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RESEARCH ARTICLE

Inhibition of mammalian carbonic anhydrase isoforms I, II and VI with thiamine and thiamine-like molecules

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Abstract

Here we determined the *in vitro* inhibitory effects of 5-(2-hydroxyethyl)-3,4-dimethylthiazolium iodide (**1**), 3-Benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride (**2**) and thiamine (**3**) on human erythrocyte carbonic anhydrase I, II isozymes (hCA I and hCA II) and secreted isoenzyme CA VI. K_I values ranged from 0.38 to 2.27 μM for hCA I, 0.085 to 0.784 μM for hCA II and 0.062 to 0.593 μM for hCA VI, respectively. The compounds displayed relatively strong actions on hCA II, in the same range as the clinically used sulfonamidesethoxzolamide, zonisamide and acetazolamide.

Keywords: Carbonic anhydrase, thiamine, inhibition

Introduction

Carbonic anhydrase (EC 4.2.1.1., CA) has been a well-characterized pH regulatory enzyme in most tissues including erythrocytes[1]. CA catalyzes the reversible hydration of CO_2 to HCO_3^- and H^+. At least 16 CA isozymes have so far been described in mammals, the most active ones as catalysts for carbon dioxide hydration being CA II and CA IX[2–5]. The first one is primarily found in red blood cells but also in many other secretory tissues of the gastrointestinal tract, kidneys, lungs, eye, central nervous system etc[3,6]., whereas the second one is a tumor-associated isoform[3–7].

Carbonic anhydrase inhibitors are a class of pharmaceuticals that suppress the activity of carbonic anhydrase. Their clinical use has been established as antiglaucoma agents, diuretics, antiepileptics in the management of mountain sickness, gastric and duodenal ulcers, neurological disorders, or osteoporosis. The connections between carbonic anhydrase and cancer is known for approximately 15 years, since two tumor-associated membrane carbonic anhydrase isozymes (CA IX and CA XII) have been identified cloned, and sequenced[8–10]. Many reports have been published on the role of CA IX in tumor physiology. In addition to its role in the control of tumor pH, there are evidences that CA IX can also influence other processes in the cell microenvironment that promote cell proliferation, invasion, and metastasis[11–13].

Other CA isoforms are found in a variety of tissues where they participate in several important biological processes such as acid-base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and electrolyte secretion[4,14].

As the inhibitors of CA enzymes are very important leads for medicinal chemistry applications[15–17], in this paper we report the inhibitory effects of compounds 1–3, sulfonamidesethoxzolamide (EZA), zonisamide (ZNA) and acetazolamide (AZA) against human CA I, II and VI isoforms. The reason for selection of these compounds is that 2-hydroxyethyl-4-methylthiazolium derivatives were shown to be bioactive molecules[18].

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Materials and methods

5-(2-hydroxyethyl)-3,4-dimethylthiazolium iodide, 3-Benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride, thiamine (2-[3-[(4-Amino-2-methyl-pyrimidin-5-yl) methyl]-4-methyl-thiazol-5-yl] ethanol), Sepharose 4B, protein assay reagents, 4-nitrophenylacetate were obtained from Sigma-Aldrich Co. (Germany). All other chemicals were analytical grade and obtained from Merck.

Purification of carbonic anhydrase isozymes by affinity chromatography

Purification of hCA I and hCAII were previously described[14–16]. Fresh non-citrated human whole blood obtained from the Blood Center of the Research Hospital at Atatürk University. The blood samples were centrifuged at 5000 rpm for 15 min and precipitant were removed. The serum was isolated. The pH was adjusted to 8.7 with solid Tris. Sepharose-4B-aniline-sulfanilamide affinity column equilibrated with 25 mM Tris-HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris-HCl/22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase (hCA VI) isozyme was eluted with 0.25 M H_2NSO_3H/25 mM Na_2HPO_4 (pH = 6.7[19,20]).

CA inhibition assay

The effect of increasing concentrations of compounds 1–3, AZA, ZNA, and EZA on HCA isozyme activity was determined colorimetrically using CO_2-hydration method of Khalifah[21]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Tris-HCl (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), at 25°C, following the CA-catalyzed CO_2-hydration reaction for a period of 10–80 s (the uncatalyzed reaction needs around 60–100 s in the assay conditions, whereas the catalyzed ones are of around 6–10 s). The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of kinetic parameters. For each inhibitor, tested in the concentration range between 0.01 and 100 μM, 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 inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) dimethyl sulfoxide (which is not inhibitory at these concentrations), and dilutions up to 0.001 μM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay in order to allow for the formation of the E-I complex. The curve-fitting algorithm allowed us to obtain the IC_{50} values, working at the lowest concentration of substrate of 1.7 mM, from which K_I values were calculated by using the Cheng–Prusoff equation[22]. The catalytic activity (in the absence of inhibitors) of these enzymes was calculated from Lineweaver–Burk plots, as reported earlier, and represent the mean from at least three different determinations. Enzyme concentrations in the assay system were 9.2 nM for hCA I, 7.3 nM for hCA II, and 7.5 nM for hCA VI. Enzymes used here were purified from human blood as described earlier.

Protein determination

Protein quantity was determined spectrophotometrically at 595 nm during the purification steps according to the Bradford method, using bovine serum albumin as a standard[23].

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzymes. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli procedure[24].

Results and discussion

Purification of the two CA isozymes used here was performed with a simple one-step method by a Sepharose-4B-aniline-sulfanilamide affinity column chromatoghrapy[16–19]. hCA I was purified approx. 102-fold with a specific activity of 874.1 $EUmg^{-1}$ and an overall yield of 62.4%, hCA II was purified approx. 685-fold with a specific activity of 5863.3 $EUmg^{-1}$ and an overall yield of 58.7% and 63-fold with a specific activity of 248 $EUmg^{-1}$ and an overall yield of 29.2%. Inhibitory effects of compounds **1**–**3** and EZA, ZNA and AZA on enzyme activities were tested under *in vitro* conditions; K_I values were calculated from the Cheng–Prus off equation and are given in Table[122].

We report here the first study on the inhibitory effects of compounds 1–3 of type on the hydratase activity of hCA I, II and VI. EZA, ZNA and AZA, CAI were used as negative controls in our experiments, and for comparison purposes. The data in Table 1 show the following regarding inhibition of hCA I, II and VI with compounds 1–3 and EZA, ZNA and AZA:

1. Against the slow cytosolic isozyme hCA I, compound **1** behaves as a moderate inhibitor, with a K_I value of 2.27 μM. Compound **2** and thiamine **3** showed better

Table 1. K_I values obtained from regression analysis graphs for hCA I, hCA II and hCA VI in the presence of different inhibitors concentrations (μM).

Compound	hCA I	hCA II	hCA VI
1	2.27	0.784	0.593
2	1.15	0.105	0.128
3	0.38	0.085	0.062
EZA	0.03	0.011	0.050
ZNA	0.06	0.041	0.095
AZA	0.27	0.015	0.015

AZA, acetazolamide; EZA, sulfonamides ethoxzolamide; hCA, human erythrocyte carbonic anhydrase; ZNA, zonisamide.

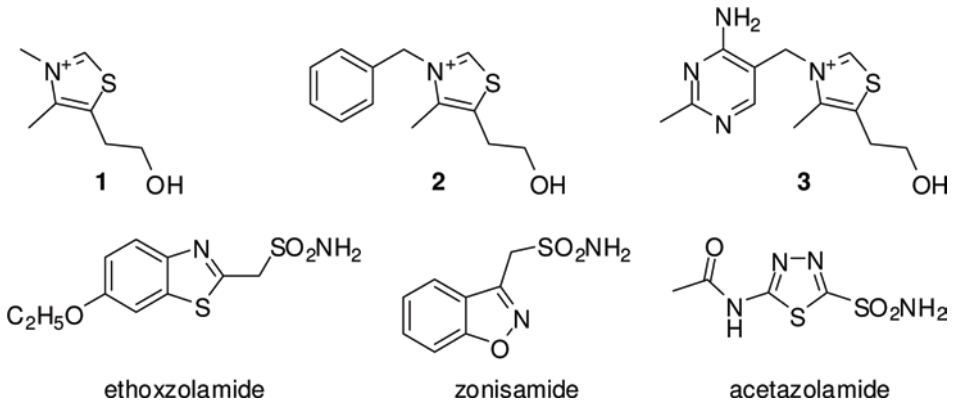

Figure 1. Structures of tested compounds and some clinical used sulfonamides. (See colour version of this figure online at www.informahealthcare.com/enz)

inhibitory activity when compared to the previously mentioned compound **1**, with K_I values of 1.15 and 0.38 μM (Table 1). Thus, the 4-amino-2-methyl-pyrimidin-5-yl moiety improves hCA I inhibitory activity. AZA is also a strong hCA I inhibitor with this assay and K_I of 0.27 μM, whereas thiamine, ZNA and EZA were more powerful inhibitors than AZA (Table 1).

2. A better inhibitory activity has been observed with compounds **2** and **3** for the inhibition of the rapid cytosolic isozyme hCA II (Table 1). Compound **1** showed moderate hCA II inhibitory activity with a K_I value of 0.784 μM (Table 1), whereas thiamine was quite an effective hCA II inhibitor, with a K_I value of 0.085 μM, (Table 1). Similar to hCA I, 4-amino-2-methyl-pyrimidin-5-yl moiety strongly influences hCA II inhibitory activity as well.
3. Compounds 1 and 2 were relatively weak inhibitors for hCA VI, whereas, 3, EZA and ZNA were moderate inhibitors of the secreted isozyme, with K_I of 0.050–0.095 μM. A better inhibitory activity has been observed with AZA for the inhibition of the secreted isozyme hCA VI (Table 1).

There are many CA inhibitors in the literature[25–34], but it is still critically important to carry out further CA inhibition studies to find novel applications for the inhibitors of these widespread enzymes.

Conclusions

Compounds **1**–**3** used in this study affect the activity of CA isozymes due to the presence of the different functional groups present in their scaffold. 4-amino-2-methyl-pyrimidin-5-yl moiety improves the inhibition capacity of the tested compounds. Our findings here contribute to discovery of novel possible CAIs of interest, in addition to the well-known sulfonamides/sulfamates/sulfamides. In addition, usage of natural molecules as inhibitors of CA or other enzymes is of particular interest for medicinal chemists. Therefore, this study may be very useful for design and biological evaluation of novel agents effective as enzyme inhibitors.

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Declaration of interest

The authors report no conflicts of interest.

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