Activation of human \(\alpha\)-carbonic anhydrase isoforms I, II, IV and VII with bis-histamine Schiff bases and bis-spinaceamine substituted derivatives

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ABSTRACT
A series of histamine bis-Schiff bases and bis-spinaceamine derivatives were synthesised and investigated as activators of four human (h) carbonic anhydrase (CA, EC 4.2.1.1) isoforms, the cytosolic hCA I, II and VII, and the membrane-associated hCA IV. All isoforms were effectively activated by the new derivatives, with activation constants in the range of 4.73–10.2 \(\mu\)M for hCA I, 6.15–42.1 \(\mu\)M for hCA II, 2.37–32.7 \(\mu\)M for hCA IV and 32 nM–18.7 \(\mu\)M for hCA VII, respectively. The nature of the spacer between the two histamine/spinaceamine units of these molecules was the main contributor to the diverse activating efficacy, with a very different fine tuning for the diverse isoforms. As CA activators recently emerged as interesting agents for enhancing cognition, in the management of CA deficiencies, or for therapy memory and artificial tissues engineering, our compounds may be considered as candidates for such applications.

1. Introduction
In previous research from our groups\textsuperscript{5,6}, we reported two novel classes of activators of the enzyme carbonic anhydrase (CA, EC 4.2.1.1): the histamine Schiff bases\textsuperscript{1} and the spinaceamine derivatives\textsuperscript{2}. As all CA activators (CAAs), these new classes of enzyme modulators also participate in the catalytic cycle of the enzyme\textsuperscript{3–6}. Indeed, CAs are metallopeptidases, usually using Zn(II) ions within their active site for performing the efficient hydration of CO\(_2\) to bicarbonate and protons. A water molecule coordinated to the zinc ion becomes highly nucleophilic, and as hydroxide ion attacks the CO\(_2\) molecule bound within the active site of the enzyme, with formation of bicarbonate coordinated to the zinc (Equation (1))\textsuperscript{7–10}. Another incoming water molecule subsequently displaces the bound bicarbonate, liberating it in solution, and leading to the formation of the acidic species of the enzyme, with water as zinc ligand Equation (1). In order to obtain the nucleophilic species of the enzyme, with the hydroxide coordinated to the zinc Equation (2), a proton transfer reaction must occur, which is the rate determining step of the entire catalytic cycle\textsuperscript{3–7,10–1}.

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

It has been demonstrated that the activators A in Equation (3) intervene in this step, providing an alternative pathway for the release of protons and formation of the zinc hydroxide species of the enzyme\textsuperscript{3–6}.

\[\begin{align*}
\text{E}\text{Zn}^{2+} = \text{OH}^- + \text{A} &\iff [\text{E}\text{Zn}^{2+} = \text{OH}^- – \text{A}] \\
&\iff [\text{E}\text{Zn}^{2+} = \text{HO}^- + \text{AH}^+] \\
&\iff \text{E}\text{Zn}^{2+} = \text{HO}^- + \text{AH}^+ \\
&\iff \text{enzyme – activator complexes} \\
\end{align*}\]
artificial tissues. Thus, there is a strong interest in designing CAAs belonging to various chemical classes, in order to detect compounds with high efficiency and eventually isomorf-selective action, considering that in humans at least 15 CA isoforms were described so far. Here, we report some new CAAs obtained by considering our previous findings, that is, histamine Schiff bases and spinacamine derivatives, which possess efficient CA activating properties.

2. Materials and methods

2.1. Chemistry

All chemicals and anhydrous solvents were purchased from Sigma-Aldrich, Merck, Alfa Aesar and TCI and used without further purification. Melting points (mp) were determined with SMP30 melting point apparatus in open capillaries and are uncorrected. FT-IR spectra were recorded using a Perkin Elmer Spectrum 100 FT-IR spectrometer. Nuclear Magnetic Resonance (1H-NMR and 13C-NMR) spectra of compounds were recorded using a Bruker Advance III 300 MHz spectrometer in DMSO-d6 and TMS as an internal standard operating at 300 MHz for 13C-NMR and 75 MHz for 13C-NMR. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F254 plates.

2.1.1. General procedure for the synthesis of bis-histamine schiff bases H1, H2, H3 and H4

Potassium hydroxide (10 mmol) was added to a stirred suspension of histamine dihydrochloride (5 mmol) in dry EtOH (10–15 ml) at room temperature. After stirring for 2 h, the precipitate salt (KCl) was filtered off and the filtrate was treated with a solution of bis-aldehydes (2.5 mmol) in dry EtOH (20–25 ml). The homogeneous mixture was stirred overnight at room temperature. The completion of the reaction was monitored by TLC and FT-IR. The excess solvent was evaporated and the oily residue was crystallized with ethyl acetate and diethylether to obtain the corresponding bis-histamine Schiff bases H1, H2, H3 and H4.

2.2. CA activation

An 8x18Mv-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic activity of various CA isoforms for CO2 hydration reaction. 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) as buffer, 0.1 M NaClO4 (for maintaining constant ionic strength), following the CA-catalysed CO2 hydration reaction for a period of 30 s at 25 °C. The CO2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each activator at least six traces of the initial 5 min 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 (at 0.1 mM) were prepared in distilled-deionised water and then stored at -20 °C. The pH of the reaction mixture was adjusted to be 7.4 by means of 0.1 M NaOH or 0.1 M HCl. The pH was determined with a pH-meter (Eutech Instruments, PHS-230, Singapore). 1H-NMR and 13C-NMR spectra were recorded using a Bruker Advance III 300 MHz spectrometer in DMSO-d6 and TMS as an internal standard operating at 300 MHz for 13C-NMR and 75 MHz for 13C-NMR. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F254 plates.

2.1.2. General procedure for the synthesis of bis-Spinacamine substituted compounds SPH1, SPH2, and SPH4

To a solution of 5 mmol of histamine dihydrochloride in 10 ml of water were added solutions of 15 mmol of sodium hydroxide (NaOH) and 2.5 mmol of appropriate bis-aldehyde derivatives in 15 ml of ethanol. The reaction mixture was heated overnight at 80 °C. The completion of the reaction was monitored by TLC and FT-IR. After that, the mixture was allowed to cool to room temperature and the formed precipitate was filtered off. The crude powders were recrystallized from hot water and dried under vacuum at 40 °C to afford SPH1, SPH2, and SPH4, which were fully characterized by FT-IR, 1H-NMR, 13C-NMR, and melting points.

1.4-Phenylethenebis(methanylylidene)bis(2-(1H-imidazol-4-yl)ethanamine) (H1): Yield: 65%; Colour: cream powder, mp: 190–192°C; FT-IR (cm⁻¹): 3117, 2926, 2849, 1648 (-C=O/C=O); 3085, 3020, 2841, 2631, 1636 (-C=O/C=O); 1512, 1434, 1398, 1347, 1279, 1135, 1034, 932, 837, 792, 751, 720, 628, 523, 435, 397, 330 (cm⁻¹).

2.1.3. General procedure for the synthesis of bis-Spinacamine substituted compounds SPH1, SPH2, and SPH4

Furan-2,5-diylibis(methanylylidene)bis(2-(1H-imidazol-4-yl)ethanamine) (H4): Yield: 32%; Colour: brown powder, mp: 114–117°C; FT-IR (cm⁻¹): 3107, 3016, 2926, 2853, 1621 (-C=N-), 1433, 1224, 816, 75 MHz, (δ ppm): 8.88 (s, 2H, -N=CH-), 7.99 (d, 2H, J = 2.2, H-2 Im), 7.74 (s, 2H, H-5 Im), 6.85 (d, 2H, J = 2.8, furan), 3.52 (t, 4H, J = 5.8, -CH2CH2-Im), 2.88 (t, 4H, J = 5.8, -CH2CH2-Im); 13C-NMR (DMSO-d6, 75 MHz, δ ppm): 166.2 (-N = CH-), 151.4, 144.9, 138.3, 134.7, 119.5, 113.2, 56.3, 31.5;

1.4-Phenylethenebis(methanylylidene)bis(2-(1H-imidazol-4-yl)ethanamine) (H1): Yield: 65%; Colour: cream powder, mp: 190–192°C; FT-IR (cm⁻¹): 3117, 2926, 2849, 1648 (-C=O/C=O); 3085, 3020, 2849, 1648 (-C=O/C=O); 1512, 1434, 1398, 1347, 1279, 1135, 1034, 932, 837, 792, 751, 720, 628, 523, 435, 397, 330 (cm⁻¹).

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dilutions up to 1 nM were made thereafter with the assay buffer. Enzyme and activator solutions were pre-incubated together for 15 min prior to assay, in order to allow for the formation of the enzyme–activator complexes. 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:

$$v = \frac{v_{max}}{1 + \left(\frac{K_M}{[S]}\right)\left(1 + \frac{[A]^*}{K_a}\right)}$$

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

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

$$v = \frac{v_0}{1 + \frac{K_A}{[E]_t + [K_A]}}$$

where $v_0$ represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator14–18. All CAs were recombinant proteins obtained as reported earlier18.

### 3. Results and discussion

#### 3.1. Chemistry

The rationale for designing CAAs presented in this work is based on our previous data which showed efficient CA VII activating effects for derivatised histamine Schiff base compounds and spinaceamine derivatives1,2. Therefore, in this work, a number of structurally diverse bis-histamine Schiff bases and bis-spinaceamine substituted compounds were synthesised according to general synthetic routes as illustrated in Scheme 1. In order to generate chemical diversity, different bis-aldehydes were chosen, possessing aromatic and heterocyclic moieties, and they were reacted with histamine leading to the bis-histamine Schiff bases and bis-spinaceamine substituted compounds SH1, H2, H3, H4, SPH1, SPH2, and SPH4 (Scheme 1). All the synthesised compounds were fully characterised by using several analytical and spectral data (see experimental part for details).

In the current work, the synthesis of a series of bis-histamine Schiff bases and bis-spinaceamine substituted compounds was carried out with some modifications of the literature procedures5. Briefly, histamine dihydrochloride was coupled with substituted aromatic and heterocyclic bis-aldehydes, leading to the formation of bis-histamine Schiff bases and ring-closure products of histamine. The structures of bis-histamine Schiff bases and bis-spinaceamine substituted compounds were confirmed by using several analytical and spectral data (FT-IR, 1H-NMR, 13C-NMR, and melting points) as described in the experimental part.

#### 3.2. CA activation

Considering the fact that the new heterocyclic derivatives H1-H4 and SPH1, 2 and 4 reported here incorporate in their molecules two functionalities with a pKa appropriate for acting as proton shuttles in the CA catalytic cycle3,12, we have investigated them as CAAs against the following four CA isoforms with important physiological functions: the three cytosolic enzymes (h = human), hCA I, II and VII19, and the membrane-associated hCA IV20. They are involved in various pathologies, both in the CNS, kidneys, eyes and other organs in which they are highly abundant21–23.

The following structure–activity relationship (SAR) can be evidenced from data of Table 1:

i. Among the four investigated isoforms, hCA VII was the most sensitive to these activators (similar to the lead compounds used for obtaining these derivatives1,2), followed by hCA IV and I, whereas hCA II was the least sensitive to the activating effects of these compounds. However, these new derivatives reported here – H1-H4 and SPH(1, 2 and 4) – were much more effective as hCA II activators compared to histamine (HST), a standard activator5 (Table 1).
Table 1. CA activation data with bis-histamines H1-H4 and bis-spinaceamines SPH(1, 2 and 4) and histamine(HST) as a standard activator, by a stopped-flow CO2 hydrase assay.

| Compound | hCA I | hCA II | hCA IV | hCA VII |
|----------|-------|--------|--------|---------|
| H1       | 4.73  | 42.1   | 3.96   | 9.02    |
| H2       | 6.15  | 30.7   | 3.28   | 18.7    |
| H3       | 18.4  | 25.9   | 10.9   | 21.3    |
| H4       | 7.13  | 20.3   | 3.45   | 0.085   |
| SPH1     | 10.2  | 8.21   | 32.7   | 0.032   |
| SPH2     | 6.29  | 6.15   | 8.12   | 0.039   |
| SPH4     | 9.87  | 19.2   | 2.37   | 0.035   |
| HST      | 2.10  | 125    | 4.03   | 37.6    |

*Ka (µM)*

*a*Mean from 3 different determinations (errors in the range of 5–10% of the reported values, data not shown).

ii. The slow cytosolic isoform hCA I was activated efficiently by H1-H4 and SPH(1, 2 and 4), with Ka's ranging between 4.73–18.4 µM. Similar activities were observed for the bis-histamine Schiff bases and the bis-spinaceamine derivatives, with the main factor influencing activity being the spacer between the two imidazole moieties. Indeed, for this isoform, the p-phenylene spacer present in H1 and the m-phenylene one, present in H2 and SPH2, led to the most effective activators (Table 1):

iii. The fast cytosolic enzyme hCA II was also effectively activated by the new derivatives, with Ka's ranging between 6.15 and 42.1 µM (compared to a Ka of 125 µM for histamine). The rationale of our drug design was in fact to introduce two proton shuttling moieties, of the histamine/spinaceamine type, in order to enhance the affinity for the enzyme and to facilitate the rate-determining step of the catalytic cycle. Although for hCA I this is not obvious, for hCA II the activating effects of the bis-derivatives investigated here are indeed much higher compared to the mono-derivatives incorporating just one proton shuttling moiety, as histamine. In fact, the best bis-activator of hCA II, compound SPH2, is 20.3 times a more effective activator compared to histamine (Table 1):

iv. The membrane-anchored hCA IV was activated by the new derivatives with activation constants ranging between 2.37 and 32.7 µM. Many of the new activators (e.g., H1, H2, H4 and SPH4) were more effective than histamine (Ka of 4.03 µM) whereas the remaining ones were slightly less effective. Again the spacer between the two imidazole(-like) units was the main factor responsible of these effects, with the 2,5-furylene one leading to effective hCA IV activators (H4 and SPH4).

v. The most activatable isoform was the brain-associated hCA VII, for which the new activators reported here showed Ka's ranging between 32 nM and 187.8 µM. One histamine bis-Schiff base (H4) and all three bis-spinaceamines SPH1, 2 and 4, were nanomolar hCA VII activators, with affinities of 32-85 nM (Table 1). Thus, for these last derivatives, the nature of the spacer had less influence on activity, as all of them show a behavior of potent activators, whereas for the histamine derivatives only the furyl-containing compound (H4) was an effective activator, with the phenylene ones H1-H3 being several orders of magnitude less effective.

4. Conclusions

We report here a small series of histamine bis-Schiff bases and bis-spinaceamine derivatives, which were synthesized by original procedures and investigated as activators of four hCA isoforms involved in a variety of diseases, the cytosolic hCA I, II and VII, and the membrane-associated hCA IV. All these isoforms were effectively activated by the new derivatives, with activation constants in the range of 4.73–10.2 µM for hCA I, 6.15–42.1 µM for hCA II, 2.37–32.7 µM for hCA IV and 32 nM -187.8 µM for hCA VII, respectively. The nature of the spacer between the two histamine/spinaceamine units of these molecules was the main contributor to the diverse activating efficacy, with a very different fine tuning for the diverse isoforms. As CA activators recently emerged as interesting agents for enhancing cognition, in the management of CA deficiencies, or for therapy memory and artificial tissues engineering, our compounds may be considered as candidates for such applications.

Disclosure statement

The authors declare no conflict of interest.

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