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RESEARCH ARTICLE

7-Amino-3,4-dihydro-1H-quinolin-2-one, a compound similar to the substituted coumarins, inhibits α-carbonic anhydrases without hydrolysis of the lactam ring

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Abstract

7-Amino-3,4-dihydro-1H-quinolin-2-one, a compound structurally similar to coumarins, recently discovered class of inhibitors of the α-carbonic anhydrases (CAs, EC 4.2.1.1) was investigated for its interaction with all human (h) CA isoforms, hCA I-XIV. The compound was not an inhibitor of the cytosolic, widespread isoform hCA II ($K_I > 10$ μM), was a weak inhibitor of hCA I, III, IV, VA, VI and XIII (K_Is in the range of 0.90–9.5 μM) but effectively inhibited the cytosolic isoform hCA VII (K_I of 480 nM) as well as the transmembrane isoforms hCA IX, XII and XIV (K_Is in the range of 16.1–510 nM). Against many CA isoforms this lactam was a better inhibitor compared to the structurally similar 4-methyl-7-aminocoumarin, but unlike this compound, the lactam ring was not hydrolyzed and the inhibition was due to the intact bicyclic amino-quinolinone scaffold. Bicyclic lactams strucurally related to coumarins are thus a new class of CA inhibitors possessing however a distinct inhibition mechanism compared to the coumarins which undergo a hydrolysis of their lactone ring for generating the enzyme inhibitory species.

Keywords

Carbonic anhydrase, coumarin, inhibitor, lactam, lactone, isoform-selectivity, quinolon-2-one

History

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Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1)[1–5] are among the fundamental catalysts found in nature, as they catalyze the hydration of CO_2 to bicarbonate and protons, being thus involved in all processes connected to pH regulation, chemosensing (of CO_2, bicarbonate, acidity, etc.), several biosynthetic pathways, electrolyte secretion in many tissues, and other physiological processes[6–15]. It is thus not unexpected that at least six genetically diverse CA families were described in organisms all over the phylogenetic tree, the α-, β-, γ-, δ-, ζ- and η-classes, which are involved in crucial physiologic and pathologic processes in these organisms[16–21]. In fact interference with the activity of these enzymes has important physiologic consequences which were exploited clinically by more than 65 years, by the use of the CA inhibitors (CAIs)[22–33]. Sulfonamides[22] constitute the main class of CAIs and they are in clinical use as antiglaucoma agents[23–28], diuretics[29], anti-obesity drugs[30,31], and for the management of hypoxic tumors which overexpress some CA isoforms[2–5,32,33].

Such multiple applications of the CAIs are due to the fact that in vertebrates 16 different CA isoforms are known[1–5], which are found in different tissues and organs, play diverse physiologic functions, and their inhibition/activation elicits different responses which are thus amenable to pharmacologic modulation[1–5,23–33].

Furthermore, in recent years these enzymes were isolated and characterized in many pathogenic bacteria, protozoa and fungi, and a possible use as anti-infectives emerged for the CAIs[18,34–39]. However the main challenge for using CAIs of the sulfonamide type in clinical settings is related to their off-target effects: the first generation such drugs (acetazolamide, methazolamide, dichorophenamide, ethoxzolamide, etc.) indiscriminately inhibit most of the mammalian CA isoforms (including the human (h) ones, hCAs), leading sometimes to serious side effects[1–5,40–42]. It is thus understandable that many alternative classes of CAIs were investigated recently, apart the sulfonamides. For example the dithiocarbamates[43] were discovered to act as CAIs, with a mechanism of action related to that of the sulfonamides, as both classes of derivatives coordinate to the metal ion from the enzyme active site and replace the water molecule/hydroxide ion acting as nucleophile in the catalytic cycle of these enzymes[1–5],

However some of the most interesting new chemotypes discovered ultimately to act as CAIs are undoubtedly the coumarins/thiocoumarins (and compounds structurally related to them, such as monocyclic five-/six-ring membered lactones)[44–51] as well as the structurally related sulfocoumarins[52–55]. These classes of CAIs possess a completely different inhibition mechanism compared to all other inhibitors investigated earlier, as demonstrated by detailed kinetic and X-ray crystallographic studies[44,45,52]. Indeed, the coumarins act as pro-drug inhibitors, being hydrolyzed by the esterase activity of the CAs within the

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active site cavity with formation of carboxylic acids which thereafter inhibit the enzyme by blocking the access to the active site. Indeed, the binding site of this class of CAIs has never been observed for any other class of inhibitors, being situated at the entrance of the enzyme active site[44,45]. On the other hand, the structurally related sulfocoumarins, in which the lactone CO moiety of the coumarin was replaced by an SO_2 group, are also acting as pro-drug inhibitors, leading after hydrolysis to sulfonic acid derivatives[52]. Unlike the hydrolyzed coumarins, the hydrolyzed sulfocoumarins bind more internally within the active site, but again they do not directly interact with the zinc ion of the enzyme. Like phenols[56] and polyamines[57], the sulfonic acids formed by hydrolysis of the sulfocoumarins anchor by means of hydrogen bonds to the zinc-coordinated water molecule which acts as nucleophile in the catalytic process[52]. But what is even more remarkable about these new classes of CAIs is that they show an excellent selectivity profile for inhibiting various CA isoforms of medicinal interest. For example among the substituted coumarins, derivatives selective for all isoforms were detected, with selectivity ratios for inhibiting the desired isoform over all other human ones of >1000 (never observed with sulfonamide CAIs)[44–51]. For sulfocumarins, derivatives with an excellent selectivity for inhibiting the tumor associated isoforms hCA IX and XII over the cytosolic ones hCA I and II were detected[52,54,55], in several series of compounds, together with a series of such derivatives which selectively inhibited hCA II over the other isoforms[53], These interesting selectivity profiles for inhibiting some and not other CA isoforms was explained by the inhibition mechanism of these derivatives which implies on one hand a hydrolytic process of the pro-drug inhibitor (which can be kinetically diverse among the different isoforms, based on their efficacy as esterases) and also due to the binding sites of the formed inhibitors of the carboxylic/sulfonic acid type. As mentioned above these inhibitors bind towards the external regions of the CA active site, which differ among the diverse isoforms, i.e. a larger number of non-conserved amino acid residues are present in those regions of the active site and not in the neighborhood of the zinc ion, where the sulfonamides and similar zinc binders are found in the enzyme-inhibitor adducts[1–5,44,45,52].

Considering all these interesting aspects related to the coumarin-type CAIs, here we explore other chemotypes structurally related to them, and include in our study a bicyclic lactam derivative, structurally rather similar to coumarins, i.e. 7-amino-3,4-dihydro-1H-quinolin-2-one. This compound has been assayed for the inhibition of all human CA isoforms with catalytic activity, hCA I - hCA XIV.

Materials and methods

Chemistry

Compounds **1**, **2** and acetazolamide (**AAZ**) used here were commercially available from Sigma-Aldrich, Milan, Italy.

CA inhibition

A stopped-flow instrument (SX.18MV-R Applied Photophysics model) was used for assaying the CA-catalyzed CO_2 hydration activity[58]. Inhibitor and enzyme were preincubated for 6 h (for **1** and **2**)[44,45] and 15 min (for **AAZ**) for allowing the complete formation of the enzyme-inhibitor adduct. IC_{50} values were obtained from dose response curves working at seven different concentrations of test compound (from 0.1 nM to 50 μM), by fitting the curves using PRISM (www.graphpad.com) and non-linear least squares methods, the obtained values representing the mean of at least three different determinations[59]. The inhibition constants (K_I) were derived from the IC_{50} values by using the Cheng-Prusoff equation, as follows: $K_i = IC_{50}/(1 + [S]/K_m)$ where [S] represents the CO_2 concentration at which the measurement was carried out, and K_m the concentration of substrate at which the enzyme activity is at half maximal. All enzymes used were recombinant, produced in *E. coli* as reported earlier[59,60]. The concentrations of enzymes used in the assay were in the range of 8.5–12.7 nM.

Results and discussion

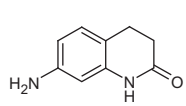
In the present paper we investigated the hCA I – hCA XIV inhibitory properties of the lactam **1**, 7-amino-3,4-dihydro-1H-quinolin-2-one, a compound bearing structural similarity with the coumarins. As coumarin counterpart we included compound **2**, 4-methyl-7-aminocoumarin, in our study as well as the standard sulfonamide inhibitor acetazolamide (**AAZ**), a clinically used and potent, non-selective CAI[1–5].

Both derivatives **1** and **2** possess a bicyclic ring system, of the lactam type in **1** and of the lactone type in **2**. They also share as a common feature the presence of an amino moiety in position 7 of the ring, but the coumarin **2** is an aromatic derivative whereas the lactam **1** has a saturated ring, as main distinction between them. Furthermore, the coumarin has a 4-methyl group which is not present in **1**, but we showed in earlier studies that small moieties (H, Me, etc.) in position 4 of coumarins as CAIs do not significantly change the inhibitory power of the corresponding derivatives[44,45].

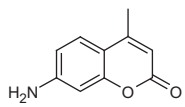
Inhibition data with compounds **1**, **2** an **AAZ** are shown in Table 1. The following structure-activity relationship can be drawn from data of Table 1:

(1) The lactam **1** did not significantly inhibit the widespread cytosolic isoform hCA II (a target for anti-glaucoma drugs[23–28] but an offtarget when other apllications of the CAIs are pursued[1–5]) showing a K_I of >10 μM. This is not such an unexpected finding, as we have reported similar features for many coumarin and sulfocoumarin derivatives, which did not significantly inhibit this isoform[44–52,54,55]. Other isoforms which were not strongly inhibited by lactam **1** were hCA I, III and XIII (cytosolic isozymes), as well as hCA IV and hCA VI (membrane associated and secreted

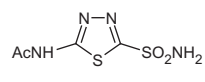
Table 1. Human CA isoforms inhibition data with compounds **1**, **2** and **AAZ** as standard, by a stopped-flow CO2 hydrase assay[49].



1



2



AAZ

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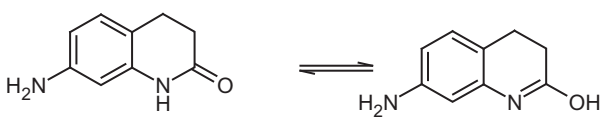
Isoform, K_I, nM*	1	2	AAZ
hCA I	6120	5560	250
hCA II	>10000	>10000	12
hCA III	3950	8440	>10000
hCA IV	9495	>10000	74
hCA VA	904	7810	63
hCA VI	3900	5695	11
hCA VII	480	185	2.5
hCA IX	124	7640	25
hCA XII	16.1	9130	5.7
hCA XIII	3810	7830	17
hCA XIV	510	188	41

*Errors in the range of ± 10% of the reported data, from 3 different assays (not shown).

isoforms, respectively), for which inhibition constants in the range of 3810–9495 nM were measured (Table 1). A slightly better inhibitory power of **1** was observed for the mitochondrial isoform hCA VA, for which a K_I of 904 nM was registered. The best inhibitory power of the lactam was observed against four isoforms, the cytosolic hCA VII and the three transmembrane isoforms hCA IX, XII and XIV. Against these enzymes the inhibition constant of **1** ranged between 16.1 and 510 nM. The best inhibited isoform was the tumor-associated hCA XII (K_I of 16.1 nM) followed by the second tumor-associated isoform, hCA IX (K_I of 124 nM). These findings are indeed remarkable, as the lactam **1** is a highly selective CAI for the tumor-associated isoforms, with very poor or insignificant inhibitory power against the widespread and diffuse isoforms hCA I and II. But what was even more remarkable was the fact that no signs of hydrolytic processes of compound **1** (in the presence of buffer alone, or buffer + CA isoforms) could be evidenced, even after incubation times as long as 24–48 h (data not shown).

(2) The coumarin **2** was also not inhibitory against hCA II (K_I of > 10 μM), as we reported earlier in a study in which the CA I, II, IX and XII inhibitory power of this compound was investigated.[51] Here we extend the earlier work to the remaining hCA Isoforms. Indeed, many of the investigated isoforms, such as hCA I, III, IV (K_I of > 10 μM), VA, VI, IX, XII and XIII were poorly inhibited by coumarin **2**, with inhibition constants in the micromolar rage (5695–9130 nM, Table 1). In fact only hCA VII and XIV were significantly inhibited by **2**, with inhibition constants of 185–188 nM. It should however be mentioned, that derivatives of **7** in which the amino moiety was derivatized by reactions with isocyanates or acyl chlorides led to compounds with low nanomolar inhibitory activity against hCA IX and XII, as shown in the earlier report[51]. Thus, the inhibition profile of compounds **1** and **2**, although structurally similar, is very different, which may be another proof that their inhibition mechanism must be different: the coumarin **2** is hydrolyzed by the esterase activity of the CA isoforms and the open form (substituted hydroxycinnamic acid) acts as inhibitor, whereas for the lactam **1**, the non-hydrolysed bicyclic compound is the inhibitor *per se*. This may be due to the fact that no peptidase activity was ever reported for the CAs, although they possess an active site metal coordination quite similar to that of effective proteases/peptidases such as for example the matrix metalloproteinases (MMPs)[61].

(3) Acetazolamide **AAZ**, a sulfonamide used clinically, is a pan-CA inhibitor, with a very promiscuous inhibition profile (Table 1)[1–5]. In fact only hCA III was not significantly inhibited (K_I of >10 μM), hCA I was inhibited with medium potency (K_I of 250 nM) whereas the remaining nine isoforms were powerfully inhibited by this sulfonamide, with K_Is in the range of 2.5–74 nM (Table 1). These results explain why **AAZ** has a wide range of side effects when administered as systemically acting antiglaucoma agent (Scheme 1)[22–28].



1a lactam tautomer **1b** lactim tautomer

Scheme 1. Tautomeric forms **1a** and **1b** of the investigated derivative **1**.

Conclusions

We investigated 7-amino-3,4-dihydro-1H-quinoline-2-one, a compound structurally similar to the coumarins, a recently discovered class of inhibitors of the α-CAs, for its interaction with all catalytically active CA isoforms, hCA I-XIV. The compound was not an inhibitor of the cytosolic, widespread isoform hCA II ($K_I > 10$ μM), was a weak, micromolar inhibitor of hCA I, III, IV, VA, VI and XIII (K_Is in the range of 0.90–9.5 μM) but effectively inhibited the cytosolic isoform hCA VII (K_I of 480 nM) as well as the transmembrane isoforms hCA IX, XII and XIV (K_Is in the range of 16.1–510 nM). Against many CA isoforms the lactam **1** was a better inhibitor compared to the structurally similar 4-methyl-7-aminocoumarin **2**, but unlike this compound, the lactam ring was not hydrolyzed and the inhibition was due to the intact bicyclic amino-quinolinone scaffold. Bicyclic lactams strucurally related to coumarins are thus a new class of CA inhibitors possessing however a distinct inhibition mechanism compared to the coumarins which undergo a hydrolysis of their lactone ring for generating the enzyme inhibitory species.

As no X-ray crystal structures are yet available for this lactam complexed to a CA isoform, we can only hypothesize its inhibition mechanism. Most probably the compound may show an inhibition mechanism typical of phenols, polyamines and hydrolyzed sulfocoumarins, which all anchor to the zinc-coordinated water molecule/hydroxide ion[52,56,57]. Indeed, through its lactam-lactim tautomers **1a**–**1b** (Scheme 1), compound **1** may anchor by means of hydrogen bonds to the zinc-coordinated water molecule/hydroxide ion either through the C = O moiety, or as the phenols, through its OH group. Further investigations are needed to verify these hypotheses, but it should be also noted that the primary 7-amino group present in **1** is also amenable to derivatization which may lead to even more interesting CAIs belonging to this novel chemotype.

Declaration of interest

The authors declare no conflict of interest. This research was financed in part by two EU projects (Dynano and Metoxia).

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