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

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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.

INTRODUCTION
Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous enzymes that interconvert carbon dioxide and bicarbonate. 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), prokaryotes, and simpler eukaryotes (such as protozoa, fungi and some bacteria). The β- and γ-class enzymes are widespread in bacteria and archaea, but are not found in eukaryotic organisms. CA inhibitors (CAIs) show pharmacological applications in pathologies in which the activity of these enzymes is dysregulated (in humans), such as edema, glaucoma, neurologic diseases (epilepsy, etc.), obesity, and some tumors, and many sulfonamide or sulphamate CAs have been in clinical use for decades. In contrast, investigation of activators of these enzymes (CAAs) have been relatively limited. 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.

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

\[
\begin{align*}
\text{H}_2\text{O} & \quad \text{E} \text{Zn}^{2+} + \text{OH}^- + \text{CO}_2 \quad \Leftrightarrow \quad \text{E} \text{Zn}^{2+} - \text{H}_2\text{O} + \text{HCO}_3^- \\
\text{E} \text{Zn}^{2+} \quad \text{OH}_2 & \quad \Leftrightarrow \quad \text{E} \text{Zn}^{2+} - \text{H}_2\text{O} + \text{H}^+ 
\end{align*}
\]

First, a reactive metal hydroxide species nucleophilically attacks a CO₂ molecule that is bound in a hydrophobic pocket within the active site of the enzyme to form a metal-bound bicarbonate. 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. 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. 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. 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). 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) may act as proton shuttle residues. For γ-CAs, Ferry’s group reported that one or two Glu residues (Glu84 and Glu62, Cam numbering system; Cam is the enzyme from Methanosarcina thermophila) act as proton shuttles in the catalytic cycle. In the presence of activators (A in Equation (3), enzyme-activator complexes can be formed, and the proton transfer reaction is intramolecular and more efficient than intermolecular transfer to
buffer molecules, which conventionally occurs in the absence of activators (Equation (3)).

\[
\text{EZN}^{2+} - \text{OH}_2 + \text{A} \iff [\text{EZN}^{2+} - \text{OH}_2 - \text{A}]
\]
\[
\iff [\text{EZN}^{2+} - \text{HO}^- - \text{A}^+] \iff \text{EZN}^{2+} - \text{HO}^- + \text{A}^+ \tag{3}
\]

enzyme-activator complexes

CAAs have been investigated in detail for human (h) x-CAs, by means of X-ray crystallography, kinetic and spectroscopic methods, and several drug design studies have also been reported. However, no drug-design CAA studies are available for bacterial, y-CAs. These enzymes have only recently been started to be investigated for their activation with amines and amino acids. 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 x- and y-class CAs from pathogenic bacteria such as \textit{Vibrio cholerae} (the enzymes included in the study were VcHAβ and VcHCAγ), \textit{Mycobacterium tuberculosis} (Rv3273, also called mtCA3), one of the three β-CAs from this bacterium was considered here) and \textit{Burkholderia pseudomallei} (BpsCAγ, a γ-CA from this pathogenic organism was used for our investigations). The amino acids used for obtaining these tripeptides, apart from 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, PA, 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. OtBu 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.

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 H₂O/acetonitrile 0.1% TFA ranging from 5% acetonitrile to 95% acetonitrile in 30 min and was found to be ≥95% (R) reported in Table 1). Nuclear magnetic resonance (NMR) spectra for 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 (N₂) as the sheath and auxiliary.

### Table 1. Characterization data for the new peptides NH₂-Xaa₁-Xaa₂-Xaa₃-NH₂ 1–6 (TFA salts).

| Compounds | Xaa₁ | Xaa₂ | Xaa₃ | Rᵢ (HPLC) μin. | MS calcld. | 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         | Glu  | 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   |

*Analytical RP HPLC: C18 linear gradient of H₂O/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/H₂O/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 Et₂O. The suspension was centrifuged and washed three times with fresh Et₂O. The crude solid was dried in high vacuum and purified on RP-HPLC.

Characterization data for new compounds

TFA-NH₂-Tyr-Phe-Asp-NH₂ (1): 81% yield; Rᵢ (HPLC) = 12.44 μin. 1H NMR (DMSO-d₆) δ: 8.78 (1H, d, NH Phe), 8.44 (1H, d, NH Asp), 7.92 (3H, s, NH₃⁺), 7.26–7.16 (7H, m, NH₂ Asp + 5H Phe aromatics), 7.14 (2H, s, NH₂ Asp amide), 7.03 (2H, dd, Tyr aromatics), 6.66 (2H, dd, Tyr aromatics), 4.58–4.52 (2H, m, CH₂ Phe + CH₂ Tyr), 3.85 (1H, m, CH₃ Asp), 3.04 (1H, dd, CH₂ Phe), 2.98–2.52 (1H, dd, CH₂ Phe + 2H, m, CH₂ Ty); MS calcld: 442.19, found: 443.31.

TFA-NH₂-His-Phe-Glu-NH₂ (2): 57% yield; Rᵢ (HPLC) = 11.57 μin. 1H NMR (DMSO-d₆) δ: 8.80 (3H, s, NH₃⁺), 8.64 (1H, d, NH Phe), 7.32 (1H, d, NH Glu), 7.24 (1H, s, CH⁻¹ His), 7.15 (2H, s, NH₂ Glu amide), 7.07 (1H, d, NH His), 6.90 (1H, d, CH⁻¹ His), 4.59 (1H, m, CH⁻¹ Phe), 4.20 (1H, m, CH⁻¹ His), 3.96 (1H, m, CH⁻¹ Glu), 3.08–2.83 (2H, m, CH₂ Phe), 2.73–2.52 (2H, m, CH₂ Glu), 2.41–2.20 (2H, m, CH₂ Glu), 1.81–1.68 (2H, m, CH₂ Glu) MS calcld: 430.21, found: 431.41.

TFA-NH₂-Glu-Ile-Thr-NH₂ (3): 83% yield; Rᵢ (HPLC) = 11.78 μin. 1H NMR (DMSO-d₆) δ: 8.54 (1H, d, NH Thr), 8.12 (3H, s, NH₃⁺), 7.92 (2H, s, NH Ile), 7.11 (2H, s, NH₂ Thr amide), 4.15 (1H, d, CH₃ Ile), 4.11 (1H, d, CH₃ Thr), 3.98 (1H, quint, CH² Thr), 3.85 (1H, m, CH³ Glu), 2.27 (2H, m, CH₂ Glu), 1.87 (2H, m, CH₂ Glu), 1.72 (1H, m, CH³ Ile), 1.43 (1H, m, CH² Ile), 1.07 (1H, m, CH³ Ile), 0.96 (3H, d,
\[ CH_3 \text{Thr}, 0.94 (3H, d, CH_3 \text{ ile}), 0.84 (3H, t, CH_3 \text{ ile}). \text{ MS calcd: 360.41, found: 461.56.} \]

TFA NH2-Gln-Asp-Ser-NH2 (4): 87% yield; \( R_t \) (HPLC) = 11.11 min.

\[ ^1H \text{ NMR (DMSO-d}_6) \delta: 8.79 (1H, d, NH Asp), 8.15 (3H, s, NH_3^+), 7.98 (1H, d, NH Ser), 7.17 (2H, s, NH_2 Glu), 7.11 (2H, s, NH_2 Ser amide), 6.44 (1H, m, CH_4 Asp), 4.13 (1H, m, CH_4 Ser), 3.52 (1H, m, CH_4 Glu), 2.75–2.56 (2H, m, CH_2 Asp and CH_2\text{ Ser}), 2.21 (2H, t, CH_2\text{ Glu}), 1.91 (2H, m, CH_2\text{ Glu}). \text{ MS calcd: 347.14, found: 348.25.} \]

\[ 3.73 (1H, m, CH_2\text{ Ser}), 1.91 (2H, m, CH_2\text{ Ser}) \]

CA enzyme activation studies

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 reaction\(^{35}\). Phenol red (at a concentration of 0.2 mM) was used as an 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 CA-catalysed CO2 hydration reaction for a period of 10 s at 25 °C. Activity of the enzyme was determined in the same manner and subtracted from the total observed rate. 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 determined in the same manner and subtracted from the total observed rate. 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 determined in the same manner and subtracted from the total observed rate. 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 determined in the same manner and subtracted from the total observed rate. 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 determined in the same manner and subtracted from the total observed rate.

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 assembly, 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-NH2 (4) and H-Asn-Asp-Ser-NH2 (5); (ii) H-Tyr-Phe-Asp-NH2 (1), H-His-Phe-Glu-NH2 (2), and H-Glu-Phe-Glu-NH2 (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-NH2 (3), which features a Glu residue at the N-terminal position. Peptides 1–6 were efficiently synthesized by following routine SPPS procedures\(^{25,27}\), 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 \( \beta^\prime \) and \( \gamma^-\)CAs from \( V. \text{cholerae} \) (VchCA\(^{\beta^\prime} \) and VchCA\(^\gamma\)), the Rv3273 CA (also called mtCA3, a \( \beta^-\)CA from \( M. \text{tuberculosis} \)) and BpsCA\(^\gamma\) (a \( \gamma^-\)CA from \( B. \text{pseudomallei} \)). 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\(^{25,24}\). The activation constants of L-Phe and L-His for the four enzymes were efficiently synthesized by following routine SPPS procedures\(^{25,27}\), 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.

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tripeptides 1–6 had $K_{\text{As}}$ in the range of 4.32 to 18.1 $\mu M$ for this CA. The most effective activator was 3 (GluLeuThr), whereas the least effective was 5 (AspAspSer). 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 ineffective case as an activator whereas the remaining amino acids were moderately potent to weak activators (activation constants from 10.0 to 30.6 $\mu M$).

(iii) VchCAγ was activated by tripeptides 1–6 with $K_{\text{As}}$ ranging between 2.74 and 14.7 $\mu M$. 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 $K_{\text{As}} > 10 \mu M$ (Table 2). For this isoform, the best activators were the simple aromatic amino acids L-His and L-Phe ($K_{\text{As}}$ of 0.73–10.1 $\mu M$) whereas L-Asp, L-Asn, L-Asc, L-Glu and L-Gln showed activities in the range of 6.37–9.21 $\mu M$. 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 $K_{\text{As}}$ ranging between 0.95 and 10.1 $\mu M$. The best activators were 5 and 2 ($K_{\text{As}}$ of 0.95 and 1.63 $\mu M$, 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 $K_{\text{A}}$ of 0.98 $\mu M$ 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 ($K_{\text{A}} > 50 \mu M$), 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 $\gamma$-Cas)?23,24

Conclusions

We discovered a very interesting class of tripeptide activators for bacterial $\beta$- and $\gamma$-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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