Investigation of piperazines as human carbonic anhydrase I, II, IV and VII activators

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ABSTRACT
Four human (h) carbonic anhydrase isoforms (CA, EC 4.2.1.1), hCA I, II, IV, and VII, were investigated for their activation profile with piperazines belonging to various classes, such as N-aryl-, N-alkyl-, N-acyl-piperazines as well as 2,4-disubstituted derivatives. As the activation mechanism involves participation of the activator in the proton shuttling between the zinc-coordinated water molecule and the external milieu, these derivatives possessing diverse basicity and different scaffolds were appropriate for being investigated as CA activators (CAAs). Most of these derivatives showed CA activating properties against hCA I, II, and VII (cytosolic isoforms) but were devoid of activity against the membrane-associated hCA IV. For hCA I, the $K_a$ were in the range of 32.6–131 μM; for hCA II of 16.2–116 μM, and for hCA VII of 17.1–131 μM. The structure-activity relationship was intricate and not easy to rationalize, but the most effective activators were 1-(2-piperidinyl)piperazine ($K_a$ of 16.2 μM for hCA II), 2-benzyl-piperazine ($K_a$ of 17.1 μM for hCA VII), and 1-(3-benzylpiperazin-1-yl)propan-1-one ($K_a$ of 32.6 μM for hCA I). As CAAs may have interesting pharmacological applications in cognition and for artificial tissue engineering, investigation of new classes of activators may be crucial for this relatively new research field.

1. Introduction
Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzymes involved in the equilibration of carbon dioxide and bicarbonate, with formation of a proton\textsuperscript{1-5}. This process can be described schematically by considering Equations (1 and 2), the first being the interconversion step between CO\textsubscript{2} and bicarbonate, and the second one, which is rate-determining for the entire catalytic cycle, regenerates the nucleophilic, zinc hydroxide species of the enzyme\textsuperscript{6-9}:

\[
\begin{align*}
\text{H}_2\text{O} & \rightleftharpoons \text{EZn}^{2+} - \text{OH}^- + \text{CO}_2 \\
\text{EZn}^{2+} - \text{OH}^- + \text{CO}_2 & \rightleftharpoons \text{EZn}^{2+} - \text{OH}_2 + \text{HCO}_3^- \\
\text{EZn}^{2+} - \text{OH}_2 & \rightleftharpoons \text{EZn}^{2+} - \text{HO}^- + \text{H}^+ 
\end{align*}
\]

For this step to occur efficiently, a proton transfer reaction must take place from the Zn(II)-bound water molecule to the external medium (Equation (2)). Generally, this process is assisted by active site amino acid residues acting as proton shuttles, for example, His residues placed in the middle or at the entrance of the active site cavity\textsuperscript{5}. In many human (h) CA isoforms, such as hCA II, IV, IX, XII, etc., this role of proton shuttle is played by His\textsuperscript{64}, but the possibility that a cluster of His residues (comprising residues 3, 4, 10, 15, and 64, hCA I numbering system) perform the shuttling has also been contemplated\textsuperscript{10}, which may explain the fact that isoforms in which the cluster is present, such as hCA II and IX are among the most effective catalysts known in Nature\textsuperscript{1,10}.

\[
\begin{align*}
\text{EZn}^{2+} - \text{OH}_2 & \rightleftharpoons \text{A} \rightleftharpoons [\text{EZn}^{2+} - \text{OH}_2 - \text{A}] \\
[\text{EZn}^{2+} - \text{OH}^- - \text{AH}^+] & \rightarrow \text{EZn}^{2+} - \text{HO}^- + \text{AH}^+ 
\end{align*}
\]

Enzyme–activator complexes

It has been shown mainly by one of our groups\textsuperscript{10} that endogenous compounds able to participate in proton shuttling processes, in a similar manner to His\textsuperscript{64}, act as CA activators (CAAs), by a mechanism described in Equation (3). The activator (A in Equation (3)) binds within the enzyme active site with formation of enzyme–activator complexes\textsuperscript{7}, in which the activator molecule participates to the rate-determining step of the catalytic cycle, i.e. the proton shuttling from the water molecule coordinated to zinc to the external medium. Kinetic data in the presence of CAAs demonstrated that the activator does not influence $K_{\text{cat}}$ (the affinity for the substrate) and has an effect only on $K_{\text{M}}$ of the enzyme-catalyzed reaction, both for the esterase and CO\textsubscript{2} hydrase activities of various CA isoforms\textsuperscript{10-15}. X-ray crystallography of CA–activator complexes, such as the histamine, noradrenaline, L-/D-His, L-/D-Phe or D-Trp bound to hCA I and hCA II confirmed that the activators bind indeed at the entrance of the active site, not far away from His\textsuperscript{64} (which is present in two conformation, the “in” conformation – which is at around 6 Å from Zn(II), and the conformation pointing towards the exit of the active site, the “out” conformation – at >8 Å from Zn(II) as shown in Figure 1\textsuperscript{11}). As seen from Figure 1, the activator binding site is indeed far away from the metal ion, in the middle part of the active site, extending

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2. Experimental

2.1 Chemistry

Compounds 1–24 were either commercially, highest purity available derivatives from Sigma–Aldrich (Milan, Italy) and were used without further purification, or were prepared as described in the literature.

2.2. Carbonic anhydrase assay

A stopped-flow method has been used for assaying the CA catalyzed CO2 hydration reaction with Phenol red as indicator, working at the absorbance maximum of 557 nm, following the initial rates of the CA catalyzed CO2 hydration reaction for 10–100 s. 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 mM 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 4), which has been fitted by non-linear least squares by using PRISM 3:

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

where [A] is the free concentration of activator.

Working at substrate concentrations considerably lower than K_M ([S] ≪ K_M), and considering that [A] 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 (5):

$$v = v_0 - K_A/\{[A]_T + 0.5\left(\{[A]_T + [E]_T + K_A\right)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of an activator. All CA isozymes used in the experiments were purified recombinant proteins obtained as reported earlier by our group.

3. Results and discussion

3.1. Chemistry

Piperazines 1–24 (Figure 3) were chosen to be investigated as CAAs as they contain the endocyclic NH group able to shuttle protons between the enzyme active site and the reaction medium.

3.2. CA activation

Activation data against four physiologically relevant hCA isoforms, hCA I, II, IV, and VII, were shown in Table 1. Indeed, hCA I, II, and IV...
are involved in a multitude of eye diseases\(^1,27\), and their inhibition is pharmacologically used for the treatment of glaucoma\(^27\), edema\(^{28}\), obesity\(^{29}\), and hypoxic tumors\(^{30}\), whereas more recently, the piperazine derivatives of great interest for memory therapy. Furthermore, Muller et al.\(^{34}\) showed that the activation of these enzymes, are of great pharmacological interest. In fact, recently CAAs were shown to potentiate cognition through the phosphorylation of the extracellular signal-regulated kinase in the cortex and the hippocampus of model animals\(^{34}\), being thus of great interest for memory therapy. Furthermore, Muller’s group showed that CAAs potentiate the initial steps of bone formation in models of artificial mineralization processes\(^{35}\).

The activation of the four CA isozymes mentioned above with the piperazine derivatives 1–24 and two standard activators (compounds A and B) shown in Table 1 allowed us to delineate the following structure-activity relationship (SAR):

\(\text{i. Although unsubstituted piperazine C was inactive as a CAA (}K_A<150\text{ }\mu\text{M against all investigated enzymes), the substituted-piperazines 1–24 showed CA activating properties against hCA I (except compounds 1, 10, 11, and 15, which had }K_A>150\text{ }\mu\text{M with activation constants ranging between 32.6 and 131 }\mu\text{M, being thus moderate – weak activators. Indeed, the leads A and B were much more potent, low micromolar activators of this isoform, with }K_A\text{ of 2.1–7.4 }\mu\text{M (Table 1)}^{18}.\)

\(\text{The best hCA I activators in the series of investigated compounds were 4, 19, 21, and 24 (}K_A\text{ of 32.6–48.1 }\mu\text{M), and they belong to variously substituted piperazines. Small variations on the core structure of these compounds generally led to a diminution of the activity. For example, 19, the best hCA I activator, carries a propionyl group on the piperazine ring and a benzyl moiety in the 3 position. Its deacylated analog, 18, was almost two times a less effective hCA I activator, with a }K_A\text{ of 73.7 }\mu\text{M, compared to 19.}\)

\(\text{The physiologically dominant cytosolic isomorf hCA II was more sensitive to activation with piperazines 1–24 investigated here compared to hCA I (Table I). Thus, only 22 was inactive (}K_A>150\text{ }\mu\text{M), and the range of the activation constants for the remaining derivatives was of 16.2–116 }\mu\text{M. A number of compounds showed }K_A\text{ in the range of 16.2–50.1 }\mu\text{M: for example, 2, 4, 14, 19, 21, 23, and 24. They belong to various chemical classes and incorporated different substituents, which demonstrates that it might be possible to design much more efficient CAAs incorporating this interesting ring. However, the simple lead compound A was a much more potent hCA II activator compared to the other piperazines investigated here, whereas histamine B was a very inefficient hCA II activator with a }K_A\text{ of 125 }\mu\text{M (Table 1). Interestingly, the best hCA II activator was 14, which has two}\)

\(\text{Table 1. CA activation of isozymes hCA I, II, and VII (cytosolic) and IV (membrane-associated) with compounds 1–24, by a stopped-flow CO}_2\text{ hydrolase assay}^{18}.\)

| Compound | Structure | R₁ | R₂ | Kₐ (µM) |
|----------|-----------|----|----|---------|
| A² | I | CH₃CH₂NH₂ | – | 7.41 | 2.30 | 24.9 | 32.5 |
| B² | – | – | – | 2.10 | 125.0 | 25.3 | 37.5 |
| C | I | H | – | >150 | >150 | >150 | >150 |
| 1 | I | Phenyl | – | >150 | 74.9 | >150 | 121.3 |
| 2 | I | 4-F-phenyl | – | 88.2 | 38.7 | >150 | 47.8 |
| 3 | I | 4-Cl-phenyl | – | 104.0 | 110.3 | >150 | 126.1 |
| 4 | I | 4-MeO-phenyl | – | 48.6 | 50.1 | >150 | 80.4 |
| 5 | I | 4-COMe-phenyl | – | 83.7 | 97.9 | >150 | >150 |
| 6 | I | 3-Cl-phenyl | – | 95.2 | 82.7 | >150 | 104.0 |
| 7 | I | 3-MeO-phenyl | – | 119.2 | 80.1 | >150 | >150 |
| 8 | I | 3-CF₃-phenyl | – | 110.2 | 77.6 | >150 | 114.5 |
| 9 | I | 2-Pyrindyl | – | 131.0 | 75.2 | >150 | 95.2 |
| 10 | I | Methyl | – | >150 | 78.4 | >150 | 97.0 |
| 11 | I | Benzyl | – | >150 | 85.3 | >150 | 98.4 |
| 12 | I | Acetyl | – | 127.4 | 109.0 | >150 | 96.4 |
| 13 | I | CH₃CH₂OH | – | 102.1 | 91.6 | >150 | 124.2 |
| 14 | I | 2-Piperidinyl | – | 62.5 | 162.0 | >150 | 49.2 |
| 15 | I | H | Methyl | >150 | 84.0 | >150 | 131 |
| 16 | I | H | Phenyl | 80.3 | 49.7 | >150 | >150 |
| 17 | I | Benzoyl | Phenyl | 75.2 | 84.5 | >150 | 35.2 |
| 18 | I | H | Benzyl | 73.7 | 116 | >150 | 17.1 |
| 19 | II | Propionyl | Benzyl | 32.6 | 36.1 | >150 | 84.0 |
| 20 | II | Benzoyl | Benzyl | 85.2 | 82.4 | >150 | 48.5 |
| 21 | II | H | COOH | 47.9 | 46.8 | >150 | 93.6 |
| 22 | III | H | CO | 115.0 | >150 | >150 | 37.1 |
| 23 | III | H | CH₂CH₂ | 79.4 | 44.6 | >150 | 98.5 |
| 24 | III | Benzyl | CH₂CH₂ | 48.1 | 33.2 | >150 | 127.0 |

Aminoethylpiperazine A and histamine B were used as standard activators.

\(^{a}\)Errors in the range of ±5–10% of the reported values (data not shown) from three different assays.

\(^{b}\)Data for A,B from Vullo et al.\(^{18}\).

\(^{c}\)Prepared as described in Guandalini et al.\(^{20}\).
potential piperidine rings that may participate in the proton shuttling processes.

iii. Surprisingly, the membrane-bound isoform hCA has not activated significantly by any of the piperazines investigated here, although the leads A and B showed medium potency efficacy with $K_A$ of 24.9–25.3 μM.

iv. The brain cytosolic isoform hCA VII was not activated by this relatively new research field.

We report here an activation study of four physiologically and pharmacologically relevant CA isoforms, hCA I, II, IV, and VII with a rather large series of piperazines and their derivatives. The compounds were included in order to investigate whether the fine tuning of the basicity correlated with the various shapes of these molecules may lead to efficient activators, considering the fact that the only piperazine investigated till now as activator (4-(2-piperidinyl)-piperazine) showed such interesting properties. hCA I was activated by most of the investigated derivatives, with activation constants of 32.6–131 μM; hCA II with activation constants of 16.2–116 μM, whereas the membrane-bound isoform hCA IV was not activated by the investigated piperidines. The brain-associated cytosolic isoform hCA VII was activated with $K_A$ in the range of 17.1–131 μM. The structure-activity relationship was intricate and not easy to rationalize for each isoform, but the most effective activators were 1-(2-piperidinyl)-piperazine 14 ($K_A$ of 16.2 μM for hCA II), 2-benzyl-piperazine 18 ($K_A$ of 16.2 μM for hCA VII), and 1-(3-benzylpiperazin-1-yl)propan-1-one 19 ($K_A$ of 32.6 μM for hCA I). As CAAs may have interesting pharmacological applications in cognition and for artificial tissue engineering, investigation of new classes of activators as the ones reported here may be crucial for this relatively new research field.

**Disclosure statement**

The authors do not declare conflict of interest.

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