Organic & Biomolecular Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Journal Name

RSCPublishing

Org Biomol Chem

Dendrimers incorporating benzenesulfonamide moieties strongly inhibit carbonic anhydrase isoforms I-XIV

Cite this: DOI: 10.1039/xoxxoooooox

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

www.rsc.org/

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Abstract: As extention of our previous study herein we report an comprehensive investigation of poly(amidoamine) (PAMAM) dendrimers as modulators of the human carbonic anhydrase (hCA, EC 4.2.1.1) isoforms I-XIV. Interestingly inhibitory activity was observed for the non-functionalized dendrimers against the hCA I, VII, IX, XII and XIV isoforms, whereas activation properties were reported only for the cytosolic abundant hCA II. Highly efficient inhibitory action against many isoforms having medicinal chemistry applications, such as hCA II, V, VII, IX, XII and XIV, was observed for the PAMAM functionalized counterparts bearing 4, 8, 16 and 32 benzene sulfonamide moieties. Possible applications of dendrimer–CA inhibitors as therapeutic/diagnostic agents are envisaged.

Introduction

Dendrimers are highly attractive molecules for a large number of biotechnological and biomedical applications, such as catalysis, preparation of synthetic enzymes, drug delivery and gene transfection systems, contrast agents for magnetic resonance imaging or as optical sensors.[1-9]

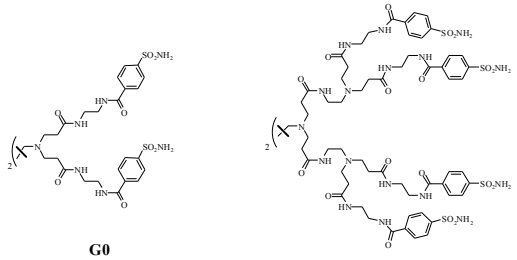
Poly(amidoamine) (PAMAM) dendrimers, which consist of repetitively branched subunits of amide and amine moieties, are thoroughly characterized and commercially available. Moreover in virtue of their interesting physico-chemical properties, versatility and ease of derivatization, PAMAM dendrimers are widely used for many of such applications.[10-13] Indeed, the branched tree-like concentric layers of the dendrimers, referred to as 'generations', allow a precise number of various functional groups to be incorporated in the macromolecule, which thereafter may act as a platform for controlling the interactions with the receptor, enzyme or tissue. In addition, the particular three-dimensional architecture that the functionalized dendrimer generations adopt may be also exploited both for targeting nano-drugs to different tissues or cell compartments as well as for enhancing bioavailability of some drugs.[10-13]

Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes involved in many crucial physiologic processes. They possess a rather large versatility as catalysts, using as substrates CO_2, COS, CS_2, cyanamide, carboxylic, phosphoric and thiocarboxylic esters.[14-16] However the physiologic reaction that they catalyse, the simple but fundamental reversible hydration of carbon dioxide to bicarbonate and protons, seems to be the only one with applications for the drug design of inhibitors and activators with therapeutic utility.[14-16]

In fact the CAs are widespread enzymes in organisms all over the phylogenetic tree, so far six genetic families encoding them are reported: α-, β-, γ-, δ-, ζ- and η-CA classes.[16-21] In vertebrates, including humans, at least 15 different α-CA isoforms were described,[14,15] which are involved in a variety of physiologic/pathologic functions, such as pH and CO_2 homeostasis, respiration and transport of CO_2/bicarbonate, electrolyte secretion in many tissues/organs, biosynthetic reactions (e.g., gluconeogenesis, lipogenesis and ureagenesis in which bicarbonate, and not CO_2, acts as a substrate for the carboxylation reaction), bone resorption, calcification, tumorigenicity, etc.[14-21]

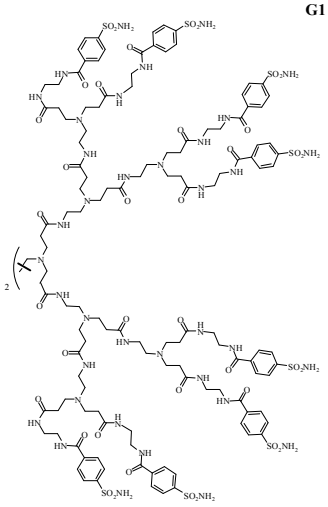
The 12 catalytically active human (h) isoforms (hCAs) can be grouped in four different subclasses depending on their subcellular localization: hCA I, II, III, VII and XIII are located in the cytosol, hCA IV, IX, XII and XIV are membrane-associated, hCAs VA and VB are found in mitochondria, whereas hCA VI is secreted in saliva and milk.[14-16,20] The disregulated activity of these enzymes leads to a variety of diseases, such as retinal/cerebral oedema (in which hCA I is involved); glaucoma, epilepsy, oedema, high altitude sickness (hCA II seems to be the main, but not the only isoform involved in these conditions); oxidative stress (hCA III); retinitis pigmentosa (hCA IV); obesity (hCA VA/VB); cariogenesis (hCA VI); epilepsy

(hCA VII); tumorigenesis (hCA IX and XII; but hCA XII is also involved in glaucoma); sterility (hCA XIII) and various retinopathies (in which hCA XIV is the main isoform involved).[14-20] As a consequence, many CA isoforms are established drug targets, with their inhibitors having a range of pharmacological applications. Indeed, sulfonamide CA inhibitors (CAIs) are in clinical use for the treatment of some of these conditions for decades.[14,15]

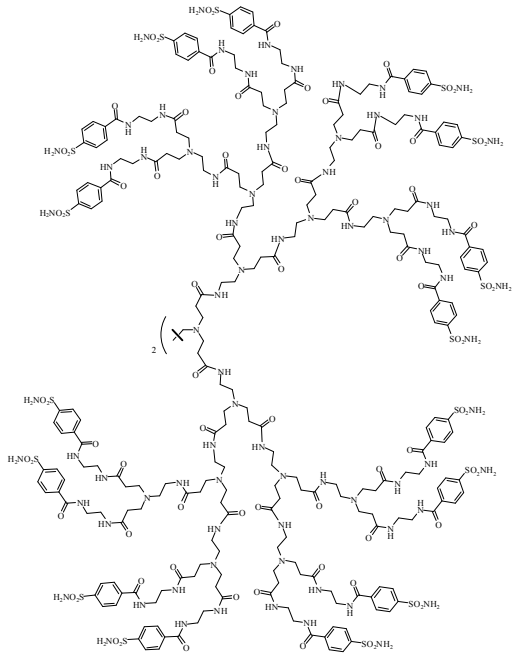


G0

G1



G2



G3

Scheme 1. Structures of functionalized dendrimers **G0-G3** investigated here as CAIs.

The sulfonamide CAIs are effective drugs for the management of many such diseases, but the large number of CA isoforms and their high affinity for the classical inhibitors of the sulfonamide type (of which acetazolamide, **AAZ**, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide is the best known representative),[14] lead to side effects, mainly due to the non selective enzyme inhibition in other tissues/ organs than the targeted one.[14-18,23,24] This is the reason why various new approaches have been investigated for the designing of selective CAIs, such as the use of nanoparticles (NPs).[25] Au(0) NPs decorated with aromatic sulfonamide functionalities, showed very interesting properties in inhibiting *in vitro* and *in vivo* the tumor/associated CA isoforms hCA IX and XII.[25] However, dendrimers were only recently investigated as CAIs, but only against isoforms hCA I, II, IX and XII.[22] This is the reason why we report here an extension of our previous study,[22] including all catalytically active human isoforms (hCA I-XIV) for the investigation of their interaction with non-functionalized and sulfonamide-derivatized PAMAM dendrimers.

Results and Discussion

Chemistry

Four generations of commercially available PAMAM dendrimers **G0-G3**, which incorporate free aminoethyl moieties, have been used for preparing the corresponding sulfonamide-dendrimers **G0-G3**.[22] The dendrimers **G0-G3** thus obtained incorporate 4, 8, 16 and 32 sulfonamide moieties. In our previous work we demonstrated that they show excellent hCA II and XII inhibitory properties, as well as antiglaucoma effects *in vivo*, in an animal model of the disease.[22]

Enzyme inhibition with non-functionalized and functionalized PAMAM dendrimers

We investigated the kinetic behaviour of the non-functionalized PAMAM dendrimers **G0–G3** with the 11 catalytically active hCA isoforms, hCA I-XIV (Table 1).

Table 1: CA inhibition data against isoforms hCA I-XIV with the non-functionalized PAMAM dendrimers **G0-G3**, by a stopped-flow CO_2 hydrase assay.[23]

	K_I (μM)*			
	G0	**G1**	**G2**	**G3**
hCA I	>50	0.41	2.71	2.89
hCA II	>50	A	A	A
hCA III	>50	>50	>50	>50
hCA IV	>50	>50	>50	>50
hCA VA	>50	>50	>50	>50
hCA VI	>50	>50	>50	>50
hCA VII	>50	6.73	4.34	4.76
hCA IX	7.00	4.11	7.64	>50
hCA XII	0.55	2.91	0.87	3.59
hCA XIII	>50	>50	>50	>50
hCA XIV	5.75	12.1	8.97	14.6

*Errors in the range of ±5 % of the reported values, from three different assays.
A = activator. A weak activating effect was observed with the following activation constants (K_A = 75.9 μM for **G1**; K_A = 14.5 μM for **G2**; K_A = 10.8 μM for **G3**).

The reason of this investigation was the fact that the starting material dendrimers **G0-G3**, incorporate terminal free aminoethyl moieties, which could interact within the CA active sites in at least two ways:

(i) as CA activators (CAAs), thus enhancing the rate of the CO_2 hydration reaction,[26] or (ii) acting as CAIs, by a mechanism of action elucidated earlier by us for the polyamines such as spermine and its derivatives.[27] Indeed, many CAAs belong to the amine or amino acid chemotypes,[26] and they bind at the entrance of the CA active site, participating in the rate-determining step of the catalytic cycle, i.e., the transfer of protons between the water coordinated to the metal ion (the nucleophile promoting catalysis) and the environment. In general this step is achieved by the participation of a His residue located in the middle of the active site cavity (usually His64), which acts as a proton shuttle. By means of kinetic and X-ray crystallographic experiments we showed that many CAAs of the amine/amino acid type bind at the entrance of the active site and participate in additional proton transfer processes.[28] On the contrary, amines such as spermine and its congeners, anchor to the zinc-coordinated water molecule, thus blocking the reaction catalysis and acting as efficient CAIs. Such a mechanism has been confirmed by kinetic and X-ray crystallographic experiments. The kinetic data reported in Table 1, and relative to not functionalized PAMAM dendrimers **G0–G3**, indeed show such compounds can interact with CAs both as CAIs or CAAs. In particular hCA II was the only isoform activated, with PAMAMs **G1-G3** acting as weak – medium potency activators (K_As comprised between 10.8–75.9 μM; Table 1). Interestingly **G0** showed no action on the same isoform. All four generations of non-derivatized dendrimers did not show any activity as inhibitors neither as activators against the hCA III, hCA IV, hCA VA, hCA VI and hCA XIII (Table 1). Interestingly **G0** showed to be a weak inhibitor against the membrane associated isoform such as the hCA IX, XII and XIV, with inhibition constants in the range of 0.55 – 7.00 μM, Table 1). **G1** showed inhibitory activity against hCA I, hCA VII, hCA IX, hCA XII and hCA XIV, with K_Is ranging between 0.41 and 12.1 μM. The same isoforms were inhibited by **G2** (K_Is ranging between 0.87 and 8.97 μM) whereas **G3** inhibited hCA I, VII, XII and XIV (K_Is ranging between 2.89 and 14.6 μM). It is obvious that there are no regularities between the inhibitory activity and the increase of the molecular weight (generation) of the dendrimers.

Table 2: CA inhibition data against isoforms hCA I-XIV with sulfonamide functionalized PAMAM dendrimers **G0-G3** and acetazolamide (**AAZ**) as standard, by a stopped-flow CO_2 hydrase assay.[23]

	K_I (nM)*				
	G0	**G1**	**G2**	**G3**	**AAZ**
hCA I	24.1	12.0	10.8	10.5	250
hCA II	10.4	3.1	0.93	0.07	12
hCA III	14000	nt	nt	nt	20000
hCA IV	60.8	18.2	2.5	0.81	74
hCA VA	247	64.2	10.9	0.62	63
hCA VI	533	426	87.9	2.8	11
hCA VII	35.6	19.4	1.6	0.05	2.5
hCA IX	34.7	20.5	8.6	5.1	25
hCA XII	9.3	1.1	0.94	0.06	5.7
hCA XIII	345	261	53.4	2.5	16
hCA XIV	436	75.5	7.9	0.87	41

*Errors in the range of ±5 % of the reported values, from three different assays.
.nt = not tested

The inhibition data of the sulfonamide functionalized dendrimers **G0-G3** (and acetazolamide as standard inhibitor) are reported in Table 2. The obtained data show that the dendrimeric derivatives incorporating sulfonamide moieties possess highly effective inhibitory properties against all 11 isoforms investigated here, except hCA III (Table 2). In particular the inhibitory power against the hCA I increased with the generation, starting from a K_I of 24.1 nM for **G0** to 12.0 nM for **G1** and 10.8 – 10.5 nM for **G2** and **G3** respectively. A more evident correlation between the inhibitory power and the increase of the PAMAM-CAI based generations was observed for hCA II: **G0** and **G1** derivatives were highly effective, low nanomolar inhibitors (K_Is of 10.4 nM and similar to those of **AAZ** for **G0**, and of 3.1 nM for **G1**). The **G2** dendrimer derivative was a sub-nanomolar inhibitor (K_I 0.93 nM), whereas **G3** reached picomolar values (K_I 0.07 nM) (Table 1). This type of increase of the inhibitory power with the generation of the dendrimer was in fact seen for all the remaining isoforms, with **G0** being the least effective and **G3** the most effective inhibitor in all cases (Table 2). hCA III, an isoform normally resistant to sulfonamide inhibitors,[29] represented the exception among the investigated isoforms and only **G0** was assayed, showing weak inhibitory properties similar to **AAZ** (K_Is of 14 and 20 μM respectively). **G0** showed to be an effective inhibitor of hCA IV, VII, IX and XII, with K_Is in the range of 9.3 – 60.8 nM. The progressive increase of the inhibitory power from **G0** to **G3** led the last dendrimer to act as low nanomolar – picomolar inhibitor against these isoforms, with K_Is in the range of 0.05 – 5.1 nM. For isoforms such as hCA VA, VI, XIII and XIV, the initial inhibitory power of **G0** was worse compared to the previously discussed enzymes, in the range of hundreds of nM (more precisely 247 – 533 nM), with the progressive increase of the inhibition to **G1**, **G2** and **G3**, which showed K_Is in the range of 0.87 – 2.8 nM. In all cases the **G3** sulfonamide dendrimer was much more effective as a CAI compared to the monovalent inhibitor acetazolamide (Table 2).

Table 3: Multivalent effects of functionalized PAMAM dendrimers **G0-G3** against the hCA I-XIV isoforms.

	G0	**G1**	**G2**	**G3**	**1** (K_I nM)*
hCA I	871 (217)	1750 (218.9)	1944 (121.5)	2000 (62.5)	21000
hCA II	15.4 (3.8)	51.6 (6.5)	172 (10.8)	2285.7 (71.4)	160
hCA III	0.11 (0.029)	NA NA	NA NA	NA NA	1600
hCA IV	40.3 (10.1)	134.6 (16.8)	980 (61.3)	3024.7 (94.5)	2450
hCA VA	171.7 (42.9)	660.4 (82.6)	3889.9 (243.1)	0.62 (68387.1)	42400
hCA VI	1.53 (0.38)	1.91 (0.24)	9.25 (0.58)	290.4 (9.1)	813
hCA VII	2.25 (0.56)	4.1 (0.52)	50 (3.13)	1600 (50)	80
hCA IX	0.95 (0.24)	1.61 (0.20)	3.8 (0.24)	6.5 (0.20)	33
hCA XII	0.34 (0.09)	2.91 (0.36)	3.4 (0.21)	53.3 (1.7)	3.2
hCA XIII	0.12 (0.03)	0.16 (0.02)	0.81 (0.05)	17.2 (0.54)	43
hCA XIV	6.65 (1.66)	38.4 (4.8)	367.1 (22.9)	3333.3 (104.2)	2900

*Errors in the range of ±5 % of the reported values, from three different assays.
.NA = Not Applicable; rp: relative potency = K_I(**1**)/K_I(Gn); In brackets rp/n: relative potency/number of sulfonamide units.

For the first time we report a complete investigation of the multivalency effects for the **G0-G3** derivatized dendrimers in comparison to a benezene sulfonamide prototype such as the

chemically stable compound ethylaminobenzene sulfonalmide **1** on the hCA I-XIV isoforms (table 3). In general all tested compounds showed remarkable inhibitory potency (rp) as well as relative potency (rp/n) improvements**,** which are indicative of strong multivalency effects. The only exceptions are represented by the rp/n of **G2** and **G3** for hCAI and of **G1** for hCA VI which showed a drop of 97.4, 156.4 and 0.14 points relative to the best performing dendrimer generation within the same enzymatic isoform. Of particular importance are the multivalent effects observed for the biological relevant hCA isorforms such as hCA II and IV which are involved, along with hCA XII with the liquor formation in the glaucomatous eyes, the mitochondrial hCA VA and the renal abundant hCA XIV. Therefore the appropriate functionalization of dendrimers whith sulfonamides indeed represents a promising tool for the treatment of pathologies.

Experimental

Chemistry. Dendrimers **G0-G3** were synthesized as described earlier.[22]

CA inhibition. A stopped-flow instrument (SX.18MV-R Applied Photophysics model) was used for assaying the CA-catalyzed CO_2 hydration activity.[23] Inhibitor and enzyme were preincubated for 15 min for allowing the complete formation of the enzyme-inhibitor adduct. IC_{50} values were obtained from dose response curves working at eight different concentrations of test compound (from 0.01 nM to 50 μM), by fitting the curves using PRISM (www.graphpad.com) and non-linear least squares methods, the obtained values representing the mean of at least three different determinations. The inhibition constants (K_I) were derived from the IC_{50} values 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.[27-29] The concentrations of enzymes used in the assay varied between 8.4 nM and 12.8 nM.

Conclusions

This is the first study reporting the interaction of dendrimers incorporating sulfonamide moieties and their CA inhibitory properties against all the catalytically active mammalian isoforms hCA I-XIV. By using the PAMAM core we obtained four generations (**G0–G3**) of dendrimers incorporating 4, 8, 16 and 32 benzenesulfonamide. The dendrimers showed excellent enzyme inhibitory properties and multivalent effects against all the four physiologically relevant CA isoforms, hCA I-XIV, some of which are established drug targets. As CAs are ubiquitous enzymes with many biomedical applications, our findings may be extended for targeting different other disease/conditions in which these enzymes are involved, among which glaucoma, obesity, epilepsy and cancer.

Acknowledgments. We thank The Distinguished Scientist Fellowship Program (DSFP) at KSU for funding this project.

Notes and references

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