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RESEARCH ARTICLE

2-Amino-3-cyanopyridine derivatives as carbonic anhydrase inhibitors

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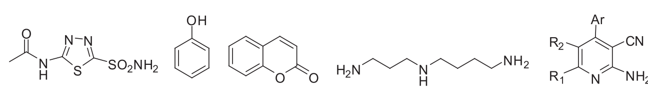
Abstract
Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous enzymes that catalyze the hydration of CO_2 to bicarbonate and protons. Inhibition of CAs has been clinically exploited for the treatment of various classes of diseases for decades, but investigating new classes of inhibitors continues to be important. We have synthesized a series of 2-amino-3-cyano-4-heteroaryl (**5a–l**) compounds and characterized the structures by NMR, IR and elemental analyses. We tested the ability of these compounds to inhibit two metalloenzyme human carbonic anhydrase (hCA, EC 4.2.1.1) isozymes, hCA I and hCA II. Compounds **5d** and **5b** showed the best inhibition activity against hCA I (IC_{50}: 33 and 34 μM, respectively), and compound **5d** showed the best activity against hCA II (IC_{50}: 56 μM).

Keywords: 2-amino-3-cyanopyridines, 4-furyl, 4-thienyl, carbonic anhydrase, enzyme inhibition

Introduction

Carbonic anhydrase (CA) is a zinc metalloenzyme that catalyzes the reversible reaction of CO_2 and water: $CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$. It was discovered in 1932 in the red blood cells following the realization that the uncatalyzed rate of HCO_3^- dehydration was too low to support CO_2 excretion during the time blood spent at the gas exchange surface[1–4].

As an enzyme, CA is of broad interest because it has one of the fastest known reaction rates. The turnover number (k_{cat}, exceeds 1×10^6 s^{-1} for some CA isoforms[1–4]). Furthermore, the reaction catalyzed by CA is fundamental to a wide array of physiological processes, including calcification, photosynthesis, respiration, metabolism, cell growth and ionic, acid-base and fluid balance[1–4]. This diversity of functions suggests that CA may have been among the earliest enzymes to appear, and CA seems to be almost ubiquitously expressed in living organisms. Five genetically unrelated families (α, ß, γ, δ and ξ) of CA isoforms exist[5]. The α-CA family is the best studied group, although recent reviews indicate rapid advancement of knowledge about other CA families[6–11].



1 2 3 4 5

The classes of CA inhibitors (CAIs) are known: the metal complexing anions, and the substituted sulfonamides such as acetazol amide[1] and their bioesters (sulfamates, sulfamides), which bind to the metal ion of the enzyme either by substituting for the non-protein zinc ligand to generate a tetrahedral adduct or by participating in the metal coordination sphere to generate trigonal-bipyramidal species[12]. Phenols[2] which bind to the zinc-coordinated water molecule/hydroxide ion from active site are also very important inhibitors that display competitive inhibition[13,14]. Coumarines[3] and thiocoumarines which have an inhibition mechanism independent of Zn (II) because they don't have proton-shutting moieties in their molecules are the third class

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of inhibitors, CAIs[15–17]. In the recent studies polyamines such as spermine and spermidine[4] are reported a new class of CAI inhibitors[18,19]. The Zn (II) ion in CAs is critical for the inhibition of these enzymes. Three mechanisms of inhibition have been proposed, one of which involves anchoring of the inhibitor to the Zn (II) bound solvent molecule (a water or hydroxide ion). Phenols and polyamines bind in this way[18–21].

A critical problem in the design of CA inhibitors with pharmacological applications for the treatment and prevention of various diseases is related to the high number of CA isoforms (16 in mammals), the diffuse localization of CAs in many tissues/organs, and the lack of isozyme selectivity of the presently available inhibitors[22,23].

The N-heteroaromatic pyridine is incorporated into the structure of many pharmaceuticals. Among these, cyanopryridines and aminocyanopyridines substituted with different alkyl and aryl groups[5] were found to have antimicrobial[24], anti-inflammatory, analgesic and antipyretic properties[25]. 2-Thienyl-4-furyl-pyridine and 2-thienyl-4-furyl-6-aryl pyridine derivatives exhibited strong inhibitory activity against the nuclear enzymes topoisomerase I and II and inhibitory activities and cytotoxicity against several human cancer cell lines[26]. These compounds are also valuable intermediates in the syntheses of a variety of biologically active heterocyclic compounds[27].

However, aminocyanopyridine derivatives bearing cycloalkanes at C5–C6 along with substituted five membered hetereocycles at C4 have not yet been tested as for inhibitory activity against CAs. We sought to fill in this gap in the literature by examining these derivatives.

Materials and methods

Chemicals

Sepharose 6B, protein assay reagents and 4-nitrophenylacetate were obtained from Sigma–Aldrich Co. (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany). All other chemicals were obtained from Merck (Merck KGaA, Darmstadt, Germany). All the chemicals investigated in the study were reagent grade and were further purified only as necessary. All organic solvents used in this study were purified according to standard methods. Elemental analyses were carried out with a LECO-CHNS-932 instrument. ^{1}H and ^{13}C-NMR spectra were recorded on a Varian 200 MHz spectrometer using TMS (Tetramethylsilane) as an internal standard. IR spectra were recorded on a Mattson-5000 FT-IR instrument in KBr pellets. Melting points were determined with a Gallenkamp melting point apparatus.

General method for preparation of 3a-b

Intermediates **3a–b** were prepared according to the methods in the literature[28].

General method for preparation of 5a–l

Compound **3a, b** (0.005 mol) was suspended in benzene (10 ml), and ammonium acetate (0.0075 mol) and cycloalkanone **4** (0.005 mol) were added. The flask was fitted with a reflux condenser and a water separator, and the mixture was refluxed for 6 h. Then, the solvent was evaporated, and the mixture was re-dissolved in chloroform (150 ml) and washed with water (2 × 50 ml). The organic phase was dried with $MgSO_4$, filtered off and recrystallized from ethyl acetate[28]. All aminocyanopyridines (**5a–l**) were prepared by this procedure (Scheme 1).

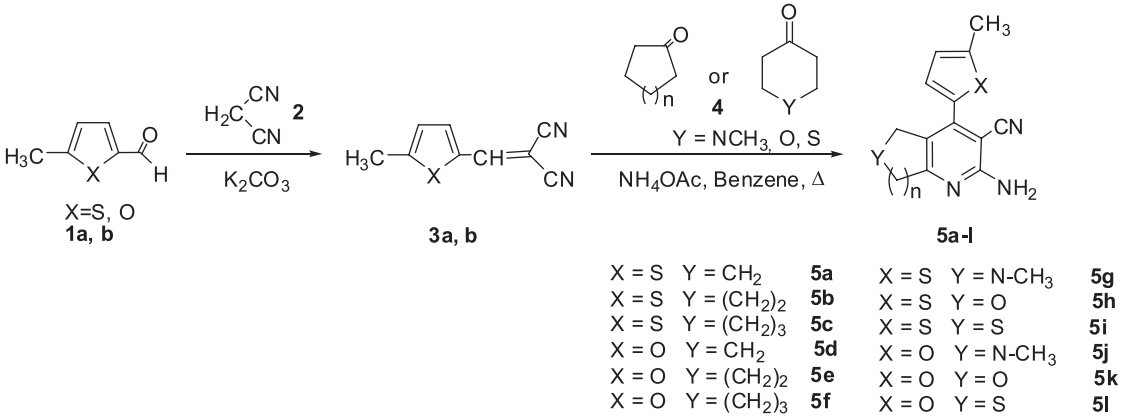
*2-Amino-6,8-dihydro-4-(5-methylfuran-2-yl)-5H-thiopyrano[3,4-b]pyridine-3-carbonitrile (**5l**); m.p: 222–225 °C, 48% yield*

IR cm^{-1} 3412–3321 (NH_2), 3175 (CH Aryl), 2923 (CH Alkyl), 2210 (CN).

^{1}H-NMR (DMSO-d_6, δ, ppm) 2.39 (s, 3H, CH_3), 2.92–2.99 (m, 4H, $2CH_2$), 3.33 (s, 2H, CH_2), 6.35–6.37 (dd, J: 3.3, 0.98 Hz, 1H Ar-H), 6.76 (bs, 2H, NH_2), 6.84–6.96 (d, J: 3.3 Hz, 1H, Ar-H).

^{13}C-NMR (DMSO-d_6, ppm) 15.0, 26.7, 27.7, 35.8, 87.1, 109.9, 117.1, 118.5, 118.8, 141.6, 146.8, 155.9, 160.6, 163.0.

El. Anal. Predicted: C, 61.97; H, 4.83; N, 15.49; S, 11.82. Observed: C, 61.64; H, 4.81; N, 15.44; S, 11.64.



Scheme 1. Synthesis of 2-amino-3-cyanopyridines (**5a–l**).

Purification of CA isozymes from human erythrocytes by affinity chromatography

The two CA isozymes were purified via a simple single-step method using Sepharose-4B-L-tyrosine-sulfanilamide affinity gel chromatography.

Erythrocytes were purified from fresh human blood obtained from the blood centre of the research hospital at Ataturk 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 hemolyzed 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 Tris.

Sepharose 4B was activated with CNBr, then filtered with a Buchner funnel and washed with cold 0.1 M $NaHCO_3$ buffer (pH 10). A saturated solution of L-tyrosine in the same buffer was coupled to the activated Sepharose 4B resin for 90 min, with magnetic stirring. The affinity gel was obtained by coupling diazosulfanilamide to the Sepharose 4B-L-tyrosine. Sulfanilamide (25 mg) was suspended in 10 ml of ice-cold 1 M HCl, and sodium nitrite (75 mg in 5 ml ice-cold water) was added to the suspension. After the mixture had been allowed to react for 10 min, the diazosulfanilamide was mixed with 40 ml of the Sepharose 4B-L-tyrosine suspension. The pH was adjusted to 9.5 with 1 M NaOH and the mixture was stirred gently for 3 h at room temperature. The coupled red Sepharose derivative was then washed with 1 l of water, followed by 200 ml of 0.05 M Tris-sulfate (pH 7.5). After this Sepharose-4B-L tyrosine-sulfanilamide affinity column had been equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7), the hemolysate was applied. 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[29].

Hydratase activity assay

The hydratase activity of hCA I and hCA II was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson[30]. The activity of CO_2-hydratase in enzyme units (EU) was calculated by using the equation $[(t_o - t_c)/t_c]$ where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

Esterase activity assay

CA 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[31]. The enzymatic reaction 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 (total volume, 3.0 mL). A reference measurement was obtained by preparing the mixture without the enzyme solution. All measurements were made in triplicate. The inhibitory effects of **5a**, **5b**, **5c**, **5d**, **5e**, **5f**, **5g**, **5h**, **5i**, **5j**, **5k** and **5l** were examined over a range of concentrations. The activity of hCA I was measured at the following cuvette concentrations of the test compounds: **5a**(0.001–1000 mM), **5b** (6–49 μM), **5c** (6–47 μM), **5d** (14–56 μM), **5e** (39–88 μM), **5f** (38–89 μM), **5g** (0.001–1000 mM), **5h** (0.001–1000 mM), **5i** (155–675 μM), **5j** (0.001–1000 mM), **5k** (0.001–1000 mM) and **5l** (125–756 μM). The activity of hCA II was measured at the following cuvette concentrations of the test compounds: **5a** (0.001–1000 mM), **5b** (0.037–0.112 μM), **5c** (0.035–0.082 μM), **5d** (0.042–0.056 μM), **5e** (0.001–1000 mM), **5f** (0.001–1000 mM), **5g** (0.001–1000 mM), **5h** (0.001–1000 mM), **5i** (0.001–1000 mM), **5j** (0.001–1000 mM), **5k** (0.001–1000 mM) and **5l** (0.001–1000 mM). The activity of the control cuvette (in the absence of inhibitor) was set to 100%. An Activity (%)-[Inhibitor] graph was constructed for each compound. The K_i values were determined from a series of experiments using three different inhibitor concentrations and 4-nitrophenylacetate as the substrate at five different concentrations (0.15–0.75 mM) to construct Lineweaver–Burk curves[32].

Protein determination

The yield of protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard[33].

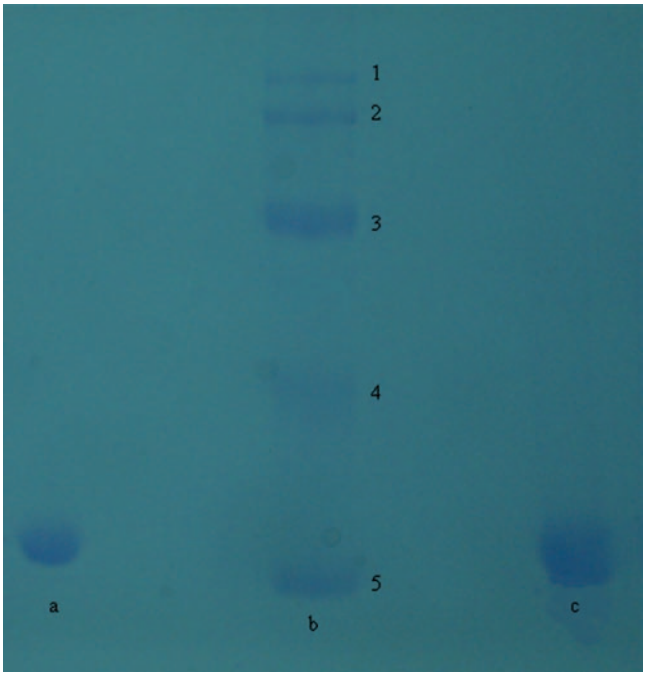


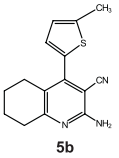
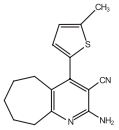
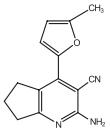
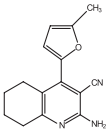
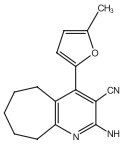
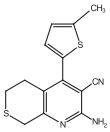
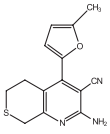
Figure 1. PAGE of the purified CA isozymes. Lane b Standards: E.coli β-galoksidaz (116 kDa), rabbit muscle phosphorylase b (97 kDa), bovine albumin (66 kDa); ovalbumin (45 kDa), bovine erythrocytes CA (29 kDa), lane a: CA I, lane c: CA II.

Table 1. Purification of hCA I and CA II.

Step	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Recovery (%)	Purification (Fold)
Hemolysate	149.00	44.00	17.26	759.44	6556.00	8.63	100	1.00
hCA I	483.00	9.00	0.48	4.32	4347.00	1006.25	66	116.60
hCA II	833.00	5.00	0.14	0.70	4165.00	5950.00	64	689.46

Mean value from at least 3 different measurements. Errors were in the range of ±3–5% of the obtained value (data not shown).

Table 2. Inhibitory activity of compounds **5a–l**.

Entry	Compound	IC_{50} hCA I (μM)	IC_{50} hCA II (μM)	Ki hCA I (μM)	Inhibition type	Ki hCA II (μM)	Inhibition type
1	 **5b**	34	99	31 ± 7.5	Competitive	182 ± 33.5	Competitive
2	 **5c**	40	77	30.6 ± 5.7	Competitive	73 ± 14.5	Competitive
3	 **5d**	33	56	23.8 ± 5.2	Competitive	41 ± 7.5	Competitive
4	 **5e**	48	–	89 ± 9	Competitive	–	–
5	 **5f**	53	–	58.5 ± 11.8	Competitive	–	–
6	 **5i**	55	–	38.4 ± 8.1	Competitive	–	–
7	 **5l**	53	–	50.3 ± 14.2	Competitive	–	–
8	SA[a]	–	–	25.5 ± 4.8	Competitive	0.023 ± 0.0045	Competitive

[a]SA (sulfanilamide) was used as a positive control. Result of positive control similar to Vomasta et al[42].
Mean value from at least 3 different measurements. Errors of IC_{50} were in the range of ± 3–5% of the obtained value (data not shown).

SDS polyacrylamide gel electrophoresis

The purity of the enzymes was confirmed using SDS polyacrylamide gel electrophoresis (Figure 1). It was carried out according to Laemmli procedure[34].

Results and discussion

Chemistry

We have previously reported the synthesis and antimicrobial evaluation of various 2-amino-3-cyanopyridine derivatives[28]. The synthesis of pentasubstituted pyridine derivatives (Scheme 1) was based on previously proposed methods[35]. First, a Knoevenagel base-catalyzed condensation was used to produce two ylidenemalononitriles (**3a**, **b**) in high yield. Then, twelve 2-amino-3-cyanopyridines (**5a–I**) were synthesized in 30–71% yield by reacting the ylidenemalononitriles (**3a, b**) with cycloalkanones[4] in the presence of ammonium acetate.

CA purification and assay

CA has been purified from many different organisms and the effects of various chemicals, pesticides, and drugs on its activity have been investigated[36–39]. In this study, hCA I and II were purified from human erythrocytes (Table 1). The inhibitory effects of a series of 2-amino-3-cyanopyridines were tested *in vitro*. The inhibitor concentrations that caused 50% inhibition (IC_{50}) were determined from % activity versus [Inhibitor] plots, and the K_i values were calculated from Lineweaver–Burk plots (Table 2)[33,34].

CA inhibition

We prepared twelve 2-amino-3-cyanopyridine derivatives with the structures shown in Scheme 1 and evaluated their ability to inhibit hCA I and hCA II over a wide range of concentrations (0,001–1000 mM). Seven of the twelve compounds (**5b**, **5c**, **5d**, **5e**, **5f**, **5i**, **5l**) inhibited hCA I, and three of them (**5b**, **5c**, **5d**) inhibited hCA II. When we analyzed the successful inhibitors in detail, we found that five of the six structures that contained a cycloalkyl ring (**5a**–**f**) inhibited hCA I, and three of them inhibited hCA II. However, compound **5a** did inhibit either hCA I or hCA II. Compounds **5e** and **5f** inhibited hCA I, but not hCA II. Only two of the six heterocyclic structures (**5g**–**l**) inhibited hCA I, and none inhibited hCA II. Although numerous reports have shown that sulfones inhibit CAs, thiopyrano-containing compounds **5i** and **5l** were only weak inhibitors of hCA I.

Our compounds were weak to moderate inhibitors of hCA I and hCA II. The effects of the six compounds that contain heterocyclic rings were the weakest. Our results are consistent with previous reports that phenols (amines) are weak inhibitors of hCA I and hCA II[15–19,40].

Spermine was found bound deep within the hCAII active site, in his inhibition mechanism, nevertheless not directly coordinated to the metal ion. The terminal ammonium group in spermine is anchored to the Zn(II) bond water molecule/hydroxide ion similarly to by means of hydrogen bond phenol[2] and this terminal ammonium group forms a second hydrogen bond with the OH of Thr199 at pH 7.4.

pK_a values of the polyamines like spermidine and spermine and their derivatives are in the range of 7.9–10.9[18–21]. Aminopyridines are weak bases with pK_a values between 6 and 9. Therefore, they can exist in neutral and protonated for at physiological pH. Aminopyridines pharmacophore and one or more amine groups, being by positive charge on the protonated nitrogen, is suitable for hydrogen bonding[41]. Possible inhibiton mechanisms of the synthesized compounds can be similar to polyamines like spermine. Ammonium group in synthesized aminocyanopyridines (**5b**, **5c**, **5d**, **5e**, **5f**, **5i**, **5l)** can be formed both with the Zn(II) bound water/hydroxide ion and second hydrogen bond between OH of Thr199 at physiological pH.

Conclusions

We have synthesized twelve 2-amino-3-cyanopyridine derivatives incorporating biologically active cycloalkyl moieties at the C5–C6 positions and furan and thiophene derivatives at the C4 position of the molecule and evaluated the ability of these compounds to inhibit hCA I and hCA II. Seven of the twelve molecules (**5b**, **5c**, **5d**, **5e**, **5f**, **5i**, **5l**) inhibited hCA I, and three of them inhibited hCA II (**5b**, **5c**, **5d**). The best inhibitors of hCA I were compounds **5d**, **5b** and **5c**, which exhibited IC_{50} values of 33, 34 and 40 μM, respectively. The best inhibitor of hCA II was compound **5d**, with an IC_{50} value of 56 μM.

The compounds that contained five- and six-membered cycloalkyl rings at C5–C6 were more active than the compounds that contained heterocyclic (O, S, N) rings. The results indicated that the more increase there is in the ring size of cycloalkyl the more decrease there is the inhibition effect of molecules and this may attributed to the steric hindrance.

Of the compounds that contained six-membered heterocyclic rings, only those with sulfur as the heteroatom exhibited an inhibitory effect. Most of the effective inhibitors contained sulfur atoms.

Declaration of interest

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