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Activation of carbonic anhydrase isoforms involved in modulation of emotional memory and cognitive disorders with histamine agonists, antagonists and derivatives

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ABSTRACT
Carbonic anhydrases (CAs, EC 4.2.1.1) activators were shown to be involved in memory enhancement and learning in animal models of cognition. Here we investigated the CA activating effects of a large series of histamine based compounds, including histamine receptors (H1R – H4R) agonists, antagonists and other derivatives of this autacoid. CA activators may be thus useful for improving cognition as well as in diverse therapeutic areas (phobias, obsessive-compulsive disorder, generalised anxiety, post-traumatic stress disorders), for which activation of this enzyme was recently shown to be involved.

1. Introduction

CO₂ is generated in most metabolic processes, being one of the simplest molecules involved in crucial physiologic processes in all life kingdoms. The carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes which catalyse its interconversion to bicarbonate (Equation (1))

\[ \text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{HCO}_3^- \]

The reaction also occurs without a catalyst, but at physiological pH values it is exceedingly slow for meeting metabolic needs, as CO₂ is a poorly water-soluble gas, which can also damage cellular components (e.g. membranes, mitochondria, etc.). On the other side, its conversion to water-soluble ions (bicarbonate and protons) counteracts this effect, and although interfering with the pH balance, is used to control homeostasis and metabolism, making CAs crucial enzymes in many physiological and pathological conditions. In fact, in vertebrates at least 16 CA isoforms belonging to the α-CA genetic family are known, whereas in other organisms all over the phylogenetic tree at least 7 other CA families were described so far, the β-, γ-, δ-, ε-, η-, θ- and r-CAs. In humans 15 CAs are expressed, 12 of which are catalytically active: the cytosolic CA I-III, VII and XIII, the membrane-associated CA IV (located on the luminal surface of cerebral capillaries and associated with the blood-brain barrier, being present also in the cortex, hippocampus and thalamus). The mitochondrial CA VA is expressed in astrocytes and in neurons, whereas CA VB seems to be absent in the SNC. CA VII and VIII are present in high levels throughout the cortex, hippocampus and thalamus, although CA VIII is acatalytic, whereas CA VII shows a good enzymatic activity with CO₂/bicarbonate as substrates. The catalytic CA X is expressed in the myelin sheath, whereas CA XI (also acatalytic) is present in the neural cell body and astrocytes. CA IX and CA XII are transmembrane proteins overexpressed in many neurologic cancers, whereas CA XIII seems not to be present in the brain. CA XIV is expressed in nuclei and nerve tracts associated with pontine, medullary and hippocampal functions being also located on the plasma membrane of some neurons and on axons of mammalian brain.

The human central nervous system (CNS), as well as the choroid plexus, contains a multitude of CA isoforms, although their particular functions are not yet completely understood. We will consider here mainly the CAs present in CNS, as the compounds investigated here for modulating their activity (i.e. the CA activators – CAs) may also have interesting applications in therapy, which started to be considered only recently. The nervous system CA isoforms comprise: the cytosolic CA I (expressed in the motor neurons in the spinal cord), CA II (present in the choroid plexus, oligodendrocytes, myelinated tracts, astrocytes and myelin sheaths); CA III (in the choroid plexus), the membrane-associated CA IV (located on the luminal surface of cerebral capillaries and associated with the blood-brain barrier, being present also in the cortex, hippocampus and thalamus). The mitochondrial CA VA is expressed in astrocytes and in neurons, whereas CA VB seems to be absent in the SNC. CA VII and VIII are present in high levels throughout the cortex, hippocampus and thalamus, although CA VIII is acatalytic, whereas CA VII shows a good enzymatic activity with CO₂/bicarbonate as substrates. The catalytic CA X is expressed in the myelin sheath, whereas CA XI (also acatalytic) is present in the neural cell body and astrocytes. CA IX and CA XII are transmembrane proteins overexpressed in many neurologic cancers, whereas CA XIII seems not to be present in the brain. CA XIV is expressed in nuclei and nerve tracts associated with pontine, medullary and hippocampal functions being also located on the plasma membrane of some neurons and on axons of mammalian brain.

The most investigated CAs are the amino acids, the biogenic amines (histamine, serotonin, catecholamines and their derivatives), and to some extent also the oligopeptides or small
proteins, although these activators were less investigated\textsuperscript{20}. The CAAs were demonstrated to participate in the catalytic cycle of the enzyme, forming enzyme-activator complexes, as described in Equation (2)\textsuperscript{20}.

\[
E Z n^{2+} + O H^{-} + A \rightarrow [ E Z n^{2+} + O H^{-} + A ] \rightarrow [ E Z n^{2+} + O H^{-} + A ] \rightarrow [ E Z n^{2+} + O H^{-} + A ]
\]

(2)

The activator molecule forms a complex with the enzyme, binding in an active site region distinct of that of the classical CA inhibitors\textsuperscript{24,25}, which generally bind to the metal ion\textsuperscript{4,6}. The activator molecule must incorporate proton shuffling moieties, which take part in the rate-determining step of the catalytic cycle, i.e. the transfer of protons from the zinc-coordinated water molecule to the external reaction medium, with formation of the nucleophilic, zinc hydroxide species of the enzyme\textsuperscript{20}. In the wild type enzyme, this proton shuffling is achieved by residue His64 (in many CA isoforms), found within the middle of the active site cleft, and which possess the imidazole moiety able to transfer protons to the pH range of 6–8\textsuperscript{20–24}. His64 was shown to possess two conformations: the in one, orientated towards the bottom of the active site, and the out one, orientated towards the external part of the active site, favouring thus the proton wiring\textsuperscript{20,24}. In such processes, within the enzyme-activator complexes, the proton transfer becomes intramolecular, being more efficient compared to the intermolecular transfer to buffer molecules (which are not bound within the enzyme cavity)\textsuperscript{20}. X-ray crystallography has been performed on several other hCA I/II – activator complexes, among which those with histamine, L- and D-His, L- and D-Phe, D-Trp, L-adrenaline as well as pyridinium derivatives of histamine\textsuperscript{20,23–26}. A schematic representation of the activators bound to CA is shown in Figure 1.

Thus, histamine was the main compound used to obtain new CAAs\textsuperscript{27}, but many of its rather simple derivatives as well as drugs belonging to the histamine receptors (H\textsubscript{1}R, H\textsubscript{2}R, H\textsubscript{3}R and H\textsubscript{4}R) agonists/antagonists, were not yet investigated for their potential activating effects. Here we report the first such study, including in our investigations 28 such derivatives which have been assayed as activators of four pharmacologically significant isoforms, hCA I, II and VII (cytosolic isoforms) and hCA IV (membrane-anchored enzyme).

2. Materials and methods

2.1. Chemistry

Histamine 1 and compounds 2–30 were commercially available, highest purity reagents from Sigma-Aldrich, Milan Italy.

2.2. Carbonic anhydrase activation

A stopped-flow method\textsuperscript{26} has been used for assaying the CA catalysed CO\textsubscript{2} hydration activity with Phenol red as indicator, working at the absorbance maximum of 557 nm, following the initial rates of the CA-catalysed CO\textsubscript{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 uncatalysed 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\textsubscript{A}), defined similarly with the inhibition constant (K\textsubscript{I}), was obtained by considering the classical Michaelis–Menten Equation (3), which has been fitted by nonlinear least squares by using PRISM 3:

\[
v = v_{\text{max}} / \left\{ 1 + K_{\text{M}} / [S] (1 + [A]/K_{A}) \right\}
\]

(3)

where [A]\textsubscript{f} is the free concentration of activator.

Working at substrate concentrations considerably lower than K\textsubscript{M} ([S]<K\textsubscript{M}), and considering that [A]\textsubscript{f} can be represented in the form of the total concentration of the enzyme ([E]\textsubscript{t}) and activator ([A]\textsubscript{t}), the obtained competitive steady-state equation for determining the activation constant is given by Equation (4):

\[
v = v_{0} K_{A} / \left\{ K_{A} + \left( [A]_{t} - 0.5 \left( [A]_{t} + [E]_{t} + K_{A} \right) \right) - \left( [A]_{t} + [E]_{t} + K_{A} \right)^{2} - 4[A]_{t} [E]_{t}^{1/2} \right\}
\]

(4)

where v\textsubscript{0} represents the initial velocity of the enzyme-catalysed reaction in the absence of an activator\textsuperscript{29–32}. Enzyme concentrations in the assay system were in the range of 6.5–12.0 nM.

3. Results and discussion

As mentioned above, histamine 1 (Figure 2) was one of the first CAAs to be investigated in detail\textsuperscript{24}, but except for histidine (L- and D-enantiomers), other histamine derivatives were not yet assayed for their potential CA activating effects.

Considering the relatively large number of histamine receptors (H\textsubscript{1}R-H\textsubscript{4}R) as well as the huge number of agonists/antagonists developed for the management of various disorders, among which allergies, gastritis and gastric ulcers, narcolepsy, acute unilateral vestibulopathy, and atopic dermatitis\textsuperscript{33,34}, there is a large number of compounds incorporating fragments of the histamine chemotype as well as a wealth of structural modifications which mimic this autacoid.

Some of these compounds, possessing structures 2–30 (Figure 1), were included in our study for investigating their possible CA activating effects against four pharmacologically relevant human isoforms, hCA I, II, IV and VII. The compounds were numbered according to their similarity to the lead histamine 1 and are: the H\textsubscript{1}R agonist 2-(2-aminoethyl)thiazole 6; the H\textsubscript{2}R agonists imiprindine 16 and nordimiprind 19; the H\textsubscript{3}R agonists N-ethylhistamine 3, z-methylhistamine 4, methimemep 8, pyroxfan 9, imetit 14, VUF16839 23; the H\textsubscript{4}R antagonists pyrilamine 24, loratadine 29;
the H₂R antagonists metiamide 12, cimetidine 13, ranitidine 17, tiotidine 18, zolantidine 20; the H₂R antagonists ciproxifan 10, clobenpropit 15, ABT239 22, GSK189254A 28, GSK334429B 30; the H₃R antagonists JNJ39758979 25, JNJ7777120 26, A940894 27; the mixed modulators of the histaminergic system Nₘ-methylhistamine 2, 4-methylhistamine 5, 1-methylhistidine 7, burimamide 11, beta-histine 21.

In some of these compounds, such as the methyl-histamines 2–5, the thiazoyl derivative 6 or τ-methyl-His 7, the histamine chemotype is readily observable, whereas the remaining compounds incorporate more drastic changes of the basic structure, but all of them possess moieties which can in principle shuttle protons in the pH range of 6–8 which, as mentioned earlier, lead to CA activation.

The following structure-activity relationship (SAR) can be worked out from the data reported in Table 1 for activation of the four isoforms hCA I, II, IV and VII:

1. Compounds 17, 20, 22, 25–30 did not induce any activation of the tested CA isoforms (Kₐ > 100 μM). Consistently, these derivatives do not possess the histamine chemotype in their structures and/or other moieties that clearly make CA

Figure 2. Histamine 1 and derivatives 2–30 acting as histamine receptors agonists/antagonists (for reviews see refs. 33, 34) investigated here as CAAs.
Table 1. hCA I, II, IV and VII activation with compounds 2–30 (Figure 2) by a stopped-flow CO₂ hydrase assay. Histamine 1 used as standard.

| Compound | hCA I | hCA II | hCA IV | hCA VII | BBB crossing | Central action | Ref |
|----------|-------|--------|--------|---------|--------------|----------------|-----|
| 1        | 2.1   | 125    | 25.3   | 37.5    | –            | +              | 35  |
| 2        | 0.11  | 8.91   | 3.21   | 2.07    | –            | +              |     |
| 3        | 3.1   | 0.43   | 7.6    | 0.23    | +            | +              | 36  |
| 4        | 0.12  | 0.082  | 2.91   | 1.25    | +            | +              | 37  |
| 5        | 0.36  | 5.4    | 5.13   | 0.39    | +            | +              |     |
| 6        | 0.87  | 7.45   | 4.66   | 0.12    | +            | +              |     |
| 7        | 0.052 | 0.57   | 13.9   | 0.19    | +            | +              |     |
| 8        | 3.16  | 5.24   | 4.66   | 0.12    | +            | +              | 38  |
| 9        | 3.15  | 7.66   | 8.01   | 0.52    | +            | +              | 39  |
| 10       | 4.29  | 9.9    | 8.12   | 0.11    | +            | +              | 40  |
| 11       | 0.88  | 8.39   | 9.07   | 0.43    | +            | +              | 41  |
| 12       | 0.98  | 8.75   | 9.62   | 1.01    | +            | +              | 42  |
| 13       | 8.79  | 6.3    | 8.54   | 0.59    | +            | +              | 43  |
| 14       | 4.25  | 8.31   | 8.05   | 1.00    | +            | +              | 44  |
| 15 >100  | 2.1   | 6.59   | 5.31   | 0.04    | +            | +              | 45  |
| 16       | 0.72  | 2.14   | 3.3    | 0.10    | +            | +              | 46  |
| 17 >100  | >100  | >100   | >100   | >100    | +            | +              | 47  |
| 18 >100  | >100  | >100   | >100   | >100    | +            | +              | 48  |
| 19       | 1.36  | 6.91   | 9.08   | 5.21    | +            | +              | 49  |
| 20 >100  | >100  | >100   | >100   | >100    | +            | +              | 50  |
| 21 >100  | >100  | >100   | >100   | >100    | +            | +              | 51  |
| 22 >100  | >100  | >100   | >100   | >100    | +            | +              | 52  |
| 23 >100  | >100  | >100   | >100   | >100    | +            | +              | 53  |
| 24 >100  | >100  | >100   | >100   | >100    | +            | +              | 54  |
| 25 >100  | >100  | >100   | >100   | >100    | +            | +              | 55  |
| 26 >100  | >100  | >100   | >100   | >100    | +            | +              | 56  |
| 27 >100  | >100  | >100   | >100   | >100    | +            | +              | 57  |
| 28 >100  | >100  | >100   | >100   | >100    | +            | +              | 58  |
| 29 >100  | >100  | >100   | >100   | >100    | +            | +              | 59  |
| 30 >100  | >100  | >100   | >100   | >100    | +            | +              | 60  |

*Kₐ (μM)*

iv. The other cytosolic isoform investigated here, hCA VII, was the most effectively activated one by the compounds investigated in this study. Indeed, a wide subset of KAs were detected in a submicromolar range (from 0.10 to 45.5 μM) for some of these derivatives. All of them showed much better activation profile than the reference compound histamine (KA of 37.5 μM) towards hCA VII. Contrariwise to hCA I and II, the most efficient CAAs were not detected among the methylhistamine derivatives 2–5: N-methylhistidine 7, N-methylpi-peridine 8 and the aryl ether 10, showed KAs ranging between 110 and 190 nM. The bis-imidazole 16 stood out as the most effective hCA VII activator of the study with a KA of 25.3 μM. Of note, the imidazole/thiazole swap led to the most effective activation increase with respect to the lead, with a KA of 1.02 μM in case of derivative 6.

Among the remaining derivatives, only the bis-imidazole 16 reported an improved KA over the lead towards this isoform (KA of 0.72 μM). In fact, the presence of S-linked thioureas worsened the activation efficacy by 2- (14, KA of 4.25 μM) or 4-fold (13, KA of 8.79 μM) with respect to the lead histamine. Oddly, the S-linked thiourea 19 showed a 2-fold improved KA compared to histamine, although bearing a dimethylamino group in place of the imidazole ring. As an exception, the N-rich compound 24 interestingly activated hCA I just two times less than histamine, in spite of a completely diverse structure. On the contrary, all histamine derivatives here reported showed a superior hCA II activation efficacy with respect to the lead (KA > 100 μM). Among mostly low micromolar CAAs (KAs in the range 2.1–13.5 μM), derivatives 3, 4 and 7 stood out as the most potent hCA II modulators of the study (KAs in the range 82 nM–0.57 μM). In particular, the α-methylation of histamine, as in 4, induced the largest increase of efficacy, up to a KA of 82 nM, when compared to the ineffective (as CAA) lead molecule.

activation possible. As a unique exception, compound 30 reported a 5.06 μM selective activation of hCA VII. It should be stressed that other derivatives, such as 18, 19, 21, 23 and 24, do not directly include imidazole-like scaffolds in their chemical structure, but showed however to possess significant CA activation profiles in a low micromolar range (KAs between 1.36 and 45.5 μM) and are thus included in the SAR discussion. These compounds possess however protanotable moieties of the secondary amine or guanidine type in their molecule, which like the imidazole may shuttle protons and thus act as CAAs.

The cytosolic and ubiquitous hCA isoforms I and II were quite efficiently activated by most active derivatives (that are 2–16, 18, 19, 21, 23 and 24), in a low micromolar to submicromolar range. Intriguingly, derivatives 15, 21 and 23 did not produce any hCA I activation up to 100 μM, whereas 18 did not activates neither hCA I nor hCA II. The methylation of histamine 1 at position N: (2), α (4), 4 (5) and the imidazole/thiazole swap (6) increased up to one order of magnitude the hCA I activation profile (from 2.1 to 0.11 μM). The presence of an extra proton transfer group (COOH) as in 7 (N-methyl-histidine) further improved 2-fold the KA (52 vs 110 nM) against hCA I compared to compound 2. The N-methylation of histamine (3, KA of 3.1 μM) slightly decreased the activation efficacy of the molecule, situations also encountered for the inclusion of the aliphatic amine into a cycle (8, KA of 3.16 μM) or the amine/ether swap (as in 9 and 10, KAs of 3.15 and 4.29 μM). Substituting the amine group with N-linked thioureas as in 11 and 12 improved 2-fold the KAs towards hCA I with respect to histamine.

Table 2 also include the literature references regarding the compounds ability to cross the BBB, which presumably should also lead to brain CA activating effects, as well as evidences for their action at central level.

4. Conclusions and future perspectives

In the present study, we investigated the CA activating effects of a series of histamine receptors agonists/antagonists (compounds 2–30 in Figure 2) towards four hCA isoforms expressed in human
brain, that are CA I, II, IV and VII. Though all derivatives possess moieties which can in principle shuttle protons in the pH range of 6–8, a consistent subset of them (17, 20, 22, 25–30), not having the histamine chromophore in their structure, did not report any activation effect for the tested CA isoforms (Km >100 μM). hCA I and II were effectively activated by methylhistamine derivatives (2–7), whereas more intriguing SAR were observed for hCA VII with more lipophilic groups (as in 6, 8 or 10) promoting greater and more selective isoform activation. Of note, a subset of selective hCA VII activators was identified, that could serve to drive the identification and optimisation of new brain specific CAAs. We are currently witnessing a second youth period for CAAs, because of innovative pharmacological studies spurring researchers to take into account these lately neglected agents for their potential clinical relevance in the treatment of emotional memory disorders, including the improvement of the clinical efficacy of exposure-based treatments of obsessive-compulsive disorders, phobias, generalised anxiety, and post-traumatic stress disorder. The here gathered data might also provide more insights on the pharmacodynamics of therapeutically used histamine modulators, whose therapeutic action and/or side effects could be related to polypharmacology. Overall, this work might bring new lights on the intricate relationship between CA activation and brain physiology.

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