Bioorganic & Medicinal Chemistry Letters 21 (2011) 710–714

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

# Carbonic anhydrase inhibitors. Inhibition studies with anions and sulfonamides of a new cytosolic enzyme from the scleractinian coral *Stylophora pistillata*

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ARTICLE INFO

*Article history:*
Received 31 October 2010
Revised 25 November 2010
Accepted 30 November 2010
Available online 4 December 2010

*Keywords:*
Carbonic anhydrase
Coral
*Stylophora pistillata*
Anion inhibitor
Sulfonamide
Carbon fixation

ABSTRACT

The catalytic activity and the inhibition of a new coral carbonic anhydrase (CA, EC 4.2.1.1), from the scleractinian coral *Stylophora pistillata*, STPCA-2, has been investigated. STPCA-2 has high catalytic activity for the physiological reaction being less sensitive to anion and sulfonamide inhibitors compared to STPCA, a coral enzyme previously described. The best STPCA-2 anion inhibitors were sulfamide, sulfamic acid, phenylboronic acid, and phenylarsonic acid ($K_I$s of 5.7–67.2 μM) whereas the best sulfonamide inhibitors were acetazolamide and dichlorophenamide ($K_I$s of 74–79 nM). Because this discriminatory effect between these two coral CAs, sulfonamides may be useful to better understand the physiological role of STPCA and STPCA-2 in corals and biomineralization processes.



Carbonic anhydrases (CA, EC 4.2.1.1) are ubiquitous metalloenzymes that catalyze the reversible hydration of carbon dioxide into bicarbonate and protons: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. There are at least five classes of CAs with polyphyletic origin:[1–6] the α-class (in vertebrates, invertebrates, bacteria, and some chlorophytes), the β-class (in eubacteria and chlorophytes), the γ-class (archea and some eubacteria), and the δ- and ζ-family (present only in marine diatoms).[1–6]

Scleractinian corals have been of particular interest for research over the past two decades owing to their fundamental role in reef formation and maintenance of this highly diverse ecosystem in oligotrophic tropical regions. This ecological success comes from two major features of corals: (i) the precipitation of an aragonitic calcium carbonate ($CaCO_3$) skeleton by a process of biomineralization, and (ii) the symbiotic association that many of them establish with photosynthetic dinoflagellates from the genus *Symbiodinium* (commonly named zooxanthellae). Numerous studies have demonstrated the involvement of CAs in both processes.[7] With regards to biomineralization, CAs are involved in the inorganic carbon supply for calcification and/or the regulation of pH at the calcification site.[8–11] A secreted CA in the coral *Stylophora pistillata*, named STPCA,[15] has been recently cloned, and some of its biochemical properties have been determined.[12–14] Due to its specific expression in calcifying cells, this enzyme has been suggested to play a key role in biomineralization.[15] Concerning symbiosis, it has been shown that inorganic carbon (Ci) from the surrounding seawater is supplied to the symbiont's photosynthetic machinery through a $CO_2$-Concentrating Mechanism (CCM) in which CAs play a crucial role, similar to a broad array of marine symbiotic processes (for review see[16,17]). The role of CAs in symbiosis is to increase the $CO_2$ availability for Ci transport and/or photosynthesis and thus allow symbiotic dinoflagellates to maintain high rates of carbon fixation in an intracellular environment.[16,17]

We have recently cloned, sequenced and localized a new α-CA from the coral *S. pistillata*, named STPCA-2.[18] Compared to STPCA, which is a secreted isoform and seems to play a direct role in biomineralization, STPCA-2 is mainly located in the cytoplasm surrounding the zooxanthellae. The present work is aimed at determining the catalytic activity and the inhibition profile of recombinant STPCA-2 with the major classes of CA inhibitors: the anions and the sulfonamides.[19–27]

CA activity and the inhibition assays were performed on coral recombinant STPCA-2 obtained as described earlier.[18] The kinetic parameters for the $CO_2$ hydration reaction by STPCA-2, as well as for STPCA and for the human isoforms, hCA I, hCA II, and hCA VI

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doi:10.1016/j.bmcl.2010.11.124

**Table 1**
Kinetic parameters for the $CO_2$ hydration reaction[42] catalyzed by the human cytosolic α-hCA isozymes I–III, the mitochondrial isozymes hCA VA and VB, the secreted isoform hCA VI (full length) and the transmembrane isozymes hCA IX (catalytic domain), hCA XII (catalytic domain), the STPCA and STPCA-2 at 20 °C and pH 7.5 in 10 mM HEPES buffer, and their inhibition data with acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide),[28] a clinically used drug

| Isozyme | Activity level | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($M^{-1}$ $s^{-1}$) | $K_I$ (acetazolamide) (nM) |
|---|---|---|---|---|
| hCA I | Moderate | $2.0 \times 10^5$ | $5.0 \times 10^7$ | 250 |
| hCA II | Very high | $1.4 \times 10^6$ | $1.5 \times 10^8$ | 12 |
| hCA VI | Moderate | $3.4 \times 10^5$ | $4.9 \times 10^7$ | 11 |
| STPCA | Moderate | $3.1 \times 10^5$ | $4.6 \times 10^7$ | 16 |
| STPCA-2 | High | $5.6 \times 10^5$ | $8.3 \times 10^7$ | 74 |

are shown in Table 1. The results show that STPCA-2 has a higher activity level compared to STPCA, but lower than the cytosolic isoform hCA II. Moreover STPCA-2 is inhibited by acetazolamide, but less than hCA II, hCA VI, and STPCA. Relative to the kinetic parameters and the inhibition by acetazolamide, it can be concluded that STPCA-2 shows characteristics intermediate between those of the cytosolic hCA II, and the secreted coral enzyme STPCA.

We tested interactions of purified recombinant STPCA-2 with the first major class of CA inhibitors, the anions.[19–27] We included anions such as the typical metal poisons cyanide, (thio)cyanate, hydrogen sulfide, azide, etc., but also studied the interaction of this new enzyme with anions which show less propensity to complex metal ions in solution such as sulfate, nitrate, perchlorate, etc.[19–28]

In the present study, buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, and sulfate) were of highest purity available, and were used without further purification. Sulfamide, sulfamic acid, phenylboronic acid, and phenylarsonic acid were also commercially available compounds.

The data in Table 2 show the inhibition of STPCA-2 with a set of physiological and non-physiological anions (literature inhibition data of human isozymes I, II, VI, and STPCA are also included in the table for the sake of comparison, since they are useful for a better understanding of the significance of STPCA-2 inhibition data).[19–27] The following can be observed for STPCA-2 inhibition with anions:

(i) A large number of physiologically relevant, as well as non-physiological anions, such as the halides (fluoride, chloride, and bromide) and pseudohalides (cyanate, thiocyanate, isocyanide), carbonate, nitrate, bisulfite, and sulfate showed a very similar inhibition behavior, with $K_I$s in the range of 0.33–0.99 mM. The least effective STPCA-2 anion inhibitor was nitrate that showed a $K_I$ of 0.99 mM.

(ii) The most effective inhibitors in this series were sulfamide, sulfamic acid, phenylboronic acid, and phenylarsonic acid with $K_I$s in the range of 5.7–67.2 μM. Thus, STPCA-2 has an anion inhibition profile which is completely different from the cytosolic isozymes (hCA I and hCA II). Indeed, hCA I and hCA II generally have a much lower affinity for most of these anions compared to hCA VI, STPCA, and STPCA-2. On the other hand, STPCA-2 shows similar anion inhibition characteristics with STPCA: same range of $K_I$ with fluoride, chloride, cyanate, thiocyanate, cyanide, nitrate, bisulfite, and sulfate. However, there are also some differences between the two coral enzymes. The inhibition constants of these two isozymes for iodide show a greatly different $K_I$ ratio (around 3600), whereas STPCA-2 and hCAII show a similar $K_I$ (33 mM and 26 mM, respectively). Sulfamic acid, phenylboronic acid, and phenylarsonic acid were more effective as STPCA-2 inhibitors than as STPCA inhibitors (i.e., 95, 83.9, and 11.6-fold, respectively).

**Table 2**
Inhibition constants of anionic inhibitors against isozymes hCA I, II, and VI (human, α-CA class enzymes), and the CA from the coral *Stylophora pistillata*, STPCA and STPCA-2, for the $CO_2$ hydration reaction, at 20 °C[42]

| Inhibitor | $K_I$ (mM)[a] | | | | |
|---|---|---|---|---|---|
| | hCA I[b] | hCA II[b] | hCA VI[b] | STPCA[c] | STPCA-2[d] |
| $F^-$ | >300 | >300 | 0.60 | 0.62 | 0.92 |
| $Cl^-$ | 6 | 200 | 0.72 | 0.50 | 0.53 |
| $Br^-$ | 4 | 63 | 0.73 | 0.0097 | 0.96 |
| $I^-$ | 0.3 | 26 | 0.81 | 0.0090 | 33.0 |
| $CNO^-$ | 0.0007 | 0.03 | 0.69 | 0.59 | 0.69 |
| $SCN^-$ | 0.2 | 1.6 | 0.89 | 0.68 | 0.51 |
| $CN^-$ | 0.0005 | 0.02 | 0.07 | 0.58 | 0.86 |
| $N_3^-$ | 0.0012 | 1.5 | 0.07 | 0.52 | 4.68 |
| $HCO_3^-$ | 12 | 85 | 0.80 | 0.45 | 7.81 |
| $CO_3^{2-}$ | 15 | 73 | 0.69 | 0.010 | 0.24 |
| $NO_3^-$ | 7 | 35 | 0.76 | 0.56 | 0.99 |
| $NO_2^-$ | 8.4 | 63 | 0.82 | 0.77 | 3.15 |
| $HS^-$ | 0.0006 | 0.04 | 0.71 | 0.58 | 3.94 |
| $HCO_3^-$ | 18 | 89 | 14.2 | 0.41 | 0.43 |
| $SO_4^{2-}$ | 63 | >200 | 9.9 | 0.91 | 0.33 |
| $H_2NSO_2NH_2$ | 0.31 | 1.13 | 0.07 | 0.010 | 0.0057 |
| $H_2NSO_3H$ | 0.021 | 0.39 | 0.09 | 0.81 | 0.0085 |
| Ph-$B(OH)_2$ | 58.6 | 23.1 | 0.82 | 0.68 | 0.0081 |
| Ph-$AsO_3H_2$ | 31.7 | 49.2 | 13.9 | 0.78 | 0.0672 |

[a] Mean from three different assays.
[b] Human recombinant isozyme, data from Ref. 44.
[c] data from Ref. 12.
[d] This work.

Because sulfonamides and their bioisosteres are the most important class of CA inhibitors, we performed inhibition studies on purified recombinant STPCA-2 with a series of 37 sulfonamides/sulfamates, among which well-known, clinically used compounds.[28]

Sulfonamides investigated for the inhibition of the coral STPCA-2 of types **1–22** are shown in Figure 1. Compounds **1–6**, **11**, **12**, **18**, and **22** are commercially available, whereas **7–10**,[29] **13–17**,[30] **19**,[20] **20**,[31] and **21**,[32] were prepared as reported earlier by this group. The 15 clinically used compounds such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA** (an orphan drug),[28] topiramate **TPM**, sulpiride **SLP**, indisulam **IND**,[33] zonisamide **ZNS**, celecoxib **CLX**, valdecoxib **VLX**, sulthiame **SLT**, and saccharin **SAC** are commercially available compounds.

Inhibition data of STPCA-2 with sulfonamides **1–22** and the 15 clinically used compounds **AAZ–SAC** are shown in Table 3, together with hCAI, hCAII, hCAVI, and STPCA inhibition data.

The following structure activity relationship (SAR) should be noted regarding the data of Table 3:

(i) The majority of investigated compounds showed low potency as STPCA-2 inhibitors, with inhibition constants in the range of 169–902 nM. These compounds include simple benzene-mono- (**1–10**, **19–22**) and 1,3-di-sulfonamide derivatives (**11** and **12**), 1,3,4-thiadiazole-2-sulfonamide

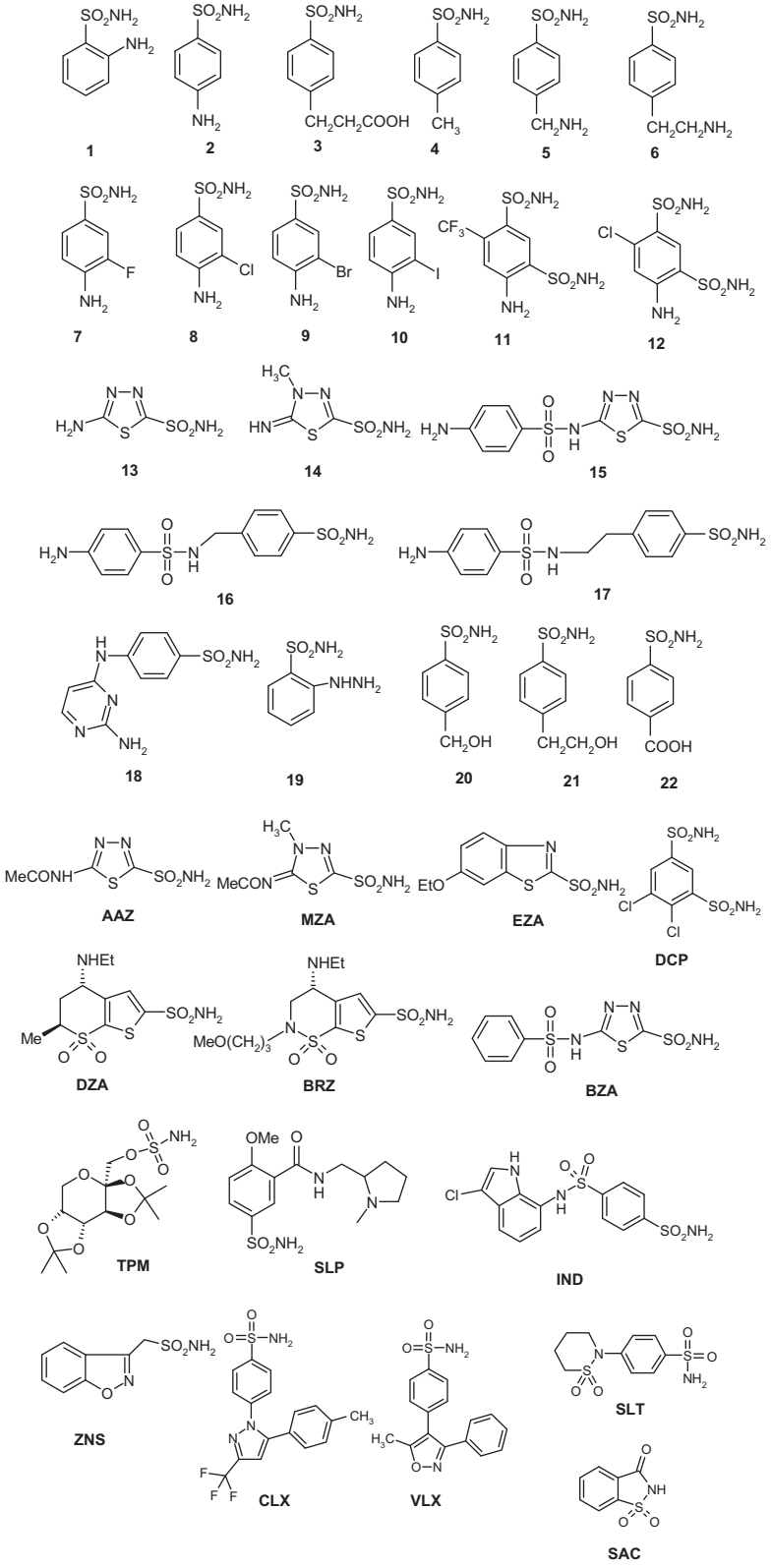


**Figure 1.** Chemical structure of sulfonamide/sulfamate inhibitors used in this study.

derivatives (**13** and **14**), heterocyclic compounds **15–18**, and also the clinically used drugs **BRZ**, **BZA**, **TPM**, **SLP**, **IND**, **ZNS**, and **CLX**.

(ii) A second group of derivatives, such as **AAZ**, **MZA**, **EZA**, **DCP**, **DZA**, **SLT**, and **SAC** showed medium STPCA-2 inhibitory properties, with inhibition constants in the range of 74–

**Table 3**
hCA I, II, VI, STPCA, and STPCA-2 inhibition data with sulfonamides **1**–**22** and the 15 clinically used derivatives **AAZ**–**SAC**. Data of isoforms I and II are from Ref. 43, data of hCA VI from Ref. 26, and date of STPCA from Ref. 13

| Inhibitor | $K_I$[a] | | | | |
|---|---|---|---|---|---|
| | hCA I[b] (nM) | hCA II[b] (nM) | hCA VI[b] (nM) | STPCA (nM) | STPCA-2 (nM) |
| 1 | 45400 | 295 | 772 | 553 | 675 |
| 2 | 25000 | 240 | 941 | 364 | 300 |
| 3 | 6690 | 495 | nt | 256 | 294 |
| 4 | 78500 | 320 | 1582 | 614 | 516 |
| 5 | 25000 | 170 | 4800 | 82.5 | 508 |
| 6 | 21000 | 160 | 813 | 94.3 | 577 |
| 7 | 8300 | 60 | 96 | 75.1 | 493 |
| 8 | 9800 | 110 | 1097 | 87.5 | 551 |
| 9 | 6500 | 40 | 4680 | 104 | 540 |
| 10 | 6000 | 70 | 1024 | 88.3 | 695 |
| 11 | 5800 | 63 | 955 | 367 | 481 |
| 12 | 8400 | 75 | 608 | 295 | 840 |
| 13 | 8600 | 60 | 798 | 105 | 361 |
| 14 | 9300 | 19 | 740 | 91.9 | 357 |
| 15 | 6 | 2 | 73 | 27.6 | 333 |
| 16 | 164 | 46 | 55 | 29.8 | 661 |
| 17 | 185 | 50 | 24 | 19.7 | 902 |
| 18 | 109 | 33 | 86 | 25.4 | 868 |
| 19 | 24000 | 125 | 1090 | 2510 | 650 |
| 20 | 55 | 80 | 6680 | 768 | 642 |
| 21 | 21000 | 125 | 4150 | 770 | 701 |
| 22 | 23000 | 133 | 887 | 435 | 607 |
| AAZ | 250 | 12 | 11 | 16.0 | 74 |
| MZA | 50 | 14 | 10 | 21.2 | 132 |
| EZA | 25 | 8 | 43 | 39.4 | 105 |
| DCP | 1200 | 38 | 79 | 431 | 79 |
| DZA | 50000 | 9 | 10 | 18.1 | 113 |
| BRZ | 45000 | 3 | 0.9 | 48.2 | 169 |
| BZA | 15 | 9 | 93 | 20.4 | 214 |
| TPM | 250 | 10 | 45 | 29.1 | 367 |
| SLP | 12000 | 40 | 0.8 | 430 | 415 |
| IND | 31 | 15 | 47 | 163 | 394 |
| ZNS | 56 | 35 | nt | 259 | 645 |
| CLX | 50000 | 21 | nt | 34.2 | 690 |
| VLX | 54000 | 43 | nt | 28.7 | 5710 |
| SLT | 374 | 9 | nt | 45.2 | 123 |
| SAC | 18540 | 5950 | 935 | 40.3 | 104 |

[a] Errors in the range of 5–10% of the shown data, from three different assays.
[b] Human recombinant isozymes, stopped-flow $CO_2$ hydrase assay method, pH 7.5, 20 mM Tris–HCl buffer.[33]

123 nM. All of these compounds are clinically used drugs. The most effective inhibitors were acetazolamide **AAZ** and dichorophenamide **DCP** (74 and 79 nM, respectively).

(iii) It may be observed that STPCA-2 has a completely different inhibition profile as compared to the human cytosolic isozymes I and II or the secreted isozymes hCA VI and STPCA. Furthermore, in general, the sulfonamides are less effective on STPCA-2 than on STPCA. The best example is shown by valdecoxib **VLX**. This compound is ineffective on STPCA-2 with a $K_I$ of about 5.7 μM and is 200-fold more efficient on STPCA. Saccharin **SAC**, an intramolecularly acylated sulfonamide, weakly inhibits hCA I, hCA II, and hCA VI but acts as an effective STPCA and STPCA-2 inhibitor (40–104 nM range versus micromolar range). It should be noted that SAC is also less effective on isoforms hCA III,[34] hCA IX,[35] hCA XII.[35]

In conclusion, we performed the first inhibition study on a new coral, CAII-like enzyme. We have tested a large series of anions and sulfonamides/sulfamate for their interaction with the catalytic domain of STPCA-2. The recombinant scleractinian coral *S. pistillata* CA II-like isoform, STPCA-2, showed a high catalytic activity for the physiological reaction of $CO_2$ hydration to bicarbonate, being slightly less active than the human isozyme hCA II. Contrary to the secreted isoform STPCA, STPCA-2 was less sensitive to bromide and iodide inhibition ($K_I$ = 0.96 and 33 mM, respectively, vs 9.7 and 9 μM). The best STPCA-2 anion inhibitors were sulfamide, sulfamic acid, phenylboronic acid, and phenylarsonic with a $K_I$ in the range of 5.7–67.2 μM. Concerning inhibition with sulfonamides, some clinically used derivatives (acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, benzolamide, and sulpiride, or indisulam, a compound in clinical development as antitumor drug), as well as the sulfamate antiepileptic drug topiramate were used. Some simple amino-/hydrazine-/hydroxy-substituted aromatic/heterocyclic sulfonamides have also been included in the study. All types of activity have been detected, with low potency inhibitors ($K_I$s in the range of 123–5710 nM), or with medium potency inhibitors ($K_I$s in the range of 74–113 nM). The clinically used sulfonamides, such as ethoxzolamide, showed the best inhibition with medium potency, with $K_I$s in the range of 74–113 nM. When compared to STPCA, STPCA-2 is less sensitive to sulfonamides.

Such inhibition data may give some information on metabolic pathways involving CAs. Indeed, in metabolically active tissues, and especially in situations requiring efficient ion transport, many CA isoforms interact with bicarbonate transporters or biosynthetic enzymes to form spatially and functionally orchestrated protein complexes called metabolons.[36] For example, CAs can improve the transmembrane movement of bicarbonate or other membrane-impermeable anions which are transported by integral membrane proteins including the $Cl^-/HCO_3^-$ anion exchangers, $Na^+$-coupled $HCO_3^-$ co-transporters, and SLC26A transporters.[36,37] One of the role of CAs (which physically interact with these anion

transporters) in these metabolons is to increase the local availability of bicarbonate (or other anions, such as sulfate or carboxylates) and thereby accelerate their transmembrane flux. Via this mechanism, various CAs contribute to the modulation of pH at both sides of plasma membranes. It is thus clear that in addition to their catalytic activity, most CAs are also involved in much more complex processes in which they interact with anions present in rather high concentrations in various tissues, as well as with their transporters. In corals, due to a Carbon Concentrating Mechanism (see[38] for review), and due to the value of intracellular pH[45] the inorganic carbon is under the form of bicarbonate in the cytosol of the coral cells.[39] This pool of bicarbonate is used by the symbiotic zooxanthellae for photosynthesis. Bicarbonate has to be dehydrated to $CO_2$ probably thanks to a proton pump[41] and a CA.[40] This CA could be a CA II-like enzyme (i.e., STPCA-2) since we have shown that this enzyme is located in symbiotic coral cells[18] where bicarbonate could be stocked.

It is also interesting to note that as global warming processes continue or even accelerate, in parallel with an increase of $CO_2$ concentration in the atmosphere and subsequently in seawater, this may lead to the disequilibrium of symbiosis. Thus, experiments performed on living corals with specific inhibition of enzymes involved in symbiosis will help to better understand the mechanisms of symbiosis and especially the role that CAs play in such a process, and surely warrant further studies.

## Acknowledgments

This research was financed in part by a grant of the 6th Framework Programme of the European Union (DeZnIT project), by an Italian FIRB Project (MIUR/FIRB RBNE03PX83_001) and by the Centre Scientifique de Monaco Research Program, which is supported by the Government of Principality of Monaco.

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