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RESEARCH ARTICLE

Multimeric xanthates as carbonic anhydrase inhibitors

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Abstract

The field of multivalent inhibition of enzymes is growing exponentially from the first reported multivalent effect on a glycosidase enzyme. However, the investigations have generally remained restricted to carbohydrate-processing enzymes. Carbonic anhydrases are ubiquitous metallo-enzymes involved in many key biological processes, that catalyze the reversible hydration/dehydration of CO_2/HCO_3^-. This study reports the first synthesis of multimeric xanthates addressing the selectivity and potency of CA multivalent inhibition. Six multivalent compounds containing three, four, and six xanthate moieties were prepared and assayed against four relevant CA isoforms together with their monovalent analogues. Some of the multimers were stronger inhibitors than the monomeric species. For hCA I, the two best molecules **18** and **20** showed an improvement of the ligand affinity of 4.8 and 2.3 per xanthate units (valence-corrected values), respectively, which corresponds to a clear multivalent effect. Moreover, the biochemical assays demonstrated that the multimeric presentation of xanthates, also affected the selectivity of the relative inhibition among the four CAs assayed.

Keywords

Carbonic anhydrases, enzyme inhibitors, multivalent inhibitors, xanthates

History

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Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1), which are ubiquitous Zn-based metallo-enzymes catalyzing the reversible hydration/dehydration of carbon dioxide/bicarbonate, play an essential role in numerous physiological processes. In animals, they are critical in vital processes such as respiration, pH regulation, biosynthesis and calcification. Fifteen different human CA (hCA) isoforms and CA-related proteins (CARPs), belonging to the α-class and differing mainly in their location within the cell and tissues, have been reported to date[1]. CA inhibitors (CAIs) are already employed as diuretics and anti-glaucoma drugs. Furthermore, they are promising as anticonvulsant, anti-obesity, anticancer, and anti-virulent agents, among others[2]. Some CAIs, i.e. coumarins[3] and lacosamide[4], inhibit the enzyme activity by occluding the entrance of the catalytic site and ortho-substituted benzoic acid derivatives can inhibit CA without entering the active site at all[5]. However, most CAIs act by binding either to the Zn(II) cation, inside of the active site and crucial for the catalytic activity, or to the water molecule coordinated to it[6]. Namely, the classical sulfonamides and their isosteres, phenols, thiophenols, polyamines, and metal complexing anions. Belonging to this last group of anionic compounds, several families of CAIs displaying the CS_2^- zinc-binding group (ZBG) have been recently reported[7–10]. By crystallography, it has been proven that this ZBG coordinates the Zn(II) from the CA active site through one of the sulphur atoms; the same S forms a hydrogen bond with the OH of amino acid residue Thr199 and other S interacts also through hydrogen bond with the NH group of the same residue[7]. This binding mode explains the good inhibition rates of these compounds for various CA tested to date[8]. Three representative families containing this ZBG, i.e. dithiocarbamates, trithiocarbonates, and xanthates (Figure 1) have been intensively investigated.

They display a wide range of inhibitory powers depending on the organic moiety present in the molecule and its orientation within the active site cavity, which promote distinct interactions with the surrounding amino acids[7]. Therefore, as the active site of all the CA isoforms is highly conserved[11], these kind of recently discovered CAIs offer the possibility of designing more selective and potent agents[8]. Herein we targeted the synthesis and study of multivalent alkali-metal xanthates as CAIs from distinct polyols.

Multivalency has gained much importance in the last years, particularly in the fields of biomedicine and bioorganic synthesis. The critical role of multivalency in essential biological phenomena is only starting to be unravelled now[12]. Many natural processes, particularly at the membranes interface, benefit from the multivalent display of biological entities to boost the efficacy of binding events. Especially, carbohydrate–protein interactions are notably weak at the monomer level, but can be tremendously enhanced if the ligand is displayed in a multivalent way[13]. Scientists have extensively studied the biophysical interactions among natural systems and created a plethora of artificial multivalent systems in order to understand the underlying mechanisms of multivalency[14–22]. The most scrutinized systems are likely lectin–carbohydrate dyads. Lectins are non-processing but only carbohydrate-binding proteins often expressing multiple binding sites. Since some biologically relevant lectins are involved in pathological processes, their inhibition can be of

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therapeutic interest[20–24]. For instance, the adhesion of pathogenic bacteria to cells can be inhibited to prevent the bacterial infections[25–32]. Nevertheless, the field of multivalent enzyme inhibition (MEI) has been barely explored until 2009, when a clear multivalent effect (and an increase in selectivity) was determined in α-mannosidase inhibition by iminosugar clusters[33]. The term ''multivalent effect'', generally only employed with carbohydrate-binding proteins, could then be applied to the field of carbohydrate-processing enzymes. After this groundbreaking result, considerable amount of research has been reported, although almost exclusively with carbohydrate-processing enzymes such as glycosidases[34–42] and glycosyltranferases[43].

In a recent work, we reported the inhibitory study of coumarin-derivatized fullerene hexakis-adducts toward a set of biologically relevant hCA isoforms[44]. These multimeric compounds did not show strong multivalent effects, however they were always more potent than the respective monovalent analogues. Furthermore, the selectivity of the multimeric species varied with respect to the monovalent ones. MEI seems to be highly depending on the structure of the multivalent inhibitor: the valency, the topology, the structure of the central core and the length and rigidity of the spacer to which each inhibitor is grafted to the scaffold are parameters that must be fine-tuned to optimize a multivalent inhibition. Herein, we decided to explore both different multivalent scaffolds and CAI. A small collection of xanthate-derivatized compounds were prepared from multivalent polyol-scaffolds (Figure 2). The multivalent xanthates contained either three, four or six functionalities. In order to asses a possible multivalent effect, the monovalent analogues for each of the linkers used were also prepared. All the compounds were assayed *in vitro* for inhibition against the cytosolic hCA I and II as well as the transmembrane, tumor-associated hCA IX and XII. Interestingly, hCA IX and XII could be targeted by multivalency since crystallographic studies have demonstrated that they both present a dimeric extracellular domain[45,46]. Therefore, inhibition of these two isoforms could be enhanced by multivalent mechanisms such as chelation if an appropriate design of the inhibitor is achieved. Other few previous works have explored the multivalent inhibition of CAs. For instance, Whitesides and collaborators studied the dependence of avidity on spacer length in bivalent model systems[47]. Also, very recently, Winum and co-workers analyzed the inhibition of multivalent CAIs-decorated silica nanoparticles[48]. Nevertheless, as far as we know, this study reports the first synthesis of multimeric xanthates as CAIs, designed to address the selectivity and the potency of CA multivalent inhibition.

Figure 1. Metal complexing anions containing the ZBG CS_2^-: dithiocarbamates, trithiocarbonates and xanthates.

Materials and methods

Chemistry

All the synthetic procedures are described in the Experimental Supporting Information (ESI). NMR spectra of all the non-previously reported compounds are also shown in the ESI.

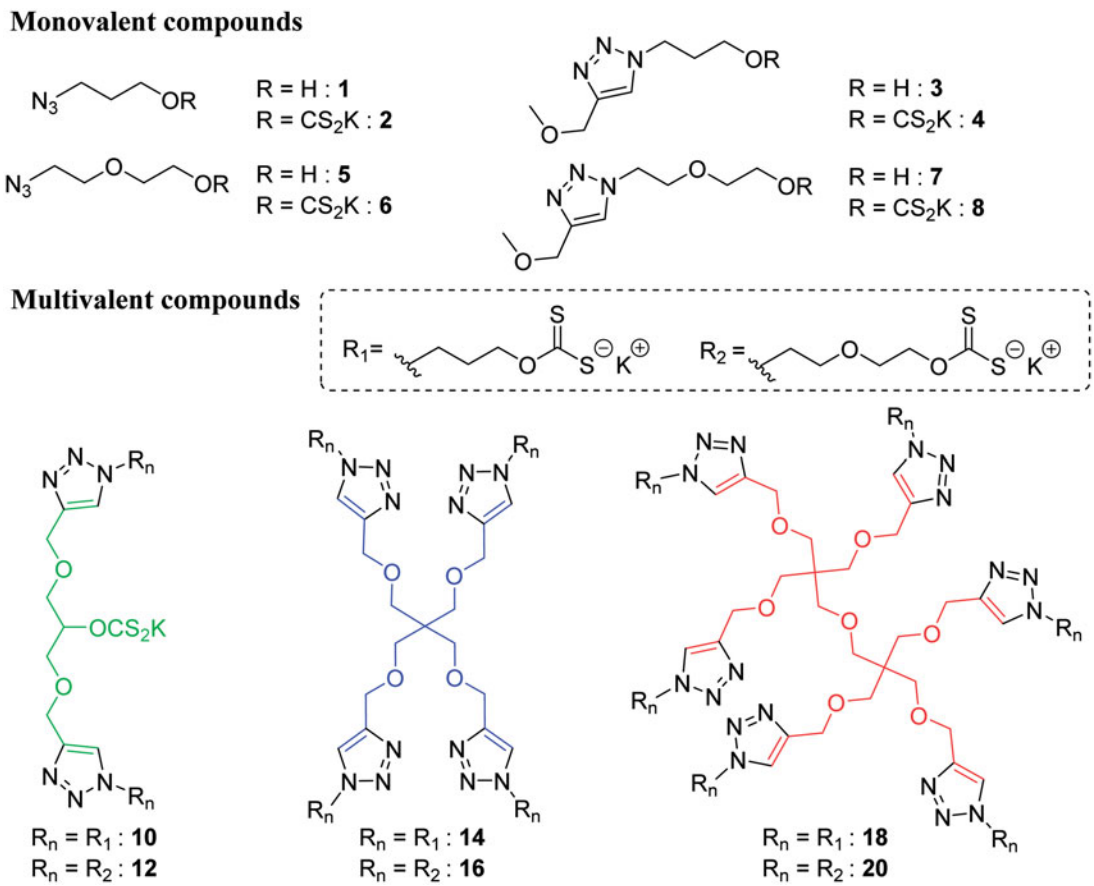


Figure 2. Multimeric compounds synthesized for this study and their monovalent counterparts.

CA inhibition

An SX.18MV-R Applied Photophysics stopped-flow instrument was used for assaying the CA-catalyzed CO_2 hydration activity by using the method of Khalifah[49]. Inhibitor and enzyme were pre-incubated for 15 min prior to assay. IC_{50} values were obtained from dose response curves working at seven different concentrations of test compound (from 0.1 nM to 50 μM), by fitting the curves using PRISM (www.graphpad.com) and non-linear least squares methods, values representing the mean of at least three different determinations, as described earlier by us[50,51]. The inhibition constants (K_I) were then derived by using the Cheng–Prusoff equation, as follows: $K_i = IC_{50}/(1 + [S]/K_m)$ where [S] represents the CO_2 concentration at which the measurement was carried out, and K_m the concentration of substrate at which the enzyme activity is at half maximal. All enzymes used were recombinant, produced in *E. coli* as reported earlier. The concentrations of enzymes used in the assay were: hCA I, 10.1 nM; hCA II, 8.4 nM; hCA IX, 7.9 nM and hCA XII, 12.5 nM[52,53].

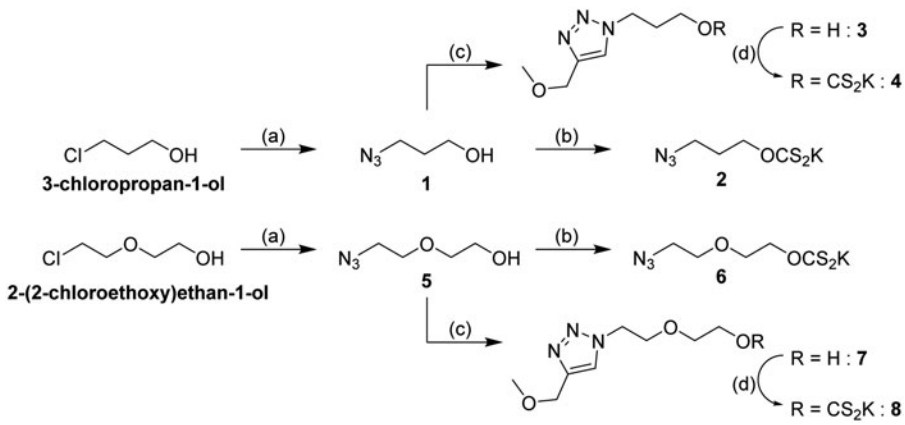
Results

Chemistry

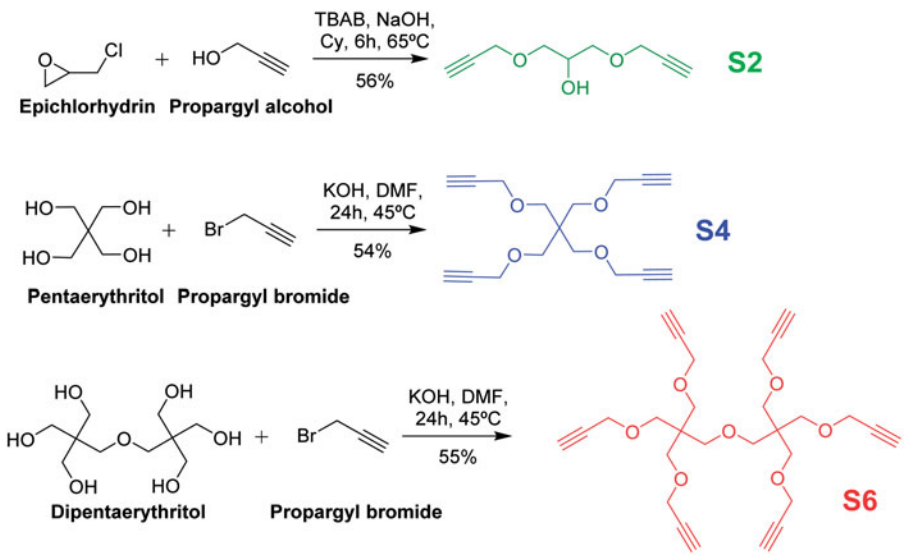
The preparation of the monovalent analogues, as shown in Scheme 1, started from commercially available 3-chloropropan-1-ol and 2-(2-chloroethoxy)ethan-1-ol and followed parallel pathways. Both molecules were azidated by substitution of the chloride leaving group to provide **1** and **5**, respectively, which were engaged in the synthesis of two distinct types of molecules. On the one hand, **1** and **5** were reacted with KOH and CS_2 in Et_2O, as reported in the literature for simple hydroxyl groups[8], giving the desired monovalent xanthates **2** and **6** as yellowish precipitates. In addition, **1** and **5** were clicked to 3-methoxyprop-1-yne to afford **3** and **7**, which were transformed in the monovalent xanthates analogues **4** and **8**, respectively, through reaction with CS_2 and KOH in DMSO/H_2O 10:1. This last procedure was optimized since the use of the same conditions as for the monovalent analogues **2** and **6**, did not afford the desired analogues in good yields. Compounds **1**–**8** were assayed against the hCAs studied to determine the importance of the presence of a triazole moiety and to assess the existence of a possible multivalent effect when compared to multimers.

The synthesis of the multivalent xanthates started with the preparation of the multivalent alkyne-derivatized scaffolds that would be later functionalized to finally provide the desired xanthates clusters. As shown in Scheme 2, the three scaffolds were obtained in one step from commercially available starting materials in good to very good yields in comparison to previously reported reactions in bibliography[54–56].

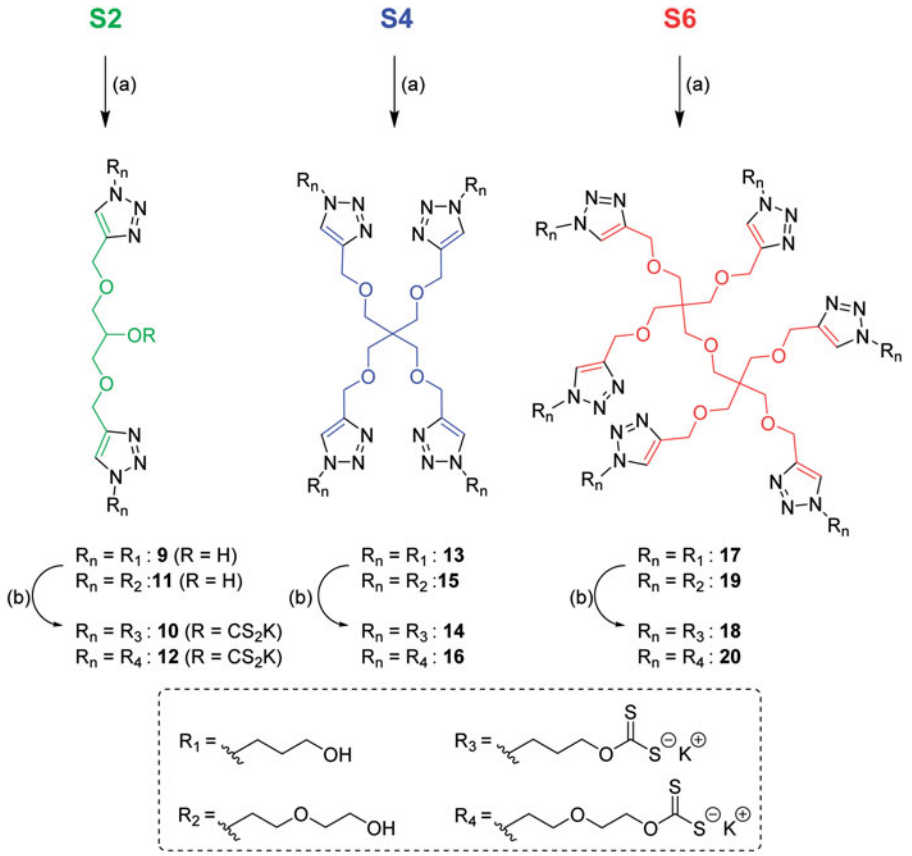
The synthesis of the multivalent xanthates from the formerly produced scaffolds was achieved in two steps, as depicted in Scheme 3, since the direct click of the azidated xanthates **2** and **6** was not feasible. Indeed, xanthates strongly coordinates Cu, thus a



Scheme 1. Synthesis of the monovalent analogues. Reagents and conditions: (a) NaN_3, H_2O, 18 h, 80 °C (1: from 3-chloropropan-1-ol, 95%; 5: from 2-(2-chloroethoxy)ethan-1-ol, 99%); (b) CS_2, KOH, Et_2O, 3 h, r.t. (2: from 1, 82%; 6: from 5, 83%); (c) 3-methoxyprop-1-yne, $CuSO_4$/NaAsc, THF/H_2O, o/n, r.t. (3: from 2, 99%; 7: from 6, 99%); (d) CS_2, KOH, DMSO/H_2O 10:1, 2 h, r.t. (4: from 3, 84%; 8: from 7, 77%).



Scheme 2. Synthesis of the alkyne-derivatized multivalent scaffolds S2, S4 and S6.



Scheme 3. Synthesis of the multivalent xanthates. Reagents and conditions: (a) 1 or 5, $CuSO_4$/NaAsc, 1,4-dioxane/H_2O, o/n, r.t. or 2 h at 80 °C (9: from 1, 70%; 10: from 5, 67%; 11: from 1, 96%; 12: from 5, 79%; 13: from 1, 90%; 14: from 5, 90%); (b) CS_2, KOH, DMSO/H_2O, 2 h, r.t. (15: from 9, 90%; 16: from 10, 89%; 17: from 11, 97%; 18: from 12, 71%; 19: from 14, 74%; 20: from 14, 83%).

high excess of Cu complex must be added to catalyze the click reaction but it cannot be fully removed afterwards. Therefore, the first step was straightforward and involved the grafting of the two different hydroxyl azidated-linkers **1** and **5** through CuAAC reaction. The click reaction was either realized overnight at r.t. or under microwave irradiation at 80 °C for 2 h with similar outcomes. After purification of the products by simple co-precipitation and removal of the residual Cu salts by scavenging (QuadrasilMP®, Sigma Aldrich, St. Louis, MO), the xanthates were prepared with the optimized conditions in very good to excellent yields, considering the number of reactions per molecule.

In order to develop an efficient methodology for the preparation, isolation and characterization of the desired multivalent xanthates, an intensive optimization of the procedure was required. It is worth to note that, as commented above, xanthates are not very stable molecules; they are known to be sensitive to acidic pH, high temperature, humidity and time[57]. The degradation process occurs through complex radical reactions giving the starting free hydroxyl and CS_2 as products, among others. Contrarily to the synthesis of the monovalent analogues, herein it was not intended that the xanthate products precipitated during the course of the reaction. Precipitation would provide a mixture of partially functionalized polyols. Therefore, after screening of solvents, DMSO was eventually selected as the preferred solvent because it can solubilize the final compounds, thus avoiding premature precipitation of partially functionalized polyols.

The completion of the reaction could be followed by ^{1}H NMR in DMSO-d_6 thanks to the chemical shifts of the peak of the CH_2 next to the hydroxyl groups ($\delta \approx 3.60$ ppm) which was converted into -OCS_2K ($\delta \approx 4.30$ ppm). For the first tests, 1 eq/-OH of KOH and CS_2 were added, but the reactions were very slow and not complete after several days. When an excess of both KOH and CS_2 was used, the reactions were complete in 2 h, but some side-products were always produced and were found impossible to be separated by selective precipitation. Based on these observations, we eventually developed a satisfactory procedure which included an excess of CS_2, since it can be removed by evaporation, and one equivalent of KOH per hydroxyl groups. Importantly, the KOH was finely crushed before the reaction and a few drops of water were added to the reactant mixture at the beginning of the reaction. After 2 h at r.t., the reactions were complete and the products able to be selectively precipitated with THF. The precipitates were washed three times with THF. To remove the last impurities and the traces of DMSO, the precipitates were dissolved in MeOH and rapidly precipitated again with Et_2O. The products were extensively dried under high vacuum to remove any traces of solvent which could lead to degradation. The characterization of all the molecules was carried out unambiguously by ^{1}H NMR, ^{13}C NMR and HRMS.

CA Inhibition assays

The monovalent compounds **1**–**8** and the multivalent compounds **9**–**20** were assayed *in vitro* for inhibition against the four physiologically relevant CA isoforms, the cytosolic hCA I and II as well as the transmembrane, tumor-associated hCA IX and XII. The results for the compounds which presented some inhibition are shown in Table 1. Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) was used as a standard in the assay[49].

Among the monovalent compounds, the alcohols **1**, **3**, **5** and **7** did not show any inhibitory effects against the four CA isoforms, as expected. The xanthates **2**, **4**, **6** and **8** on the other hand showed inhibitory effects (Table 1). The isoform more sensitive to these compounds was hCA I, for which the monovalent xanthates showed K_Is ranging between 7.5 and 51.7 μM. The organic scaffold did not show much influence in the case of xanthates **2**, **6**

Table 1. Inhibition constants (K_Is) by a stopped flow CO_2 hydrase assay, with acetazolamide AAZ as standard drug[48].

Compound	Valency (xanthate)	K_I (μM)[a]					
		hCA I			hCA II	hCA IX	hCA XII
			rp[b]	rp/n[c]			
2	1	**7.5**	–	–	>100	>100	**9.4**
4	1	**51.7**	–	–	>100	>100	>100
6	1	**7.9**	–	–	>100	>100	**93.9**
8	1	**7.5**	–	–	>100	**9.9**	>100
11	–	**70.8**	–	–	>100	>100	**15.9**
14	4	**82.9**	0.6	–	>100	>100	>100
16	4	>100	–	–	>100	**8.1**	>100
18	6	**1.80**	29	4.8	**68.9**	**10.0**	>100
20	6	**0.53**	14	2.3	**66.1**	**39.0**	>100
AAZ	–		0.25		0.012	0.025	0.006

Bold numbers are the values for the compounds with a $K_i < 100$ μM.
[a]Mean from three different assays. Errors were in the range of ±10% of the reported values (data not shown).
[b]Relative potency calculated as the ratio of K_I values of the monovalent controls (**4** or **8**) to the multivalent compounds.
[c]Corrected relative potency (rp/valency) calculated as the ratio of rp to the valency of the multivalent compounds.

and **8**, which had very similar inhibition constants whereas **4**, which incorporates the substituted triazole moiety (present also in **8**) but a shorter linker between the ring and the ZBG (compared to **8**), was an order a magnitude a weaker CAI compared to the other monovalent xanthates prepared here. hCA II was not at all inhibited by these xanthates (and alcohols) up to 100 μM concentrations in the assay system, and the same situation was observed for the inhibition of hCA IX, except for **8**, which was a low micromolar inhibitor (K_I of 9.9 μM). Against hCA XII xanthates **2** and **6** showed some degree of inhibition (K_Is of 9.4 – 93.9 μM).

For the multivalent compounds **9**–**20** reported here, the inhibition data were again more interesting against the slow cytosolic isoform hCA I. Molecules **9**, **10**, **12**, **13**, **15**, **17** and **19** did not show any inhibitory effect. Against isoform hCA I, the alcohol **11** and the xanthate **14** were weak micromolar inhibitors (K_Is of 70.8 – 82.9 μM). On the other hand, the polyxanthates derived from S6, **18** and **20**, showed low-micromolar or submicromolar inhibitory action (K_Is of 0.53 – 1.80 μM). Thus, in this case a very interesting multivalent effect is observed for the inhibition of hCA I, since the corresponding monomeric compounds were much less effective enzyme inhibitors compared to the multivalent xanthates. Indeed, the relative potency values of **18** and **20** were found to be 29 and 14, respectively (Table 1). Taking into account the valency of these hexameric species, the enhancement of the affinity per xanthate unit corresponds to 4.8 and 2.3, which is already significant for multivalent enzyme inhibitors. The same two xanthates, **18** and **20**, were also inhibiting hCA II, but orders of magnitude less efficiently compared to hCA I (K_Is of 66.1–68.9 μM). None of the other multivalent derivatives possessed inhibitory activity against this isoform. For hCA IX, three multivalent xanthates (**16**, **18** and **20**) showed inhibition, with K_Is ranging between 8.1 and 39.0 μM. Interestingly, hCA XII was not inhibited by the multivalent xanthates, and only the alcohol **11** showed a weak inhibitory action.

Discussion and conclusions

We designed monovalent alcohols/xanthates **1**–**8** and three series of multivalent derivatives **9**–**20** in order to investigate multivalent inhibition of CA isoforms involved in crucial physiologic and pathologic processes. An optimized procedure for the synthesis of multivalent xanthates was developed providing all the desired molecules in very good to excellent yields. Starting from readily available commercial materials, all the molecules, even the complex multimeric xanthates, were produced in three or less steps. The characterization was performed unambiguously by ^{1}H NMR, ^{13}C NMR and HRMS.

Some of the multivalent compounds, containing three, four, and six xanthate moieties showed interesting CA inhibitory activity, mainly against hCA I, an isoform whose physiologic function is still poorly understood. Together with their monovalent analogues, some of the multimers were significantly stronger inhibitors than the monomeric species although no strong ''multivalent effect'' was found in general (as compared to carbohydrate-lectin interactions). All four CA isoforms on which we tested the xanthates have been crystallized alone or in adduct with various classes of inhibitors. The two cytosolic isoforms hCA I and II are monomers whereas the two transmembrane ones are dimers with two accessible active sites. However, as shown from the data in the Table 1, both monomeric as well as dimeric isoforms were inhibited in a rather similar manner by the multivalent derivatives reported in the paper. The best hCA I inhibitors in the reported series had inhibition constants of 0.53 – 1.80 μM, and they were two orders of magnitude less efficient against the physiologically dominant cytosolic isoform hCA II. Thus, an interesting class of hCA I-selective inhibitors was detected in this work. Nonetheless, the biological assays demonstrated that the multimeric presentation of xanthates, indeed affected the selectivity of the relative inhibition among the four CAs assayed, and may open new directions in the research of isoform selective CAIs binding to the metal ion from the enzyme active site.

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Declaration of interest

The authors report no conflict of interest. We thank the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme ''DyNano'' FP7/2007–2013/under REA grant agreement no. 289033 for the financial support.

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Supplementary material available online
Supplementary Information