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Carbonic Anhydrase-Encoded Dynamic Constitutional Libraries: Toward the Discovery of Isozyme-Specific Inhibitors

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A constitutional dynamic library (CDL) was generated under thermodynamic control by using the amino-carbonyl/imine interconversion as reversible chemistry, combined with noncovalent bonding within the active site of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1). Considering the pharmacological importance to find isoform-selective CA inhibitors (CAIs), two of the 15 human (h) isoform, i.e., hCAI and hCA II, have been subjected to a parallel screening of the same CDL. The use of parallel constitutional screening of CDL chemistry for the discovery of enzyme inhibitors is straightforward and it might provide initial insights toward the generation of efficient classes of selective, high affinity inhibitors. We demonstrate here that the high selectivity and specificity of inhibiting the hCA I and hCA II isozymes with some of the detected hits may be used to describe a complex constitutional behavior through component selection from the dynamic library, driven by the selective binding to the specific isoform active site. These results also point to the possibility of modulating the drug discovery methods by constitutional recomposition induced by a specific enzymatic target.

Introduction

Carbonic anhydrases (CA[a]) represent an important class of ubiquitously expressed zinc metalloenzymes catalyzing the reversible hydration of carbon dioxide to bicarbonate and a proton.[1−3] The clinically available pharmacological agents known to date present weak inhibition selectivity toward of different CA isoforms inducing important side effects. Thus, the development of isozyme-specific inhibitors is currently a great challenge for obtaining novel types of drugs acting in specific physiologic/pathologic processes.[1] Much progress has been achieved in the past decade for identifying selective CA inhibitors (CAIs) by means of rational drug design.[2,3] The emergence of numerous families of selective CA inhibitors against several pharmacologically relevant isozymes are based on specific strategies including X-ray crystal structures for some enzyme−inhibitor complexes.[4] They mostly address major basic structural elements such as the zinc coordinating function[5,6] or the nature of the hydrophobic residue[7−10] lying to the hydrophobic pocket standing above active metal ion binding site. Among the 13 catalytically active α-CA isozymes currently known and studied as the drug targets, human carbonic anhydrases hCA I and hCA II are considered the most selective isoforms. Their inhibition has already offered important biomedical options in the development of antiglaucoma, antiepileptic, antiobesity, or anticancer drugs.

Dynamic combinatorial chemistry (DCC)[11−13] has been extensively implemented during the past decade as a powerful approach in drug discovery[14] that gives access to rapid and attractive identification of ligands and inhibitors for receptors and enzymes. The dynamic combinatorial approach is based on a shift of chemical equilibrium of a library of reversibly connected molecular components encompassing all possible combinations, driven by a biomolecular (molecular) target that favors the amplification of the fittest constituent forming the most stable noncovalent supramolecular entities with the target. DCC has been successfully implemented in a variety of biological systems nonexhaustively including lectins,[15,16] acetylcholinesterase,[17,18] neuraminidase,[19,20] galactosyltransferase,[21] glycosidase,[22] DNA,[23,24] etc.

Carbonic anhydrases (CA) have been one of the early addressed biological targets for which the DCC[25−29] may offer a complementary route to high-throughput combinatorial methods.[30] The first example in this field has been pioneered by Lehn et al., who reported a library of 12 constituents containing different Zn^{2+} complexing groups and various aromatic moieties connected by the reversible imino-bond, generating thus a hydrophobic sulphonamide inhibitor possessing high affinity toward the bovine carbonic anhydrase (bCA II, EC 4.2.1.1).[25] Then, the feasibility of this concept has been extended by Nguyen et al.[26] and Poulsen et al.,[27−29] including a kinetic approach and a thermodynamic approach based of cross-metathesis reversible reaction, all of which addressing the same challenge: discovery of small molecule

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[a] Abbreviations: CDL, constitutional dynamic library; DCC, dynamic combinatorial chemistry; DCL, dynamic combinatorial libraries; CA, carbonic anhydrase; CAI, carbonic anhydrase inhibitors; hCA I, human carbonic anydrase I; hCA II, human carbonic anydrase II; bCAII, bovine carbonic anydrase II; ZBG, zinc-binding groups.

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inhibitors of bCA II, an easily accessible and inexpensive enzyme but not very useful for discovering human CA inhibitors (the two enzymes differ a lot from the point of view of binding inhibitors, crystallographically and kinetically).[1]

One major advantage with reversible dynamic combinatorial libraries (DCLs) over their irreversible counterparts is their potential adaptability to express the sorting constituent in response to an external selection pressure.[11,12] Change in the output constituents of a DCL under the pressure of internal and external factors (stimuli) based on constitutional dynamics[31] express the system adaptation to a given situation.[32] Herein, we report a such CDL that is susceptible to change its composition (output expression) through component selection driven by the selective binding to human hCAI and hCA II isozymes. Considering the pharmacological importance to find selective CAIs isozymes for a specific inhibitor, the human hCAI and hCA II isozymes, known to be more selective (specific) than bovine carbonic anhydrase (bCA II), are subjected to a parallel screening of the same CDL[21] by using the aminocarbonyl/imine interconversion as reversible chemistry. In particular, the use of CDL chemistry for the inhibitors discovery might provide initial insights toward the strategy of generation of efficient classes of selective active compounds, against human carbonic anhydrases, for instance.

Results and Discussion

For achieving this objective, to design a constitutional dynamic library of inhibitors against the ubiquitous and pharmacologically highly relevant isoforms hCAI and hCA II, imine generation and exchange has been used to produce a prototypical DCL.[32−35] In the present study, our choice went to imine libraries of inhibitors resulting from the reversible assembly of a series of amines bearing specific zinc-binding groups (ZBGs) with a set of hydrophobic aldehyde counterparts potentially interacting with the hydrophobic pocket of the enzyme. Subsequent reduction reaction with $NaBH_3CN$ stopped the dynamic exchange at equilibrium, leading thus to the corresponding libraries of amines (Figure 1).

To generate dynamic combinatorial library, a set of three aldehydes, **1**−**3**, and two amines, **A** and **B** (Figure 1), were selected. Aromatic aldehydes bearing electron withdrawing moieties and primary amines are known to form high yield Schiff's bases in water. They are specifically chosen to arrange the interactional components in a given geometry, favorably interacting within the enzyme active site of the different CA isozymes, leading to effective inhibitors.

The structural variability involving different substitution patterns at the aromatic rings is essential for obtaining DCC libraries with diverse binding affinities to the enzyme. Thus, the structural core components were chosen to contain a sulfonamide group (amine **A**) as one of the best ZBG allowing to generate potent CAIs,[37] the sulfonic acid (aldehyde **2**), which is a weak ZBG compared to the sulfamoyl one. For example *p*-toluenesulfonate has a K_I of 460 μM against hCA II, whereas the corresponding sulfonamide of 11 μM[38] and carboxylic acid (aldehyde **3**) moieties also have weak zinc binding functions.[39] Two aromatic hydrophobic moieties such as phenyl (amine **B**) and 2-fluorophenyl (aldehyde **1**) have been selected in order to probe the hydrophobic interactions on the hydrophobic pocket above the active enzyme site (Figure 1).

Equilibration of amines **A**−**B** with aldehydes **1**−**3** was expected to produce a set of six imines in equilibrium with the six initial amines components. The distribution of this mixture has been strongly altered by the addition of human hCAI and hCA II isozymes. After reduction, most of initial primary and secondary amines of the DCL together with the corresponding alcohols resulted by reduction of initial

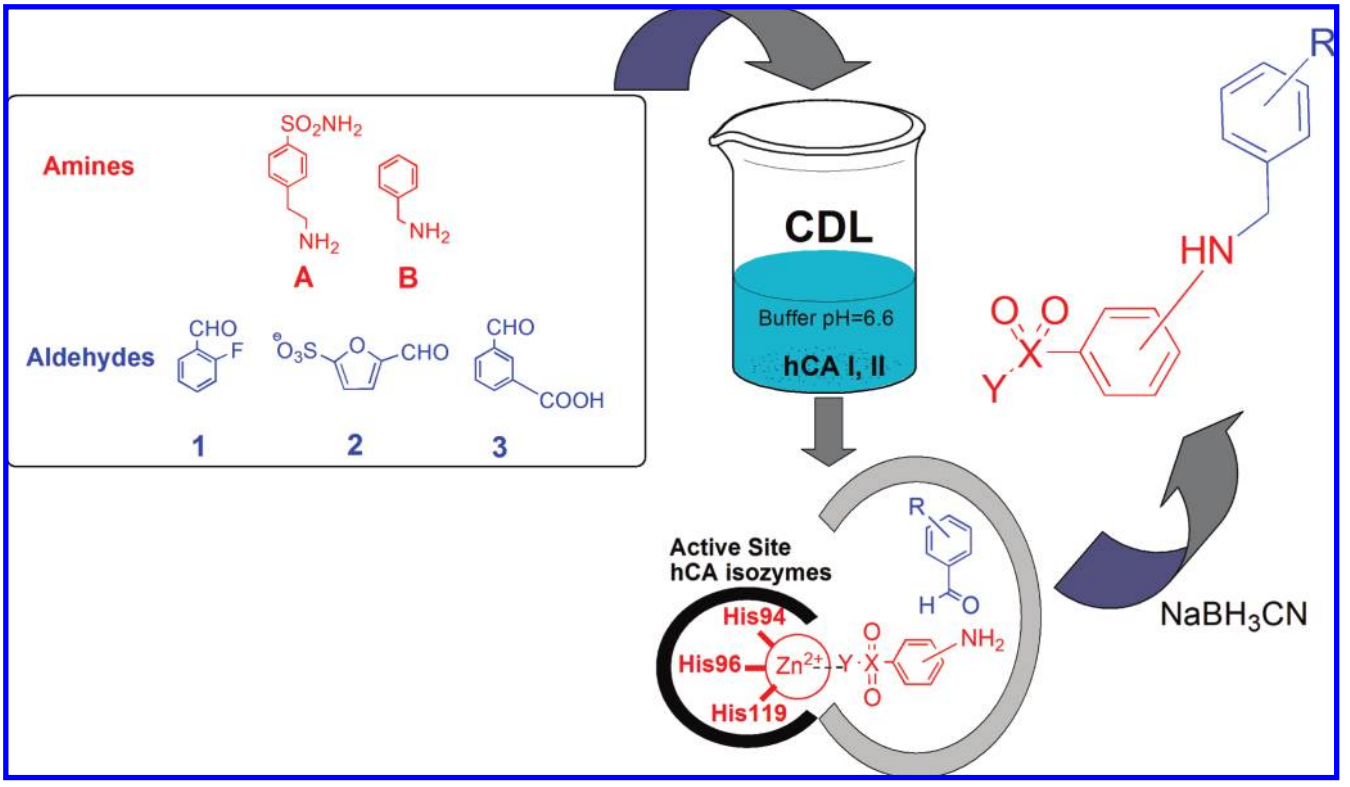

Figure 1. Constitutional dynamic chemistry applied to human carbonic anhydrase hCAI and hCA II isozymes and elaboration of constitutional dynamic library (CDL).

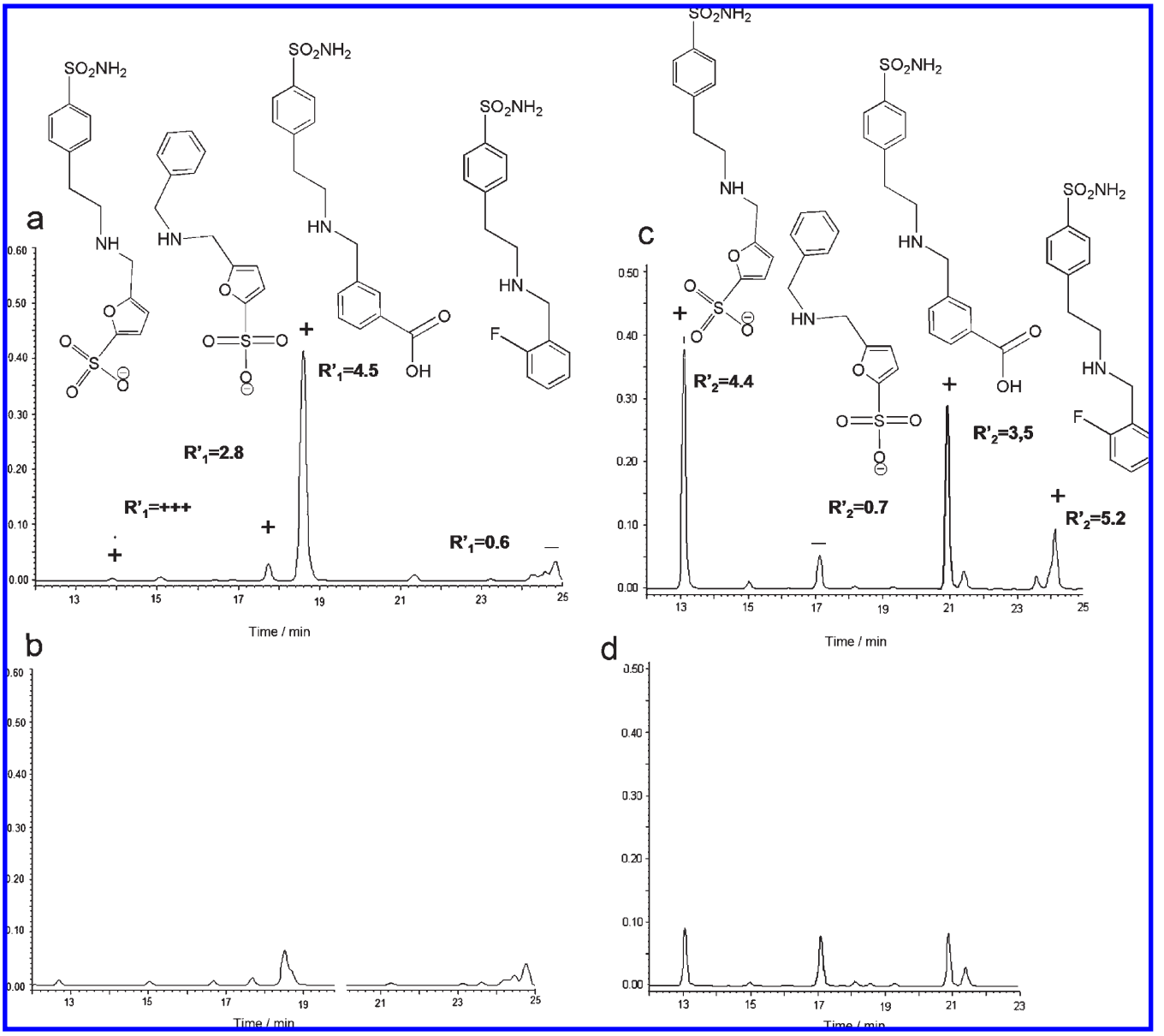

Figure 2. HPLC analysis of generation and screening of the CDL library: (a) in the presence and (b) in the absence of human hCA I isozyme and (c) in the presence and (d) in the absence of human hCA II isozyme.

aldehydes have been identified by comparison of the retention time in HPLC and UV profiles with those of each pure compound. Surprisingly, compound **1B** was not detected in the mixture.

Simultaneously, the six imines $\mathbf{1A_{im}}-\mathbf{3A_{im}}$, $\mathbf{1B_{im}}-\mathbf{3B_{im}}$ and six secondary amines **1A**−**3A**, **1B**−**3B** compounds have been synthesized separately and their inhibition constant K_I against catalytically active human hCA I and hCA II cytosolic isozymes have been determined. Inhibition data correlated with the results obtained from this library generation and screening process, as well as the standard sulphonamide acetazolamide **AZA**, are shown in Figures 2 and 3 and Table 1.

As a general rule, there are also differences of hCA I inhibitory activity between the stereochemically rigid imino compounds and the corresponding saturated mobile amino compounds. It is interesting to note that a rigid imino linker would result in poorer binding, decreasing the inhibitory properties. The difference in inhibition is very important, for compounds $\mathbf{1A_{im}}$/**1A** and $\mathbf{3A_{im}}$/**3A** with the amino derivatives being respectively 3 and 2 times better hCA I inhibitors as compared to imino compounds. This rule is no more valuable in the case of very selective hCA II isozyme for which both imino/amino compounds present almost the same the inhibitory properties.

The following structure−activity/CDL evolution relationship can be drawn by considering data of Table 1:

(a) In the presence of hCA I only two amines: **3A** and **2B** were amplified (Figures 2 and 3). The amine **3A** presented an important amplification above 350% and very good inhibition constants, confirming the strong inhibition power of sulfonamide group combined to hydrophobic/H bonding effects of the carboxylic group of the component **3** present in hydrophobic pocket. There are several cases of compounds containing COOH moieties, which act as very effective hCA I/II inhibitors, the best studied one being furosemide, for which an X-ray crystal structure is available. This compound is a good inhibitor of both hCA I (K_I of 62 nM)

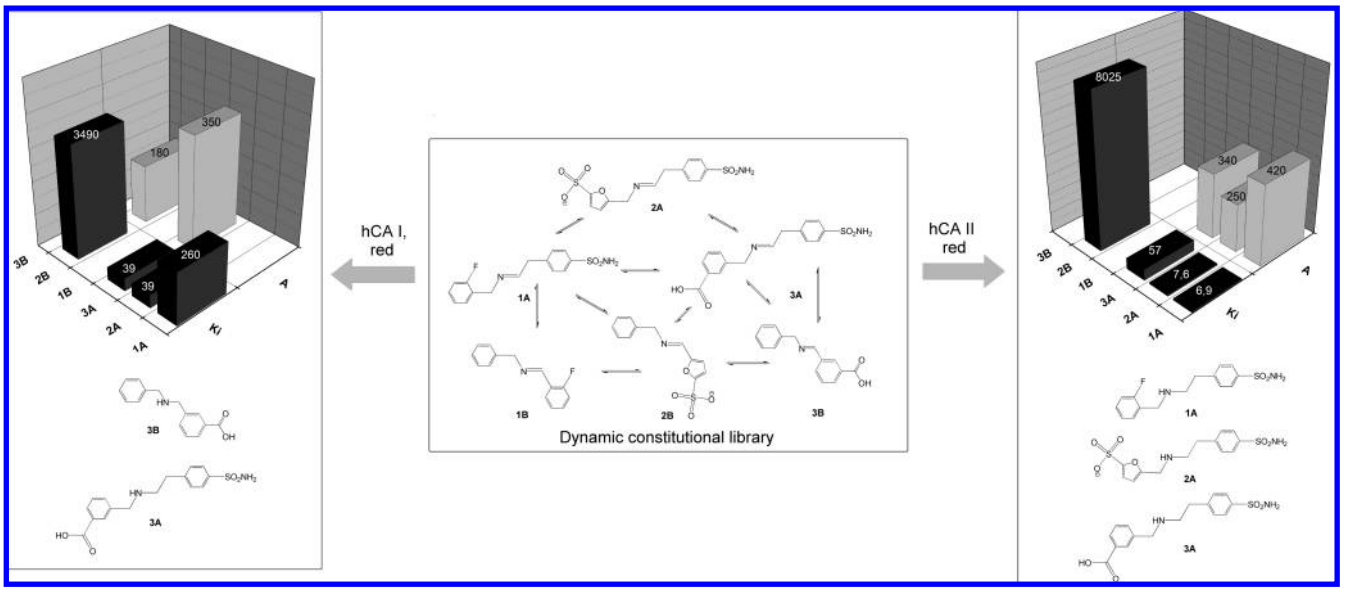

Figure 3. Inhibition constants K_I and the amplification of the constitutional dynamic library (CDL) against catalytically active human hCA I and hCA II cytosolic isosymes.

Table 1. Inhibition Constants K_I against hCA I and hCA II of Pure Compounds **1A**−**3A** and **1B**−**3B**, the Relative Peak Area, and the Amplification of the CDL in the Presence of hCA I and hCA II

	K_I (nM)[a]		relative peak area, RPA[b]		amplification factor[c] %	
inhibitors	hCA I	hCA II	hCA I	hCA II	hCA I	hCA II
1A$_{im}$	620	7.2				
1A	260	6.9	0.6	5.2		420
2A$_{im}$	35	4.9				
2A	39	7.6		4.4		340
3A$_{im}$	65	51				
3A	39	57	4.5	3.5	350	250
1B$_{im}$	> 100000	> 100000				
1B	> 100000	> 100000				
2B$_{im}$	8960	> 100000				
2B	3490	8025	2.8	0.7	180	
3B$_{im}$	> 100000	> 100000				
3B	> 100000	> 100000				
AZA[d]	250	12				

[a] From 3 different determinations. Errors: ±5−10% of the shown value. [b] Relative value of the peak area is calculated using the ratio between the experimental peak area in the presence and in the absence of CA: RPA = S_{CA}/S_0 [c] We speak about *amplification* when the peak area in the presence of CA is higher than in the absence of CA. It is calculated as following: $A\ \% = [(S_{CA}-S_0)/S_0] \times 100$. [d] Acetazolamide (AZA) was used as standard drug for inhibition constant determination.

and of hCA II (K_I of 65 nM).[40] It is very interesting to note the selective formation of amine **3A** in the presence of **1A** and **2A** compounds. The presence of 2-fluoride substituent in aldehyde component **1** leads to a dramatic decrease of enzyme inhibitory activity, resulting in poorer binding and thus reduced reactivity into the enzyme hydrophobic site. Moreover, the amine **2A**,[41] presenting the similar inhibitory activity against hCA I with **3A**, is practically not formed in this CDL, probably as a result of the weak reactivity of the furansulfonic aldehyde **2**.

(b) The strong inhibition power of sulfonamide group is confirmed by the strong amplification of compounds **1A**−**3A** in the presence of hCA II one of the most active (selective) isoforms of CA family. The selective amplification of all the possible combinations of component **A** against the similar combinations of component **B**, which are not present in the reactional mixture in the presence of hCA II, is probably due to the strong inhibitory power of amines **1A**−**3A**, sterically better fitting into the enzyme active site than **1B**−**3B** (Table 1). Surprisingly, the hydrophobic component **1A** with the same inhibition against hCA II like **2A** (amplification of 340%), presented the higher amplification of 420%. It is in fact documented that the fluorine atoms present in CAIs may form hydrogen bonds and other favorable interactions with amino acid residues from the active site, leading thus to enhanced stability of the enzyme−inhibitor complex.[42,43] In the same time, the presence of 3-carboxyl substituent in aldehyde component **3** leads to a 10 times weaker hCA II inhibitory activity, resulting in poorer enzyme binding and thus reduced reactivity (amplification of 250%), probably due to a clash between this moiety and some amino acid residue form the hCA II active site, as evidenced earlier by this group for a sugar sulfamide derivative.[44] We conclude that the specific hydrophobic or H bonding binding sites present in the hydrophobic pocket of the enzyme connected to the sulfonamide group of

component **A** control the composition of the resulted CDL by the synergistic inhibition/reactional phenomena as a result of the optimized enthalpic/entropic factors. The strong enthalpic effect of the sulphonamide binding seems to be not reliable to the compounds **1B**−**3B** presenting low inhibition activity.

Conclusion

In this study, it has been shown that a fine analysis can be performed to identify new inhibitors and to evaluate their relative affinities toward the physiologically relevant human carbonic anhydrase hCA I and hCA II isozymes. A dynamic combinatorial library of six components can be generated under thermodynamic control by imine formation and exchange combined with noncovalent bonding within the enzyme binding site. The observed high selectivity and specificity of hCA I and hCA II isozymes may be used to describe the complex behavior displayed by the constitutional recomposition of a dynamic library under the distinct and specific templating effect of the two enzymes.[21,31,45] In this context, the constitutional dynamic library (CDL) presented in this paper is susceptible to change its composition (output expression) through component selection driven by the selective binding to human hCAI and hCA II isozymes. Among all possible imines formed, active compounds of appropriate geometry can be easily identified in competitive reactional conditions. This method enables the identification of a series of three sulfonamide inhibitors **1A**−**3A** presenting a good inhibition and potent formation with high amplification factors (250−420%) in the presence of hCA II isosyme. Moreover, the dynamic screening process was beneficial to finely identify compound **2B**, as potent inhibitor of hCA I isosyme. The compound **3A**, which might represent the better compromise between entropic/enthalpic factors as a result of a combined binding effects such as the sulphonamide zinc-coordinating function of the component A and the hydrophobic/H-bonding of the component **4** present in hydrophobic pocket. Finally, once the fittest structural features have been found, the results illustrate the response of such a dynamic constitutional system to the presence of two different enzymatic effects, demonstrating thus the adaptive behavior of the system under the pressure of external factors. Such features are of great interest for the development of drugs targeting enzymes family with many isoforms such as the family of the carbonic anhydrases. Indeed, a large number of representatives (13 catalytically active isoforms in mammals) playing fundamental physiological and pathological functions are known for the mammalian CAs, and these findings can be used as a paradigm in nonconventional drug design studies aimed at obtaining compounds with selectivity for some isoforms and thus drug candidates with reduced side effects. Our findings may be thus relevant to the general drug design research, especially when enzyme families with a multitude of members and with similar active site features are targeted.

Experimental Section

Materials and Methods. Carbonic anhydrase hCA I or hCA II isosymes from human erythrocytes (Sigma), benzylamine 99.5% (Sigma), 4-(2-aminoethyl)benzenzsulfonamide, 99% (Across Organics), 2-fluorobenzaldehyde 98%, 3-carboxy-benzaldehyde 95% (Fluka), and 5-formyl-2-furansulfonic acid sodium salt hydrates 97% (Alfa Aesar) were purchased and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on an ARX 300 MHz Bruker spectrometer in CD_3OD and D_2O with the use of the residual solvent peak as reference. The assignments were made on the base of the COSY and NOESY spectra. Mass spectrometric studies were performed in the positive ion mode using a quadrupole mass spectrometer (Micromass, Platform II). Samples were continuously introduced into the mass spectrometer through a Waters 616HPLC pump. The temperature (60 °C) and the extraction cone voltage (V_c = 5−10 V) were usually set to avoid fragmentations. The purity of synthesized imine and amine compounds was determined by HPLC method (see below and Supporting Information), confirming >95% purity.

General Procedure of Imine 1−3A$_{im}$ and 1−3B$_{im}$ Synthesis. One mmol of the starting aldehyde (**1**−**3**) and 1 mmol of corresponding amine (**A**, **B**) were dissolved in a CH_3CN and stirred at 70 °C for 18 h. The reaction mixture was than evaporated and the crude product was dissolved in a minimum of CH_3CN and precipitated in hexane or ethylic ether. The purity of synthesized imine compounds was determined by HPLC method (see below and Supporting Information), confirming >95% purity.

General Procedure of Amine 1−3A and 1−3B Synthesis. To a solution of imine (1 mmol) in methanol, we added drop by drop a methanolic solution of sodium borohydride ($NaBH_4$) (10 mmol). After 4 h, the product was purified by a semipreparative C18 silica column by using the methanol as eluent. The purity of synthesized imine compounds was determined by HPLC method (see below and Supporting Information), confirming >95% purity.

^{1}H NMR and ESI MS characterization data of the most amplified compounds **1**−**3A** and **2B** are the following:

***N*-(2-Fluorobenzyl)(4-sulphonamide phenyl)ethanamine (1A).** Yield 32%. ^{1}H NMR (300 MHz, D_2O) δ = 7.78 (td, 1H, CH-5′), 7.71 (d, 2H, CH-3,5), 7.38 (td, 1H, CH-2′), 7.32 (d, 2H, CH-6,2), 6.95−7.01 (m, 2H, CH-3′,4′), 3.3 (t, 2H, CH_2−N), 2.90 (t, 2H, CH_2−Ar). $C_{17}H_{15}FN_2O_2S$, mol wt: 308.36. ES-MS: *m*/*z* (%) *m*/*z* 309.2 (90) MH^+, 349.1(90) $[MCH_3CNH]^+$.

***N*-(5-Methane-2-furansulfonicacid)(4-sulfonamidephenyl)-ethanamine (2A).** Yield 46%. ^{1}H NMR (300 MHz, D_2O) δ = 7.81 (d, 2H, CH-3,5), 7.44 (d, 2H, CH-2,4), 6.78 (d, 1H, CH furane), 6.61 (d, 1H, CH furane), 3.29 (t, 2H, CH_2−N), 3.07 (t, 2H, CH_2−Ar). $C_{15}H_{13}N_2O_6S_2$, mol wt: 359.38. ES^--MS: *m*/*z* 359 (100) M^-.

***N*-(3-Carboxybenzyl)(4-sulphonamidephenyl)ethanamine (3A).** Yield 36%. ^{1}H NMR (300 MHz, D_2O) δ = 7.90 (s, 1H, CH-6′), 7.83−7.03 (m, 3H, CH-4′,3,5), 7.45 (m, 4H, CH-2′,3′,2,6), 3.72 (t, 2H, CH_2−N), 2.79 (t, 2H, CH_2−Ar), 2.03 (s, 2H, NH_2). $C_{18}H_{16}N_2O_4S$, mol wt: 334.38. ES^--MS: *m*/*z* 333.3 (100) MH^-.

***N*-(5-Methane-2-furansulfonicacid)(phenyl)methanamine (2B).** Yield 46%. ^{1}H NMR (300 MHz, D_2O) δ = 7.39 (m, 5H, CH Ar 2−6), 6.78 (d, 1H, CH furane), 6.60 (d, 1H, CH furane), 4.16 (s, 2H, CH_2). $C_{14}H_{10}NO_4S$, mol wt: 266.28. ES^+-MS: *m*/*z* 267.1 (100) MH^+. ES^--MS: *m*/*z* 266.2 (100) M^-.

For ^{1}H NMR and ESI MS characterization data of the nonamplified compounds, see Supporting Information.

Synthesis of DCL Libraries. The reactions were performed in the presence of hCA I or 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. The aldehydes and amines reacted reversibly to form imines with percentages that vary according to the reactivity of the starting amines and aldehydes and to their affinity for the casting site on CA.[46] Three reactions were performed with and without enzyme (hCAI or hCAII) in a sodium phosphate solution at pH 6.5 (20 mM phosphate buffer). Stock solution in DMSO of three aldehydes (10 mM) and the two amines (100 mM) were added to three3 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. Prior to analyzing the library, the mixture was left for 2 h to be separated from CA by decantation. The thermal denaturation of the enzyme (2 min at 80 °C) was also tested to ensure the release from casting site of some possible tightly bound ligands.

HPLC Screening Method. The chromatography was performed on a diode array detector. An Atlantis C_{18} reverse-phase column was used (4.6 mm × 100 mm, 3 μm), under a binary gradient of CH_3CN and buffer solution at pH 3.1. The purity of synthesized imine and amine compounds have been determined by HPLC. The buffer solution was prepared by dissolving KH_2PO_4 in distillated 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 hCAI or 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. The overall conversion of the initial aldehydes is 100%, 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.[25,46]

CA Inhibition Assay. An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument was used to assay the catalytic/inhibition of various CA isozymes as reported by Khalifah.[36] Phenol Red (at a concentration of 0.2 mM) was 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 or $NaClO_4$ (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 (see Supporting Information for the synthesis of pure compounds details) for 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 nonlinear least-squares methods using PRISM 3 and represent the mean from at least three different determinations.

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Supporting Information Available: HPLC screening method details; UV, NMR, and ESI-MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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