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NO-releasing esters show carbonic anhydrase inhibitory action against human isoforms I and II

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ABSTRACT

Carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs) are a class of pharmaceuticals used as antiglaucoma agents, diuretics, antiepileptics, in the management of mountain sickness, gastric and duodenal ulcers, neurological disorders, or osteoporosis. We report here the inhibitory capacities of some organic nitrates against two human (hCA) isozymes, hCA I and hCA II. The IC_{50} values of compounds **1–12** against hCA I ranged between 7.13 mM and 124 mM, and against hCA II between 65.1 μM and 0.79 mM. Nitrate esters are thus interesting hCA I and II inhibitors, and might be used as leads for generating enzyme inhibitors eventually targeting other isoforms which have not been assayed yet for their interactions with such agents.

1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc-containing metaloenzymes which participate in the maintenance of pH homeostasis in the human body, catalyzing the reversible hydration of carbon dioxide in a two-step reaction, to yield bicarbonate and protons.[1,2] Sixteen CA isozymes have been described so far, that differ in their subcellular localization, catalytic activity, and susceptibility to different classes of inhibitors.[1] These isozymes play important roles in different tissues.[1–4] Some of the isozymes are cytosolic (CA I, CA II, CA III, CA VII, and CA XIII), others are membrane bound (CA IV, CA IX, CA XII, and CA XIV), two are mitochondrial (CA VA and CA VB), and one is secreted in the saliva and milk (CA VI).[3–6] CAs are expressed in a variety of tissues and have crucial roles in several important biological processes, including acid-base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis, and body fluid formation.[1,2,4] CA I and CA II are the major isozymes that are present at high concentrations in the cytosol in erythrocytes, and CA II has the highest turnover rate of all the CAs, together with CA IX.[3] Many of the CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited to treat several disorders including edema, glaucoma, obesity, cancer, epilepsy, and osteoporosis.[1–5]

The inhibitory effects of different sulfonamide derivatives, anions, metal ions, phenols, and various drugs have been investigated up to now against many mammalian, bacterial and fungal CAs and other enzymes.[5,7–10] As isoform CA II is the physiologically most relevant one, its inhibitors are used for several applications, in particular for the treatment of glaucoma, epilepsy, and as diuretics. Other compounds, targeting isoforms IX and XII, have applications as antitumor agents/diagnostic tools.[1–3] Therefore, discovery of novel CA inhibitors (CAIs) targeting various isoenzymes has gained attention nowadays.[1–5]

Nitroaromatics and the structurally related *N*-oxides constitute an important class of therapeutic agents against a variety of protozoan and bacterial infections of humans and animals,[11] as well as prodrugs (bioreductive agents), useful for the treatment or imaging of hypoxic tumors.[11–15] In our previous study, we demonstrated the inhibitory ability of organic nitrates on glutathione reductase activity and proposed that the molecules could be used as antimalaria agents.[16] For this reason, it seemed reasonable to investigate the effects of such molecules on the CA activity. In the current study, aiming toward the discovery of novel CA inhibitors, we report the synthesis of 12 organic nitrate derivatives and the evaluation of their ability to inhibit hCA I and II isoforms.

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2. Results and discussion

2.1. Chemistry

Organic nitrates **1–12**, possessing mono- or polycyclic scaffolds, have been investigated as CAIs in this study (Fig. 1). It is known that inorganic nitrite/nitrate show inhibitory activity against all mammalian, bacterial and fungal CAs investigated so far.[17–22] Very recently, one of our groups reported sulfonamide CAIs also incorporating NO-donating moieties, as nitrate esters, which behaved as very potent CAIs and also show excellent in vivo activity as antiglaucoma agents, in experimental animals.[22] It has been hypothesized that the strong enzyme inhibitory activity of such agents (against isoforms CA I, II, and IV) are due to the dual inhibition of CAs by both the sulfonamide moiety (coordinating to the Zn(II) ion from the enzyme active site) as well as the released NO, which may bind itself to the enzyme or nitrosylate it, and thus contribute to the inhibition, by a mechanism of action not well understood at this moment. In order to get more insights to the level of the nitrate moiety contribution to the CA inhibition (due to the release of NO), we decided to investigate here compounds which do not possess the sulfamoyl zinc-binding group, but only the nitrate ester one. Thus, the mono- or bis-nitrates **1–12**, possessing various mono or polycyclic scaffolds have been included in this study.

2.2. CA purification, assay, and inhibition

The purification of the two CA isozymes was performed with a simple one step method by a Sepharose-4B-aniline-sulfanilamide affinity column. hCA I was purified, 103.43-fold with a specific activity of 895.74 EU mg^{-1} and overall yield of 61% and the hCA II enzyme was purifed, 756.35-fold with a specific activity of 6550 EU mg^{-1} and overall yield of 57% (Table 1).[23–25] Inhibitory effects of organic nitrates on enzyme activities were tested under in vitro conditions; I_{50} values were calculated from plots of %Activity − [Inhibitor] and are given in Table 2. K_i values were calculated from Lineweaver–Burk plots and are given in Table 3.[26,27]

It is known that CA has been purified many times from different organisms and the effects of various chemicals, pesticides, and drugs have been investigated on its activity.[18,19,28–32] In this study, CA I and II were purified from human erythrocytes, and the activity of the effluents were determined by the hydratase method, with CO_2 as substrate and further kinetic studies were performed using the esterase activity method, with 4-nitrophenyl acetate (NPA) as substrate. We report here a study on the inhibitory effects of organic nitrates on the CA esterase activity of isoforms hCA I and II. Data of Tables 2 and 3 show the following, regarding inhibition of hCA I and II with nitrates **1–12**:

(i) Against the slow cytosolic isozyme hCA I, compounds **1–3** behave as weak, millimolar inhibitors, with I_{50}s in the range of 113–129 mM. A second group of derivatives, including **4–12** showed better inhibitory activity as compared to the previously mentioned organic nitrates, with I_{50}s in the range of

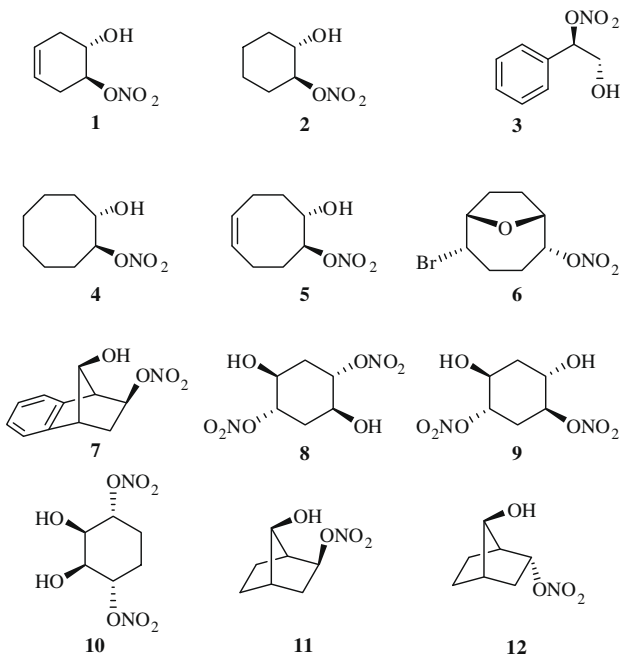


Figure 1. Structures of the investigated molecules **1–12**.

Table 2
I_{50} values for the in vitro inhibition of hCA I and hCA II with new synthetized organic nitrates **1–12**, by the esterase method with 4-NPA as substrate

Inhibitor	I_{50} values for hCA I (mM)	I_{50} values for hCA II (μM)
trans-(1*S*(*R*),6*S*(*R*))-6-Hydroxycyclohex-3-enyl nitrate (**1**)	124	790
trans-(1*S*(*R*),2*S*(*R*))-2-Hydroxycyclohexyl nitrate (**2**)	113	560
trans-(*R*(*S*))-2-Hydroxy-1-phenylethyl nitrate (**3**)	129	632
trans-(1*S*(*R*),2*S*(*R*))-2-Hydroxycyclooctyl nitrate (**4**)	23	181
trans-(1*S*(*R*),8*S*(*R*),*Z*)-8-Hydroxycyclooct-4-enyl nitrate (**5**)	21	243
(1*S*(*R*),2*S*(*R*),5*R*(*S*),6*R*(*S*))-5-Bromo-9-oxabicyclo[4.2.1] nonan-2-yl nitrate (**6**)	12.1	110
9(*R*(*S*))-Hydroxy-1,2,3,4-tetrahydro-1,4-methano-naphthalen-2(*R*(*S*)-yl nitrate (**7**)	10.23	98.1
(1*R*(*S*),2*R*(*S*),4*R*(*S*),5*R*(*S*))-2,5-Dihydroxycyclo-hexane-1,4-diyl dinitrate (**8**)	11.23	113
(1*S*(*R*),3*S*(*R*),4*S*(*R*),6*S*(*R*))-4,6-Dihydroxycyclo-hexane-1,3-diyl dinitrate (**9**)	10.7	86.3
(1*R*(*S*),2*R*(*S*),3*S*(*R*),4*S*(*R*))-2,3-Dihydroxycyclo-hexane-1,4-diyl dinitrate (**10**)	13.71	129
(2*S*(*R*),7*R*(*S*))-7-Hydroxybicyclo[2.2.1] heptan-2-yl nitrate (**11**)	9.1	73.1
(2*R*(*S*),7*R*(*S*))-7-Hydroxybicyclo[2.2.1] heptan-2-yl nitrate (**12**)	7.13	65.1

Table 1
Summary of purification procedure for hCA I and hCA II

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity	Specific activity (EU mg^{-1})	Yield (%)	Purification factor
Haemolysate	136	40	15.7	628	5440	8.66	100	1
hCA I	421	8	0.47	3.76	3368	895.74	61	103.43
hCA II	786	4	0.12	0.48	3144	6550	57	756.35

Table 3
K_i values obtained from regression analysis graphs for CA I and CA II in the presence of different inhibitors concentrations

Inhibitor	K_i values for hCA I (mM)	Inhibition type	K_i values for hCA II (μM)	Inhibition type
1	274	Noncompetitive	1371	Noncompetitive
2	221	Noncompetitive	986	Noncompetitive
3	288	Uncompetitive	1143	Noncompetitive
4	45.2	Noncompetitive	267.2	Noncompetitive
5	41.8	Noncompetitive	541.4	Noncompetitive
6	22.5	Noncompetitive	223.2	Uncompetitive
7	18.7	Competitive	195.6	Competitive
8	19.9	Uncompetitive	239.1	Competitive
9	19.3	Uncompetitive	172.2	Competitive
10	23.8	Uncompetitive	266.8	Uncompetitive
11	17.2	Competitive	132.7	Competitive
12	13.6	Competitive	121.4	Competitive

7.13–23 mM (corresponding K_is of 13.6–45.2 mM, Table 3). These compounds incorporate moieties leading to an acidification of the OH groups, except for **6**, form the organic nitrates scaffold (such as the bromine atom in the 5-position in compound **6** or the two nitro moieties present in **8**, **9**, and **10**), as well as the bulkier scaffolds present in **6**, **7**, and especially **11**, **12**. Molecules **11** and **12** are stereoisomers and have similar CA inhibitory activity, which indicates that acidification is the main factor for these molecules. Molecules **6–12** were among the best inhibitors in this series of organic nitrates. Data of Table 3 also show that similarly to nitro-containing sulfonamides,[33] some of the investigated organic nitrates act as competitive inhibitors with 4-NPA as substrate, that is, they bind in the same regions of the active site cavity as the substrate. However the binding site of 4-NPA itself is unknown, but it is presumed to be in the same region as that of CO_2, the physiological substrate of this enzyme.[5] Data of Table 3 also show that similarly to salicylic acid derivatives investigated earlier by us,[21] the investigated nitrates **1**, **2**, **4**, **5**, and **6** act as noncompetitive inhibitors with 4-NPA as substrate, that is, they bind in different regions of the active site cavity as compared to the substrate. Compounds **3**, **8**, **9**, and **10** on the other hand showed a behavior of uncompetitive inhibition with 4-NPA as substrate (Table 3).

(ii) A rather similar activity of these compounds has been observed also for the inhibition of the rapid cytosolic isozyme, hCA II (Tables 2 and 3). Thus, a first group of derivatives, **1**, **2**, and **3** showed modest hCA II inhibitory activity with I_{50}s in the range of 560–790 μM (corresponding to K_is of 986–1371 μM, Table 3), whereas the remaining nine derivatives, that is, the same compounds acting as efficient hCA I inhibitors, showed I_{50}s in the range of 65,1–243 μM (the corresponding K_is were in the range of 121.4–541.4 μM, Table 3). Structure–activity relationship was thus quite similar in these small groups of nitrate derivatives, for both the inhibition of hCA I and II, although differences of affinity between the two isozymes are evident. Again most of these compounds acted as noncompetitive inhibitors with 4-NPA as substrate, except for **7**, **8**, **9**, **11**, and **12** which were competitive inhibitors and **10** which acts as uncompetitive inhibitor with each isoenzymes (Table 3).

We hypothesize that CAs (which as we show above, possess esterase activity against several substrates), hydrolyses these organic nitrates leading to nitrate anions and cyclic alcohols/diols, as illustrated in Figure 2. Previously, studies showed that K_i values of nitrate anions were determined to be of 7.0 mM for hCA I and 35 mM for hCA II.[17] As these values are quite different of those reported here, it can be thought that this reaction does not occur extensively or it occurs only slightly (for some compounds and some isoforms, e.g., **12** with hCA I). Another study supporting this idea came from Steele et al.[12] who showed that organic nitrates present in NO-donating sulfonamide conjugates, and acting as potent CAIs, have the nitrate ester non-hydrolyzed in the CA-sulfonamide adduct, by means of X-ray crystallography.[22]

Thus, even if recent studies showed that some nitro-containing sulfonamides have selective inhibitory effects on particularly tumor-related CA isoenzymes[11] it is critically important to explore further classes of potent CAIs in order to detect compounds with a different inhibition profile as compared to the sulfonamides and their bioisosteres and to find novel applications for the inhibitors of these widespread enzymes, in particular the tumor-related isoforms.

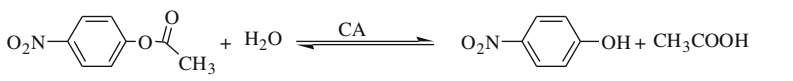
3. Conclusions

Organic nitrates **1–12** affect the activity of CA isozymes due to the presence of the different functional groups (ONO_2, Br, and OH) in their structures. Our findings indicate thus another class of possible CAIs of interest, in addition to the well-known sulfonamides/sulfamates/sulfamides, although their mechanism of CA inhibition remains rather elusive at this moment. Indeed, some organic nitrates investigated here showed effective CA I and II inhibitory activity, in the micromolar range, by the esterase assay method. These findings point out that substituted organic nitrates may be used as leads for generating more potent CAIs eventually targeting other isoforms which have not been assayed yet for their interactions with such agents.

4. Experimental

4.1. Chemicals

Sepharose-4B, protein assay reagents, 4-nitrophenylacetate, and chemicals for electrophoresis were purchased from Sigma–Aldrich



4-Nitrophenol acetate 4-Nitrophenol (*1*)

(*2*)

Figure 2. The esterase reaction of CA in in vitro conditions (*1*) and estimated hydrolysis reaction of the nitrate esters (*2*). Reaction (*2*) does not seem to occur in the conditions of experiments reported in this paper.

Co. All other chemicals were of analytical grade and obtained from Merck.

4.2. Purification of carbonic anhydrase isozymes from human erythrocytes by affinity chromatography

Erythrocytes were purified from fresh human blood obtained from the Blood Center 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 vols 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 Tris. Firstly, Sepharose-4B was oxidized by $KMnO_4$ and subsequently activated by $SOCl_2$. After that, aniline was attached to the activated gel as a spacer arm and finally diazotized sulfanilamide was clamped to the *para* position of aniline molecule as ligand. The hemolysate was applied to the prepared Sepharose-4B-aniline-sulfanylamide 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 °C.[34]

4.3. Hydratase activity assay

Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson.[24] CO_2–hydratase activity as an enzyme unit (EU) was calculated by using the equation $(t_0\text{-}t_c/t_c)$ where t_0 and t_c are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

4.4. Esterase activity assay

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25 °C using a spectrophotometer (CHEBIOS UV–VIS) according to the method described by Verpoorte et al.[25] The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris–SO_4 buffer (pH 7.4), 1 mL 3 mM 4-nitrophenylacetate, 0.5 mL H_2O, and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of compounds **1**–**12** were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. hCA I enzyme activities were measured for *trans*-(1*S*(*R*),6*S*(*R*))-6-hydroxycyclohex-3-enyl nitrate (**1**) (50–200 mM), *trans*-(1*S*(*R*),2*S*(*R*))-2-hydroxycyclohexyl nitrate (**2**) (50–200 mM), *trans*-(*R*(*S*))-2-hydroxy-1-phenylethyl nitrate (**3**) (50–200 mM), *trans*-(1*S*(*R*),2*S*(*R*))-2-hydroxycyclooctyl nitrate (**4**) (10–30 mM), *trans*-(1*S*(*R*),8*S*(*R*),*Z*)-8-hydroxycyclooct-4-enyl nitrate (**5**) (10–40 mM), (1*S*(*R*),2*S*(*R*),5*R*(*S*),6*R*(*S*))-5-bromo-9-oxabicyclo[4.2.1] no- nan-2-yl nitrate (**6**) (5–30 mM), 9(*R*(*S*))-hydroxy-1,2,3,4-tetrahydro-1,4-methano-naphthalen-2(*R*(*S*)-yl nitrate (**7**) (5–30 mM), (1*R*(*S*),2*R*(*S*),4*R*(*S*),5*R*(*S*))-2,5-dihydroxycyclo-hexane-1,4-diyl dinitrate (**8**) (5–30 mM), (1*S*(*R*),3*S*(*R*),4*S*(*R*),6*S*(*R*))-4,6-dihydroxycyclohexane-1,3-diyl dinitrate (**9**) (5–30 mM), (1*R*(*S*),2*R*(*S*),3*S*(*R*),4*S*(*R*))-2,3-dihydroxycyclo-hexane-1,4-diyl dinitrate (**10**) (5–35 mM), (2*S*(*R*),7*R*(*S*))-7-hydroxybicyclo[2.2.1] heptan-2-yl nitrate (**11**) (5–30 mM), and (2*R*(*S*),7*R*(*S*))-7-hydroxybicyclo[2.2.1]heptan-2-yl nitrate (**12**) (5–30 mM) at cuvette concentrations and hCA II enzyme activities were measured for *trans*-(1*S*(*R*),6*S*(*R*))-6-hydroxycyclohex-3-enyl nitrate (**1**) (500–900 μM), *trans*-(1*S*(*R*),2*S*(*R*))-2-hydroxycyclohexyl nitrate (**2**) (300–700 μM), *trans*-(*R*(*S*))-2-hydroxy-1-phenylethyl nitrate (**3**) (500–800 μM), *trans*-(1*S*(*R*),2*S*(*R*))-2-hydroxycyclooctyl nitrate (**4**) (100–300 μM), *trans*-(1*S*(*R*),8*S*(*R*),*Z*)-8-hydroxycyclooct-4-enyl nitrate (**5**) (150–400 μM), (1*S*(*R*),2*S*(*R*),5*R*(*S*), 6*R*(*S*))-5-bromo-9-oxabicyclo[4.2.1] nonan-2-yl nitrate (**6**) (50–300 μM), 9(*R*(*S*))-hydroxy-1,2,3,4-tetrahydro-1,4-methano-naphthalen-2(*R*(*S*)-yl nitrate (**7**) (50–200 μM), (1*R*(*S*),2*R*(*S*), 4*R*(*S*),5*R*(*S*))-2,5-dihydroxycyclo-hexane-1,4-diyl dinitrate (**8**) (50–200 μM), (1*S*(*R*),3*S*(*R*),4*S*(*R*),6*S*(*R*))-4,6-dihydroxycyclo-hexane-1,3-diyl dinitrate (**9**) (50–250 μM), (1*R*(*S*),2*R*(*S*), 3*S*(*R*),4*S*(*R*))-2,3-dihydroxycyclo-hexane-1,4-diyl dinitrate (**10**) (50–250 μM), (2*S*(*R*),7*R*(*S*))-7-hydroxybicyclo[2.2.1] heptan-2-yl nitrate (**11**) (50–200 μM), and (2*R*(*S*),7*R*(*S*))-7-hydroxybicyclo[2.2.1]heptan-2-yl nitrate (**12**) (30–150 μM) at cuvette concentrations. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor an %Activity – [Inhibitor] graph was drawn. To determine K_i values, three different inhibitor concentrations were tested; In these experiments, 4-nitrophenylacetate was used as substrate at five different concentrations (0.15–0.75 mM). The Lineweaver–Burk curves were drawn.[28]

4.5. Protein determination

Protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard.[26]

4.6. 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. A 20 mg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without the dye.[26]

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