	15.4	30.6	0.73	1.73

*Mean ± standard error, from three different assays.
[a]From Refs.[28–31].

tripeptides **1**–**6** had K_As in the range of 4.32 to 18.1 μM for this CA. The most effective activator was **3** (GluIleThr), whereas the least effective was **5** (AsnAspSer). Tripeptide **2** was the next most effective activator after **3**. These latter two peptides both have one Glu residue, albeit in opposing positions (amino-terminal vs carboxy-terminal). Considering the simple amino acid derivatives of Table 2, L-Glu was in this case ineffective as an activator whereas the remaining amino acids were moderately potent to weak activators (activation constants from 10.0 to 30.6 μM).

(iii) VchCAγ was activated by tripeptides **1**–**6** with K_As ranging between 2.74 and 14.7 μM. The most effective activator was **6**, which incorporates two Glu residues in the sequence, followed by **2**, which has one such carboxy-terminal residue. The remaining tripeptides were less effective activators, with K_As > 10 μM (Table 2). For this isoform, the best activators were the simple aromatic amino acids L-His and L-Phe (K_As of 0.73–1.01 μM) whereas L-Asp, L-Asn, L-Glu and L-Gln showed activities in the range of 6.37–9.21 μM. Thus, the SAR is rather challenging to delineate for this enzyme and with this series of activators.

(iv) BpsCAγ was efficiently activated by tripeptides **1**–**6** with K_As ranging between 0.95 and 10.1 μM. The best activators were **5** and **2** (K_As of 0.95 and 1.63 μM, respectively), which do not share much in similarity except that in both sequences there is one acidic amino acid residue, Asp in **5**, and Glu in **2**. The most ineffective activator was **1**, which does not incorporate such a residue. However, it is interesting to note that L-Asn with a K_A of 0.98 μM was the most effective activator among the simple amino acids considered in the study. Indeed, this latter activation constant was one order of magnitude lower than that for L-Asp, whereas such an important difference is not seen for the L-Glu/L-Gln pair (Table 2).

(v) A very interesting observation is the fact that the human isoforms hCA I and II were not at all activated by tripeptides **1**–**6** investigated here (K_A > 50 μM), although they are highly activated by some of the amino acids, such as L-His, and L-Phe. hCA II is in fact sensitive only to these two amino acids, whereas hCA I is also activated by L-Asp, L-Asn, L-Glu (but not L-Gln) and of course, L-His and L-Phe (there are X-ray crystal structures for adducts of hCA I/II with some of these two amino acids, which proved in detail the activation mechanism of α-Cas)[23,24].

Conclusions

We discovered a very interesting class of tripeptide activators for bacterial β- and γ-class CAs, which do not interfere with the activity of the off-target, human isoforms hCA I and II. These activators incorporate aromatic amino acid residues, as well as acidic (Asp and Glu) residues in their sequence which may have roles in the rate-determining proton-transfer processes in the catalytic mechanism of these enzymes. The activity of the tripeptides differ both across the two classes of enzymes and between particular members of each class from different pathogens, such as *V. cholerae, M. tuberculosis* and *B. pseudomallei*. Overall, these tripeptides may be useful as tools for investigating the role of these enzymes in key bacterial processes such as invasion, colonization and pathogenicity, which are currently poorly understood.

Disclosure statement

No potential conflict of interest was reported by the authors.

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