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BRIEF REPORT

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New isoxazolidinyl-based *N*-alkylethanolamines as new activators of human brain carbonic anhydrases

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ABSTRACT

Carbonic anhydrases (CAs) are widespread metalloenzymes which catalyse the reversible hydration of carbon dioxide (CO_2) to bicarbonate (HCO_3^-) and a proton, relevant in many physiological processes. In the last few years, the involvement of CA activation in different metabolic pathways in the human brain addressed the research to the discovery of novel CA activators. Here, a new series of isoxazoline-based amino alcohols as CA activators was investigated. The synthesis and the CA activating effects towards four human CA isoforms expressed in the human brain, that are hCAs I, II, IV and VII, were reported. The best results were obtained for the (methyl)-isoxazoline-amino alcohols **3** and **5** with K_A values in the submicromolar range (0.52–0.86 μM) towards hCA VII, and a good selectivity over hCA I. Being hCA VII involved in brain function and metabolism, the newly identified CA activators might be promising hit compounds with potential therapeutic applications in ageing, epilepsy or neurodegeneration.
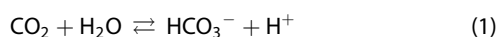
Introduction

Carbonic anhydrases (CAs) are a superfamily of ubiquitous metalloenzymes responsible for the catalysis of the reversible hydration of carbon dioxide (CO_2) to bicarbonate (HCO_3^-) by proton transfer mechanism[1]. This simple reaction is essential to different biological processes, and it is represented by Equation (1):

$$CO_2 + H_2O \rightleftarrows HCO_3^- + H^+ \qquad (1)$$

Although this reaction may also occur spontaneously, at physiological pH values it is too slow to guarantee metabolic needs and maintain homeostasis. In that context, CA catalysis becomes relevant in covering all the physiological processes speeding up the equilibrium of CO_2/HCO_3^- interconversion.
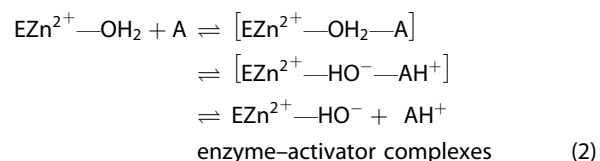
Generally, CAs are phylogenetically classified into different subgroups: α-, β-, γ-, δ-, ζ-, η-, θ-, and ι-[2]. Human CAs belong to the α-class, and are represented by 15 isoforms of which 12 are catalytically active[3]. The catalytic core is maintained in all the α-isoforms and it is constituted by a Zn^{2+} ion arranged in tetrahedral coordination with three *His* residues and a water molecule.

So far, CAs have been deeply explored as targets for the design of CA inhibitors[4–6]. In fact, CA inhibitors are in advanced pharmacological studies and some of them, in the last decade, reached clinical use for the treatment of different pathologies such as glaucoma[7], epilepsy[8] and obesity[9]. Unfortunately, most of the CA inhibitors lack selectivity of action against the different CA isoforms[10] but also over other zinc metalloenzymes such as matrix metalloproteinases, due to the common structural requirement of a zinc-binding group in the inhibitor structure[11–14].

However, recently the less explored class of CA activators (CAAs) is gaining increased attention, principally due to the recent biological findings on CA activation[15]. Some human isoform deficiencies are related to the development of specific pathological conditions such as osteopetrosis, hyperammonemia, hyperchlorhydrias and cystic fibrosis[16–20] Furthermore, the activation of CAs has been reported to increase memory[21,22] and promote bone mineralisation[23]. Recent evidence focused on the involvement of CA activation in the treatment of neurological and neurodegenerative diseases, proposing an effective role of CA activation in the strengthening of synaptic efficacy[24].

In 1990, the general enzymatic mechanism of action of CAAs was reported[25]. The generation of a ternary enzyme-substrate-activator complex [Equation (2)] is due to the non-competitive binding of the activator to the enzyme in the active site at the middle edge of CA, assisting the proton shuttling[26].

$$EZn^{2+}\!-\!OH_2 + A \rightleftarrows [EZn^{2+}\!-\!OH_2\!-\!A]$$
$$\rightleftarrows [EZn^{2+}\!-\!HO^-\!-\!AH^+]$$
$$\rightleftarrows EZn^{2+}\!-\!HO^- + AH^+$$
$$\text{enzyme–activator complexes} \qquad (2)$$

The discovery of the activation mechanism turned on the interest in the activation target and many studies regarding CAAs have been carried out[27].

A CAA is usually a small molecule with the structural requirement of a basic moiety necessary to mimic the proton shuttling mechanism. Thus, the first molecules to be tested as CAAs have been amino acids and their respective amines, identifying histamine (Figure 1) as the prototypical CAA[28]. Therefore, at first, the CAA drug design was focused on histamine analogues, including histamine-inspired molecules, and His combined with its β-alanine dipeptide derivative carnosine (Figure 1)[29]. In the following years, with the purpose to

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Figure 1. General structures of some reported CAAs.

explore the field of CAA Structure-Activity Relationships (SARs), different design approaches focused on amines and amino acid structures but were not strictly related to the parent compounds[27]. Among new scaffolds, β-arylchalcogeno amines containing sulfur, selenium and tellurium, structurally related to the psychoactive drug amphetamine[30], amino alcohol oxime ethers[31] and neuronal nitric oxide synthase (nNOS) inhibitor heterocycles conjugated with amino acids, represent the mainly investigated alternative to classic CAAs[32].

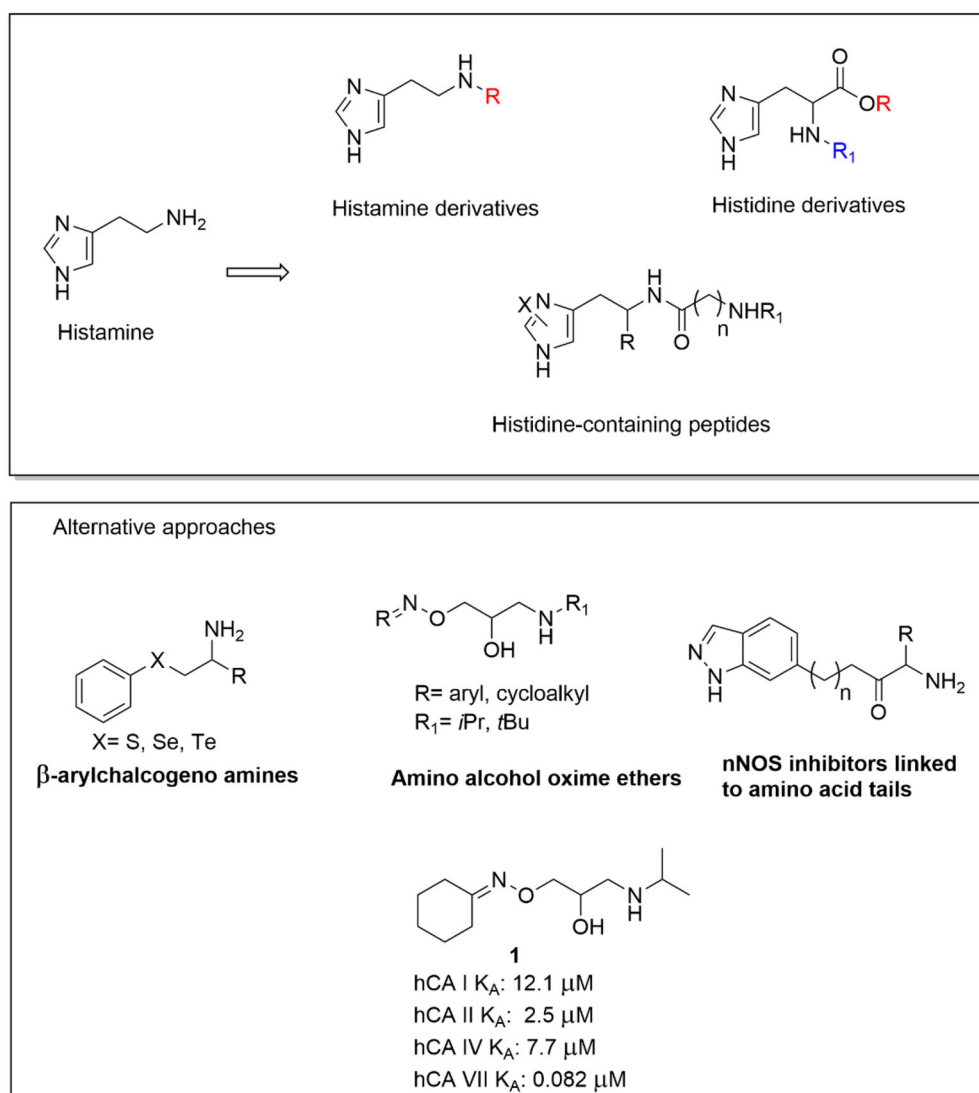Of note, a large series of amino-alcohol derivatives, inspired by the lead CAA β-amino alcohol timolol, has been recently reported (Figure 1)[31]. Timolol is selective for hCA I and hCA II isoforms, binding the active site entrance through the formation of a ternary complex with enzyme and CO_2[33]. The new amino alcohol series of CAAs were obtained by ring opening of differently substituted epoxides with isopropylamine or *tert*-butylamine. All the amino alcohol derivatives showed good activation properties, reporting K_A values spanning from a micromolar to a nanomolar range. In particular, compound **1** (Figure 1) turned out to be highly selective for hCA II and hCA VII, thus opening new perspectives in the use of amino alcohol scaffolds as potential CAAs.

Based on these promising results, we extended our SAR studies to an in-house library of isoxazoline amino alcohols, composed of diasteroisomeric isoxazolinyl-N-alkylethanolamines (**2–23**, Table 1),

some of them previously reported as antagonists of β-adrenergic receptors[34–36]. Taking into consideration that β-1 adrenergic receptor blockade was reported to have a positive effect on memory performance[37], we here hypothesized that a dual activity of this class of compounds on CA activation and β-adrenergic receptor blockade could be beneficial for improving memory and cognitive functions. In this new series, an isoxazoline ring replaced the oxime moiety, as a conformationally restrained alternative structure, to explore the fitting inside the binding site, and gain new information about the SAR of amino alcohol CA activators. Similarly to the previously described amino alcohol series, these isoxazolines presented isopropyl or *tert*-butyl substituents in R_1 (Table 1).

Here, we present our studies on amino alcohols as CAAs, describing the synthesis, NMR characterization and CA activating effects of this new series of isoxazoline-based amino alcohols towards four hCA isoforms mainly expressed in the human brain.

Materials and methods

Chemistry

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III HD 400 MHz spectrometer. Chemical shifts (δ) are reported in parts

Table 1. Activation data of human CA isoforms I, II, IV and VII with isoxazoline amino alcohols **2–23** and histamine as reference CAA by a stopped-flow CO_2 hydrase assay[38].

General structure: isoxazoline ring with R at position 3, and a side chain $-CH(OH)-CH_2-NHR_1$.
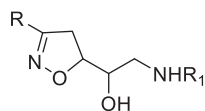
Compound				K_A (µM)[a]			
Code	R	R_1	Stereoisomer	hCA I	hCA II	hCA IV	hCA VII
2	CH_3-	*i*-Pr	syn	12.4	4.15	15.6	0.86
3	CH_3-	*i*-Pr	anti	27.6	3.89	7.61	0.52
4	CH_3-	*t*-Bu	syn	11.9	3.06	9.08	0.69
5	CH_3-	*t*-Bu	anti	48.1	3.52	14.9	0.53
6	cyclohexyl	*i*-Pr	syn	11.7	2.6	6.36	4.98
7	cyclohexyl	*t*-Bu	syn	25.8	3.51	7.93	5.12
8	phenyl	*i*-Pr	syn	47.5	2.8	25.5	5.97
9	phenyl	*i*-Pr	anti	>100	2.85	19.2	6.15
10	phenyl	*t*-Bu	syn	49.6	1.74	35.7	8.26
11	phenyl	*t*-Bu	anti	63.5	2.01	24.8	7.06
12	2-Cl-phenyl	*i*-Pr	syn	40.6	3.75	11.1	1
13	2-Cl-phenyl	*i*-Pr	anti	53.1	3.03	10	1.23
14	2-Cl-phenyl	*t*-Bu	syn	29.8	2.9	9.67	2.3
15	2-Cl-phenyl	*t*-Bu	anti	43.6	2.67	5.98	2.84
16	H_3CO-, Cl-phenyl	*i*-Pr	syn	38.1	1.53	6.23	2.82
17	H_3CO-, Cl-phenyl	*t*-Bu	syn	47.8	2.09	5.8	2.67
18	H_3CO-, Cl-phenyl	*t*-Bu	anti	>100	2.54	7.93	2.89
19	Cl-, OCH_3-phenyl	*i*-Pr	syn	49.1	2.77	17.6	5.8
20	Cl-, OCH_3-phenyl	*i*-Pr	anti	48	1.93	6.05	5.69
21	Cl-, OCH_3-phenyl	*t*-Bu	syn	47.3	2.69	6.02	6
22	3-Cl-phenyl	*i*-Pr	syn	>100	1.84	4.35	3.8
23	3-Cl-phenyl	*i*-Pr	anti	>100	3.05	12.7	6.51
Histamine	–	-	–	2.1	125	25.3	37.5

[a]From three different assays (errors within ± 10% of the reported values).

per million and coupling constants (*J*) are reported in hertz (Hz). ^{13}C NMR spectra were fully decoupled. The following abbreviations were used to explain multiplicities: singlet (s), doublet (d), triplet (t), double doublet (dd), broad (br), and multiplet (m). Chromatographic separations were performed on silica gel columns by flash column chromatography (Kieselgel 40, 0.040 0.063 mm, Merck). Reactions were followed by thin-layer chromatography (TLC) on Merck aluminium silica gel (60 F254) sheets that were visualised under a UV lamp. Evaporation was performed *in vacuo* (rotating evaporator). Sodium sulphate was always used as the drying agent. Commercially available chemicals were purchased from Sigma-Aldrich.

Compounds prepared according to literature procedures

As mentioned in the introduction, compounds **2–5**, **8–11**, **12–15**, and **22–23** [34–36] (Table 1) have been already reported as antagonists of *β*-adrenergic receptors. Here, we detail an updated characterisation by ^{1}H NMR and ^{13}C NMR spectra.

(syn)-2-(isopropylamino)-1-(3-methyl-4,5-dihydroisoxazol-5-yl)ethan-1-ol maleate (2): ^{1}H NMR (400 MHz, DMSO-d6) δ: 6.01 (s, 2H); 4.40–4.34 (m, 1H); 3.66–3.63 (m, 1H); 3.00–2.89 (m, 3H); 2.75–2.73 (m, 1H); 1.91 (s, 3H); 1.19–1.18 (m, 6H).^{13}C NMR (100 MHz, DMSO-d6) δ: 167.2; 155.5; 136.1; 80.6; 68.2; 49.4; 47.4; 19.9; 19.2; 12.6.

(anti)-2-(isopropylamino)-1-(3-methyl-4,5-dihydroisoxazol-5-yl)ethan-1-ol maleate (3): ^{1}H NMR (400 MHz, DMSO-d6) δ: 8.45, 8.34 (2 × bs, 2H); 6.02 (s, 2H); 5.76–5.75 (d, *J* = 5.2 Hz, 1H); 4.45, 4.43 (dddd, J_1 = 4.0 Hz, J_2 = 8.0 Hz, J_3 = 15.2 Hz, 1H); 3.78–3.77 (m, 1H); 3.02 (dd, J_1 = 7.2 Hz, J_2 = 18 Hz, 1H); 2.89 (dd, J_1 = 7.6 Hz, J_2 = 16.8 Hz, 1H); 1.90 (s, 3H); 1.23 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d6) δ: 167.2; 155.6; 136.1; 80.3; 67.7; 49.8; 45.9; 18.8; 18.1; 12.6.

(syn)-2-(tert-butylamino)-1-(3-methyl-4,5-dihydroisoxazol-5-yl)ethan-1-ol maleate (4): ^{1}H NMR (400 MHz, DMSO-d6) δ: 8.27 (bs, 2H); 6.01 (s, 2H); 5.85–5.84 (m, 1H); 4.43–4.37 (m, 1H); 3.69–3.67 (m, 1H); 3.06–2.97 (m, 3H); 2.75–2.71 (m, 1H); 1.92 (s, 3H); 1.28 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d6) δ: 167.1; 155.7; 136.1; 80.3; 67.7; 56.4; 43.7; 25.0; 12.6.

(anti)-2-(tert-butylamino)-1-(3-methyl-4,5-dihydroisoxazol-5-yl)ethan-1-ol maleate (5): ^{1}H NMR (400 MHz, DMSO-d6) δ: 8.39, 8.30 (2 × bs, 2H); 6.01 (s, 2H); 5.73 (d, *J* = 5.2 Hz, 1H); 4.45 (dddd, J_1 = 4.0 Hz, J_2 = 8.0 Hz, J_3 = 15.2 Hz, 1H); 3.75–3.73 (m, 1H); 3.87–2.83 (m, 4H); 1.91 (s, 3H); 1.28 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d6) δ: 167.1; 155.5; 136.1; 80.2; 68.1; 56.2; 43.3; 27.1; 25.1; 12.5.

(syn)-2-(isopropylamino)-1-(3-phenyl-4,5-dihydroisoxazol-5-yl)ethan-1-ol maleate (8): ^{1}H NMR (400 MHz, DMSO-d6) δ: 8.34 (bs 2H); 7.69–7.66 (m, 2H); 7.49–7.47 (m, 3H); 6.01 (s, 2H); 5.98–5.97 (m, 1H); 4.68–4.62 (m, 1H); 3.86–3.83 (m, 1H); 3.49 (dd, J_1 = 10.6 Hz, J_2 = 17.2 Hz, 1H); 3.43 (dd, J_1 = 9.6 Hz, J_2 = 17.2 Hz, 1H); 3.11–3.09 (m, 1H); 2.91–2.86 (m, 1H); 1.24 (dd, J_1 = 3.6 Hz, J_2 = 6.4 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d6) δ: 167.6; 157.1; 136.6; 130.7; 129.6; 129.4; 127.1; 82.1; 67.7; 50.4; 46.7; 36.2; 19.4; 18.6.

(anti)-2-(isopropylamino)-1-(3-phenyl-4,5-dihydroisoxazol-5-yl)ethan-1-ol oxalate (9): ^{1}H NMR (400 MHz, DMSO-d6) δ: 7.68–7.64 (m, 2H); 7.48–7.42 (m, 3H); 4.98 (bs, 1H); 4.66 (dddd, J_1 = 4.4 Hz, J_2 = 8.8 Hz, J_3 = 15.2 Hz, 1H); 3.57–3.53 (m, 1H); 3.30–3.23 (m, 1H); 2.76–2.69 (m, 1H); 2.65 (dd, J_1 = 4.8 Hz, J_2 = 12 Hz, 1H); 2.57 (dd, J_1 = 8 Hz; J_2 = 11.6 Hz, 1H); 0.97 (dd, J_1 = 1.2 Hz, J_2 = 6 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d6) δ: 156.4; 129.8; 129.6; 128.7; 126.4; 82.4; 70.9; 49.1; 48.2; 36.0; 22.8.

(syn)-2-(tert-butylamino)-1-(3-phenyl-4,5-dihydroisoxazol-5-yl)ethan-1-ol oxalate (10): ^{1}H NMR (400 MHz, DMSO-d6) δ: 7.68–7.66 (m, 2H); 7.45–7.44 (m, 3H); 4.66 (td, *J* = 4.8 Hz, *J* = 9.2 Hz, 1H); 3.59–3.55 (m,1H); 3.35–3.33 (m, 2H); 2.58 (dd, J_1 = 4.4 Hz, J_2 = 11.2 Hz,

1H); 2.52–2.47 (m, 1H); 1.29 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d6) δ: 156.3; 129.8; 129.7; 128.8; 126.4; 82.6; 70.7; 49.7; 45.1; 35.2; 28,8.

(anti)-2-(tert-butylamino)-1-(3-phenyl-4,5-dihydroisoxazol-5-yl)ethan-1-ol oxalate (11): ^{1}H NMR (400 MHz, DMSO-d6) δ: 7.66–7.65 (m, 2H); 7.46–7.42 (m, 3H); 4.70 (dddd, J_1 = 4.4 Hz, J_2 = 8.8 Hz, J_3 = 12.8 Hz, 1H); 3.51–3.24 (m, 2H); 2.60–2.59 (m, 2H); 1.03 (s, 9H).^{13}C NMR (100 MHz, DMSO-d6) δ: 156.8; 130.3; 130.1; 129.2; 126.9; 82.8; 72.0; 50.2; 45.0; 36.5; 29.2.

(syn)-1-(3-(2-chlorophenyl)-4,5-dihydroisoxazol-5-yl)-2-(isopropylamino)ethan-1-ol maleate (12): ^{1}H NMR (400 MHz, DMSO-d6) δ: 8.38 (br s, 2H); 7.62–7.58 (m, 2H); 7.52–7.43 (m, 2H); 6.03–6.01 (m, 1H); 6.02 (s, 2H); 4.71–4.65 (m, 1H); 3.88–3.86 (m, 1H); 3.56 (dd, J_1 = 10.4 Hz, J_2 = 17.2 Hz, 1H); 3.51 (dd, J_1 = 7.6 Hz, J_2 = 17.6 Hz, 1H); 3.39–3.32 (m, 1H); 3.12–3.08 (dd J_1 = 2.4 Hz, J_2 = 10.4 Hz, 1H); 2.92 − 2.87 (m, 1H); 1.25–1.22 (dd, J_1 = 4 Hz, J_2 = 6.4 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d6) δ: 167.2; 156.0; 136.1; 131.6; 131.4; 130.7; 130.6; 128.5; 127.5; 81.8; 67.1; 49.9; 46.2; 38.4; 18.8; 18.1.

(anti)-1-(3-(2-chlorophenyl)-4,5-dihydroisoxazol-5-yl)-2-(isopropylamino)ethan-1-ol maleate (13): ^{1}H NMR (400 MHz, DMSO-d6) δ: 8.34 (br s, 2H); 7.62–7.57 (m, 2H); 7.51–7.42 (m, 2H); 6.02 (s, 2H); 5.92 (b s, 1H); 4.73 (dddd, J_1 = 4 Hz, J_2 = 7.6 Hz, J_3 = 11.2 Hz, 1H); 3.88–3.86 (m, 1H); 3.61 (dd, J_1 = 11.2 Hz, J_2 = 17.2 Hz, 1H); 3.38 (dd, J_1 = 11.2 Hz, J_2 = 17.2 Hz, 1H); 3.35-3.31 (m, 1H); 3.10 (dd, J_1 = 2.4 Hz, J_2 = 12.4 Hz, 1H); 2.98–2.95 (m, 1H); 1.25–1.22 (dd, J_1 = 4.4 Hz, J_2 = 6.4 Hz). ^{13}C NMR (100 MHz, DMSO-d6) δ: 167.2; 155.9; 136.1; 131.6; 131.3; 130.7; 130.6; 128.5; 127.5; 81.7; 67.6; 49.8; 45.8; 38.5; 18.9; 18.1.

(syn)-2-(tert-butylamino)-1-(3-(2-chlorophenyl)-4,5-dihydroisoxazol-5-yl)ethanol maleate (14): ^{1}H NMR (400 MHz, DMSO-d6) δ: 8.35 (bs, 2H); 7.63–7.58 (m, 2H); 7.53–7.43 (m, 2H); 6.02–6.01 (m, 1H); 6.02 (s, 2H); 4.73–4.67 (m, 1H); 3.87–3.85 (m, 1H); 3.55–3.52 (m, 2H); 3.09–3.07 (m, 1H); 2.85–2.82 (m, 1H); 1.48 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d6) δ: 167.2; 156.1; 136.2; 131.6; 131.4; 130.7; 130.6; 128.5; 127.6; 81.8; 67.4; 56.5; 43.5; 38.3; 25.1.

(anti)-2-(tert-butylamino)-1-(3-(2-chlorophenyl)-4,5-dihydroisoxazol-5-yl)ethanol maleate (15): ^{1}H NMR (400 MHz, DMSO-d6) δ: 8.31 (bs, 2H); 7.63–7.57 (m, 2H); 7.52–7.43 (m, 2H); 6.01 (s, 2H); 5.89 (bs; 1H); 4.73–4.67 (m, 1H); 3.87–3.85 (m, 1H); 3.63 (dd, J_1 = 11.2 Hz, J_2 = 17.2 Hz, 1H); 3.42 (dd, J_1 = 7.6 Hz, J_2 = 17.2 Hz, 1H); 3.09–3.07 (m, 1H); 2.85–2.82 (m, 1H); 1.48 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d6) δ: 167.6; 156.3; 136.4; 132.0; 131.8; 131.2; 131.1; 129.0; 128.0; 82.1; 68.5; 56.9; 43.5; 39.0; 25.5.

(syn)-1-(3-(3-chlorophenyl)-4,5-dihydroisoxazol-5-yl)-2-(isopropylamino)ethan-1-ol maleate (22): ^{1}H NMR (400 MHz, DMSO-d6) δ: 8.28 (bs, 2H); 7.69–7.65 (m, 2H); 7.57–7.49 (m, 2H); 6.02 (s, 2H); 6.02–6.01 (m, 1H); 4.72–4.66 (m, 1H); 3.87–3.84 (m, 1H); 3.52–3.25 (m, 2H); 3.11–3.08 (m, 1H); 2.90–2.84 (m, 1H); 1.24–1.22 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d6) δ: 167.7; 156.3; 136.6; 134.1; 131.7; 131.3; 130.5; 126.7; 125.7; 82.6; 67.7; 50.3; 46.6; 35.9; 19.4; 18.6.

(anti)-1-(3-(3-chlorophenyl)-4,5-dihydroisoxazol-5-yl)-2-(isopropylamino)ethan-1-ol maleate (23): ^{1}H NMR (400 MHz, DMSO-d6) δ: 8.32 (bs, 2H); 7.68–7.64 (m, 2H); 7.56–7.49 (m, 2H); 6.01 (s, 2H); 5.86 (bs, 1H); 4.76-4.68 (m, 1H); 3.86–3.84 (m,1H); 3.57–3.48 (m, 1H); 3.45–3.43 (m, 1H); 3.11–3.08 (m, 1H); 3.00–2.84 (m, 1H); 1.25–1.22 (dd, J_1 = 4 Hz, J_2 = 6.4 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d6) δ: 167.2; 155.8; 136.17; 133.6; 131.3; 130.8; 129.9; 126.2; 125.2; 81.9; 67.9; 49.8; 45.9; 35.9; 18.9; 18.2.

General procedure for the synthesis of cyclohexyl- isoxazolidinyl oxirane 27b or chlorophenyl isoxazolidinyl oxiranes 27e and 27f

The proper aldoxime **24b**, **24e** or **24f** (1 eq) was added to a stirred mixture of *N*-chlorosuccinimide (2 eq) in anhydrous CHCl₃ and pyridine at room temperature. After 15 min the reaction

mixture was treated with an excess of 1,3-butadiene (3 eq) at 0 °C, and successively a solution of Et₃N (1.5 eq) in dry CHCl₃ was added dropwise and stirred for 3 h. Then the reaction mixture was washed with water and brine and evaporated to give a crude oil of the alkenes **26b, 26d or 26e,** used directly in the next step without further purification.

A stirred mixture of the resulting crude alkene **26b, 26 d,** or **26e** (1 eq) and NaHCO₃, (2 eq) in anhydrous CH₂Cl₂ was cooled to 0 °C and a solution of 70% *m*-chloroperoxybenzoic acid (2 eq) in anhydrous CH₂Cl₂ was added dropwise. The mixture was stirred under a nitrogen atmosphere at room temperature for 72 h and then filtered. The resulting solution was washed with 3% of aqueous K₂CO₃, Na₂S₂O₃ 1 N and brine, dried with Na₂SO₄, and evaporated. The crude residue, consisting almost exclusively of a 1:1 mixture of the diastereomeric *anti*- and *syn*-epoxide **27b, 27e and 27f**, was submitted to MPLC on silica gel, eluting with a 4:2:1 hexane/CHCl₃/AcOEt mixture, isolating pure the *anti* and *syn* epoxides.

(syn)-3-cyclohexyl-5-(-oxiran-2-yl)-4,5-dihydroisoxazole (27b): (47% from oxime **24b**) ¹H NMR (400 MHz, CDCl₃): 4.67–4.54 (1H, m), 3.74–3.68 (m,1H), 3.02–2.73 (2H, m), 2,77–2,75 (m, 2H), (1.81–1.64 (6H, m), 1.34–1.20 (5H, m).

3-(4-chloro-2-methoxyphenyl)-5-(-oxiran-2-yl)-4,5-dihydroisoxazole (27f): (**27f,** *anti*): (41% from oxime **24f**). ¹H NMR (400 MHz, CDCl₃): 7.70–7.69 (m, 1H); 7.63–7.61 (m, 1H); 7.25–7.23 (m, 1H); 4.44 (dddd, $J = 4.2$ Hz, $J = 7.6$ Hz, $J = 9.0$ Hz, 1H); 3.3-2.75 (m, 4H), 2.62 (dd, $J = 2.3$ Hz, $J = 4.2$ Hz, 1H); (**27f,** *syn*): (35% from oxime 24f). ¹H NMR (400 MHz, CDCl₃): 7.70–7.69 (m, 1H); 7.63–7.61 (m, 1H); 7.25–7.23 (m, 1H); 4.44 (dddd, $J = 4.2$ Hz, $J = 7.6$ Hz, $J = 9.0$ Hz, 1H); 3.38-2.93 (m, 3H), 2.65 (d, $J = 3.8$ Hz, 2H)

3-(2-chloro-4-methoxyphenyl)-5-(oxiran-2-yl)-4,5-dihydroisoxazole (27e): (**27e,** *anti*), (40% from oxime 23e); ¹H NMR (400 MHz, CDCl₃): 7.70–7.69 (m, 1H); 7.63–7.61 (m, 1H); 7.25–7.23 (m, 1H); 4.48 (dddd, $J = 4.6$ Hz, $J = 8.1$ Hz, $J = 10.0$ Hz, 1H), 3.32–2.72 (m, 4H), 2.65 (dd, $J = 2.8$ Hz, $J = 4.0$ Hz, 1H); (**27e,** *sin*) (40% from oxime 23f); ¹H NMR (400 MHz, CDCl₃): 7.70–7.69 (m, 1H); 7.63–7.61 (m, 1H); 7.25–7.23 (m, 1H); 4.52 (dddd, $J = 4.2$ Hz, $J = 7.9$ Hz $J = 9.8$ Hz, 1H), 3.28–2.89 (m, 3H), 2.70 (d, $J = 3.9$ Hz, 2H)

Synthesis of cyclohexyl-isoxazolidinyl-N-alkyl-ethanolamines 6 and 7

A solution of syn epoxide **27b** (1 eq) and *i*-PrNH₂, or *t*-BuNH₂ (4.6 eq) in a mixture of 1:2 anhydrous benzene/EtOH was stirred at room temperature for 72 h, and then evaporated to dryness. The crude oily residue was taken up in 10% aqueous HCl and brine, and the resulting mixture was washed with Et₂O, alkalinised with solid K₂CO₃, and then extracted with CHCl₃. The organic phases were collected and evaporated and the residue was dissolved in diethyl ether and treated with a small excess of oxalic acid. The oxalic salts **6** and **7**, have been obtained pure by crystallisation process in a 4:1 Et₂O/EtOH mixture.

(syn)-1-(3-cyclohexyl-4,5-dihydroisoxazol-5-yl)-2-(isopropylamino)ethanol oxalate (6) (75% yield), m.p.: 124–126 °C. ¹H NMR (400 MHz, DMSO-d₆) δ: 8.65 (br s, 1H); 4.38–4.32 (m, 1H); 3.76–3.72 (m, 1H); 3.33–3.27 (m, 1H); 3.02–2.96 (m, 3H); 2.81–2.71 (m, 1H); 2.37–2.35 (m, 1H); 1.79–1.61 (m, 5H); 1.29–1.22 (m, 11H). ¹³C NMR (100 MHz, DMSO-*d₆*) δ: 162.8; 80.4; 67.7; 50.3; 47.1; 37.3; 36.9; 30.4; 25.9; 25.6; 19.1; 18.6.

(syn)-2-(tert-butylamino)-1-(3-cyclohexyl-4,5-dihydroisoxazol-5-yl)ethan-1-ol oxalate (7): (67% yield) m.p.: 182–185 °C. ¹H NMR (400 MHz, DMSO-d₆) δ: 8.99 (bs, 1H); 8.48 (bs, 1H); 6.02–5.87 (m, 1H); 4.40–4.34 (m, 1H); 3.75–3.71 (m, 1H); 3.55–3.50 (m, 2H); 3.03–2.95 (m, 3H); 2.76–2.72 (m, 1H); 2.40–2.36 (m, 1H); 1.78–1.61

(m, 5H); 1.29–1.10 (m, 14H). ¹³C NMR (100 MHz, DMSO-d₆) δ: 162.3; 79.8; 67.5; 56.3; 44.0; 36.8; 36.4; 29.9; 25.5; 25.2; 24.9.

General procedure for the synthesis of chloro-methoxyphenyl-isoxazolidinyl-N-alkyl-ethanolamines 16-21

A solution of the proper *syn* or *anti* epoxide **27e** or **27f** (1 eq) and *i*-PrNH₂, or *t*-BuNH₂ (4.6 eq) in a mixture of 1:2 anhydrous benzene/EtOH was stirred at room temperature for 72 h, and then evaporated to dryness. The crude oily residue was taken up in 10% aqueous HCl and brine, and the resulting mixture was washed with Et₂O, alkalinised with solid K₂CO₃, and then extracted with CHCl₃. The organic phases were collected and evaporated and the residue was dissolved in diethyl ether and treated with a small excess of proper organic acid. The isoxazolidinyl-N-alkyl-ethanolamine salts **16–21**, have been obtained pure by crystallisation process in a 4:1 Et₂O/EtOH mixture.

(syn)-1-(3-(2-chloro-4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl)-2-(isopropylamino) ethan-1-ol maleate (16): (73% yield) m.p.0.183–184 °C. ¹H NMR (400 MHz, DMSO-d₆) δ: 8.32 (bs, 2H); 7.70–7.69 (m, 1H); 7.64–7.61 (m, 1 H); 7.26–7.24 (m, 1H); 6.02 (s, 2H); 5.96 (bs, 1H); 4.66–4.60 (m, 1H); 3.91 (s, 3H); 3.83–3.81 (m, 1H); 3.49–3.40 (m, 2H); 3.09–3.07 (m, 1H); 2.89–2.84 (m, 1H); 1.24-1.21 (dd, $J_1= 4.4$ Hz, $J_2= 6.4$ Hz, 6H). ¹³C NMR (100 MHz, DMSO-d₆) δ: 167.2; 155.8; 155.4; 136.1; 127.8; 126.9; 122.5; 121.5; 113.0; 81.7; 67.2; 56.4; 49.9; 46.2; 35.8; 18.9; 18.1.

(syn)-2-(tert-butylamino)-1-(3-(2-chloro-4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl) ethan-1-ol oxalate (17): (67% yield) m.p: 210–212 °C. ¹H NMR (400 MHz, DMSO-d₆) δ: 7.72–7.71 (m, 1H); 7.65–7.62 (m, 1H); 7.26–7.24 (m, 1H); 4.69–4.64 (m, 1H); 3.91 (s, 3H); 3.87–3.83 (m, 1H); 3.49–3.39 (m, 2H); 3.09–3.06 (m, 1H); 2.84–2.78 (m, 1H); 1.29 (s, 9H). ¹³C NMR (100 MHz, DMSO-d₆) δ: 165.3; 156.3; 155.9; 128.3; 127.4; 123.0; 121.9; 113.5; 82.2; 67.9; 56.9; 56.7; 44.1; 36.2; 25.5.

(anti)-2-(tert-butylamino)-1-(3-(2-chloro-4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl) ethan-1-ol oxalate (18) (65% yield) m.p.: 205–207 °C. ¹HNMR (400 MHz, DMSO-d₆) δ: 7.70–7.69 (m, 1H); 7.63–7.61 (m, 1H); 7.25–7.23 (m, 1H); 4.71–4.63 (m, 1H); 3.91 (s, 3H); 3.67–3.65 (m, 1H); 3.43 (dd, $J_1= 10.8$ Hz, $J_2= 16.8$ Hz, 1H); 3.30 (dd, $J_1= 8.8$ Hz, $J_2= 17.2$ Hz, 1H); 2.87–2.83 (m, 1H); 2.77-2.63 (m, 1H); 1.17 (s, 9H). ¹³C NMR (100 MHz, DMSO-d₆) δ: 167.2; 155.8; 155.4; 136.2; 127.8; 126.9; 122.6; 121.5; 113.1; 81.6; 68.3; 56.5; 56.4; 43.3; 36.2; 25.1.

(syn)-1-(3-(2-chloro-4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl)-2-(isopropylamino) ethan-1-ol maleate (19) (85% yield) m.p.:140–141 °C. ¹H NMR (400 MHz, DMSO-d₆) δ: 8.31 (bs, 2H); 7.56–7.50 (m, 2H); 7.20–7.18 (m, 1H); 6.01 (s, 2H); 5.97–5.95 (m, 1H); 4.63–4.57 (m, 1H); 3.85 (s, 3H); 3.82–3.77 (m, 1H); 3.47–3.40 (m, 2H); 3.09–3.06 (m, 1H); 2.89–2.83 (m, 1H); 1.24–1.22 (dd, $J_1= 2$ Hz, $J_2= 3.6$ Hz, 6H). ¹³C NMR (100 MHz, DMSO-d₆) δ: 167.6; 156.6; 155.3; 136.6; 131.4; 128.4; 124.8; 120.2; 114.8; 82.2; 68.9; 56.7; 50.3; 38.6; 19.4; 18.6.

(anti)1-(3-(4-chloro-2-methoxyphenyl)-4,5-dihydroisoxazol-5-yl)-2-(isopropylamino) ethan-1-ol maleate (20) (80% yield) m.p.:180–181 °C. ¹H NMR (400 MHz, DMSO-d₆) δ: 8.63 (br s, 2H); 7.71–7.70 (m, 1H); 7.70–7.48 (m, 1H); 7.19–7.17 (m, 1H); 6.02 (s, 2H); 5.92 (br s, 1H); 5.86–5.85 (m, 1H); 4.70–4.64 (m, 1H); 3.91–3.90 (m, 1H); 3.84 (s, 3H); 3.55–3.47 (m, 1H); 3.39–3.30 (m, 1H); 3.06–2.93 (m,1H); 1.24 (m, 6H). ¹³C NMR (100 MHz, DMSO-d₆) δ: 164.9; 156.6; 155.3; 131.4; 128.4; 124.8; 120.3; 114.7; 82.2; 68.0; 56.7; 44.1; 38.5; 31.2; 25.6.

(sin)-2-(tert-butylamino)-1-(3-(4-chloro-2-methoxyphenyl)-4,5-dihydroisoxazol-5-yl) ethan-1-ol maleate (21) (70%yield)

m.p: 163–164 °C. ^1H NMR (400 MHz, DMSO-d$_6$) δ: 8.31 (bs, 2H); 7.56–7.55 (m, 1H); 7.52–7.49 (m, 1H); 7.20–7.18 (m, 1H); 6.02 (s, 2H); 5.97 (bs, 1H); 4.66–4.60 (m, 1H); 3.84 (s, 3H); 3.83–3.81 (m, 1H); 3.48–3.43 (m, 2H); 3.35–3.34 (m, 1H); 3.08–3.05 (m, 1H); 2.83–2.77 (m, 1H); 1.29 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ: 167.2; 156.2; 154.9; 136.1; 131.0; 128.0; 124.3; 119.8; 114.3; 81.7; 67.5; 56.5; 56.3; 43.5; 38.0; 30.7; 25.0.

Carbonic anhydrase activation

A Stopped Flow method[38] has been used for assaying the CA-catalysed CO_2 hydration activity with Phenol red as an indicator, working at the absorbance maximum of 557 nm, following the initial rates of the CA-catalysed CO_2 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 and dilutions up to 0.1 nM were done thereafter with the assay buffer. The activation constant (K$_A$), defined similarly with the inhibition constant (K$_I$), was obtained by considering the classical Michaelis–Menten equation [Equation (3)], which has been fitted by non-linear least squares by using PRISM 3:

$$v = v_{max}/\left\{1 + K_M/[S] \left(1 + [A]_f/K_A\right)\right\} \tag{3}$$

where $[A]_f$ is the free concentration of the activator

Working at substrate concentrations considerably lower than K_M ([S]≪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(4):

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

where v_0 represents the initial velocity of the enzyme-catalysed reaction in the absence of an activator. Enzyme concentrations in the assay system were in the range of 7.0–16.0 nM.

Results and discussion

Chemistry

As mentioned in the introduction, compounds **2–5**, **8–15** and **22–23** (Table 1) have been prepared according to literature procedures. The same reported procedure was applied to synthesise the new compounds **6–7**, **16–21**, as reported in Scheme 1.
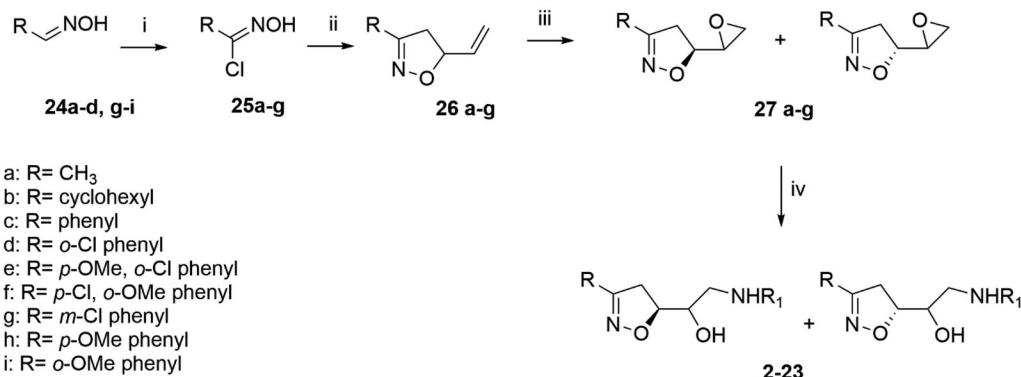
The chlorination of the proper aldoximes **24a–d, g–i,** conducted by treatment with *N*-chlorosuccinimide in DMF, afforded the corresponding hydroxamyl chlorides **25a–d** and **g–i**. For the methoxy phenyl aldoximes **24h** and **24i**, an unexpected electrophilic addition of chlorine on the *o*- or *p*- aromatic position concerning the methoxy moiety, occurred. Probably, chlorine overreaction was due to the improved susceptibility of the *p*- or *o*-OMe substitution in the aromatic ring to the initial chlorination process. In fact, the substitution of a chlorine atom on the aromatic ring is easily promoted by the presence of a methoxy group in a general electrophilic aromatic substitution. Nevertheless, the synthesis was completed and the *p*- and *o*- methoxy chlorophenyl hydroxamyl chlorides **25e–f** were used in the following reaction steps. The treatment by triethylamine and 1,3-butadiene of the proper hydroxamyl chlorides **25a–g**, afforded the corresponding vinyl-2-isoxazolines **26a–g**. The following oxidation of isoxazolines **26a–g** with *m*-chloroperoxybenzoic acid yielded a mixture of *anti* and/or *syn* **27a–g** epoxides which were separated by flash chromatography. Thus, aminolysis conducted by treatment of epoxides **27a–g** with an excess of *i*-PrNH$_2$ or *t*-BuNH$_2$ yielded the corresponding *anti* and/or *syn* **2–23** amino alcohols purified by crystallization as organic salts (maleate, fumarate or oxalate).

The *anti* or *syn* configurations have been assigned by ^1H NMR comparison with the already published compounds, deeply characterized by NMR and crystallographic studies. Moreover, by analyzing the ^1H NMR and the bi-dimensional COSY spectra, it was possible to detect how the difference in the configuration affected the Δ chemical shift of diastereotopic vicinal protons of the hydroxyl group (OH–CH–**CH$_2$**–NH–, protons **D**, Figure 2). Generally, each proton of methylene **D** (H$_{D1}$ and H$_{D2}$) presented a chemical shift sparing from 3.2 to 3.6 ppm, characterized by a double doublet signal, due to the couplings HD1-HD2 and HD-C. Interestingly, in the *anti* configuration two separated D proton double doublets with $\Delta_{HD1-HD2}$ of 0.20 ppm were detected at 3.60 and 3.40 ppm (Figure 2). Otherwise, in the *syn* configuration a superimposition of the two D proton double doublet signals with a consequent $\Delta_{HD1-HD2}=0$ was evident (Figure 2). This difference in Δ chemical shift is probably due to the different coupling between C and D protons caused by the different configurations.

Carbonic anhydrase activation

The novel isoxazoline-based amino alcohol series (**2–23**) has been tested on four physiologically relevant hCAs (hCA I, hCA II, hCA IV and hCA VII), mainly expressed in the human brain. Focussing on the central nervous system (CNS), the cytosolic hCA I is mainly expressed by motoneurons, sited in the human spinal cord[39], hCA

Scheme 1. Reactions and conditions: (i) *N*-Chlorosuccinimide, DMF 15 min, rt; (ii) Et$_3$N, 1,3-butadiene, CHCl$_3$, 0 °C-rt, 3 h; (iii) *m*-Cl-perbenzoic acid, DCM, 0 °C-rt, 72 h; (iv) iPrNH$_2$, tBuNH$_2$, Benzene/EtOH, rt, 72 h.
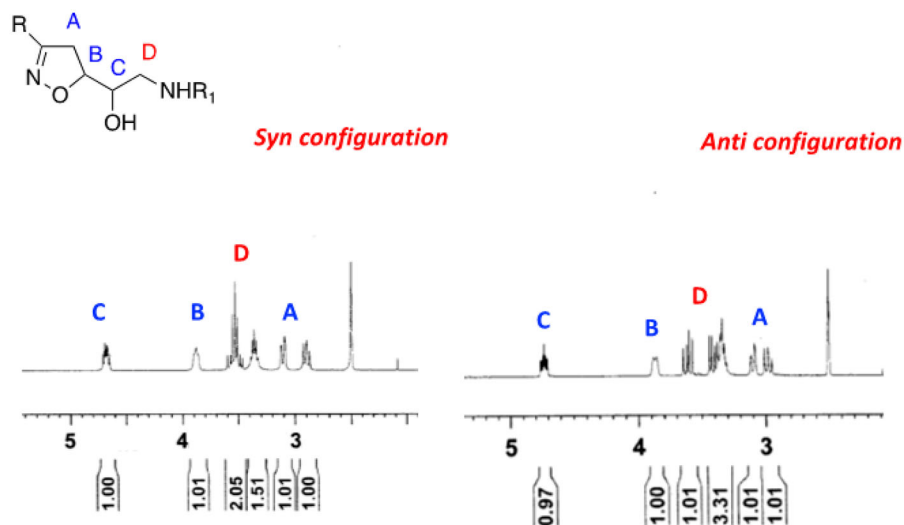
Figure 2. Superimposition of **12** (left) and **13** (right) to identify the different chemical shifts of methylene D protons.

II is located both in the choroid plexus, and in oligodendrocytes, myelinated tracts, astrocytes and myelin sheaths and hCA IV is expressed on the luminal surface of cerebral capillaries, associated with the blood–brain barrier, and in a different area of the cortex, thalamus and hippocampus[40,41]. The membrane-associated hCA VII might be considered the brain-target CA, because is predominantly expressed in the brain, in the cortex, thalamus and hippocampus, while it is almost absent in the other human tissues[42].

The CA activation data of human brain CA isoforms (hCA I, hCA II, hCA IV and hCA VII), with isoxazoline-based amino alcohols (**2–23**) are reported in Table 1. Histamine has been tested as a reference compound. In general, all the new CA activators presented activity towards hCA II, hCA IV and hCA VII in the low micromolar range, and poor activity towards hCA I. The data evidenced no significant differences between *tert*-butyl and isopropyl derivatives for the activation of all the hCA isozymes, thus indicating a minor importance of the R_1 amino substituent in determining the affinity for the enzymes.

Regarding the cytosolic isoform hCA I, the activation promoted by isoxazoline amino alcohols was weak, presenting K_A values ranging from 11.7 to >100 μM. In particular, a lack of hCA I activity (K_As > 100 μM) was demonstrated by the *p*-OMe-*o*-Cl phenyl isoxazoline **18** and the phenyl isoxazoline **9**. Furthermore, the *m*-Cl phenyl-isoxazoline-isopropylamines **21** and **22** showed K_As > 100 μM for hCA I and a 2-fold decrease of activity concerning their *o*-Cl analogues **12** and **13**. It can be noted a preferred *o*-chloro substitution to maintain hCA I activity.

All the compounds activated cytosolic hCA II with potency in the low micromolar range (K_As from 1.53 to 4.15 μM). hCA II was activated more than hCA I and hCA IV by all the isoxazoline amino alcohols of this new series, without any exception. Interestingly, compounds **9**, **18**, and **22** reported almost a 50-fold enhancement of activation efficacy of hCA II when compared to hCA I (hCA I K_A >100/hCA II 1.84–2.85 μM). Importantly, this new series of compounds showed an improved activity of at least 40-fold compared to histamine (K_A of 125 μM), the reference compound which is a weak activator of this isoform.

As for activity towards the membrane-associated isozyme hCA IV, all K_As were in a rather flat low micromolar range, from 4.35 to 25.5 μM values, with no K_A value measured in the submicromolar range. No significant differences exist between *tert*-butyl and isopropyl derivatives and between different configurations, for the activation of hCA IV. Notably, the reference CAA histamine was a

poorer activator of hCA IV (K_A of 25.3 μM) than the tested isoxazolines, with the only exception of derivative **10** with a K_A of 35.7 μM against hCA IV.

The other isoform investigated here, hCA VII, was rather potently activated by all of the compounds reported in this work (K_As in the range 0.52–8.26 μM). The most interesting data were those reported for the methyl-substituted isoxazoline derivatives **2–5**, able to activate hCA VII in a submicromolar range (K_A values 0.52–0.86 μM). These compounds presented also a moderate selectivity over the other tested isoforms. The small size of the methyl group for the aryl substituents of other derivatives seems to be significant to address activity and selectivity towards hCA VII isoform.

Notably, configuration affected the hCA VII activity data in terms of activity and selectivity. In particular, a slight improvement (1.3–1.7 fold) of hCA VII activity was reported for the *anti*-diastereoisomers **3** and **5** compared to their *syn* analogues **2** and **4**. Moreover, compounds **3** and **5** demonstrated a significantly improved selectivity over hCA I concerning **2** and **4**. In fact, *syn*-methyl-isoxazoline **2** presented a 13-fold selectivity hCA VII/hCA I versus the 53-fold selectivity hCA VII/hCA I of *anti*-methyl-isoxazoline **3**, and the *syn*-*tert*-butylamine **4** showed a 15-fold selectivity hCA VII/hCA I versus the 90-fold selectivity hCA VII/hCA I of *anti*-*tert*-butylamine **5.**

Overall, among all tested derivatives the best results were achieved with the methyl-substituted isoxazoline **3**, showing a 72-fold higher activity for hCA VII (K_A = 0.52 μM) concerning histamine and a 53-fold selectivity over hCA I.

hCA VII is one of the most representative CA expressed in the brain, and because of its predominant presence in brain tissues, selective hCA VII-targeting can be considered an area of interest in the research of more effective agents for treating neurodegeneration-related diseases.

Conclusions

In the present study, the synthesis and CA activating properties of a new series of isoxazoline-based amino alcohols towards four CAs, that are hCA I, hCA II, hCA IV and hCA VII, mainly expressed in the human brain are reported. All the tested compounds induced a consistent activation of the tested CAs, with K_A value ranges spanning from low to intermediate micromolar range.

All compounds showed a generally poor affinity for hCA I, and a good activity profile for hCA II and hCA VII, main targets in CNS diseases. Specifically, hCA VII targeting was achieved by the (methyl)-isoxazoline-amino alcohols **2–5** with K_A values in the sub-micromolar range (0.52–0.86 μM), but only with compounds in *anti*-configuration, **3** and **5**, a selectivity over hCA I was achieved. These molecules represent a new chemotype in the field of CA activators and deserve further investigation. Owing to the wide CNS expression of hCA VII, such molecules could be useful as a tool to study the physiological role of this enzyme in diseases involved in CNS such as epilepsy, ageing and neurodegeneration.

Disclosure statement

Funding

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