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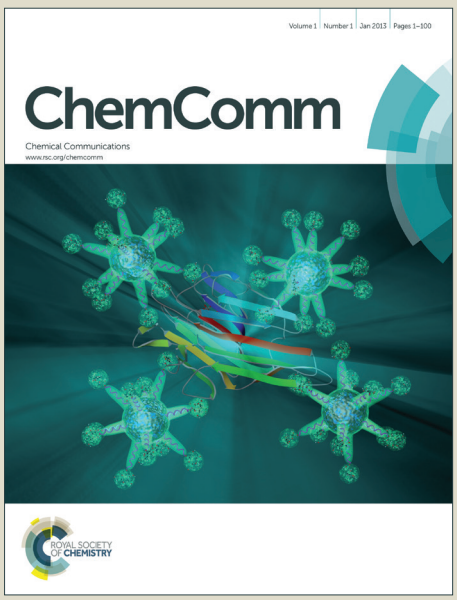





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# Dominant behaviours in the expression of human of Carbonic Anhydrase hCA I activity

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**Here we describe the screening via Dynamic Deconvolution of DCLs of inhibitors (CAIs) and activators (CAAs) of hCA I. The inhibitory effects dominate over the activating ones, while the CAAs may be identified in the absence of CAIs.**

Constitutional Dynamic Chemistry (CDC)[1-8] and its application Dynamic Combinatorial Chemistry (DCC)[9-18] are new evolutional approaches to obtain *chemical diversity*. By virtue of the reversible interchanges, a Dynamic Combinatorial Library - DCL, virtually forming all possible combinations of building components, can adapt to a biotarget, that can be used to select an active ligand. Lectins,[19-22] acetylcholinesterase,[23,24] neuraminidase,[25,26] galactosyltransferase,[27] glycosidase,[28] DNA,[29,30] have been used as biotargets. Carbonic Anhydrase (CAs)[30-34] catalysing the reversible hydration of carbon dioxide to bicarbonate and a proton, has been one of the early addressed biotarget for DCC.[5] The pioneering work of Lehn *et al.*,[35] demonstrated that a known inhibitor of the bovine CA (bCA II, EC4.2.1.1) was amplified form a DCL and the feasibility has been further proven by other groups.[36-39] Various DCLs generated under thermodynamic control have been evaluated by our groups for their relative inhibition toward the physiologically relevant human CAs: hCA I and hCA II, the most active isoforms and studied as a drug targets.[40] They are able to differentiate amplified binders, under the specific binding effect of the two isozymes.[41] The literature survey allow the following conclusions to be made: a) important progress has been achieved in the past two decades for identifying CAIs by using DCLs; b) no DCLs studies were dedicated to the chemistry of CAAs so far; c) the use of DCC for the discovery of CAIs/CAAs might provide insights in the discovery of efficient classes of active compounds, against 16CA isoforms known nowadays in humans.[31] Herein, we report DCLs of components susceptible to selective binding to the hCA I, both as inhibitors or as activators, subjected to a parallel screening by using the amino-carbonyl/imine reversible chemistry. We investigate whether the competitive generation of potent CAIs and CAAs could be selectively expressed as an *independent* (linear), an *interfering* (crossover) or a *dominant* behaviour of the above mentioned events. The reversible formation of a Schiff's base is an advantageous reaction for generating DCLs, because the formation and component interchange processes are faster in slightly acidic aqueous solutions (PBS buffer, pH=6.5).

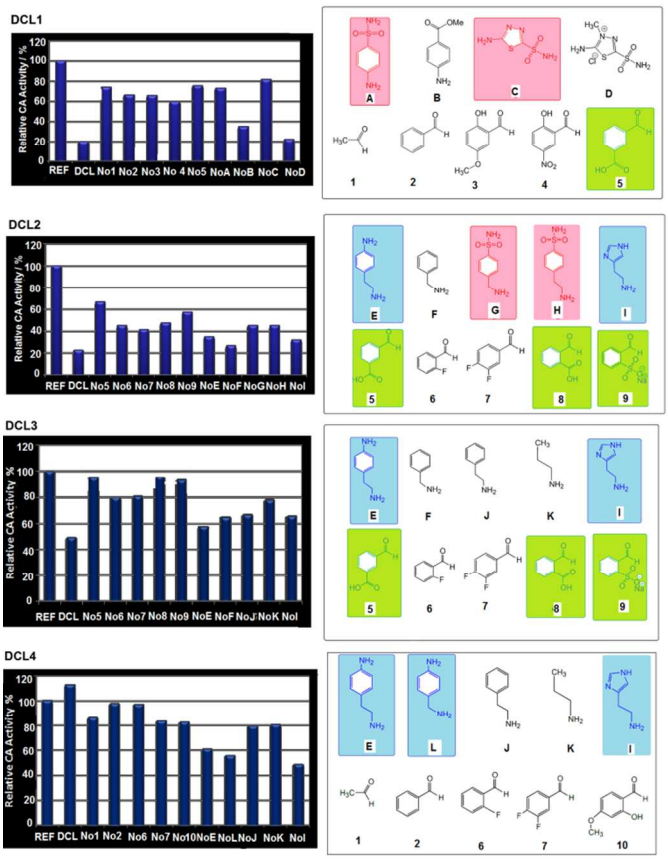
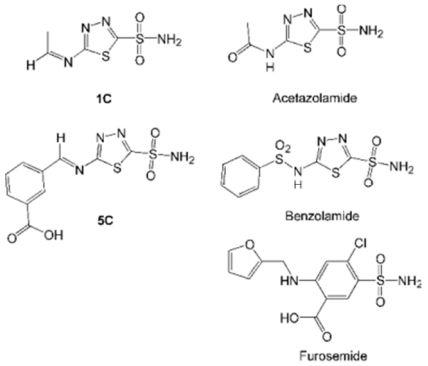


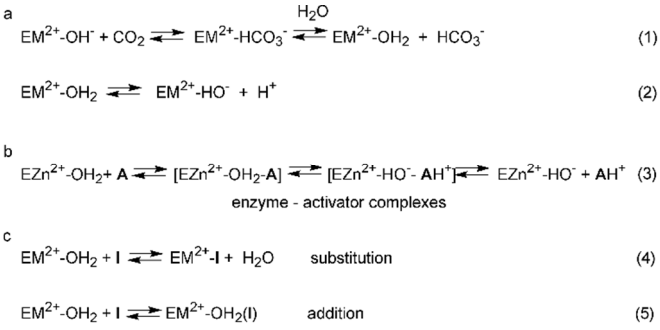

**Fig. 1.** Dynamic Deconvolution of the **DCL1-4**. **DCL1** contains mixtures of active strong-inhibitor-components (pink); **DCL2** containing mixtures of active strong-inhibitor (pink) and activator (blue) components; **DCL3** containing mixtures of active low-inhibitor (green) and activator (blue) components and **DCL4** containing mixtures of activator (blue) components. All libraries contain inactive components represented in black supposed to not bind the active metal site but interacting in the hydrophobic pocket of the enzyme. In the presence of strong metal binding site inhibitors, the low-inhibitors might be located in the hydrophobic pocket of the enzyme.

However, the quantitative analysis of the final DCL mixture became very complicate and time-consuming, when using large numbers of building blocks.[40,41] One more efficient way is the Dynamic Deconvolution procedure reported by Ramström and Lehn,[20,23] based on the sequential, one by one removal of the starting components of a given DCL, followed by the determination of the enzyme activity. A decrease in the inhibition/ activation effects compared with the presence of all components of the DCL, is indicative that the removed component is an important part of an active molecule that can be generated from the DCL. The **DCL1-4** and the corresponding deconvoluted libraries have been generated in the presence of hCA I, from all building components and from all except one component, respectively, and then we measured the enzymatic activity. The choice of the DCL components mostly address major basic structural elements, such as strong of low zinc coordinating function[42-44] or the nature of the residues[45] lying via donor and acceptor H-bonding, or hydrophobic interactions within the hydrophobic pocket of the enzyme.[46] The resultant relative activities to the reference samples of hCAI without any component, are presented in Figure 1. The **DCL1,** containing active strong-inhibitor-components, showed 80% relative inhibition of the reference hCAI activity, indicating the presence of active CAIs in the equilibrated mixture (Figure 1). On removal of a specific building block from the **DCL1**, a decrease in inhibition indicates that the component omitted, contributed to the inhibition of hCA I. For the **DCL1** all the aldehyde counterparts showed important effects, but the largest effects were observed when either **1** or **5** have been removed from the pool. Conversely, the amine counterparts **A** or **C** showed important effects, so that the most active combinations as CAIs are **1A**, **5A**, **1C** or **5C**. In fact, compounds **1C** and **5C** have a structural similarity to two clinically used compounds, acetazolamide **AAZ** and benzol-amide **BZA** (an orphan drug), (Scheme 1) which are highly effective inhibitors of hCA I.[31] Then, by mixing strong inhibitor-type (**G**, **H**) and activator-type (**E**,**I**) components together with hydrophobic (**6**,**7**) and H-bonding (**5**, **8**, **9**) counterparts within **DCL2,** the hCA I activity showed 78% relative inhibition when compared with the reference one (Figure 1). As previously observed, most hydrophobic components proved to be active but the largest effect is arising on removal of the H-bonding components **5** and **9**, binding the hydrophobic pocket. The fluorinated components **6** and **7** are less active, in accordance with their low inhibition activity on hCA I ($K_I$=620 nM).[41] More important, the inhibitor- components **G** and **H** showed when removed from the pool, *dominant* effects at the expense of the activator-ones **E** and **I**- see the detailed discussion bellow (Scheme 2) about mechanistic behaviours governing this assumption. The most active inhibitors of **DCL2** are amino reduced analogues of **5G**, **9G**, ($K_I$=65 nM)[41] **5H** and **9H** ($K_I$=35 nM for a similar 5-propylaminofuran-2-sulfonate inhibitor)[41].




**Scheme 1.** Structural similarity between the potent inhibitors discovered herein and clinically used drugs as inhibitors of hCAI.

These compounds confirm the strong inhibitory power of the –$SO_2NH_2$ group combined to H bonding interactions of the –COOH and –$SO_3H$ groups in hydrophobic pocket. Amazingly, on removal of the strong-inhibitor components **G** and **H**, in the **DCL3**, the data show less effective, but still inhibition (50%) of the hCA I activity compared to **DCL1** and **DCL2** (20%) (Figure 1). Within the **DCL3** the carboxylic-type **5**, **8** and sulfonic-type, **9** used as hydrophobic components show multiple expression and exhibit within this context differently low-inhibitory effects, as proved by almost recovery of the reference activity when these components have been removed from the pool. Moreover their inhibitory activity is still *dominant* at the expense of the activator **E** and **I** components. Some reports described the low-inhibition of CAs with carboxylate, acetate or salicylic acid, with inhibition constants in the range of mM.[46a] There are also several cases of compounds containing -COOH moieties the best studied one being furosemide (Scheme 1), which act as very effective hCAI ($K_I$ of 62 nM) and of hCAII ($K_I$ of 65 nM) inhibitors.[46b] On the complete removal of both strong- (**G** and **H** in **DCL2**) and low-inhibitor (**5**, **8** and **9** in **DCL3**) components within **DCL4**, 10% relative activation activity of the hCAI has been observed (Figure 1). This is reminiscent with the fact that components **E** and **I** are effective activators of hCAI, only in the absence of the inhibitor-type components. On the removal of a component from the **DCL4**, a decrease in activation indicates that the omitted component contributed to the activation of hCA I. For the **DCL4** all the aldehyde counterparts show some effects but the largest effects were observed when components **1**, **7** or **8** have been removed from the pool. Similar to the amine counterparts **E** or **I**, the fragment **L** show important effects, so that the most active derivatives **1E**, **7E**, **10E**, **1L**, **7L**, **10L**, **1I**, **7I** or **10I** combine the activation power of the amine[47] and imidazole[48] groups for which both a hydrophobic and H-bonding effects lead to interactions with the active site as shown by means of X-ray crystallography for the adduct of hCAI with L-His.[48]




**Scheme 2.** a) The $CO_2$ hydration mechanism with carbonic anhydrase-CA; b) The activation and c) the inhibition CA mechanisms in the presence of Activator **A** and Inhibitor **I**, molecules respectively.

The physiological reaction catalysed by the CAs involves the nucleophilic attack metal-bound hydroxide on the $CO_2$, optimally positioned and orientated in a hydrophobic binding pocket of the enzyme. Bicarbonate formed in this way is then replaced by a water molecule, with generation of the catalytically inactive (acidic) form of the enzyme $EM^{2+}$-$OH_2$ (*Eq. 1*), where $M^{2+}$ is $Zn^{2+}$ for hCA I (α-CAs), the isoform discussed here (Scheme 2a). In order to regenerate the catalytically active form, a proton transfer reaction occurs from the water bound to $Zn^{2+}$ within the enzyme active site, to the external medium. In most α-CAs this step (*Eq. 2*) is assisted by an active site amino-acid residue (e.g. His 64), placed in the middle of the cavity or at the entrance of the active site.[47] In the presence of activators Eq. *2* becomes *3*, it has been demonstrated that they participate in the proton transfer processes (Scheme 2b). The activator-binding site is

placed at the entrance of the active site cavity, in a region exposed to the solvent. Most CAAs investigated so far showed μM affinity (although some nM CAAs were also reported).[49-51] Differently, the inhibitors bind deep within the active site and coordinate to the $Zn^{2+}$ ion (Scheme 2c, *Eq. 4,5*).[52,53] This explains why many strong CAIs have low nM affinity. Such a difference in the binding sites of the CAAs compared to the CAIs also explain our present findings why the inhibitory effects dominate over the activating ones in DCLs containing both inhibitors and activators of the hCA I.

Our findings show that DCLs-Carbonic Anhydrase story may reserve novel surprises, relevant to the general drug design research, especially when enzyme families like CAs with a multitude of members. The present study revealed a new paradigm: if compounds of agonistic inhibitor and activator activities are formed, the Dynamic Deconvolution,[20,23] may lead to the discovery of the inhibitory set of components expressed at the expense of the activators ones. This shed light on the dominant mechanistic inhibition behaviours. Moreover the simplicity of the Dynamic Deconvolution strategy and of its analysis can be easily led to valuable simple mechanistic insights on inhibitory/activatory relative synergistic affinities toward the h CAs. This contribution adds several new behaviours to the systematic rationalization and prediction of novel CA active compounds.

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## Notes and references

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