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Carbonic anhydrase inhibitors: Two-prong versus mono-prong inhibitors of isoforms I, II, IX, and XII exemplified by photochromic *cis*-1,2-α-dithienylethene derivatives

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ABSTRACT

We investigated the inhibition of five physiologically relevant CA isoforms with photochromic *cis*-1,2-α-dithienylethene-based compounds incorporating either a benzenesulfonamide and Cu(II)-iminodiacetic acid (IDA)-, bis-benzenesulfonamide-, bis-Cu(II)-IDA-, and bis-ethyleneglycol-methyl ether moieties, in both their open- and closed-ring forms. For hCA I the best inhibitors were the mono-prong bis-sulfonamide and the bis-Cu-IDA complexes (K_Is of 2–3 nM) in their open form. For hCA II, best inhibitors were the open and closed forms of the mono-prong bis-sulfonamide (K_Is of 13–18 nM). hCA IX was moderately inhibited by these compounds (K_Is of 9–376 nM) whereas hCA XII and XIV were less susceptible to inhibition (K_Is of 1.12–16.7 μM).

The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes present in prokaryotes and eukaryotes, encoded by at least five distinct, evolutionarily unrelated gene families: the α-CAs (in prokaryotes from the bacteria domain, algae, cytoplasm of green plants, and vertebrates with 15 isozymes presently known in humans), the β-CAs (predominantly in bacteria, fungi, algae, and chloroplasts of both mono- as well as dicotyledons), the γ-CAs (in archaea and some bacteria), the δ-CAs, found so far only in marine diatoms, and the recently characterized ξ-CAs, which are cadmium enzymes also present in marine diatoms.[1–8] CAs catalyze the conversion of CO_2 to the bicarbonate ion and protons. The active site of most CAs contains a zinc ion, which is essential for catalysis. The CA reaction is involved in many physiological and pathological processes, including respiration and transport of CO_2 and bicarbonate between metabolizing tissues and lungs; pH and CO_2 homeostasis; electrolyte secretion in various tissues and organs; biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis); bone resorption, calcification; and tumorigenicity.[3]

Two main classes of CA inhibitors (CAIs) are known: the metal-complexing anions, and the substituted sulfonamides and their bioesters (sulfamates, sulfamides), which bind to the metal ion of the enzyme either by substituting the non-protein zinc ligand to generate a tetrahedral adduct or by addition of the metal coordination sphere, generating trigonal-bipyramidal species.[3,4] However a critical problem in the design of CAIs with pharmacological applications in the treatment and prevention of various diseases is related to the high number of isoforms (16 in mammals), their rather diffuse localization in many tissues/organs, and the lack of isozyme selectivity of the presently available inhibitors.[3–5] By attaching amino acid moieties with coordinating metal ion properties to the sulfonamide, compounds with interesting activity were obtained. Indeed, the polyamino acyl derivatized sulfonamides with tails such as IDA (iminodiacetic acid), EDTA (ethylenediamino tetraacetic acid), or DTPA (diethylenetetraamino pentaacetic acid) as well as their Zn(II), Cu(II), or Al(III) complexes were shown to act as very potent CAIs against several isozymes such as CA I, II, IV, and IX.[9] The idea of this approach is to assure a high binding affinity of the inhibitor towards the enzyme through the supplementary interaction between the metal present in the CAI and amino acid residues from the active site, such as His 64, and therefore increase the inhibitory effect.[3,9,10]

Another interesting aspect for this type of compounds is to control this effect by an external input, such as light, to have an alternative medicinal tool for influencing enzyme inhibition.[10] Molecules constructed from the photochromic diarylethene scaffold decorated with the Cu(II)-IDA (copper(II)-iminodiacetic acid) complex[11] and a sulfonamide zinc-binding group, were recently shown to act as potent inhibitors for the human isoform CA I (hCA I). In such compounds, the enzyme inhibition can be also reg-

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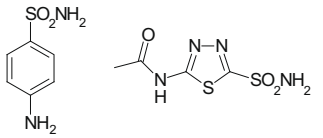
ulated by light.[9,10] Herein, we extend the earlier inhibition studies, and report the ability of some of these photochromic compounds to selectively inhibit physiologically relevant hCA isozymes such as hCA I, II (cytosolic, widespread isoforms) as well as hCA IX, XII, and XIV (the first two are transmembrane, tumor-associated isozymes recently shown to represent promising anticancer targets).[2–5]

The synthesis and photochromic properties of compounds **1**–**4** (Scheme 1) employed in this study were recently reported[10] and will be not discussed here. Irradiation[12] of the *cis*-1,2-α-dithienylethene (DTE) scaffold of the 'open' isomers **1o**–**4o** with the appropriate wavelengths (312 nm and >420 nm) induce the ring closure due to the cycloaddition reaction, with formation of the 'closed' isomers **1c**–**4c** (Scheme 1). In order to test the efficacy of two-prong versus mono-prong derivatives for obtaining isozyme-selective CAIs, the compound **1o**/**1c** incorporating a benzenesulfonamide head and a copper-IDA tail, together with the corresponding bis-sulfonamides **2o**/**2c** (incorporating two equivalent benzenesulfonamide heads) will be compared for their interactions with various CA isozymes. For assessing the role of the Cu-IDA moiety for the binding of the inhibitors to these enzymes, the compounds **3o**/**3c** (incorporating two equivalent Cu-IDA moieties but no sulfonamide zinc-binding group) were also used in our experiments, together with the presumably non-CA ligands **4o**/**4c**, which incorporate methoxy-ethyleneglycol moieties instead of the benzenesulfonamide/IDA moieties present in the CA ligands **1**–**3**, which should have no specific affinity for the CA active site, as they lack structural elements that can coordinate to the Zn(II) ion or interact with His residues involved in the catalytic cycle.[13]

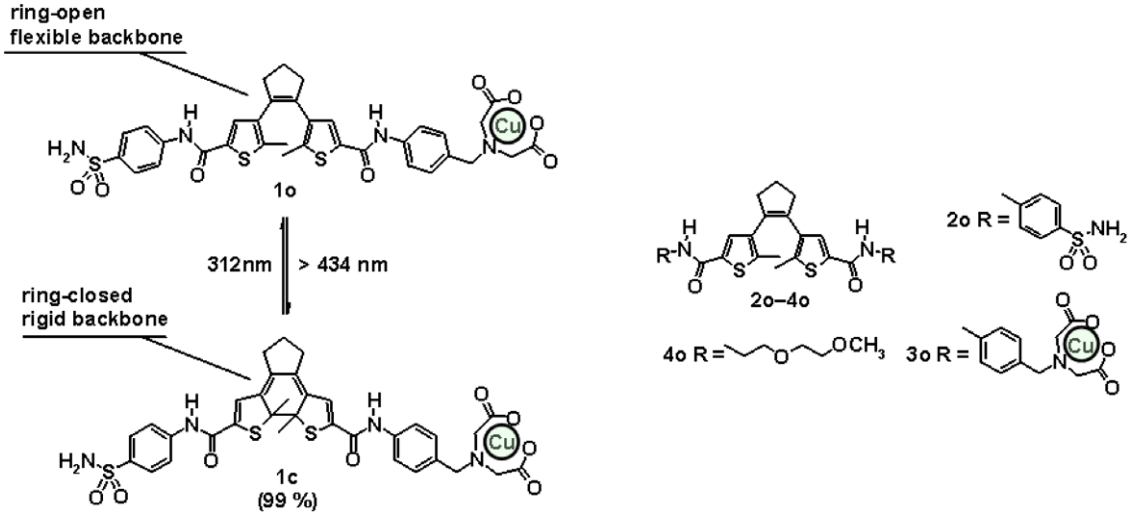
The ring-open isomers **1o** and **2o** show some structural flexibility due to the free rotation around the C–C single bonds joining the two thiophene heterocycles to the central cyclopentene ring, which allows the compounds to adopt a geometry appropriate for binding to the enzyme by means of the sulfonamide moiety (which will be anchored to the Zn(II) ion from the enzyme active site, in the case of both **1o** and **2o**) as well as by the sulfonamide moiety and the copper(II) ion (for **1o**), which may bind to His64 (or another His residue belonging to the His cluster present in the active site of several CA isozymes, such as CA I, II, IX, and XII among others).[13] On the other hand, in the 'closed' isomers **1c**/**2c**, the free rotation around the C–C single bond is no longer possible (due to its incorporation in the 6-membered ring), the flexibility of the compounds being drastically reduced.[10] Thus, the main difference between the open and closed isomers is the drastic change in flexibility of the molecule. Along with this conformational change, the spatial distance between the two recognition groups present in **1o**/**1c**, i.e., the sulfonamide zinc-binding moiety and the copper-IDA moiety that recognized His residues, are changed as well upon irradiation.[10]

Inhibition data against five physiologically relevant CA isozymes, i.e., the cytosolic, ubiquitous hCA I and II, the transmembrane hCA IX, hCA XII (tumor-associated isoforms), and hCA XIV, with compounds **1**–**4** (in open (o) and closed (c) forms) as well as the clinically used sulfonamide standards sulfanilamide SA and acetazolamide AZA, are presented in Table 1.[14,15]



The following structure–activity relationship (SAR) can be observed for the inhibition of various CA isozymes with compounds **1**–**4**, SA, and AZA:

(i) Against the slow cytosolic isoform hCA I, compounds **1**–**3** (in both open or closed form) act as very potent inhibitors, with inhibition constants in the range of 2–18 nM, in contrast to the DTE-methoxyethylenoxy-ethyl scaffold **4o**/**4c** which is devoid of any enzyme inhibitory activity. The simple sulfonamides SA and AZA are also rather weak inhibitors of hCA I, with K_Is in the range of 250 nM–25 μM, as already reported earlier.[4,15] It may be observed that for the sulfonamide derivatives **1** and **2**, the open, more flexible isomers **1o** and **2o** are 3.5–9 times more potent hCA I inhibitors as compared to the corresponding closed isomer **1c** and **2c**, respectively, as already documented earlier by some of us.[10] However, two remarkable new findings emerged here. First, the symmetrical bis-sulfonamide derivative **2o** (mono-prong derivative) is two times a better hCA I inhibitor as compared to the two-prong derivative **1o**, although the Cu-IDA moiety should in principle allow a supplementary binding[16] to the His64 residue situated in the middle of the active site cavity and thus further stabilization of the enzyme–inhibitor adduct, over the corresponding bis-sulfonamide



Scheme 1. Reagents: *Left.* Asymmetric two-prong photochromic inhibitor **1o** (opened) and the photo-induced conversion of the *cis*-diarylethene-scaffold with formation of the 'closed' isomer **1c**. *Right.* The symmetric compounds **2** (monoprong, bis-sulfonamide), **3** (bis-Cu-IDA complex), and **4** (used as a control).

Table 1
CA inhibition data against isoforms I, II (cytosolic) and IX, XII and XIV (transmembrane enzymes) with the photochromic compounds **1–4** (**o** = opened, **c** = closed isomers) and standard sulfonamide sulfanilamide **SA** and acetazolamide **AZA**

Inhibitor	K_I** (nM)				
	hCA I	hCA II	hCA IX	hCAXII	hCA XIV
1o	4	27	54	1120	7400
1c	14	136	62	3550	2200
2o	2	13	75	4300	16,700
2c	18	18	94	4700	2700
3o	3	40	9	2600	15,900
3c	3	51	376	5000	2600
4o	>10,000	>100,000	2080	>100,000	4800
4c	>10,000	>100,000	>100,000	>25,000	1600
SA	25,000	240	294	37	5400
AZA	250	12	25	5.7	41

** Mean value from at least 3 different measurements.[14] Errors were in the range of ±5% of the obtained value (data not shown).

2o, which cannot interact with His64 or other His residues belonging to the His cluster present in this isoform.[13] Thus, this is a clear-cut example that the two-prong approach[17] is not a general one for designing tight binding CAIs, and more than ever isozyme-selective such compounds, since a bis-sulfonamide devoid of Cu-IDA moieties (**2o**) acts as a better inhibitor than the two-prong, structurally similar compound (**1o**). But what is even more interesting is the fact that the bis-Cu-IDA derivatives **3o** and **3c** showed equipotent, extremely strong hCA I inhibitory activity (K_I of 3 nM), which is intermediate between that of the two-prong inhibitor **1o** (K_I of 4 nM) and the mono-prong bis-sulfonamide **2o** (K_I of 2 nM). Furthermore, the closed ring copper(II) complex **3c** is by far the best hCA I inhibitor among the closed derivatives **1–3** examined here, with the two-prong **1c** (K_I of 14 nM) and bis-sulfonamide **2c** (K_I of 18 nM) compounds being much less effective inhibitors. We cannot explain these data without an X-ray crystal structure for the adduct of hCA I with **3o** or **3c**, but presumably these derivatives may bind to some of the His residues present within hCA I active site (His64, His67, His200, and His243)[13] which lack in other CA isoforms among those investigated here, without interaction with the catalytically critical Zn(II) ion from the enzyme cavity.

(ii) The ubiquitous, physiologically dominant[2] isozyme hCA II is also inhibited significantly by sulfonamides **1**, **2**, AZA and the copper complexes **3** (K_Is in the range of 12–136 nM), being less susceptible to inhibition with sulfanilamide SA (K_I of 240 nM) and not at all inhibited by the scaffold of **4o**/**4c** (Table 1). Again SAR is very interesting, with the open isomers **1o**–**3o** being always better hCA II inhibitors than the corresponding closed ones **1c**–**3c**. As for hCA I, the two-prong compounds **1o**/**1c** are less effective hCA II inhibitors as compared to the mono-prong bis-sulfonamides **2o**/**2c**, but unlike hCA I, the copper complexes **3** are generally weaker inhibitors than the sulfonamides **1** and **2**. It should be also noted that simple sulfonamides such as sulfanilamide and acetazolamide are much better hCA II than hCA I inhibitors, whereas for sulfonamides **1** and **2** incorporating the DTE scaffold, just the opposite is true.

(iii) The tumor-associated isoform hCA IX is moderately inhibited by compounds **1**, **2**, **3c,** and SA (K_Is in the range of 54–376 nM) being strongly inhibited only by the open copper complex **3o** and AZA (K_Is of 9–25 nM). For this isoform, the two-prong inhibitors **1o**/**1c** showed better inhibitory activity as compared to the mono-prong ones **2o**/**2c**, but unexplainably the best inhibitor does not possess a sulfonamide moiety (**3o**). The differences of inhibitory activity between **3o** and **3c** are also quite important, with the closed isomer being 41.7 times less inhibitory than the open one.

(iv) The transmembrane isozymes hCA XII and XIV showed less susceptibility to be inhibited by the compounds investigated here, with inhibition constants in the range of 1.12–15.9 μM, orders of magnitude higher than for the isozymes discussed earlier. As for hCA IX, also for hCA XII and XIV the two prong inhibitors were slightly more effective than the mono-prong ones, whereas the copper complexes **3** showed also a weak inhibitory capacity. Whereas derivatives **4** showed no notable interaction with the enzymes discussed above (except **4o** with hCA IX), these ethers seem to inhibit appreciably (in the low micromolar range) hCA XIV. Further studies are warranted to understand the inhibition mechanism of **4** against hCA XIV since this compound does not possess an obvious structural motif that should interact with the CA active site.

In conclusion we investigated the inhibition of five physiologically relevant CA isoforms with DTE-based compounds incorporating either a sulfonamide and Cu-IDA, bis-sulfonamide, bis-Cu-IDA and bis-ethyleneglycol-methyl ether moieties, in both their open- and closed-ring forms. For hCA I the best inhibitors were the mono-prong bis-sulfonamide and the bis-Cu-IDA complexes (K_Is of 2–3 nM) in their open form. For hCA II, best inhibitors were the open and closed forms of the mono-prong bis-sulfonamide (K_Is of 13–18 nM). hCA IX was moderately inhibited by these compounds (K_Is of 9–376 nM) whereas hCA XII and XIV were less susceptible to inhibition (K_Is of 1.12–16.7 μM). This study clearly proves the lack of usefulness of the two-prong approach for designing both tight-binding as well as isozyme-selective CA inhibitors.

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as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. For each inhibitor 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 (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM 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 inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier,[9] and represent the mean from at least three different determinations. Mammalian CA isozymes were prepared in recombinant form as reported earlier by our group.[15]

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