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To cite this article: Emir Alper Türkoğlu, Murat Şentürk, Claudiu T. Supuran & Deniz Ekinci (2017) Carbonic anhydrase inhibitory properties of some uracil derivatives, Journal of Enzyme Inhibition and Medicinal Chemistry, 32:1, 74-77, DOI: [10.1080/14756366.2016.1235043](https://doi.org/10.1080/14756366.2016.1235043)

To link to this article: <https://doi.org/10.1080/14756366.2016.1235043>



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Published online: 18 Jan 2017.



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RESEARCH ARTICLE



Carbonic anhydrase inhibitory properties of some uracil derivatives

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ABSTRACT

Inhibitors of carbonic anhydrase (CA) have been carried out in many therapeutic applications, especially antiglaucoma activity. In this study, we investigated some uracil derivatives (**4–12**) to inhibit human CA I (hCA I) and II (hCA II) isoenzymes. The K_i values of the compounds **4–12** are in the range of 0.085–428 μ M for hCA I and of 0.1715–645 μ M against hCA II, respectively. It is concluded from the kinetic investigations, all compounds used in the study act as competitive inhibitors with substrate, 4-NPA. Uracil derivatives are emerging agents for the inhibition of carbonic anhydrase which could be used in biomedicine.

ARTICLE HISTORY

Received 15 August 2016
Revised 7 September 2016
Accepted 7 September 2016

KEYWORDS

Carbonic anhydrase;
hydroxyl; inhibitor; uracil
derivatives

Introduction

Uracil, one of the pyrimidine bases, is commonly present in ribonucleic acid (RNA)¹. It is chemically weak acid and exposed to two tautomeric forms at pH 7.0. This amide-imidic acid tautomeric shifts from lactam which is the amide tautomer to the imidic acid tautomer is referred to as the lactim structure (Figure 1)². Uracils are used in the area of drug discovery due to their bioactivities, drug similarity in the perspectives of synthetic accessibility and ability. And it is reported that uracils show anti-viral and anti-tumour characteristics in addition to bactericidal, herbicidal and insecticidal features³.

5-Fluorouracil (5-FU) (**1**) is an anti-tumour agent used for the treatment of cancerous tumours in colon or/and breast⁴. Though 5-FU as a single agent has gained medical achievement, the molecule has been chemically modified with different processes to create new derivatives. Therefore, modified syntheses can improve their therapeutic indexes due to their well-known side effects². 6-Amino-5-chlorouracil (**2**) and 6-amino-5-bromouracil (**3**) were the first thymidine phosphorylase inhibitors to be generated. However, their relatively less favourable IC_{50} values did not allow them to be developed into drug candidates².

Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes involving in numerous vital biochemical/physiological processes^{5,6} based on reversible hydration/dehydration process of CO_2/HCO_3^- . The enzyme is found in all living beings of the three domains of life⁸ with six evolutionary gene families⁹. 15 CA isoenzymes encoded by α -CA gene family described in humans¹⁰. Some of these human CA isoenzymes such as CA I, II, III, VII and XIII are cytosolic, some forms, CA IV, IX, XII and XIV, are membrane bound, two ones as CA VA and VB are mitochondrial and CA VI is found in saliva. The last three forms of human CAs (CA VIII, X and XI) are determined as noncatalytic⁵.

The testing of CA inhibitors is carried out for the treatment of clinically important cases¹¹. Many chemical ligands have been used to inhibit CAs such as anions¹², bischalcones¹³, coumarins¹⁴,

benzenesulfonamides¹⁵ and phenoles¹⁶. And uracil derivatives have also been carried out to inhibit the catalytic activity of CAs¹⁷.

Experimental

Orotic acid (**9**), isoorotic acid (**10**), 6-Amino 1,3-Dimethyluracil (**11**), 5,6-Diamino 1,3-Dimethyluracil (**12**), and other chemicals were obtained commercially from Sigma-Aldrich.

Purification of human carbonic anhydrase isozymes by affinity chromatography

Erythrocytes were purified from fresh human blood obtained from the Blood Centre of the Research Hospital at Atatürk University. The blood samples were centrifuged at 1500 rpm for 15 min and the plasma and buffy coat were removed. The red cells were isolated and washed twice with 0.9% NaCl, and hemolysed with 1.5 volumes of ice-cold water. The ghost and intact cells were removed by centrifugation at 20 000 rpm for 30 min at 4 °C. The pH of the hemolysate was adjusted to 8.7 with solid Tris¹⁸. Firstly, benzoyl chloride and stirred for four hours at room temperature in CH_2Cl_2 cellulose. After the spacer arm cellulose added as a benzyl group and finally diazotized sulfanilamide clamped to the para position of benzyl group as ligand. The hemolysate was applied to the prepared cellulose-benzyl-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 I and hCA II) isozymes were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa /0.5 M $NaClO_4$ (pH 5.6), respectively. All procedures were performed at 4 °C¹⁶.

CA inhibition

Enzyme activity was determined spectrophotometrically by following the change in absorbance at 348 nm of 4-nitrophenylacetate

to 4-nitrophenylate over a period of 3 min at 25 °C^{19–21}. The enzymatic reaction contained 1.4 mL 0.05 M Tris-SO₄ buffer (pH 7.4), 1 mL 3 mM 4-nitrophenylacetate, 0.5 mL H₂O and 0.1 mL enzyme solution, in a total volume of 3.0 mL²². Inhibitory effects of compounds **4–12** were compared with phenolic compounds **13–15**. Different inhibitor concentrations were used and all compounds were tested in triplicate at each concentration used. Control cuvette activity was acknowledged as 100% in the absence of inhibitor^{22–24}. An Activity% – [Inhibitor] graph was drawn for each inhibitor^{22–24}. The curve-fitting algorithm allowed to obtain the IC₅₀ values, working at the lowest concentration of substrate of 0.15 mM, from which K_i values were calculated^{25,26}. The catalytic activity of these enzymes was calculated from Lineweaver–Burk plots, as reported earlier, and represent the mean from at least three different determinations. The CA I and II isoenzymes used here were purified from human blood as described earlier^{19–24}.

Results and discussion

Some research groups have studied with phenol and several phenolic compound types on 12 mammalian CA isoenzymes to show inhibitory properties of phenols²⁷. We have extended previous investigations with some phenolic compounds which are globally used in food and medical industries. These kinds of compounds in different phenolic structures with antioxidant characteristics are essential in quenching reactive oxygen species^{28–29}. These type of compounds show several medical activities such as anticancer, anticarcinogenic, antimutagenic, antibacterial, antiviral or anti-inflammatory⁵. Phenol, phenolic compounds and hydroxybenzoic acid derivatives are widely put into effect as prodrugs and/or drugs. Salicylic acid possesses the ability to reduce fevers and ease aches and pains. To date these medicinal features have been known and used as anti-inflammatory drug^{27–34}.

In the present study we have purified CA I and II (hCA I and hCA II) from human erythrocytes and examined the *in vitro* inhibition effects of above mentioned uracil derivatives **4–12** on these

enzymes (Table 1), using the esterase activity of hCA I and II, with 4-NPA as substrate. Uracil derivatives **4** and **5** were synthesized from commercially available uracil. For this purpose uracil was converted to the brom uracil by using 1.1 Eq bromine in DMF at 120 °C¹⁷. Condensation of brom uracil **2** with methanesulfonyl chloride, p-toluenesulfonyl chloride and acetic anhydride to afford uracil derivatives **3** which has antitumour activity, **4**, and **5** in comparable yield^{35,36}, respectively. The other tested compounds **6–15** were purchased from Sigma-Aldrich.

(i) Against the slow cytosolic isozyme hCA I, compounds **4–8**, and **15** behave as weak inhibitors, with K_i values in the range of 316.2–464 μM, similarly to the structurally related compounds **13** and **14** (K_is of 795 and 4003 μM). It is interesting that compounds **9–12** were much better hCA I inhibitors as compared to the corresponding compounds **7** and **9** from which it was derived (Figure 2). Kinetic investigations (Lineweaver–Burk plots, data not shown) indicate that similarly to sulfonamides and inorganic anions^{12–16}, all the investigated natural compounds act as competitive inhibitors with 4-NPA as substrate.

(ii) A better inhibitory activity has been observed with compounds **9–12** investigated here for the inhibition of the rapid cytosolic isozyme hCA II (Table 1). Structure-activity relationship (SAR) is thus quite sharp for this small series of hydroxylic compounds: compounds **9–12** are effective leads, with two mono or di hydroxy moieties is already a submicromolar hCA II inhibitor. This effect is maintained when different groups are present in the *meta* position to the phenol OH moiety, such as in resorcinol. The best hCA II

Table 1. hCA I and II inhibition data some compounds, by an esterase assay with 4-nitrophenylacetate as substrate.^{12b}

Compound	K _i (μM)*	
	hCA I	hCA II
4 ^a	428	645
5 ^a	10.83	28.88
6 ^a	57.76	NE
7 ^a	49.51	NE
8 ^a	316.2	166.4
Orotic acid (9)	0.7325	1.682
Isorotic acid (10)	0.4585	1.432
6-Amino 1,3-Dimethyluracil (11)	0.085	0.1715
5,6-Diamino 1,3-Dimethyluracil (12)	0.1035	0.2360
13 ^b	795	7.7
14 ^b	4003	9.9
15 ^b	10.2	5.5

^aFrom Ref. 17. ^bFrom Ref. 27. NE: No-effect.

*Mean from at least three determinations. Errors in the range of 1–3% of the reported value (data not shown).

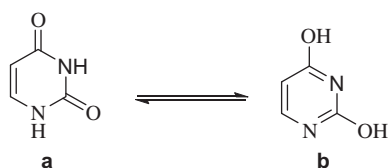


Figure 1. Tautomeric forms of Uracil: lactam-lactim.

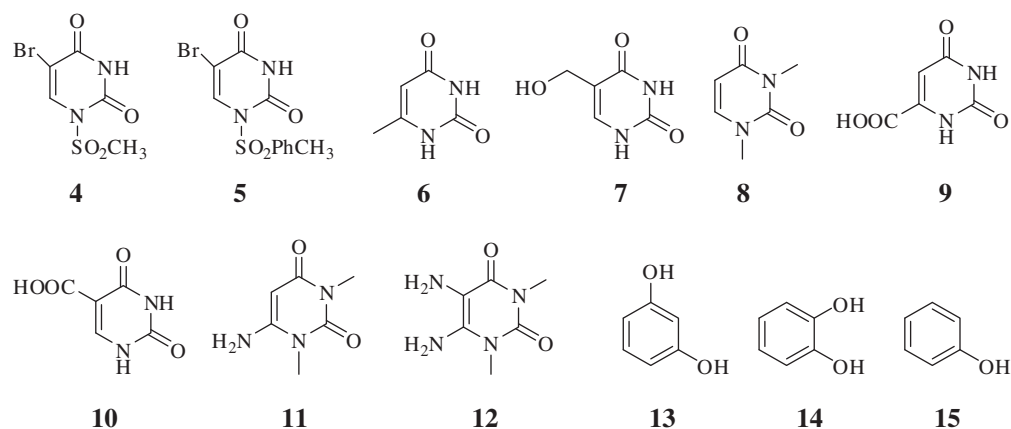


Figure 2. Structure of tested compounds.

inhibitor in this series of derivatives were compound **11** with a K_i of 0.1715 μM .

In a recent study it was reported that catechol and resorcinol²⁷ act as a CAI inhibitor, and could represent the starting point for a new class of inhibitors that may have advantages for patients with sulfonamide allergies. The sulfonamide zinc-binding group is thus superior to the thiol one (from the thioxolone hydrolysis product) for generating CA inhibitors with a varied and sometimes isozyme-selective inhibition profile against the mammalian enzymes. However, it is still important to explore further classes of potent CAIs in order to detect compounds with different inhibition profiles.

Compounds **3–12** used in this study affect the activity of CA isozymes due to the presence of the different functional groups (CH_3 , OH, Br, COOH , NH_2 , mesityl, and tosyl) present in their scaffold. Therefore, our findings indicate another class of possible CAIs of interest, in addition to the well-known inhibitors, the phenols/biphenyl diphenols bearing bulky *ortho* moieties in their molecules. Some hydroxylic compounds investigated here exhibited effective hCA I and II inhibitory activity, in the low-micromolar range, by the esterase method which usually gives K_i -s an order of magnitude higher as compared to the CO_2 hydrase assay. These findings point out that substituted hydroxylic compounds may be used as leads for generating potent CAIs eventually targeting other isoforms.

Disclosure statement

The authors report no declarations of interest

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