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RESEARCH ARTICLE



## A class of carbonic anhydrase I – selective activators

Erol Licsandru<sup>a</sup>, Muhammet Tanc<sup>b</sup>, Istvan Kocsis<sup>a</sup>, Mihail Barboiu<sup>a</sup> and Claudiu T. Supuran<sup>b</sup>

<sup>a</sup>Adaptive Supramolecular Nanosystems Group, Institut Europeen des Membranes, University of Montpellier ENSCM-UMR CNRS 5635, Montpellier, France; <sup>b</sup>Department of Neurofarba and Laboratorio di Chimica Bioinorganica, Sezione di Chimica Farmaceutica e Nutraceutica, Università degli Studi di Firenze, Sesto Fiorentino (Florence), Italy

### ABSTRACT

A series of ureido and bis-ureido derivatives were prepared by reacting histamine with alkyl/aryl-isocyanates or di-isocyanates. The obtained derivatives were assayed as activators of the enzyme carbonic anhydrase (CA, EC 4.2.1.1), due to the fact that histamine itself has this biological activity. Although inhibition of CAs has pharmacological applications in the field of antiglaucoma, anticonvulsant, anticancer, and anti-infective agents, activation of these enzymes is not yet properly exploited pharmacologically for cognitive enhancement or Alzheimer's disease treatment, conditions in which a diminished CA activity was reported. The ureido/bis-ureido histamine derivatives investigated here showed activating effects only against the cytosolic human (h) isoform hCA I, having no effect on the widespread, physiologically dominant isoform hCA II. This is the first report in which CA I-selective activators were identified. Such compounds may constitute interesting tools for better understanding the physiological/pharmacological effects connected to activation of this widespread CA isoform, whose physiological function is not fully understood.

### ARTICLE HISTORY

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### Introduction





The carbonic anhydrases (CAs, EC 4.2.1.1) represent a superfamily of metalloenzymes, with six distinct genetic families known to date, the  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -,  $\zeta$ -, and  $\eta$ -CAs, all of the which efficiently catalyze the reaction between CO<sub>2</sub> and water, with the formation of bicarbonate and protons<sup>1–11</sup>. The inhibition and the activation of CAs are well-understood processes: most types of classical inhibitors bind to the metal center within the enzyme active site<sup>12–21</sup>, whereas the activators bind at the entrance of the active site cavity and participate in proton shuttling processes between the metal ion-bound water molecule and the environment<sup>22–24</sup>. This leads to enhanced formation of the metal hydroxide, catalytically active species of the enzyme<sup>1,21–24</sup>.

The substrates/reaction products involved in the CA catalyzed reaction, i.e. CO<sub>2</sub>, bicarbonate and protons, are essential molecules/ions in many important physiologic processes in all life kingdoms (*Bacteria*, *Archaea*, and *Eukarya*), throughout the tree of life, and for this reason, relatively high amounts of these enzymes are present in different tissues/cell compartments of most investigated organisms<sup>1–11</sup>.

Sulfonamides are the most important class of CA inhibitors (CAIs)<sup>25–43</sup>, with at least 20 such compounds in clinical use for decades, or in clinical development<sup>44–79</sup>. Sulfonamide/sulfamate CAIs are used as diuretics, antiglaucoma, anticonvulsant, and anti-obesity agents<sup>80–87</sup>, whereas the anticancer and anti-infective use of such derivatives started to be investigated only recently<sup>3,4</sup>. Furthermore, in the last period, the use of CAIs for the management of neuropathic pain<sup>88</sup>, organ preservation without ischemia

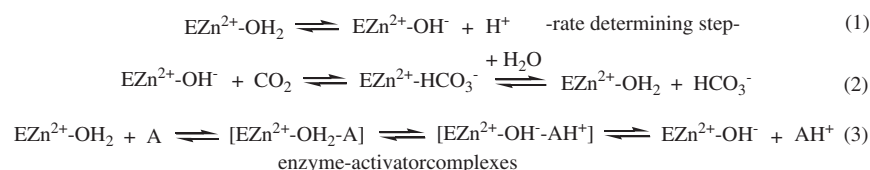
reperfusion injury<sup>89</sup>, and the management of cerebral ischemia<sup>90</sup> were also reported, extending thus the applications of these pharmacological agents. However, the activators of CAs (CAAs), although known for decades<sup>23,24</sup>, do not have at this moment pharmacological applications. This is due to several reasons, the first of which has to do with the catalyzed reaction (Scheme 1).

As mentioned above, the catalytically effective species of all CAs has a metal hydroxide species within the active site, which for the  $\alpha$ -CAs is a zinc hydroxide species generated from a water molecule bound to the Zn<sup>2+</sup> ion (Equation (1) in Scheme 1). This is also the rate-determining step for the catalytic cycle of many CAs and it is assisted by buffers present in the medium as well as by an amino acid residue from the middle of the active site cavity, His64, which has a pK<sub>a</sub> of about 7 and may shuttle protons between the active site and the environment<sup>23,24</sup>. The second step (Equation (2) in Scheme 1) involves the nucleophilic attack of the zinc hydroxide to the CO<sub>2</sub> molecule bound in a hydrophobic pocket, with formation of bicarbonate coordinated to zinc, which is thereafter replaced by an incoming water molecule, with formation of the acidic species of the enzyme, with water as the fourth zinc ligand<sup>1–3,23,24</sup>. Many CAs are highly effective catalysts, with turnover numbers of  $>10^8$  s<sup>-1</sup>, close to the limit of diffusion-controlled processes<sup>1–3</sup>. Thus, many researchers in the period starting with 50s until the 90s were reluctant to admit that CAs may have activators. Only in 1997, we reported the first X-ray crystal structure of an activator bound to the human (h) CA isoform hCA II. This activator was histamine<sup>23</sup>. The activator was found bound at the entrance of the CA active site cavity, with the imidazole moiety participating in shuttling protons between the active site and

**CONTACT** Mihail Barboiu  [mihail-dumitru.barboiu@univ-montp2.fr](mailto:mihail-dumitru.barboiu@univ-montp2.fr)  Adaptive Supramolecular Nanosystems Group, Institut Europeen des Membranes, University of Montpellier ENSCM-UMR CNRS 5635, Place Eugene Bataillon CC047, Montpellier, F-34095, France; Claudiu T. Supuran  [claudiu.supuran@unifi.it](mailto:claudiu.supuran@unifi.it)  Department of Neurofarba and Laboratorio di Chimica Bioinorganica, Sezione di Chimica Farmaceutica e Nutraceutica, Università degli Studi di Firenze, Sesto Fiorentino (Florence), Italy

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**Scheme 1.** CA-catalyzed CO<sub>2</sub> hydration reactions (steps 1 and 2), and CA activation with an activator molecule A.

the bulk solvent, thus acting as a second proton shuttle of the enzyme in addition to His64, whereas the amino group from the aminoethyl moiety of histamine did not participate in any interaction with the enzyme active site<sup>23</sup>.

The second reason why CAAs do not have for the moment pharmacological applications is due to their difficult pharmacology. Indeed, it has been reported that some CAAs (such as phenylalanine or imidazole) administered to experimental animals may produce an important pharmacological enhancement of synaptic efficacy, spatial learning, and memory, proving that this class of relatively unexplored enzyme modulators may have important applications in conditions in which learning and memory are impaired, such as for example aging or Alzheimer's disease<sup>91,92</sup>. One must also mention that it was reported that the levels of CA are significantly diminished in the brain of patients affected by Alzheimer's disease<sup>93</sup> and these facts strongly support the involvement of different brain CA isozymes in cognitive functions. However, no clinical trials for the use of CAAs for the management of these conditions were done at this moment. One should mention that the chemistry and biochemistry of this class of derivatives was thoroughly investigated, with a large number of activators classes (mainly histamine, catecholamine, and amino acid derivatives<sup>94–104</sup>) reported and several CA – activator adducts X-ray crystal structures available<sup>23,24,95–97</sup>. However, few isoform-selective CAAs are known to date. Here we report a class of CA I-selective CAAs, based on the histamine scaffold, which has been derivatized by using cyanate/dicyanate chemistry.

## Materials and methods

### Chemistry

All the compounds were synthesized following the general procedure described below. The amine (30 mmol) was mixed with the corresponding amount of isocyanate, under sonication (1 eq.: 1eq. for the monourea compounds and 2 eq. for the diurea compounds). The mixture was solubilized in 10 ml of tetrahydrofuran (THF), 5 ml of ethylacetate, and 10 ml of dimethylacetamide. The reaction mixture was heated to 120 °C for 15 min. When the precipitation began, 5 ml of acetonitrile were added and the heating was maintained for another hour. The resulting product (a white powder) was filtered and washed with methanol. The exceptions of the protocol were compounds **1**, **6**, **10**, and **11** for which the reaction temperature was 60 °C and compounds **2**, **7**, **12**, and **13** for which the reaction temperature was 80 °C. Compound **1** is soluble in the reaction mixture and, therefore, the purification procedure consisted in evaporation of the solvent under vacuum in a round bottomed flask and recrystallization from CHCl<sub>3</sub>. Alternatively a microwave reactor has been used. The procedure was the following: the isocyanate was dissolved in 5 ml of acetonitrile and added over the amine in the microwave reactor. The reaction was performed at 140 °C under energetic stirring, for 15 min. The product was filtered and washed with methanol. In the case of compounds **1**, **6**, **10**, and **11**, the temperature was 50 °C and for compounds **2**, **7**, **12**, and **13** was 90 °C. For

compound **1**, the purification method was the same. Ureas **1–22** were characterized using <sup>1</sup>H NMR methods and mass spectrometry.

### 1-(2-(1H-imidazol-4-yl)ethyl)-3-butylurea **1**: (mass spectrometry, ES M<sup>+</sup> = 211.1)

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 0.86 (t, 3H, CH<sub>3</sub>CH<sub>2</sub>); 1.30 (m, 4H, CH<sub>3</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>); 2.58 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 2.96 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.21 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH), 5.75 (s mod, 1H, NHCH<sub>2</sub>); 5.83 (s mod, 1H, NHCH<sub>2</sub>); 6.78 (s, 1H, C CHNH imidazole); 7.55 (s, 1H, N CHNH imidazole).

### 1-(2-(1H-imidazol-4-yl)ethyl)-3-hexylurea **2**: (mass spectrometry, ES M<sup>+</sup> = 239.1)

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 0.86 (t, 3H, CH<sub>3</sub>CH<sub>2</sub>); 1.24–1.34 (m, 8H, CH<sub>3</sub>(CH<sub>2</sub>)<sub>4</sub>CH<sub>2</sub>); 2.57 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 2.96 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.21 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH), 5.75 (s mod, 1H, NHCH<sub>2</sub>); 5.84 (s mod, 1H, NHCH<sub>2</sub>); 6.75 (s, 1H, C CHNH imidazole); 7.54 (s, 1H, N CHNH imidazole).

### 1-(2-(1H-imidazol-4-yl)ethyl)-3-octylurea **3**: (mass spectrometry, ES M<sup>+</sup> = 267.1)

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 0.86 (t, 3H, CH<sub>3</sub>CH<sub>2</sub>); 1.25–1.34 (m, 12H, CH<sub>3</sub>(CH<sub>2</sub>)<sub>6</sub>CH<sub>2</sub>); 2.58 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 2.95 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.21 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH), 5.74 (s mod, 1H, NHCH<sub>2</sub>); 5.82 (s mod, 1H, NHCH<sub>2</sub>); 6.76 (s, 1H, C CHNH imidazole); 7.52 (s, 1H, N CHNH imidazole).

### 1-(2-(1H-imidazol-4-yl)ethyl)-3-dodecylurea **4**: (mass spectrometry, ES M<sup>+</sup> = 323.2)

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 0.85 (t, 3H, CH<sub>3</sub>CH<sub>2</sub>); 1.25–1.33 (m, 20H, CH<sub>3</sub>(CH<sub>2</sub>)<sub>10</sub>CH<sub>2</sub>); 2.57 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 2.93 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.21 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH), 5.75 (s mod, 1H, NHCH<sub>2</sub>); 5.83 (s mod, 1H, NHCH<sub>2</sub>); 6.75 (s, 1H, C CHNH imidazole); 7.50 (s, 1H, N CHNH imidazole).

### 1-(2-(1H-imidazol-4-yl)ethyl)-3-octadecylurea **5**: (mass spectrometry, ES M<sup>+</sup> = 407.3)

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 0.86 (t, 3H, CH<sub>3</sub>CH<sub>2</sub>); 1.24–1.33 (m, 32H, CH<sub>3</sub>(CH<sub>2</sub>)<sub>16</sub>CH<sub>2</sub>); 2.58 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 2.95 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.21 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH), 5.75 (s mod, 1H, NHCH<sub>2</sub>); 5.82 (s mod, 1H, NHCH<sub>2</sub>); 6.78 (s, 1H, C CHNH imidazole); 7.56 (s, 1H, N CHNH imidazole).

### 1-(Butane-1,4-diyl)bis(3-(2-(2H-imidazol-4-yl)ethyl)urea) **6**: (mass spectrometry, ES M<sup>+</sup> = 363.2)

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 1.32 (m, 4H, CH<sub>3</sub>(CH<sub>2</sub>)<sub>2</sub>CH<sub>2</sub>); 2.58 (t, 4H, NHCH<sub>2</sub>CH<sub>2</sub>); 2.95 (q, 4H, CH<sub>2</sub>CH<sub>2</sub>NH);

3.22 (q, 4H, CH<sub>2</sub>CH<sub>2</sub>NH), 5.78 (s mod, 2H, NHCH<sub>2</sub>); 5.88 (s mod, 2H, NHCH<sub>2</sub>); 6.76 (s, 2H, C CHNH imidazole); 7.51 (s, 2H, N CHNH imidazole).

**1-(Hexane-1,6-diyl)bis(3-(2-(2H-imidazol-4-yl)ethyl)urea) 7: (mass spectrometry, ES M<sup>+</sup> = 391.2)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 1.22–1.36 (m, 8H, CH<sub>3</sub>(CH<sub>2</sub>)<sub>4</sub>CH<sub>2</sub>); 2.57 (t, 4H, NHCH<sub>2</sub>CH<sub>2</sub>); 2.92 (q, 4H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.22 (q, 4H, CH<sub>2</sub>CH<sub>2</sub>NH); 5.76 (s mod, 2H, NHCH<sub>2</sub>); 5.85 (s mod, 2H, NHCH<sub>2</sub>); 6.76 (s, 2H, C CHNH imidazole); 7.55 (s, 2H, N CHNH imidazole).

**1-(Octane-1,8-diyl)bis(3-(2-(2H-imidazol-4-yl)ethyl)urea) 8: (mass spectrometry, ES M<sup>+</sup> = 419.3)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 1.24–1.36 (m, 12H, CH<sub>3</sub>(CH<sub>2</sub>)<sub>6</sub>CH<sub>2</sub>); 2.58 (t, 4H, NHCH<sub>2</sub>CH<sub>2</sub>); 2.95 (q, 4H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.21 (q, 4H, CH<sub>2</sub>CH<sub>2</sub>NH); 5.76 (s mod, 2H, NHCH<sub>2</sub>); 5.84 (s mod, 2H, NHCH<sub>2</sub>); 6.77 (s, 2H, C CHNH imidazole); 7.54 (s, 2H, N CHNH imidazole).

**1-(Dodecane-1,12-diyl)bis(3-(2-(2H-imidazol-4-yl)ethyl)urea) 9: (mass spectrometry, ES M<sup>+</sup> = 475.2)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 1.24–1.33 (m, 16H, CH<sub>3</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>2</sub>); 2.59 (t, 4H, NHCH<sub>2</sub>CH<sub>2</sub>); 2.95 (q, 4H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.22 (q, 4H, CH<sub>2</sub>CH<sub>2</sub>NH); 5.77 (s mod, 2H, NHCH<sub>2</sub>); 5.83 (s mod, 2H, NHCH<sub>2</sub>); 6.82 (s, 2H, C CHNH imidazole); 7.62 (s, 2H, N CHNH imidazole).

**(R)-1-(2-(1H-imidazol-4-yl)ethyl)-3-(3-methylbutan-2-yl)urea 10: (mass spectrometry, ES M<sup>+</sup> = 225.2)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 0.81 (q, 6H, CH<sub>3</sub>CHCH<sub>3</sub>); 0.92 (d, 3H, CH<sub>3</sub>CHHC<sub>2</sub>); 1.57 (h, 1H, CH<sub>3</sub>CHCH<sub>3</sub>); 2.60 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 3.21 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.45 (m, 1H, CH<sub>2</sub>CH<sub>3</sub>CHNH); 5.68 (s mod, 1H, NHCH<sub>2</sub>); 5.71 (s mod, 1H, NHCH<sub>2</sub>); 6.82 (s, 1H, C CHNH imidazole); 7.51 (s, 1H, N CHNH imidazole).

**(S)-1-(2-(1H-imidazol-4-yl)ethyl)-3-(3-methylbutan-2-yl)urea 11: (mass spectrometry, ES M<sup>+</sup> = 225.2)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 0.81 (q, 6H, CH<sub>3</sub>CHCH<sub>3</sub>); 0.92 (d, 3H, CH<sub>3</sub>CHHC<sub>2</sub>); 1.56 (h, 1H, CH<sub>3</sub>CHCH<sub>3</sub>); 2.57 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 3.20 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.45 (m, 1H, CH<sub>2</sub>CH<sub>3</sub>CHNH); 5.68 (s mod, 1H, NHCH<sub>2</sub>); 5.70 (s mod, 1H, NHCH<sub>2</sub>); 6.76 (s, 1H, C CHNH imidazole); 7.51 (s, 1H, N CHNH imidazole).

**(R)-1-(2-(1H-imidazol-4-yl)ethyl)-3-(hexan-2-yl)urea 12: (mass spectrometry, ES M<sup>+</sup> = 239.3)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 0.86 (t, 3H, CH<sub>3</sub>CH<sub>2</sub>); 0.97 (d, 3H, CH<sub>3</sub>CHHC<sub>2</sub>); 1.24 (m, 6H, CH<sub>3</sub>(CH<sub>2</sub>)<sub>3</sub>CH<sub>2</sub>); 2.57 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 3.21 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.54 (m, 1H, CH<sub>2</sub>CH<sub>3</sub>CHNH); 5.64 (s mod, 1H, NHCH<sub>2</sub>); 5.69 (s mod, 1H, NHCH<sub>2</sub>); 6.81 (s, 1H, C CHNH imidazole); 7.51 (s, 1H, N CHNH imidazole).

**(S)-1-(2-(1H-imidazol-4-yl)ethyl)-3-(hexan-2-yl)urea 13: (mass spectrometry, ES M<sup>+</sup> = 239.2)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 0.84 (t, 3H, CH<sub>3</sub>CH<sub>2</sub>); 0.97 (d, 3H, CH<sub>3</sub>CHHC<sub>2</sub>); 1.23 (m, 6H, CH<sub>3</sub>(CH<sub>2</sub>)<sub>3</sub>CH<sub>2</sub>); 2.56 (t, 2H,

NHCH<sub>2</sub>CH<sub>2</sub>); 3.20 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.54 (m, 1H, CH<sub>2</sub>CH<sub>3</sub>CHNH); 5.63 (s mod, 1H, NHCH<sub>2</sub>); 5.70 (s mod, 1H, NHCH<sub>2</sub>); 6.80 (s, 1H, C CHNH imidazole); 7.50 (s, 1H, N CHNH imidazole).

**(R)-1-(2-(1H-imidazol-4-yl)ethyl)-3-(octan-2-yl)urea 14: (mass spectrometry, ES M<sup>+</sup> = 267.2)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 0.85 (t, 3H, CH<sub>3</sub>CH<sub>2</sub>); 0.97 (d, 3H, CH<sub>3</sub>CHHC<sub>2</sub>); 1.24 (m, 10H, CH<sub>3</sub>(CH<sub>2</sub>)<sub>5</sub>CH<sub>2</sub>); 2.57 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 3.20 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.53 (m, 1H, CH<sub>2</sub>CH<sub>3</sub>CHNH); 5.67 (s mod, 1H, NHCH<sub>2</sub>); 5.69 (s mod, 1H, NHCH<sub>2</sub>); 6.82 (s, 1H, C CHNH imidazole); 7.51 (s, 1H, N CHNH imidazole).

**(S)-1-(2-(1H-imidazol-4-yl)ethyl)-3-(octan-2-yl)urea 15: (mass spectrometry, ES M<sup>+</sup> = 267.2)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 0.85 (t, 3H, CH<sub>3</sub>CH<sub>2</sub>); 0.97 (d, 3H, CH<sub>3</sub>CHHC<sub>2</sub>); 1.23 (m, 10H, CH<sub>3</sub>(CH<sub>2</sub>)<sub>5</sub>CH<sub>2</sub>); 2.57 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 3.20 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 3.49 (m, 1H, CH<sub>2</sub>CH<sub>3</sub>CHNH); 5.66 (s mod, 1H, NHCH<sub>2</sub>); 5.69 (s mod, 1H, NHCH<sub>2</sub>); 6.75 (s, 1H, C CHNH imidazole); 7.51 (s, 1H, N CHNH imidazole).

**1,1'-((1*r*,4*r*)-cyclohexane-1,4-diyl)bis(3-(2-(1H-imidazol-4-yl)ethyl)urea) 16: (mass spectrometry, ES M<sup>+</sup> = 389.3)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 1.09 (t mod, 5H, CHCH<sub>2</sub>CH<sub>2</sub>); 1.09 (dd mod, 5H, CHCH<sub>2</sub>CH<sub>2</sub>); 2.58 (t, 4H, NHCH<sub>2</sub>CH<sub>2</sub>); 3.32 (q, 4H, CH<sub>2</sub>CH<sub>2</sub>NH); 5.69 (s mod, 2H, NHCH<sub>2</sub>); 5.74 (s mod, 2H, NHCH<sub>2</sub>); 6.68 (s, 2H, C CHNH imidazole); 7.56 (s, 2H, N CHNH imidazole).

**1-(2-(1H-imidazol-4-yl)ethyl)-3-phenylurea 17: (mass spectrometry, ES M<sup>+</sup> = 231.1)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 2.66 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 2.58 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 3.31 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 6.18 (s mod, 1H, C CHNH imidazole); 6.87 (t, 1H, p-CH Ph); 7.20 (t, 2H, m-CH Ph); 7.375 (d, 2H, o-CH Ph); 7.55 (s, 1H, N CHNH imidazole); 8.48 (s, 1H, CHNH imidazole).

**1,1'-(2,2'-(1,3-phenylene)bis(propane-2,2-diyl))bis(3-(2-(1H-imidazol-4-yl)ethyl)urea) 18: (mass spectrometry, ES M<sup>+</sup> = 467.2)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 1.61 (s, 12H, CH<sub>3</sub>); 2.08 (s, 2H, CH); 2.56 (t, 4H, NHCH<sub>2</sub>CH<sub>2</sub>); 3.17 (q, 4H, CH<sub>2</sub>CH<sub>2</sub>NH); 5.80 (s mod, 2H, NHCH<sub>2</sub>); 6.24 (s, 1H, C CC phenyl); 6.75 (s, 2H, C CHNH imidazole); 7.13–7.18 (m, 3H, CCHCH phenyl and s mod, 2H, NHCH<sub>2</sub>); 7.33 (s mod, 2H, CHCHCH phenyl); 7.52 (s, 1H, N CHNH imidazole).

**(R)-1-(2-(1H-imidazol-4-yl)ethyl)-3-(1-phenylethyl)urea 19: (mass spectrometry, ES M<sup>+</sup> = 259.1)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 1.29 (d, 3H, CH<sub>3</sub>); 2.57 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 3.21 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 4.70 (qv, 1H, CH<sub>3</sub>CHNH); 5.79 (s mod, 1H, NHCH<sub>2</sub>); 6.37 (d mod, 1H, NH-CH-Ph); 6.77 (s, 1H, C CHNH imidazole); 7.17–7.33 (m, 5H, phenyl); 7.56 (s, 1H, N CHNH imidazole).

**(S)-1-(2-(1H-imidazol-4-yl)ethyl)-3-(1-phenylethyl)urea 20: (mass spectrometry, ES M<sup>+</sup> = 259.1)**

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 300 MHz) δ (ppm) = 1.29 (d, 3H, CH<sub>3</sub>); 2.60 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 3.19 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 4.72 (qv, 1H, CH<sub>3</sub>CHNH);



5.79 (s mod, 1H, NHCH2); 6.37 (d mod, 1H, NH-CH-Ph); 6.80 (s, 1H, C CHNH imidazole); 7.19–7.33 (m, 5H, phenyl); 7.61 (s, 1H, N CHNH imidazole).

**(R)-1-(2-(1H-imidazol-4-yl)ethyl)-3-(1-(4-fluorophenyl)ethyl)urea 21:**  
(mass spectrometry, ES  $M^{*+} = 277.1$ )

$^1\text{H-NMR}$  (DMSO- $d_6$ , 300 MHz)  $\delta$  (ppm) = 1.28 (d, 3H, CH<sub>3</sub>); 2.58 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 3.20 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 4.78 (qv, 1H, CH<sub>3</sub>CHNH); 5.80 (s mod, 1H, NHCH<sub>2</sub>); 6.40 (d mod, 1H, NH-CH-Ph); 6.80 (s, 1H, C CHNH imidazole); 7.13–7.18 (m, 2H, CHCCH phenyl); 7.27–7.32 (m, 2H, CHCFCH phenyl); 7.52 (s, 1H, N CHNH imidazole).

**(S)-1-(2-(1H-imidazol-4-yl)ethyl)-3-(1-(4-fluorophenyl)ethyl)urea 22:**  
(mass spectrometry, ES  $M^{*+} = 277.1$ )

$^1\text{H-NMR}$  (DMSO- $d_6$ , 300 MHz)  $\delta$  (ppm) = 1.28 (d, 3H, CH<sub>3</sub>); 2.58 (t, 2H, NHCH<sub>2</sub>CH<sub>2</sub>); 3.22 (q, 2H, CH<sub>2</sub>CH<sub>2</sub>NH); 4.72 (qv, 1H, CH<sub>3</sub>CHNH); 5.79 (s mod, 1H, NHCH<sub>2</sub>); 6.39 (d mod, 1H, NH-CH-Ph); 6.79 (s, 1H, C CHNH imidazole); 7.09–7.15 (m, 2H, CHCCH phenyl); 7.27–7.31 (m, 2H, CHCFCH phenyl); 7.58 (s, 1H, N CHNH imidazole)

### Carbonic anhydrase assay

A stopped-flow method<sup>105</sup> has been used for assaying the CA catalyzed CO<sub>2</sub> hydration activity with Phenol red as indicator, working at the absorbance maximum of 557 nm, following the initial rates of the CA-catalyzed CO<sub>2</sub> hydration reaction for 10–100 s. For each activator, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activator (0.1 mM) were prepared in distilled-deionized water with 5% DMSO and dilutions up to 0.1 nM were done thereafter with distilled-deionized water. The activation constant ( $K_A$ ), defined similarly with the inhibition constant  $K_i$ , was 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 the following equation<sup>23,24,95–104</sup>:

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

where  $v_0$  represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator. All CA isozymes used in the

experiments were purified recombinant proteins obtained as reported earlier by our group<sup>23,24</sup>.

## Results and discussion

### Chemistry

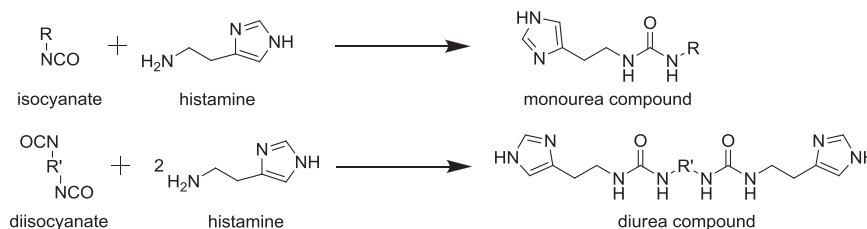
The rationale for designing new CAAs reported in this paper is based on the reported X-ray crystal structure for the hCA II – histamine adduct<sup>23</sup>. As mentioned above, the aminoethyl moiety of the activator does not make relevant contacts with the enzyme and is free to be derivatized as it points out towards the exit of the active site. In this way, the imidazole moiety of the activator can participate to the proton shuttling processes crucial for enhancing the catalytic efficiency of the enzyme, whereas the derivatized amino group may lead to a further stabilization of the enzyme-activator adduct. In an earlier work<sup>106</sup>, we showed that sulfonamido, carboxamido, and ureido/thioureido derivatives of histamine (at the aliphatic portion of the molecule) act as efficient activators of several CA isoforms, such as hCA I, hCA II, and bCA IV (b = bovine isoform). As only a few (more exactly 5) ureido derivatives of histamine were reported, all of them incorporating aromatic R moieties, here we decided to investigate a larger such series of ureas and diureas, obtained by reacting histamine with alkyl/aryl isocyanates and di-isocyanates, as describes in Scheme 2.

A rather large number of such derivatives were obtained (Table 1), which incorporate various alkyl moieties of variable length, cycloalkyl, and aryl moieties. All compounds were thoroughly characterized by physico-chemical procedures which confirmed their structure (see Materials and methods for details).

### CA activation

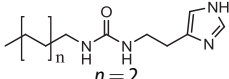
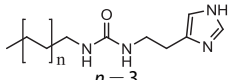
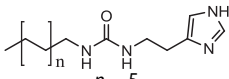
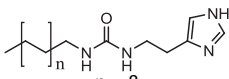
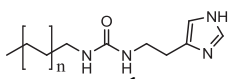
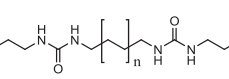
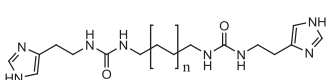
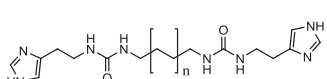
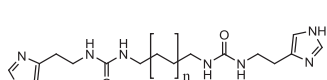
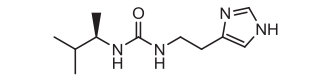
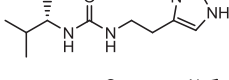
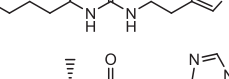
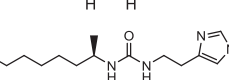
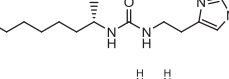
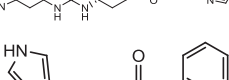
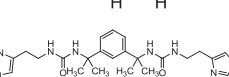


Ureas **1–22** and histamine were assayed for the activation of the physiologically most important cytosolic isoforms hCA I and II (Table 1). It should be mentioned that these are widespread isoforms in many tissues (e.g. red blood cells contain approximately 150  $\mu\text{M}$  of hCA I and 20  $\mu\text{M}$  of hCA II)<sup>1</sup>, including not only the blood but also the gastro-intestinal tract, kidneys, lungs, and the brain<sup>1,7</sup>.

Data of Table 1 show some very interesting structure-activity relationship (SAR) data for the activation of these two isoforms with histamine and its ureido/bisureido derivatives **1–22**. The most salient feature is that unlike histamine, which is a poor hCA II activator ( $K_A$  of 125  $\mu\text{M}$ ) but a rather efficient hCA I activator ( $K_A$  of 2.0  $\mu\text{M}$ ), the ureas **1–22** do not activate at all hCA II, but are all of them effective hCA I activators, with  $K_A$ s in the range of 0.73–3.4  $\mu\text{M}$  (Table 1). The second rather interesting feature of this class of CAAs is the fact that the activation constants against hCA I show a rather modest range, with a minimal variation of potency, irrespective of the rather diverse substitution pattern at the ureido moiety, or whether they are mono- or bis-urea derivatives (and as a consequence they contain one or two imidazole moieties able to participate in proton shuttling processes). Thus, the most



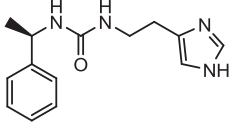
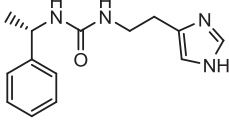
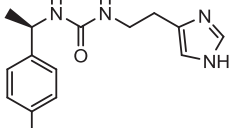
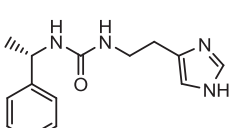
**Scheme 2.** Synthesis of ureas **1–22** from histamine and isocyanates/diisocyanates.

**Table 1.** CA activation against isoforms hCA I and II with ureas 1–22 and histamine as standard, by a stopped-flow CO<sub>2</sub> hydrase assay<sup>105</sup>.  $K_A$  = activation constant.

No.	Structure	$K_A$ hCA I ( $\mu\text{M}$ )*	$K_A$ hCA II ( $\mu\text{M}$ )*
1	 $n = 1$	3.1	>200
2	 $n = 2$	2.7	>200
3	 $n = 3$	3.0	>200
4	 $n = 5$	3.4	>200
5	 $n = 8$	2.8	>200
6	 $n = 1$	2.9	>200
7	 $n = 2$	3.1	>200
8	 $n = 3$	2.2	>200
9	 $n = 5$	1.7	>200
10	 $n = 5$	1.6	>200
11		1.5	>200
12		3.0	>200
13		2.2	>200
14		3.1	>200
15		3.0	>200
16		1.6	>200
17		1.1	>200
18		0.73	>200

(continued)

Table 1. Continued

No.	Structure	$K_A$ hCA I ( $\mu\text{M}$ )*	$K_A$ hCA II ( $\mu\text{M}$ )*
19		1.2	>200
20		1.4	>200
21		0.98	>200
22		0.97	>200
–	Histamine	2.0	125

\*Errors in the range of  $\pm 10\%$  of the reported values, from three different determinations (data not shown).

effective activator is indeed a *bis*-urea (compound **18**), which contains two imidazoles in its molecule and showed a  $K_A$  of  $0.73 \mu\text{M}$ , being thus 2.74 times a more effective hCA I activator compared to histamine. However, the other submicromolar CAAs detected here, compounds **21** and **22** ( $K_A$ s of  $0.97$ – $0.98 \mu\text{M}$ ) were monoureas, containing only one imidazole moiety. Several of the ureas investigated here (e.g. **1–8** and **12–15**) were slightly less effective hCA I activators compared with histamine, with  $K_A$ s in the range of  $2.2$ – $3.4 \mu\text{M}$ . It is difficult to explain this loss of activity, also considering the fact that some aryl-ureido histamines reported earlier<sup>106</sup>, possessing a very diverse substitution pattern compared with these compounds, possessed a much more efficient activating profile against hCA I (and also activated hCA II and bCA IV). Probably the rather long aliphatic moieties present in the ureas investigated here were detrimental for the binding of the activator at the entrance of the active site cavity, a region of the enzyme rich in hydrophilic amino acid residues<sup>1,18</sup>. However, although slightly less effective than histamine, these compounds did show activating properties against this isoform, but not at all against hCA II, which is probably even more difficult to explain. However, as explained earlier by us<sup>23</sup>, the entrance of the active site cavity of the two isoforms are very diverse, with hCA II possessing a cluster of at least 6 histidine residues (His3, 4, 10, 15, 17, and 64) which is absent in hCA I. The much more hydrophilic environment at the entrance of hCA II active site probably explains why the hydrophobic ureas reported here **1–22** do not efficiently bind to this enzyme, and do not show any CA activating effect. This is, as far as we know, the only example of isoform-selective CAA, and may be of relevance for better understanding the physiology/pharmacology of hCA I.

## Conclusions

By catalyzing the simple but highly important hydration of carbon dioxide to bicarbonate and protons, CAs are involved in critical

steps of the life cycle of many organisms, including eukaryotes, *Bacteria* and *Archaea*. A large number of CA inhibitors have pharmacological applications in the field of antiglaucoma, anticonvulsant, anticancer, and anti-infective agents, whereas activation of these enzymes is not yet properly exploited pharmacologically for cognitive enhancement or Alzheimer's disease, conditions in which a diminished CA activity was reported. We report here a series of ureido/*bis*-ureido histamine derivatives which were investigated for their activating effects against the cytosolic human (h) isoform hCA I and II. We observed that all these compounds, unlike histamine or other activator classes, show no activating effects on the widespread, physiologically dominant isoform hCA II, but were rather effective hCA I activators. This is the first report in which CA I-selective activators were identified. Such compounds may constitute interesting tools for better understanding the physiological/pharmacological effects connected to activation of this widespread CA isoform.

## Disclosure statement

One author (C. T. S.) declares conflict of interest, being author of several patents in the field of CA inhibitors/activators. This research was financed by several EU projects (Euroxy, Metoxia, DeZint, and Dynano). The other authors do not declare conflict of interest.

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