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A Selenolesterase Enzyme Activity of Carbonic Anhydrases

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Carbonic Anhydrases (CAs, E.C. 4.2.1.1) are metalloenzymes expressed on a variety of cell types. Their overexpression leads to serious pathologies, including cancer. The discovery of a series of selenolesters with high structural diversity as novel CA inhibitors is reported here. These compounds showed a remarkable *in vitro* inhibition against a panel of human CA isoforms such as hCA I, II, IX and XII. We observed that they undergo a CA mediated hydrolysis, releasing different active selenol fragments, which act as CA inhibitors. Notably, to the best of our knowledge, this is the first example of an enzyme with selenolesterase activity. In addition, X-ray crystallographic data support the proposed mechanism, proving selenolesters as novel pro-drug inhibitors with potential pharmacological applications.

Carbonic Anhydrases (CAs, E.C. 4.2.1.1) are ubiquitous metalloenzymes expressed in all life kingdoms.[1] Eight different and non-related gene families were discovered to date[1,2] and all reversibly catalyse the hydration of carbon dioxide to carbonic acid.[3] This reaction plays a pivotal role in several biological systems such as pH and CO_2 homeostasis as the main ones.[4] Inhibition of CAs was used since 70 years to treat a range of diseases including oedema, glaucoma, obesity, epilepsy, osteoporosis and, recently, hypoxic tumors in which CA IX is overexpressed.[5] The diverse kinetic profiles of 12 human CA isoforms (i.e. 3 are devoid of any activity) are the basis of the clinical applications of several kind of inhibitors. In this spot is the new born research on organoselenium compounds as modulators of CAs[6] and, selenols were shown to act as a new and potent inhibitory chemotype.[7] However the relative instability of selenols into a physiological environment makes them difficult to be considered for biomedical purposes. To overcome such an issue we envisaged to design and synthesise variously substituted selenolesters as masked selenols, which we hypothesized that might be activated by hydrolytic processes of CAs, considering the fact that these enzymes also possess esterase,[8a,b] thioesterase,[8c] sulfatase,[8d] and phosphatase[8e] activities.

Since the above mentioned hCA properties were also well suited with the versatility and the reactivity of chalcogenoesters[9] our idea of selenolesters as prodrugs of the recently discovered selenol CAI moieties was sustained.[7]

In this context, it is worthwhile mentioning that, owing to their peculiar properties and reactivity, organoselenium compounds play a key role in chemical sciences, finding wide applications in organic synthesis,[10] material science,[11] polymer chemistry,[12] medicinal chemistry, and biology.[13] A number of selenium-containing organic molecules have been indeed demonstrated to possess antioxidant,[14] anticancer,[15] and cells growth inhibitor[16] properties. Therefore, the study of novel biologically active selenium-containing systems would be highly desirable in order to develop new drug candidates.

Hereby, we report our findings on the synthesis and the study in vitro of the CA inhibitory activity of a wide variety of differently substituted and functionalised aryl- and alkyl-selenolesters. X-ray studies were thereafter undertaken in order to unravel the mechanism by which these new selenium containing potential prodrugs are activated by the enzymes.

The unique reactivity of the SeH group allowed its functionalization under very mild reaction conditions with a wide variety of electrophiles.[17] In order to develop a general and direct route towards selenolesters, we were attracted by the possibility of using aromatic and aliphatic selenols as valuable precursors of selenolate anions that, in the presence of a weak base, would react with acyl chlorides to afford the desired compounds. Indeed, the harsh reaction conditions (strong reducing agents or strong bases)[18] represent, together with the use of metal catalysts, one of the main drawbacks of the existing methodologies towards selenolesters.[19]

We began by studying the synthesis of derivative **2a** from benzeneselenol **1a** and benzoyl chloride. On the basis of our

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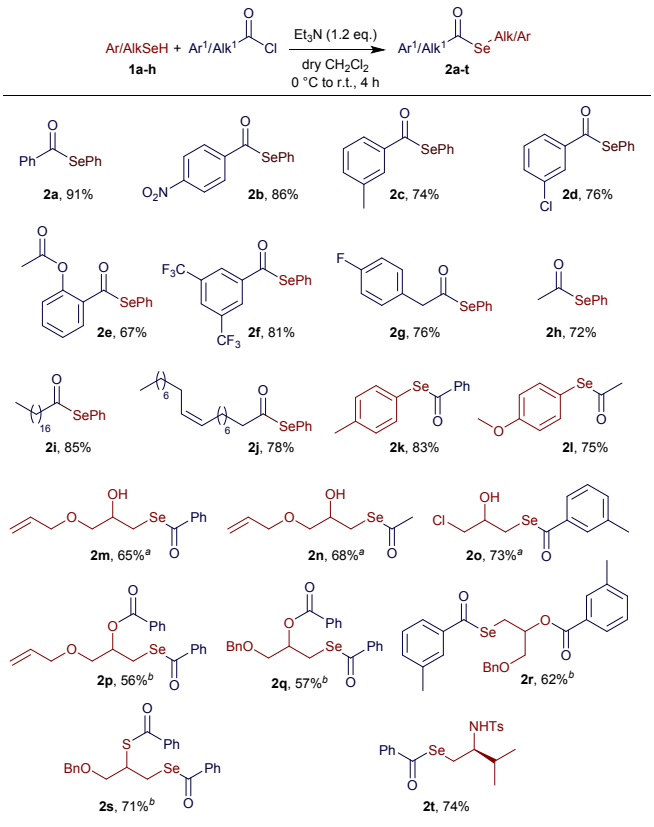
recent reports,[20] weak organic (Et_3N and $^iPr_2EtN$) and inorganic (Cs_2CO_3) bases were evaluated in order to promote the desired selenoacylation reaction. Different solvents (THF, DMF, CH_2Cl_2) and temperatures (-78 °C, 0 °C, and r.t.) were also investigated to optimise the formation of **2a**. The optimal conditions proved to be those reported in Scheme 1 with treatment of **1a** with Et_3N and benzoyl chloride in CH_2Cl_2 to provide the *Se*-phenyl benzoselenoate **2a** in 91% yield. Having in hands the optimised conditions, we next pursued the scope of this reaction with respect to the acyl chloride partner. A variety of acyl chlorides bearing electron-deficient and electron-rich aromatics smoothly reacted with benzeneselenol **1a** to afford the corresponding selenolesters **2a**-**f** in good yields (Scheme 1). The reaction was also amenable to benzyl- and saturated or unsaturated alkyl-substituted acyl chlorides. Natural-product-derived stearoyl- and oleoyl- chlorides were successfully converted into the corresponding phenylseleno-esters **2i,j** (Scheme 1).



Scheme 1. Synthesis of selenolesters from selenols and acyl chlorides. Isolated yields are reported. [a] 0.8 eq. of acyl chloride were used. [b] 2.5 eq. of acyl chloride were used.

Notably, pharmacologically relevant fluorinated moieties could also be employed in this chemistry, enabling the synthesis of compounds **2f**,**g**. Having demonstrated the versatility of the reaction towards a variety of acyl chlorides, we turned our attention to evaluating the scope of this methodology with respect to the selenol partner. Substituted aromatic selenols **1b**,**c** performed well under standard conditions, enabling the synthesis of derivatives **2k**,**l** (Scheme 1).Functionalised alkyl selenols could also be efficiently employed in this reaction, thus providing access to unprecedented classes of substituted selenolesters. β-Hydroxy-selenolesters **2m**-**o** were obtained through selective selenoacylation of the corresponding selenols **1d**,**e** under slightly modified conditions (see Scheme 1 and ESI for details). Interestingly, treatment of β-hydroxy-selenols **1d**,**f** with an excess of acyl chloride led to the formation of compounds **2p**-**r** through the esterification of both the selenol and the hydroxyl groups (see Scheme 1 and ESI for details). Similarly, this procedure allowed the synthesis of derivative **2s**, bearing a selenolester and a thiolester group. Notably, labile and further functionalisable epichlorohydrin and glycidol derivatives could also be synthesised by using this mild protocol. Finally, this approach was also extended to amino-substituted selenols; the enantioenriched β-aminoselenol **1h** was selectively converted into the corresponding selenolester **2t** under standard conditions.

To the best of our knowledge the selenolesterase activity of any known metalloenzyme has not yet been investigated, even if this process has similar reaction mechanism to the hydrolysis of esters and thioesters.

Figure 1 showed selenolesters **2a,i,n,q** having time-dependent inhibition constant (K_i) values when incubated with hCA II. The K_i continued to decrease until an incubation period of 6 h was reached. Such data suggest that the compound **2a** undergoes a chemical transformation promoted by the enzyme, presumably similar to other prodrug CA inhibitors such as coumarins and sulfocoumarins which do require a minimum of 6 h incubation time to exert the inhibitory activity.[21] Exposure of **2a** under the same conditions of solvent and temperature used for the kinetic assays up to 18 h with no enzyme, allowed to recover the starting material in quantitative yields. No traces of cleaved by-products were detected by inspection of the ^{1}H-NMR spectra of raw material. (see ESI).

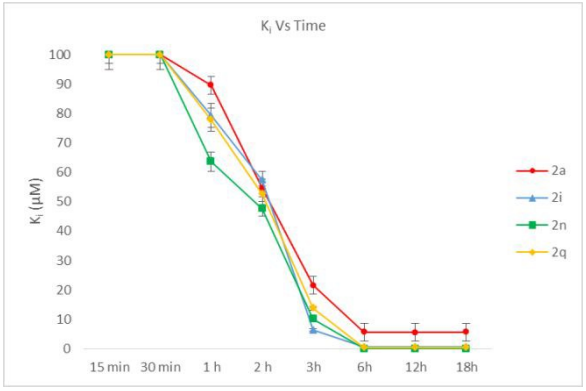


Figure 1. Inhibition constant (K_i) change for compounds **2a,l,n,q** versus time, incubated with hCA II for 1–18 h(s). Errors in the range of ± 5% of the reported values, from three different stopped-flow assays.[22]

In light of such results, the in vitro CA inhibition activities of compounds **2a-t** in comparison to the sulfonamide reference acetazolamide (**AAZ**) were measured against four

physiologically relevant hCA isozymes (i.e. the aboundant hCA I, II and the cancer-related isoforms hCA IX, XII) at 6 h incubation (Table 1).

Overall compounds **2a-t** inhibited the hCAs tested with K_I values in the low or submicromolar range with few exceptions and a rather complex structure–activity relationship (SAR). On focusing on the most potent data in Table 1, **2e** and **2i** inhibited the hCA I isoform 2.8 and 3.1 fold more potently than the reference **AAZ** followed by the seleno acyl derivatives **2h** and **2l** which showed K_I values comparable to the same sulphonamide standard CAI. As for the hCA II the **2c, 2e, 2h, 2k, 2l, 2m, 2n** and **2q** were the most effective among the series in inhibiting such an isoform with submicromolar K_I values. **2h, 2k, 2l** were also very effective inhibitors of the hCA IX isoform along with **2d** and **2g**. The second tumor associated hCA XII revealed to be strongly inhibited from all the compounds **2a-t** with an almost flat kinetic profile. On the basis of the kinetic data in Table 1 it is clear that the inhibitory activity seems unaffected by the electron-donating or electron-withdrawing nature of the substituents introduced as well as by steric considerations. The high diversity of K_i values among the series opens new possibilities for the application of these compounds to biomedical purposes by making use of the CA expressions.

Table 1: Inhibition data of compounds **2a-t** and **AAZ** against four human CA isoforms (hCA I, II, IX and XII) by a stopped flow CO_2 hydrase assay.[15]

	K_i (μM)[a]			
Cmp	hCA I	hCA II	hCA IX	hCA XII
2a	55.7	5.1	26.9	3.9
2b	64.1	8.9	14.1	0.56
2c	>100	0.09	5.0	0.09
2d	>100	3.2	1.1	0.41
2e	0.09	0.05	21.3	2.1
2f	4.6	0.45	30.2	7.6
2g	8.3	2.0	0.21	0.08
2h	0.34	0.05	1.3	7.0
2i	0.08	0.63	>100	0.09
2j	6.5	8.4	24.6	0.41
2k	>100	0.08	0.99	0.09
2l	0.37	0.55	1.1	0.08
2m	7.6	0.09	23.2	0.3
2n	0.66	0.04	>100	8.9
2o	31.5	55.4	22.6	0.09
2p	>100	44.7	>100	0.86
2q	0.71	0.52	>100	9.0
2r	>100	63.6	13.9	5.2
2s	7.2	73.7	>100	6.5
2t	>100	>100	26.6	0.77
AAZ	0.25	0.012	0.026	0.006

[a]Mean from 3 different assays, by a stopped flow technique (errors were in the range of ± 5-10 % of reported values).

X-ray investigations of compound **2a** with hCA II demonstrated in detail the real binding species within the active site of the enzyme.

We performed the experiments by soaking the native hCA II crystals in the mother liquor solution containing **2a** for 1 day. Data were collected and the electron density maps clearly showed the selenolate species and deeply bound within the enzymatic cleft adjacent to the zinc atom establishing hydrophobic interactions mainly with Val122 and Leu198 (Figures 2a and 2b). Since we reported the benzeneselenol in adduct with hCA II in our previous report[6] a superimposition of both complex was operated and showed perfect matching between the adducts (Figure 2c).

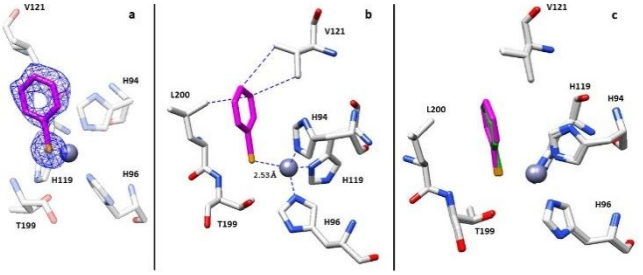


Figure 2. a) Active site region of hCA II/**2a** adduct (PDB: 6XWZ). Inhibitor showed as σA-weighted $|F_o–F_c|$ density map at 2.0 σ. **b)** Van der Waals interactions and the active site Zn^{2+}-ion coordination are shown and labelled in blue. **c)** hCA II structure with bound **2a** was superposed on to the hCA II structure with bound selenol previously reported.[6] The structure of the **2a** complex is colored with magenta whereas the structure of the selenol complex as green with the zinc cation shown as a gray sphere.

In conclusion, under our experimental conditions, most of the compounds here reported, displayed comparable *in vitro* inhibition potency with respect to parent selenols,[6] which were previously used as CA inhibitors. In fact, we explored the feasibility to design stable organoselenium compounds which, after enzyme-mediated hydrolysis, afford the corresponding differently functionalised selenols which act as carbonic anhydrase inhibitors. A wide range of differently substituted and functionalised selenolesters was synthesised their CA inhibition profile was studied. The unprecedented selenolesterase activity of different CA isoforms confirmed these compounds as potential pro-drugs. X-ray co-crystallography was also employed to further study the mechanism of such a novel CAs inhibitors.

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Conflicts of interest

There are no conflicts to declare.

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