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Carbonic anhydrase II-induced selection of inhibitors from a dynamic combinatorial library of Schiff's bases

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ABSTRACT

A dynamic combinatorial library (DCL) has been generated under thermodynamic control by using the aminocarbonyl/imine interconversion as reversible chemistry, combined with non-covalent binding within the active site of the metalloenzyme human carbonic anhydrase II (hCA II, EC 4.2.1.1). The high affinity of hCA II isozyme towards some sulfonamide inhibitors obtained here was used to select from the dynamic library specific inhibitors of this isoform. These results point out to the possibility of identifying sulfonamide amplified compounds presenting potent inhibition and high yield of formation in the presence of the isoform(s) towards which the inhibitors were designed.

The identification and the isolation of small molecules, capable of binding specifically and selectively to proteins/enzymes is a central issue situated at the boundaries of chemistry, biology, and medicine. In such a context, combinatorial chemistry has been extensively used in drug-discovery processes as a powerful tool to explore compounds, individually synthesized in parallel through high-throughput methods.[1] Dynamic combinatorial chemistry (DCC) is a new paradigm in drug discovery, that gives access to rapid ligand identification based on simultaneous implementation of reversible molecular assembly and supramolecular recognition processes.[2] The DCC screening is based on a shift of chemical equilibrium in mixtures of reversibly connected components driven by a biomolecular target, resulting in the preferred amplification of one or a few components. DCC has been used in a variety of biomolecular systems, non-exhaustively including lectins,[3] acetylcholine-esterase,[4] neuramidase,[5] galactosyltransferase,[6] glycosidase,[7] DNA,[8] etc.

Carbonic anhydrases (CAs, EC 4.2.1.1) were among the earliest targets used to successfully overcome the challenging synthetic task for which the DCC may offer a complementary route.[9–12] This strategy was pioneered by Huc and Lehn.[9] These authors[9] reported a library of 12 compounds connected by a reversible imino-bond (i.e., Schiff's bases) among which a hydrophobic sulfonamide CA inhibitor possessing excellent affinity toward bovine carbonic anhydrase (bCA II). Then, this methodology has been extended by Nguye and Huc[10] and Poulsen[11] who described the kinetic- and thermodynamic-driven formation of selective bCA II inhibitors, respectively. We recently demonstrated that the specificity of inhibiting the widely spread human (h) isoforms hCA I and hCA II may be used to describe a complex constitutional behavior through component selection from a dynamic library, driven by the selective binding to the inhibitor to a particular isoform active site, giving thus the possibility to obtain isozyme-selective CA inhibitors (CAIs).[12] Indeed, CAs represent an important group of ubiquitously expressed zinc metalloenzymes which are involved in numerous physiological and pathological processes, as they catalyze the reversible hydration of carbon dioxide to bicarbonate and a proton.[13a,b] Among the catalytically active α-CA isozymes currently studied as drug targets, hCA II is one of the most active isoforms which is extremely abundant in most human cells, playing a key role in physiological processes connected with pH homeostasis, ion transport and biosynthetic processes.[13c] Inhibition of this cytosolic isozyme has been considered as an important therapeutic strategy and has been exploited for the design of pharmacological agents for many years.

CAIs were shown to be useful in the treatment and prevention of a variety of diseases such as glaucoma, epilepsy, congestive

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heart failure, mountain sickness, gastric and duodenal ulcers, neurological disorders, osteoporosis, cancer, and obesity among others. The X-ray crystallographic structures of many adducts of this isozyme with sulfonamide, sulfamate, and sulfamide inhibitors have been reported, proving that the deprotonated binding function of the inhibitor is directly bound to the Zn^{2+} ion of the enzyme, which remains in its tetrahedral geometry with the other ligands being His94, His96, and His119 (Scheme 1).[13–16]

Compared with the previous studies on bCA II,[9–11] we propose to widen the scope of the use of the DCC approach on the discovery of CA inhibitors and especially to further explore the inhibition of human hCA II, a pharmacologically relevant isozyme for many biomedical applications (diuretics, antiglaucoma, anticonvulsant drugs, etc.).[13–16]

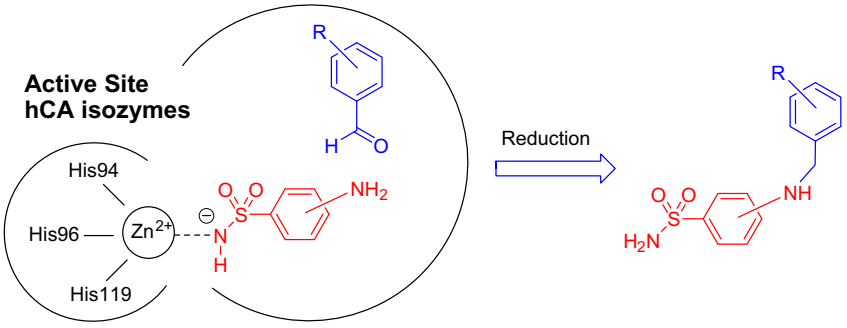
Herein we have developed a dynamic combinatorial library (DCL) of 20 initial constituents that are susceptible to binding to hCA II isozyme using aminocarbonyl/imine interconversion.[9,17,18] In order to demonstrate the use of this reversible chemistry for the CAIs discovery, the inhibition constants K_I against hCA II provided the initial insights toward the strategy of generation of efficient classes of active compounds.

To generate the library, a set of four aldehydes **1**–**4** and 10-fold excess of five amines **A**–**E** (Fig. 1) were selected. Aromatic aldehydes and amines are known to form stable Schiff's bases which are able to create favorable interactions within the enzyme active site of the different carbonic anhydrase isozymes, leading to effective inhibitors.[19]

The structural variability involving different substitution patterns at the aromatic rings is essential for obtaining DCL with diverse binding affinities to the enzyme. Thus, the structural core components were chosen to contain a sulfonamide group (one of the best zinc binding function allowing to generate potent CAIs)[13] among the starting amines (**C**–**E**), whereas the remaining ones incorporate a carboxylic acid moiety (**A**, which is a weak zinc binding function)[20] or a carboxymethyl group (**B**) function unable to bind the zinc ion within CA active site. Different aromatic aldehydes have been selected (**1**–**4**) in order to probe the hydrophobic interactions on the hydrophobic pocket above the active enzyme-site (Fig. 1). Equilibration of amines **A**–**E** with aldehydes **1**–**4** was expected to produce a set of 20 imines (by reaction of the five different amine component with the four different aldehydes) in equilibrium with the nine initial components **A**–**E** and **1**–**4**. The distribution of this mixture would be altered by addition of the hCA II isozyme (equimolar amount vs aldehyde components). After reduction with $NaBH_3CN$ stopping the dynamic exchange at equilibrium, most of the starting and final (secondary) amines of the DCL have been identified by comparison of the retention time in HPLC–UV profiles with those of each pure compound (Fig. 2).

After the reduction the chromatographic analysis of the resulted mixtures in the absence (control experiment) or in the presence of hCA II showed exclusively the presence of reduced secondary imines and the excess of starting amines. We noticed the absence of related alcohols produced by direct reduction of the aldehydes. The overall conversion of the initial aldehydes is 100% in both experiments, probably determined by the 10-fold excess of starting amines. High conversion for imine formation in water has been already observed at this stoichiometry as previously reported.[9,20]

2A, **1B**, **2B**, and **2C** were not identified in the mixture probably due to the weak reactivity of starting aromatic amines **A**–**C** substituted in *para*-position by the electron-withdrawing groups, which



Scheme 1. DCC applied to human hCA II isozyme.

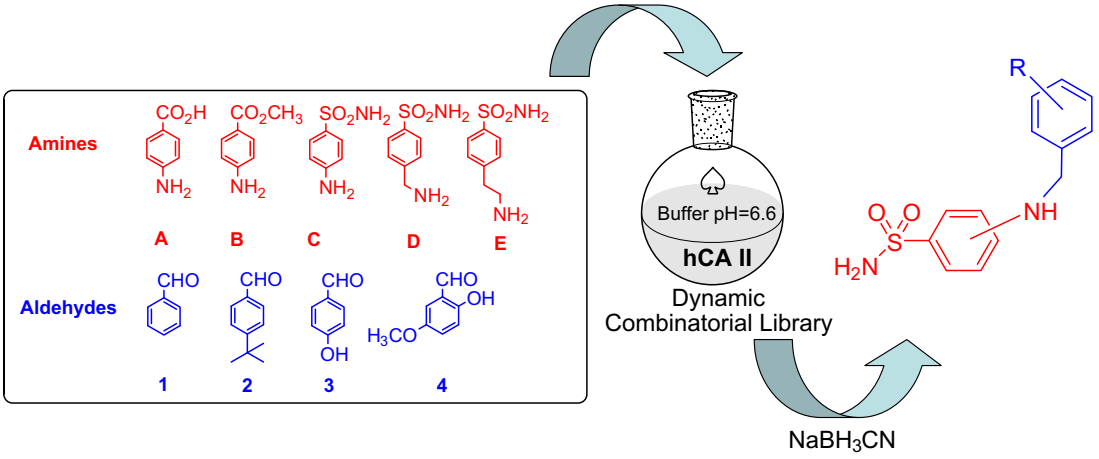


Figure 1. Elaboration of the DCL.

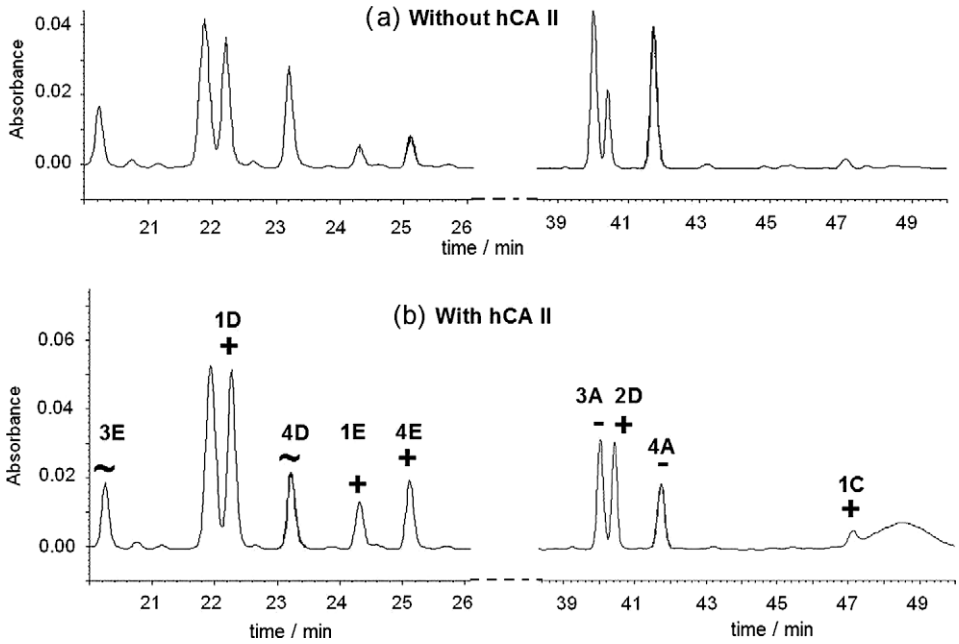


Figure 2. HPLC analysis of generation and screening of the DCL generated (a) in the absence and (b) in the presence of human hCA II isozyme, at equilibrium.

prevent the formation of the imines in aqueous media.[21] Simultaneously, the 20 possible amine compounds have been synthesized separately and their inhibition constant K_I against hCA II have been determined (Table 1). The results obtained from this library generation and screening process correlated with the experimental inhibition constant K_I are presented in Figure 3. We noticed that five amines were amplified in the presence of hCA II. The three amines **1D**, **1C**, and **2D** (red color group in Fig. 3) presented a relative peak area (RPA) above 1.5 and very good inhibition constants confirming the strong inhibition power of the sulfonamide group of **C** and **D** combined to hydrophobic components **1** and **2**. This combined effect cannot be extended to sterically much longer ethylamine **1E**, which showed a low inhibition activity. Obviously an extended linker would result in poorer binding because of a higher entropic loss upon binding, which is not compensated by the hydrophobic

Table 1
Inhibition data (K_Is) against hCA II and retention time of pure compounds **1A**–**4E**, and relative HPLC–UV peak area for the DCL generated in this study[23]

Inhibitor	K_I (nM)	Relative peak area, RPA[a]	HPLC retention time (min)
1A	123.2	0.6	51.26
2A	184.5	ND	62.32
3A	200.5	0.7	39.98
4A	142.5	0.5	41.65
1B	22,171	ND	63.50
2B	158.4	ND	64.31
3B	141.5	0.6	51.69
4B	5329.0	0.3	52.82
1C	21.4	1.6	47.23
2C	32.4	ND	63.53
3C	701.8	0.7	35.03
4C	86.6	0.8	38.00
1D	38.5	1.5	22.21
2D	24.0	1.4	40.28
3D	79.7	ND	34.98
4D	130.5	0.8	23.14
1E	160.0	2.3	23.59
2E	8.6	0.7	41.11
3E	236	1.1	20.15
4E	51.5	2.3	25.10

[a] Relative value of the peak area is calculated using the ratio between the experimental peak area in the presence and in the absence of hCA II.

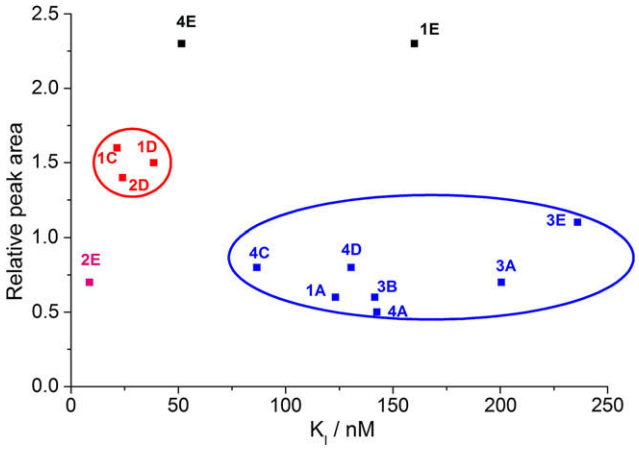


Figure 3. Relative peak area expressing the amplification of the DCL function of inhibitory power (K_I) against hCA II.

interaction of phenyl moiety within the hydrophobic pocket. However, in the meantime compound **1E** was strongly amplified from the DCL (RPA = 2.3), probably as a result of the high binding of the sulfonamide moiety to Zn^{2+} ion of the enzyme. Surprisingly, the hydrophobic and hydrogen-bonding components **2** and **3**, connected to the entropically unfavorable component **E** were both non-amplified from this DCL despite the good inhibition of **2E** against hCA II.

In the meantime a RPA of 2.3 and a very good inhibition against hCA II was experienced for compound **4E**. This derivative might represent a better compromise between unfavorable entropic loss due to the length of component **E** bearing the sulfonamide complexant propriety and the enthalpic gain of combined hydrophobic/hydrogen-bonding binding effects of component **4** present within the hydrophobic pocket. Compound **4E** would then be able to bind in a conformation suitable for fitting into the enzyme active site via the synergistic inhibition/reactional reversible processes. These facts were confirmed by the presence of almost all the possible combinations of component **4** in the mean inhibition/ forma-

tion group of components (blue color in Fig. 3). There are in fact several amino acid residues situated in the middle of the active site cavity of hCA II, such as Asn62, His64, Gln92, and Thr200, which frequently participate in strong hydrogen-bonds with various moieties of the inhibitor, strongly stabilizing the enzyme–inhibitor adduct, as shown by extensive X-ray crystallographic work.[22]

Finally, we also observed that the amplification of **1E**, **4E**, and **2D** increased from 2.3 to 2.7, 2.3 to 2.6, and 1.4 to 1.6, respectively, when the sample was heated, proving that the denaturation of enzyme totally released the inhibitor from the active site.

In conclusion, this study showed that a fine analysis can be performed to identify enzyme–inhibitors and to evaluate their relative affinities toward the relevant pharmacological target hCA II by using DCC. A DCL of 20 components has been generated under thermodynamic control by imine formation and exchange, combined with non-covalent bonding within the enzyme active site. Among all possible imines formed, the active compounds of appropriate geometry were easily identified in competitive reaction conditions. This method enabled the identification of a series of sulfonamide hCA II inhibitors (**1D**, **1C**, and **2D**) presenting a good inhibition profile and high yield formation (except **1C**), with amplification factors of 150–270%, in the presence of the enzyme. Furthermore, these data were beneficial to rapidly identify from a DCL of competitive components compound **4E**, which might represent a better compromise between entropic/enthalpic factors as a result of a combined hydrophobic/hydrogen-bonding binding effects of the component **4** present within the hydrophobic pocket of the enzyme. Finally once the structural features has been found, more precisely defined components can be developed in further studies, allowing the identification of enzyme–inhibitors with better selectivity for the target isoform. Indeed, the family of the carbonic anhydrases, with a large number of representatives (13 catalytically active isoforms in mammals) playing fundamental physiological and pathological functions, can be used as a paradigm in non-conventional drug design studies aimed at obtaining compounds with selectivity for some isoforms, and thus drug candidates with reduced side effects. Although the CA inhibitor field is a small one, our findings may be relevant to the general drug design research, especially when enzyme families with a multitude of members and with similar active site features are targeted.

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18. The reactions were performed in the presence of hCA II in water at pH 6.5. A 10-fold excess of the starting amines was used to limit side reactions between the aldehydes and the terminal amino residues groups of CA. Two reactions were performed with and without enzyme (hCAII, 0.08 mM) in a sodium phosphate solution at pH 6.5 (20 mM phosphate buffer). Stock solution in DMSO of four aldehydes (10 mM) and the two amines (100 mM) were added to aqueous solutions in order to reach the final concentration of 0.08 mM for aldehydes and 0.8 mM for amines, respectively. The clear mixture was incubated at 25 °C for 3 days before addition 0.8 mM of $NaBH_3CN$ and incubated for one more week. The thermal denaturation of the enzyme (2 min at 80 °C) was also tested to ensure the release from casting site of possible tightly bound ligands. The chromatography was performed on a diode array detector ($\lambda = 210$ nm). An Atlantis C_{18} reverse-phase column was used (4.6×100 mm, 3 μm), under a binary gradient of CH_3CN and buffer solution at pH 3.1). The buffer solution was prepared by dissolving KH_2PO_4 in distilled water. The pH was adjusted at 3.1 by addition H_3PO_4. The total concentration of the buffer was fixed at 6.8 g/l. The optimized gradient begins by 100% of buffer solution then reached 45% of buffer solution and 55% of CH_3CN in 60 min. After the reduction the chromatographic analysis of the resulted mixtures in the absence or in the presence of hCAII showed exclusively the presence of reduced secondary imines and the excess of starting amines. We noticed the absence of related alcohols produced by direct reduction of the aldehydes.
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23. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic/inhibition of various CA isozymes. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.4) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength; these anions are not inhibitory in the used concentration), following the CA-catalyzed CO_2 hydration reaction for a period of 5–10 s. Saturated CO_2 solutions in water at 25 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO–water 1:1, v/v) and dilutions up to 1 nM done with the assay buffer mentioned above. At least seven different inhibitor concentrations have been used for measuring the inhibition constant. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and represent the mean from at least three different determinations. hCA II was either recombinant enzyme produced in-house[22] or was purchased from Sigma–Aldrich (Milan, Italy).