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To cite this article: Niccolò Chiaramonte, Soumia Maach, Caterina Biliotti, Andrea Angeli, Gianluca Bartolucci, Laura Braconi, Silvia Dei, Elisabetta Teodori, Claudiu T. Supuran & Maria Novella Romanelli (2020) Synthesis and carbonic anhydrase activating properties of a series of 2-amino-imidazolines structurally related to clonidine, Journal of Enzyme Inhibition and Medicinal Chemistry, 35:1, 1003-1010, DOI: 10.1080/14756366.2020.1749602

To link to this article: https://doi.org/10.1080/14756366.2020.1749602

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Published online: 27 Apr 2020.

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Synthesis and carbonic anhydrase activating properties of a series of 2-amino-imidazolines structurally related to clonidine

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ABSTRACT
The Carbonic Anhydrase (CA, EC 4.2.1.1) activating properties of histamine have been known for a long time. This compound has been extensively modified but only in few instances the imidazole ring has been replaced with other heterocycles. It was envisaged that the imidazole ring could be a bioisoster of the imidazole moiety. Indeed, we report that clonidine, a 2-aminoimidazolinaldehyde, was found able to activate several human CA isoforms (hCA I, IV, VA, VII, IX, XI, XII and XIII), with potency in the micromolar range, while it was inactive on hCA II. A series of 2-aminoimidazoline, structurally related to clonidine, was then synthesised and tested on selected hCA isoforms. The compounds were inactive on hCA II while displayed activating properties on hCA I, VA, VII and XIII, with Ka values in the micromolar range. Two compounds (10 and 11) showed some preference for the hCA VA or VII isoforms.

1. Introduction
Carbonic anhydrases (CAs, EC 4.2.1.1) are metallo-enzymes widespread in all life kingdoms. These enzymes catalyse a plethora of reactions, among which the reversible hydration of CO2 is the most important one. The active site contains the cofactor, a metal ion (usually Zn2+) which coordinates a water molecule responsible, once activated as hydroxide ion, of the nucleophilic attack onto carbon dioxide. Eight genetically different families have been found (x;i); 15 isoforms belonging to the x class have been characterised in humans. CAs have been drug targets since more than 70 years; inhibitors of these enzyme are used for the treatment of oedema, glaucoma and epilepsy but several new therapeutic applications are under study. In recent years, the attention has been focussed also on activators of these enzymes, despite the fact that CA are among the most efficient enzymes known. In fact, genetic deficiencies of several CA isoforms were reported in the last decades (reviewed in Refs. [3,4]), and in principle a loss of function of these enzymes could be treated with CA selective activators (CAAs). In addition, there is evidence that CA activation improves cognitive performance. However, the influence of CA on these processes is complex since also inhibitors have been found to improve memory deficits in animal models (reviewed in Ref. [11]); these findings point out the need for isoform selective inhibitors or activators to elucidate the role of CA isoforms in cognitive processes. Other possible applications of CAAs could be in the formation of artificial tissues and in CO2 capture and sequestration processes.

Histamine (HST, Chart 1) was among the first reported activators, whose interaction mode was elucidated by means of X-ray crystallography. The adduct with hCA II revealed a complex network of H-bonds involving the Zn-bound water molecule, His64 and the imidazole ring of the activator, which is located far away from the metal ion, in a region approaching the edge of the active site cavity. X-ray crystallographic studies have later shown that also other activators bind in this area. As common structural feature, CAAs possess flexible tails decorated with protonable moieties, with pKa values spanning between 6 and 8. The molecule of histamine has been extensively modified, placing substituents on the imidazole C atoms and on the NH2 group, showing that the latter is not essential, since it can be largely modified to keep or improve potency (reviewed in Ref. [4]). Only in few instances the imidazole ring has been replaced by another heterocycle, such as a thiazole ring.

Chart 1. Chemical structure of CA activators.

In search for bioisosters of the imidazole moiety, our attention was attracted by the imidazole ring. This feature is present in a well-known drug, Clonidine (CLO, Chart 1), which is clinically used...
as an antihypertensive agent being an agonist at the central α2-adrenergic receptor, but it is able to interact with other targets, such as the imidazoline binding sites and the hyperpolarization-activated cyclic nucleotide gated channels. Therefore, we decided to measure the potential CA activating properties of this compound, finding that CLO behaves as CAA on several CA isoforms (Table 1). Encouraged by this positive outcome, we synthesised a series of 2-substituted imidazolines (compounds 1–24, Chart 1) and tested their activity on five different hCA isoforms. The ubiquitous cytosolic enzymes CA I and II, the mitochondrial CA VA, which is associated with the glucose homeostasis,18 the cytosolic CA VII which is particularly abundant in the CNS and has been recently demonstrated to have a protective role against oxidative damage,19 and the cytosolic CA XIII, which is particularly expressed in the reproductive organs20,21 were selected.

2. Materials and methods

2.1. Chemistry

All melting points were taken on a Büchi apparatus and are uncorrected. NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz for 1H NMR, 100 MHz for 13C). Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063–0.200 mm; Merck) or flash chromatography (Kieselgel 40, 0.040–0.063 mm; Merck). Yields are given after purification, unless differently stated. When reactions were performed under anhydrous conditions, the mixtures were maintained under nitrogen. High-resolution mass spectrometry (HR-MS) analyses were performed with a Thermo Fisher Scientific spectrometer (400 MHz for 1H NMR, 100 MHz for 13C). ESI-LCMS (m/z) uncorrected. NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz for 1H NMR, 100 MHz for 13C).

Table 1. Activation constants of Clonidine (CLO) and histamine (HST) on selected human CA isoforms, measured by means of a stopped-flow, CO2 hydrate assay.a

| Compound | I | II | IV | VA | VII | IX | XII | XIII |
|----------|---|---|---|----|-----|----|-----|-------|
| CLO      | 73.6 | >200 | 132 | 42.6 | 8.4 | 54.1 | 126 | 7.8  |
| HSTb     | 2.1 | 125 | 25.3 | 0.01 | 37.5 | 35.1 | 27.9 | 4.6  |

aErrors (data not shown) are in the range of ±5–10% of the reported values from three different assays.

Table 2. Synthetic details for the preparation of compounds 2–20 (see general formula in Scheme 1) starting from 1a (R1 = H) or 1b (R1 = Me) and amines R2R3NH.

| N | Starting material | R2 | R3 | Solvent | Reaction time (h)a | Yields (%)b |
|---|------------------|----|----|--------|-------------------|------------|
| 2 | 1a H             | –CH2Ph | THF | 7       | 94                |
| 3 | 1a H             | –(CH2)2Ph | THF | 16 | 90             |
| (S)–4 | 1a H | –(S)-(CH2)MePh | Amine | 2 | 36d             |
| (R)–4 | 1a H | –(R)-(CH2)MePh | Amine | 2 | 74d             |
| 5 | 1a H             | –(CH2)3Ph | THF | 3       | 88                |
| 6 | 1a Me            | –CH2Ph | THF | 22 | 32d            |
| 7 | 1a Me            | –(CH2)2Ph | THF | 2       | 88                |
| 8 | 1b H             | –(CH2)3Ph | Amine | 2 | 86             |
| 9 | 1b Me            | –(CH2)2Ph | Amine | 2 | 97             |
| 10 | 1b Me | –CH2Ph | Amine | 3 | 70d          |
| 11 | 1b H             | –CH2Ph | Amine | 3 | 81             |
| 12 | 1b H             | –(CH2)2Ph | Amine | 2 | 45d           |
| 13 | 1b H | –CH2C6H4Cl(4) | THF | 16 | 97            |
| 14 | 1b H | –CH2C6H4CH2(4) | THF | 4 | 89d          |
| 15 | 1b H | –CH2C6H4F(4) | Amine | 6 | 89             |
| 16 | 1b H | –CH2C6H4Cl(3) | Amine | 6 | 88d          |
| 17 | 1b H | –CH2C6H4CH2(3) | Amine | 24 | 92             |
| 18 | 1b H | –CH2C6H4Cl(3) | Amine | 16 | 87             |
| 19 | 1a H | –CH2C6H4Cl(4) | Amine | 3 | 56d          |
| 20 | 1b H | –CH2C6H4NHCH2Ph | Amine | 4 | 57d         |

aHeating at 70°C; all amines were commercially available.
bUnless otherwise stated, yields are given after trituration with diethyl ether.
cThe amine was used in excess (5 eq).
dAfter chromatographic separation.

2.1.1. General procedure for the preparation of 2-amino-imidazoline 2–20

A mixture of the appropriate intermediate (1a23 or 1b24, 0.05 g) and the amine (1 eq) was suspended in THF (5 ml) and the mixture was heated at 70°C until reaction completion (TLC monitoring, eluent CH2Cl2/CH3OH/NH4OH 90:10:1); alternatively, an excess of amine (5 eq) was used as solvent. Volatiles were evaporated under vacuum and the residue was triturated with Et2O to remove the unreacted amine, affording the desired imidazoline derivative. In some instances, additional purification by means of flash chromatography was necessary. The conditions for each compound are reported in Table 2. With this method, the following compounds were prepared.

N-Benzyl-4,5-dihydro-1H-imidazol-2-amine hydroiodide 23. White solid; m.p. 143–146°C ESI-LCMS (m/z) 176.0 [M + H]+; [1H]-NMR (D2O) δ: 3.56 (s, 4H, CH2CH2); 4.31 (s, 2H, CH2); 7.24–7.35 (m, 5H, NH Ar) ppm. [13C]-NMR (D2O) δ: 24.70 (CH2Ph); 46.56 (2CH2); 126.93 (CHAr); 128.00 (CHAr); 128.95 (CHAr); 136.25 (CAr); 159.91 (C=N) ppm.

N-Phenethyl-4,5-dihydro-1H-imidazol-2-amine hydroiodide 3. White solid; m.p. 82–83°C; ESI-LCMS (m/z) 189.7 [M + H]+.
30.68 (CH$_2$), 32.63 (CH$_3$); 42.57 (CH$_3$); 43.39 (CH$_3$); 126.22 (CH$_3$); 128.54 (CH$_3$); 140.62 (CH$_3$); 159.67 (C=N) ppm. ESI-HRMS (m/z [M + H$^+$]): calculated for C$_{13}$H$_{19}$N$_3$O$^+$ 218.1652; found 218.1654.

N-Benzyl-N-methyl-4,5-dihydro-1H-imidazol-2-amine hydroiodide 13. White solid, m.p. 84°C. [1H]-NMR (MeOD) $\delta$: 2.96 (s, 3H, CH$_3$), 3.61–3.76 (m, 4H, CH$_2$CH$_3$), 4.41 (s, 2H, CH$_2$), 7.31 (d, $J = 8.4$ Hz, 2H, Ar), 7.35 (d, $J = 8.4$ Hz, 2H, Ar), 7.42 (s, 2H, NH) ppm. [1H]-NMR (MeOD) $\delta$: 20.68 (CH$_3$); 40.90 (CH$_3$); 49.80 (CH$_3$); 45.44 (CH$_3$); 101.11 (CH$_3$); 112.68 (CH$_3$); 126.97 (CH$_3$); 128.67 (CH$_3$); 134.13 (CH$_3$); 158.22 (C=N) ppm. ESI-HRMS (m/z [M + H$^+$]): calculated for C$_{12}$H$_{17}$N$_3$O$^+$ 208.1245; found 208.1245.

1-Methyl-N-phenyl-4,5-dihydro-1H-imidazol-2-amine hydroiodide 8. White solid; m.p. 118–120°C. [1H]-NMR (CDCl$_3$) $\delta$: 3.00–3.08 (m, 5H, NCH$_2$ + NCH$_3$); 3.48 (s, 4H, 2CH$_2$); 3.65 (apparent q, $J = 6.8$ Hz, 2H, NCH$_2$); 6.96 (s, 1H, NH), 7.12–7.35 (m, 5H, Ar), 7.49 (t, $J = 5.6$, 1H, NH) ppm. [13C]-NMR (CDCl$_3$) $\delta$: 33.78 (CH$_3$); 34.99 (CH$_3$); 41.04 (CH$_2$); 45.22 (CH$_2$); 50.11 (CH$_2$); 126.79 (CH$_3$); 128.67 (CH$_3$); 129.32 (CH$_3$); 137.87 (CH$_3$); 158.22 (C=N) ppm. ESI-HRMS (m/z [M + H$^+$]): calculated for C$_{13}$H$_{19}$N$_3$O$^+$ 204.1495; found 204.1494.

N1-Dimethyl-N-phenyl-4,5-dihydro-1H-imidazol-2-amine hydroiodide 10. Purification by flash chromatography (CH$_2$Cl$_2$/CH$_3$OH/NH$_3$ 90:10:1 as eluent). Oil. [1H]-NMR (CDCl$_3$) $\delta$: 3.01 (s, 3H, CH$_3$); 3.07 (s, 3H, CH$_3$); 3.70–3.87 (m, 4H, CH$_2$CH$_3$), 4.63 (s, 2H, CH$_2$Ph); 7.22–7.35 (m, 5H, Ar), 8.10 (s, 1H, NH) ppm. [13C]-NMR (CDCl$_3$) $\delta$: 33.66 (CH$_3$); 36.97 (CH$_3$); 39.70 (CH$_3$); 40.33 (CH$_3$); 52.89 (CH$_3$); 54.71 (CH$_3$); 127.23 (CH-Ar); 128.92 (CH-Ar); 136.96 (C-Ar); 162.47 (C=N) ppm. ESI-HRMS (m/z [M + H$^+$]): calculated for C$_{13}$H$_{19}$N$_3$O$^+$ 218.1652; found 218.1649.

N-Benzyl-N,N-dimethyl-4,5-dihydro-1H-imidazol-2-amine hydroiodide 11. White solid; m.p. 130–133°C. [1H]-NMR (CDCl$_3$) $\delta$: 3.16 (s, 3H, CH$_3$); 3.58–3.66 (m, 4H, CH$_2$CH$_3$), 4.67 (d, $J = 5.6$ Hz, 2H, CH$_2$Ph), 6.87 (s, 1H, NH); 7.25–7.38 (m, 3H, Ar), 7.49 (d, $J = 7.2$ Hz, 2H, Ar), 8.21 (s, 1H, NH) ppm. [13C]-NMR (CDCl$_3$) $\delta$: 33.76 (CH$_3$); 41.31 (CH$_3$); 46.38 (CH$_3$); 50.22 (CH$_3$); 128.13 (CH$_3$); 128.34 (CH$_3$); 129.04 (CH$_3$); 135.63 (C$_6$); 158.35 (C=N) ppm. ESI-HRMS (m/z [M + H$^+$]): calculated for C$_{13}$H$_{19}$N$_3$O$^+$ 210.1339; found 210.1338.

1-Methyl-N-(3-phenylpropyl)-4,5-dihydro-1H-imidazol-2-amine hydroiodide 12. Purification by flash chromatography (CH$_2$Cl$_2$/CH$_3$OH/NH$_3$ 90:10:1 as eluent). Gum. [1H]-NMR (CDCl$_3$) $\delta$: 2.02 (p, $J = 7.6$ Hz, 2H); 2.69 (d, $J = 8.0$ Hz, 2H, CH$_3$); 2.97 (s, 3H, CH$_3$); 3.41–3.54 (m, 4H, CH$_2$CH$_3$); 3.60 (d, $J = 8.0$ Hz, 2H, CH$_3$); 7.10–7.25 (m, 5H, Ar) ppm. [13C]-NMR (CDCl$_3$) $\delta$: 30.62 (CH$_3$); 32.93 (CH$_3$); 33.77 (NCH$_3$); 40.97 (CH$_3$); 43.83 (CH$_3$); 50.11 (CH$_3$); 126.01 (CH$_3$); 128.44 (CH$_3$); 128.58 (CH$_3$); 141.25 (C$_6$); 157.91 (C=N) ppm. ESI-HRMS (m/z [M + H$^+$]): calculated for C$_{13}$H$_{19}$N$_3$O$^+$ 210.1339; bound 210.1338.

N4-Chlorobenzyl)-1-methyl-4,5-dihydro-1H-imidazol-2-amine hydroiodide 14. White solid, m.p. 167°C. [1H]-NMR (CDCl$_3$) $\delta$: 3.18 (s, 3H, CH$_3$), 3.64 (s, 4H, CH$_2$CH$_3$), 3.76 (s, 3H, OCH$_3$) ppm. [1H]-NMR (CDCl$_3$) $\delta$: 30.81 (CH$_3$), 49.08 (CH$_3$), 45.44 (CH$_3$), 50.11 (CH$_3$), 115.26 (d, $J_{CF} = 22$ Hz, CH$_3$), 129.12 (d, $J_{CF} = 8$ Hz, CH$_3$), 132.25 (C$_6$), 158.62 (C=N), 162.48 (d, $J_{CF} = 244$ Hz, CF) ppm. ESI-HRMS (m/z [M + H$^+$]): calculated for C$_{12}$H$_{17}$N$_3$O$^+$ 220.1444; found 220.1433.

N4-(Fluorobenzyl)-1-methyl-4,5-dihydro-1H-imidazol-2-amine hydroiodide 15. White solid, m.p. 157°C. [1H]-NMR (MeOD) $\delta$: 2.97 (s, 3H, CH$_3$), 3.61–3.75 (m, 4H, CH$_2$CH$_3$), 4.42 (s, 2H, CH$_2$), 7.08 (t, $J = 8.5$ Hz, 2H, Ar), 7.42 (dd, $J = 8.5, 5.4$ Hz, 2H, Ar) ppm. [13C]-NMR (MeOD) $\delta$: 30.81 (CH$_3$), 49.08 (CH$_3$), 45.44 (CH$_3$), 50.11 (CH$_3$), 115.26 (d, $J_{CF} = 22$ Hz, CH$_3$), 129.12 (d, $J_{CF} = 8$ Hz, CH$_3$), 132.25 (C$_6$), 158.62 (C=N), 162.48 (d, $J_{CF} = 244$ Hz, CF) ppm. ESI-HRMS (m/z [M + H$^+$]): calculated for C$_{12}$H$_{17}$N$_3$O$^+$ 220.1444; found 220.1433.
ter. The combined organic layers were dried (Na₂SO₄), filtered for 5 h at room T. The solvent was evaporated and diethyl ether.

N. CHIARAMONTE ET AL.

13C-NMR (D₂O) 45.24 (2CH₂); 128.31 (CHAr); 128.72 (CHAr); 129.10 (CHAr); 129.37 (CH₂); 129.80 (CH Ar); 129.37 (CH Ar); 129.80 (CH Ar); 130.81 (C Ar); 156.18 (C Ar); 162.89 (C Ar); 169.69 (C O). Oxalate ppm.

2.1.2. N1-(4,5-Dihydro-1H-imidazol-2-yl)ethane-1,2-diamine 22²²

A mixture of commercially-available 2-imidazolidinethione (0.1 g, concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5, for x-Ca²⁺) as buffers, 0.1 M NaClO₃ (for maintaining constant ionic strength), following the CA-catalysed CO₂ hydration reaction for a period of 10 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 5–10% of the reaction have been used for determining the initial velocity. The uncatalysed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activators (at 0.1 mM) were prepared in distilled-deionised water and 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 (Kₐ) defined similarly with the inhibition constant Kᵢ, can be obtained by considering the classical Michaelis–Menten equation (Equation (1)), which has been fitted by non-linear least squares by using PRISM.

\[
\v = \frac{v_{\text{max}}}{1 + \left(\frac{[A]/[S]}{K_M}\right)}(1 + \frac{[A]/[K_A]}{1})
\]

where [A]₀ is the free concentration of activator.

Working at substrate concentrations considerably lower than Kₐ (|S| ≪ Kₐ), and considering that [A]₀ can be represented in the form of the total concentration of the enzyme ([E][A]) and activator ([A][A]), the obtained competitive steady-state equation for determining the activation constant is given by Equation (2):

\[
v = \frac{v_0 K_A}{[K_A + ([A]_1 + [E]_1 + K_A^-1) - ([A]_1 + [E]_1 + K_A)^2 - 4[A]_1[E]_1^{1/2}]}
\]

where v₀ represents the initial velocity of the enzyme-catalysed reaction in the absence of activator. This type of approach to measure enzyme–ligand interactions is in excellent agreement with recent results from native mass spectrometry measurements.

3. Results and discussion

3.1. Chemistry

2-Amino imidazolines 2–20 were prepared by reacting primary and secondary amines with 2-(methylthio)-4,5-dihydro-1H-imidazole hydroiodide 1a²³ or its N⁴-methyl derivative 1b²⁴ using tetrahydrofuran or an excess of amine as solvents (Scheme 1). The final compounds were obtained as hydroiodide salts in different yields. Synthetic details are reported in the experimental section. Reactions with 3- or 4-nitrobenzyl amine or with dibenzyl amine were unsuccessful.

This method failed also to give compound 22 (step b, Scheme 2), whose synthesis was therefore attempted according to the procedure of Gomez Saint-Juan (step c, Scheme 2)³⁰ however, also

\[
\begin{align*}
\text{N} & \text{SMe} \\
\text{R₁} & \text{R₂} \\
\text{R₃} & \text{R₄} \\
\text{NH} & \text{THF or neat, Δ} \\
\text{R₂} & \text{R₄} \\
\end{align*}
\]

\[
\text{1a: R₁ = H} \quad \text{1b: R₁ = Me}
\]

Scheme 1. Synthesis of compounds 2-20.
this method was abandoned since the reaction of 1-acetyl-2-(methylthio)-4,5-dihydro-1H-imidazole 23 with aniline was not successful. Finally, 22 was prepared from 1-[(2-aminoethyl)-3-phenylthiourea29 according to Heinelt et al.28 Compound 2127 was synthesised through condensation of guanidine and ethylenediamine while 2431 was prepared by reaction of imidazoline-2-thione with benzyl bromide.

### 3.2. CA activating profile

The stopped-flow method32 has been used for assaying the CO2 hydration activity catalysed by different CA isoforms; the results are expressed as KA (activation constant, μM). The activating profile of CLO is reported in Table 1, in comparison with HST. CLO behaved as an activator on several CA isoforms (I, IV, VA, VII, IX, XII and XIII), with KA values in the range 7.8–11.1 μM. In particular, the isoforms most sensitive to CLO were CA VII and CA XIII.

Finally, inserting a CH2 unit between the exocyclic N atom and the Ph ring of 22 improved the activity: in fact, CH2 unit between the exocyclic N atom and the Ph ring of 22 improved activity compared with the sulphur analogues 1a and 23, and 24 were about 3 times more potent than the parent compound.

### 3.2.1. hCA I

The KA value of CLO on this isoform was 76.3 μM. Removal of both chlorine atoms abolished activity, since 22 (R2 = Ph) was inactive when tested up to a 150 μM concentration. On the contrary, inserting a CH2 unit between the exocyclic N atom and the Ph ring of 22 improved the activity: in fact, 2 (KA = 4.18 μM), 18 times more potent than CLO, was one of the most potent compounds on this isoform among the newly synthesised analogues. The elongation of the methylene chain gave compounds less active than 2; interestingly, a chain formed by 3 CH2 units was tolerated (5, KA = 36.7 μM) while a CH2CH2 chain was not (3, KA =150 μM). Side-chain branching abolished activity (S-4, R-4: KA >150 μM). Methylation of the exocyclic N4 atom was tolerated when R2 was a phenethyl group (7: KA = 68.6 μM) but not when R2 was a benzyl moiety (6: KA =150 μM). Also the methylation of the endocyclic N1 atom gave contrasting results, since compounds 8 and 12 were more active than their non-methylated analogues 3 and 5, but 11 (R2 = benzyl) showed a fourfold decrease of activity with respect to 2 (11: KA = 16.9; 2: 4.18 μM). Double methylation was productive for the phenethyl analogues (compare 9 with 3, 7 and 8); for the benzyl analogue 10 this modification improved the activity only with respect to 6. The aminoethyl derivative 21 (KA = 3.87 μM) was the most potent compound on the hCAI isoform; the addition of a benzyl moiety on the primary amine group abolished activity (19, KA >100 μM), and also the N-propyl analogue 20 was inactive.

Aromatic substitution on the benzyl moiety did not improve the potency: in fact, while the 4-Cl derivative 13 was equipotent with 11, a 4-OME (14) or 4-F substituent (15) increased from 2 to 3.5 times the KA values. The same substituents in the meta position reduced to a higher extent or abolished the activity. As far as the sulphur analogues 1a, 23 and 24 are concerned, the small methyl group seemed tolerated, not the bulkier benzyl moiety (24, KA >150 μM). The basicity of the amidine group appeared to be not crucial, since the NH and the N-acetyl derivatives (1a and 23, respectively) were equipotent.

### 3.2.2. hCA VA

The KA value of CLO on this isoform was 42.6 μM. The removal of both chlorine atoms did not affect activity, since 22 (R2 = Ph) was almost equipotent with CLO. Also the insertion of a CH2 unit between the exocyclic N atom and the Ph ring of 22 did not substantially modified potency (2, KA = 45.7 μM). On this isoform, the majority of the compounds showed good activating properties, with potency higher than CLO: the KA values of 24, 23, 3–5, 7–9, 11 and 12 were in the range 3.7–17.2 μM. The most potent compound was 10 (KA = 0.9 μM), a benzyl derivative carrying a methyl group on both N1 and N3 atoms; this compound was 47 times more active than CLO. The removal of the exocyclic N1-Me group decreased 4 times the activity (11, KA = 3.7 μM), while the removal of the N2-methyl group was more detrimental: as a matter of fact, compounds 6 (KA = 40.5 μM) and 2 (KA = 45.5 μM) were about 40 times less potent than 10 (KA = 0.9 μM). On the contrary, the degree of methylation did not substantially affect the potency of the phenethyl and phenyl propyl derivatives, since compounds 3, 5, 7, 8 and 12 had KA values in the range 9.9–17.2 μM. Similarly, aromatic substitution on the benzyl moiety slightly decreased the potency without substantial modulation, the KA values of compounds 13–18 being 2–4 times higher than 11. Side-chain branching (compounds 5–4 and R-4) improved the activity on this isoform, and a small enantioselectivity was observed: the R-enantiomer was twice more potent as the S-isomer. A benzyl moiety on the terminal amino group of 21 (KA = 31.2 μM) increased the activity, as analogue 19 and its N1-methyl derivative 20 were about 3 times more potent than the parent compound.

As far as the sulphur derivatives are concerned, the replacement of the N2H moiety of 2 (KA = 45.5 μM) with S (24, KA = 11.1 μM) brought a fourfold improvement in activity. Acetylation of the N1
Table 3. Activation constant (Ka) of the synthesised compounds and Clonidine (CLO) for human I, II, VA, VII and XIII Carbonic Anhydrase isoforms.

| N   | R₁   | X    | R₂   | hCA I | hCA II | hCA VA | hCA VII | hCA XIII |
|-----|------|------|------|-------|--------|--------|---------|----------|
| 1a  | H    | S    | –CH₃ | 9.61  | >150   | 38.3   | 41.9    | >100     |
| 2   | H    | NH   | –CH₂Ph| 4.18  | >150   | 45.7   | 35.2    | >100     |
| 3   | H    | NH   | –(CH₂)₂Ph| >150  | >150   | 16.7   | 18.9    | >100     |
| 5-4 | H    | NH   | (3)-CH(Me)Ph| >150  | >150   | 12.4   | 31.5    | >100     |
| 8-4 | H    | NH   | (R)-CH(Me)Ph| >150  | >150   | 4.92   | 24.2    | >100     |
| 5   | H    | NH   | –(CH₂)₂Ph| 36.7    | >100   | 9.9    | 11.4    | 24.3     |
| 6   | H    | NMe  | –CH₂Ph| >150  | >150   | 40.5   | 11.0    | >100     |
| 7   | H    | NMe  | –(CH₂)₂Ph| 68.6   | >100   | 14.9   | 2.4     | 6.5      |
| 8   | CH₃  | NH   | –(CH₂)₂Ph| 95.4   | >100   | 14.6   | 16.2    | 31.0     |
| 9   | CH₃  | NMe  | –(CH₂)₂Ph| 20.2   | >100   | 14.9   | 2.6     | 36.9     |
| 10  | CH₂₅  | NMe  | –CH₂Ph| 30.2   | >100   | 0.9    | 6.5     | 17.4     |
| 11  | CH₂₅  | NH   | –CH₂Ph| 16.9   | <100   | 3.7    | 0.9     | 19.1     |
| 12  | CH₂₅  | NH   | –(CH₂)₂Ph| 10.9   | >100   | 17.2   | 3.1     | 10.9     |
| 13  | CH₂₅  | NH   | –CH₂CH₂Cl(4)  | 17.7  | >100   | 10.5   | 22.7    | 22.9     |
| 14  | CH₂₅  | NH   | –CH₂CH₂OCH₂Cl(4)| 37.6 | >100   | 15.3   | 30.2    | 19.3     |
| 15  | CH₂₅  | NH   | –CH₂CH₂F(4)  | 59.7   | >100   | 9.3    | 17.4    | 24.4     |
| 16  | CH₂₅  | NH   | –CH₂CH₂Cl(3)| 99.2   | >100   | 10.4   | 24.0    | 37.0     |
| 17  | CH₂₅  | NH   | –CH₂CH₂OCH₂Cl(3)| >100  | >100   | 13.1   | 29.4    | 14.7     |
| 18  | CH₂₅  | NH   | –CH₂CH₂F(3)  | >100   | >100   | 14.9   | 19.4    | 23.3     |
| 19  | H    | NH   | –(CH₂)₂NHCH₂Ph| >100  | >100   | 11.0   | 41.7    | 20.1     |
| 20  | CH₃  | NH   | –(CH₂)₂NHCH₂Ph| >100  | >100   | 11.9   | 29.0    | 16.3     |
| 21  | H    | NH   | –CH₂CH₂NH₂| 3.87   | <100   | 31.2   | 91.6    | >100     |
| 22  | H    | NH   | –Ph   | >150   | >150   | 52.7   | 32.6    | >100     |
| 23  | COCH₃| S    | –CH₂| 12.7   | >150   | 15.0   | 30.9    | >100     |
| 24  | H    | S    | –CH₂Ph| >150  | >150   | 11.1   | 46.7    | >100     |
| CLO | H    | NH   | (2,6-dichloro)Ph| 76.3 | >200   | 42.6   | 8.4     | 7.8      |

*a* All compounds have been tested as HCl salts, with the exception of 21 (oxalate), 22 and 23 (free bases), 24 (HBr), and CLO (HCl).

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

Nitrogen was also favourable, as 23 was twice more potent than 1a.

### 3.2.3. hCA VII

The Ka value of CLO on this isoform was 8.4 μM. All the tested compounds showed activation properties on this isoform, with Ka values between 0.9 and 91.6 μM. The least potent compound was the primary amine 21 (Ka 91.6 μM), whose activity was however improved by adding a benzyl group on the terminal NH₂ moiety (19, Ka 41.7 μM) and a methyl group on the endocyclic N atom (20, Ka 29.0 μM). The removal of chlorine atoms of CLO reduced 16 times the activity (22, R₂ = Ph, Ka 32.6 μM) while the separation of the phenyl and N₂H moieties by means of a CH₂ unit did not substantially modified potency (3, Ka 35.2 μM). On the contrary, the potency increased by elongating the chain from 1 to 3 CH₂ units (3, Ka 35.2 μM; 5, Ka 11.4 μM) by adding a methyl group on the N₂H moiety: with the latter modification the potency of 3 (Ka 18.9 μM) and of 2 (Ka 35.2 μM) were increased 3 (6, Ka 11.0 μM) and 8 times (7, Ka 2.4 μM), respectively. Side-chain branching did not substantially affect activity, since S-4 and R-4 were equipotent with 2. Methylation on the endocyclic N atom was the most effective modification in this set of molecules: as a matter of fact, with this structural change the Ka value of 2 (Ka 35.2 μM) was reduced 39 times, and 11 (Ka 0.9 μM) resulted in the most potent compound on this isoform. The same modification was also effective on the phenylpropyl derivative 5 (Ka 11.4 μM), whose activity was increased 4 times (12, Ka 3.1 μM). The double methylation on the N₁ and N₃ atoms gave potent compounds (10, Ka 6.5 μM and 9, Ka 2.6 μM) even if the Ka values are, respectively, 7 and 3 times lower than that of 11. Aromatic substitution on the benzyl moiety of 11 was detrimental for activity, as compounds 13–18 were 19–33 times less potent than 11. As far as the sulphur analogues 1a, 23 and 24 are concerned, their Ka values were in the range 30.9–46.7 μM, not better that the other tested 2-aminoimidazole derivatives. Attempts to crystallise adducts of 7, 11 and 12 with hCA VII are ongoing.

### 3.2.4. hCA XIII

This is the isoform most sensitive to CLO among those studied (Ka 7.8 μM). As it happened on the hCA I isoform, the removal of both chlorine atoms, to give 22, abolished activity. Several other compounds resulted inactive when tested at concentrations up to 100 μM, i.e. the sulphur derivatives, the polar aminoethyl derivative 21, and all the compounds having both the N₁ and N₃ atoms as secondary amines, with the exception of the lipophilic phenylpropyl derivative 5 (Ka 24.3 μM). The activity of 21 could be restored by adding a benzyl moiety on the primary amino-group (19, Ka 20.1 μM) and a methyl group on the endocyclic N atom (20, Ka 16.3 μM). Also the methylation of the phenethyl analogue 3 on the N₁ atom re-established activity, giving 7 (Ka 6.5 μM) which resulted the most potent compound of the series on this isoform. Methylation on the endocyclic N₁ atom gave compounds 8, 11 and 12 whose potency ranged from 10.9 to 31.0 μM, the most potent being the derivative carrying a phenylpropylamino side chain (12). Methylation on both N₁ and N₃ atoms or aromatic substitution on the benzyl moiety did not improve activity.

As far as selectivity is concerned, the two compounds showing submicromolar Ka values displayed also interesting selectivity...
profiles: 10 was more active on hCA VA with respect to hCA I (33 times), II (>100 times), VII (7 times), and XIII (19 times), while 11 showed a preference for hCA VII over hCA I (19 times), II (>100 times), VA (4 times), and XII (21 times).

4. Conclusions

We have synthesised a series of 2-aminoimidazolines, structurally related to Clonidine, and tested them on five different hCA isoforms (I, II, VA, VII and XIII). As the lead compound, none of the newly synthesised molecules was active on the ubiquitously expressed CA II; on the contrary, the compounds showed activity in the micromolar range on the other tested CA isoforms. Structure–activity relationships were derived, which were different on the various isoforms, suggesting that it could be possible, in this class of compounds, to find molecules, selective for a particular CA isoform. Indeed, from these preliminary modifications it has been possible to find two compounds, 10 and 11, with a promising preference towards, respectively, CA VA and VII. Work is underway to improve both potency and selectivity, in order to find new pharmacological tool to activate specific CA isoforms in pathologies characterised by their loss of functionality.

Disclosure statement

The authors report no conflict of interest.

Funding

This work was supported by grants from the University of Florence [Fondo Ricerca Ateneo RICATEN18].

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