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Molecular Cloning, Characterization, and Inhibition Studies of a β-Carbonic Anhydrase from *Malassezia globosa*, a Potential Antidandruff Target

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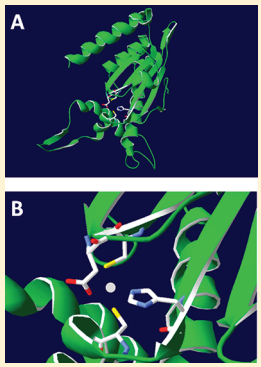
Supporting Information

ABSTRACT: A β-carbonic anhydrase (CA, EC 4.2.1.1) from the fungal pathogen *Malassezia globosa* has been cloned, characterized, and studied for its inhibition with sulfonamides. This enzyme, designated MG-CA, has significant catalytic activity in the CO_2 hydration reaction and was inhibited by sulfonamides, sulfamates, and sulfamides with K_I in the nanomolar to micromolar range. Several sulfonamides have also been investigated for the inhibition of growth of *M. globosa*, *M. dermatis*, *M. pachydermatic*, and *M. furfur* in cultures, whereas a mouse model of dandruff showed that treatment with sulfonamides led to fragmented fungal hyphae, as for the treatment with ketoconazole, a clinically used antifungal agent. These data prompt us to propose MG-CA as a new antidandruff drug target.



INTRODUCTION

Carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzymes in all life kingdoms.[1−4] In mammals, 16 different isoforms have been identified, with varying physiological importance.[4] Accordingly, these isozymes possess different catalytic activities, cellular locations, and tissue distributions. In terms of localization, five are found in the cytoplasm (CAs I−III, CA VII, CA XIII), five are membrane bound (CA IV, CA IX, CA XII, CA XIV, CA XV), two are located in the mitochondria (CA VA and CA VB), and there is a single secreted isozyme (CA VI).[4] The function of isozymes VIII, X, and XI remains unclear, but they appear to be acatalytic.[4] The isozymes are distributed throughout the human body and have been identified, among other tissues, in the brain, kidney, eye, lung, epithelium, esophagus, colon, pancreas, and heart.[4]

In addition to the mammalian isoforms, CAs from specific organisms have recently been targeted for therapeutic gain, most notably to fight infectious disease.[4−9] Thus, the CAs from *Plasmodium falciparum* (malaria), *Helicobacter pylori* (stomach ulcers/gastric cancer), *Candida albicans* (fungal infections), *Cryptococcus neoformans* (meningitis), and *Mycobacterium tuberculosis* (tuberculosis), among others have all been proposed as potential drug targets.[4−9] Whereas in mammals only the α-class of CAs have been found so far, most prokaryotes encode CAs belonging to the β-class.[1−4] These gene families are evolutionarily unrelated.[1] One of the significant differences between these classes is in the amino acid residues used to bind the catalytic Zn ion at the active site. The α-class of CAs utilizes three histidine residues, while the β-class uses an aspartate, histidine, and two conserved cysteine residues to coordinate the Zn ion ("closed active site"), or one histidine and two cysteine residues, with the fourth ligand being a water molecule/hydroxide ion ("opened active site").[3,10]

Dandruff (historically termed *Pityriasis capitis*) is a commonly occurring phenomenon, found in more than 50% of the worldwide population, and presents as an excessive shedding of dead skin cells from the scalp.[11] In individuals without dandruff, skin cells typically mature and shed in approximately 1 month.[11] However, for those with dandruff, the shedding rate is accelerated and may take only 2−7days. Dandruff can be triggered by several factors, including an increase in sebum production, irritation by organisms (particular of the *Malassezia* sp.) and individual susceptibility (hereditary factors).[11] *Malassezia* (previously known as *Pityrosporum*) are yeasts, which are found to naturally occur on the skin and scalp of most individuals. There are several recognized species including *M. globosa*, *M. furfur*, and *M. restricta*.[12] Until recently, *M. furfur* was thought to be responsible for the onset of dandruff, but instead, the scalp specific *M. globosa* was found to be the causative agent.[12] *Malassezia* are dependent on external lipids

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for growth and hence secrete lipases to break down triglycerides that occur in the sebum of human skin. Byproducts of this breakdown include unsaturated fatty acids, such as oleic acid, which penetrate the stratum corneum (the top layer of the epidermis), resulting in an inflammatory response. In susceptible individuals, this results in rapid shedding of the stratum corneum and is presented as dandruff.[11,12] Several strategies and treatments are available for dandruff, the majority of which aim to target growth of the causative fungi. A common active ingredient in antidandruff shampoos is zinc pyrithione, also known as zinc pyridinethione (**A**) (Figure 1).[13] Its effect is most likely mediated through disruption of fungal membrane activities.[13] At low concentrations, fungi are capable of overcoming the effect of zinc pyrithione. Ketoconazole (**B**) (Figure 1), present in other shampoos, is an azole antifungal agent interfering with the biosynthesis of fungal sterols.[14] Other azoles have also been used in antidandruff treatments, as they interfere with the synthesis of ergosterol, a key component of fungal cell walls.[14] Since mammals utilize cholesterol instead of ergosterol, they are not sensitive to ketoconazole and the agent is specific for fungi.[14] In general, those shampoos that contain ketoconazole have been found to be more effective as an antidandruff treatment than those containing zinc pyrithione.[14]

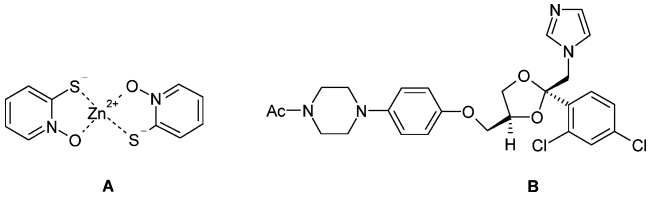

Figure 1. Two currently used ingredients in antidandruff shampoos: zinc pyrithione (**A**) and ketoconazole (**B**).

However, the effectiveness of compounds **A** and **B** for preventing/treating dandruff is not very high, and additional therapeutic strategies are being explored, such as inhibition of the lipase present in *Malassezia* spp.[15] Here, we report the molecular cloning, characterization, and in vitro/in vivo inhibition studies of a β-CA from *M. globosa*, denominated MG-CA, which we propose as a novel antidandruff target.

RESULTS AND DISCUSSION

MG-CA Cloning and Purification. The *M. globosa* protein database was searched using the *E. coli* CA sequence. BLAST analyses identified two identical sequences, XP_001730815.1 and EDP43601.1 (see Supporting Information for all the used sequences). Further sequence alignments (Figure 2), with the *E. coli* enzyme and Can2 from *Cryptococcus neoformans*, showed the zinc binding residues to be conserved among these organisms and the enzyme to belong to the β-family of CAs.[1−3] Additional sequence comparisons of XP_001730815.1/EDP43601.1 with *E. coli* CA, *C. neoformans* Can2, and the CA from *Candida albicans* showed not only the Zn binding residues to be conserved but appreciable identity throughout the sequences (i.e., 33% with Can2 and 35% with *E. coli* T2 CA). In contrast, however, no significant sequence identity was found between human (h) CA II and the *M. globosa* CA (MG-CA), as the two enzymes belong to different classes (β for the fungal enzyme and α for the human one). This MG-CA gene encodes for a 241 amino acid polypeptide with a high sequence homology to that of other β-CAs of fungal or bacterial origin such as the *E. coli* CA and Can2 (Figure 2). A GST-MG-CA fusion protein cloned, expressed, and purified as described by this group[5−10] for the β-CAs from *H. pylori, M. tuberculosis, C. neoformans*, and *Saccharomyces cerevisiae* (see Experimental Protocols for details and Figure 1 in the Supporting Information). We have demonstrated that these

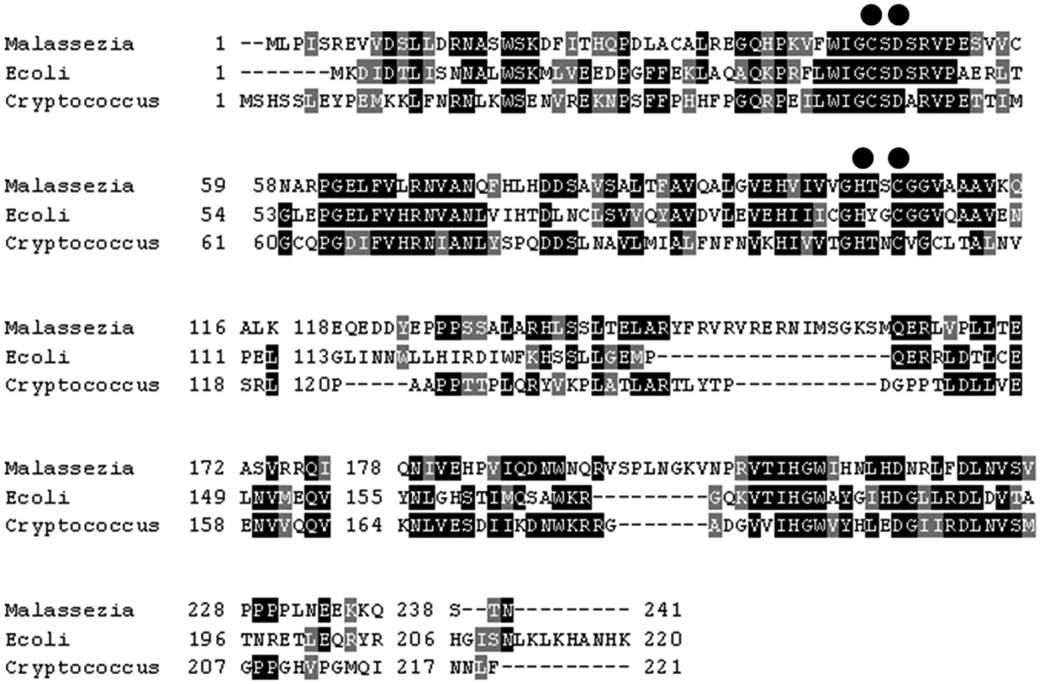

Figure 2. CLUSTAL W alignment of carbonic anhydrase sequences from *M. globosa* (XP_001730815.1/EDP43601.1), *Escherichia coli* CA (P61517) [35%], and *Cryptococcus neoformans* Can2 (Q314V7) [33%]. Numbers are the primary accession numbers and the percentage identity with *Malassezia globosa* carbonic anhydrase, and ● indicates the conserved Zn ion binding residues.

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Table 1. Characterization of *M. globosa* β-CA (MG-CA) and Comparison of Its Activity and Acetazolamide Inhibition with Those of the Human Isoforms hCA I and II, as Well as the β-CAs from *Cryptococcus neoformans* (Can2), *Candida albicans* (CaNce103), and *Candida glabrata* (CgNce103)[a]

enzyme[b]	class	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	K_I (acetazolamide) (nM)
hCA I	α	2.0×10^5	5.0×10^7	250
hCA II	α	1.4×10^6	1.5×10^8	12
Can2	β	3.9×10^5	4.3×10^7	10.5
CaNce103	β	$8.0x\ 10^5$	9.7×10^7	132
CgNce103	β	3.8×10^5	4.8×10^7	11
MG-CA[c]	β	$(8.6 \pm 0.1) \times 10^5$	$(6.9 \pm 0.2) \times 10^7$	76000 ± 450

[a]The activity was measured by a stopped flow technique[17] at 20 °C and pH 7.5 in 10 mM HEPES buffer for α-CAs. The activity of the β-CAs was measured at 20 °C, pH 8.3, in 20 mM Tris·HCl buffer and 20 mM NaCl. [b]Data for hCA I, II, Can2, CaNce103, and CgNce103 were reported earlier by this group.[4,6,7] [c]Mean ± standard error from three different determinations.

GST-fusion constructs generally lead to a high level of protein expression in *E. coli* and that the CA thus obtained is properly folded.[5−10] Indeed, as seen from Supporting Information Figure 1, MG-CA has been purified with great efficiency by using this construct.

MG-CA Catalytic Activity. We performed a kinetic investigation of purified MG-CA, comparing its kinetic parameters (k_{cat} and k_{cat}/K_M)[17] with those of thoroughly investigated CAs, such as the cytosolic, ubiquitous human isozymes hCAs I and II, as well as β-CAs from fungal pathogens such as *C. albicans* (CaNce103), *C. neoformans* (Can2), or *C. glabrata* (CgNce103) characterized[4,5,18] (Table 1).

Data from Table 1 show that, similar to other CAs belonging to the α- or β-class, the fungal enzyme MG-CA possesses appreciable CO_2 hydrase activity, with a k_{cat} of 8.6×10^5 s^{-1}, and k_{cat}/K_m of 6.9×10^7 $M^{-1}\cdot s^{-1}$. Thus, MG-CA has a similar activity for the physiologic reaction (CO_2 hydration to bicarbonate and protons) as the human hCA I isoform and the other β-class enzymes from fungal pathogens investigated earlier.[4,5,18] The data of Table 1 also show that all these enzymes, except MG-CA, were appreciably inhibited by the clinically used sulfonamide acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), whereas MG-CA was not inhibited by this compound, with an inhibition constant of 76 μM. This lack of effectiveness of **AAZ** for inhibiting a β-class enzyme has been observed only for Cab, an enzyme isolated and characterized from the Archaeon *Methanobacterium thermoautotrophicum*.[1]

Figure 2 shows an alignment of the amino acid sequence of bacterial/fungal β-CAs investigated thus far by means of X-ray crystallography and kinetic methods, such as the *E. coli* T2 enzyme[16] and Can2 from *C. neoformans*.[6] It may be observed that similar to all these β-CAs, MG-CA possesses the conserved amino acids involved in the catalytic cycle of this class of enzymes: (i) the Zn(II) binding residues Cys47, Asp49, His103, and Cys106 (based on the *M. globosa* numbering) and (ii) the Asp-Arg dyad (in this case Asp49 and Arg51), which activates the metal ion for catalysis. Thus, residues Cys47, Asp49, His103, and Cys106 (*M. globosa* CA numbering) probably coordinate the metal ion as in other β-CAs investigated so far (Figure 3), for which some of the X-ray crystal structures have also been reported.[1,6,16] As for most such CAs, when the zinc ion is coordinated by two Cys, one His, and one Asp ligand, with no catalytic water directly bound, this represents the so-called "closed active site", which cannot explain the catalytic activity of these enzymes.[3] However, as shown by Jones' group for the *M. tuberculosis* enzymes, at pH > 8.3 the fourth zinc ligand, the Asp residue, is replaced by a water molecule/hydroxide ion as a consequence of a salt bridge formation of the Asp with the conserved Arg residue nearby from the dyad mentioned above (Arg51, *M. globosa* CA numbering). Thus, the fourth metal ligand becomes a strong nucleophile (water molecule/hydroxide ion) that can attack CO_2 and transform it to bicarbonate.[3,4,16] The Asp-Arg dyad described here is thus involved in the zinc−water activation mechanism, as in all closed active site β-CAs investigated so far.[3,4,16] The homology model of MG-CA from Figure 3 also shows the typical fold of the fungal β-CAs, quite similar to that of Can2 reported recently by X-ray crystallography,[6] whereas the active site details are also conserved with those for other CAs belonging to this class, such as the enzymes isolated from bacteria (*M. tuberculosis*)[3] or fungi (Can2).[6]

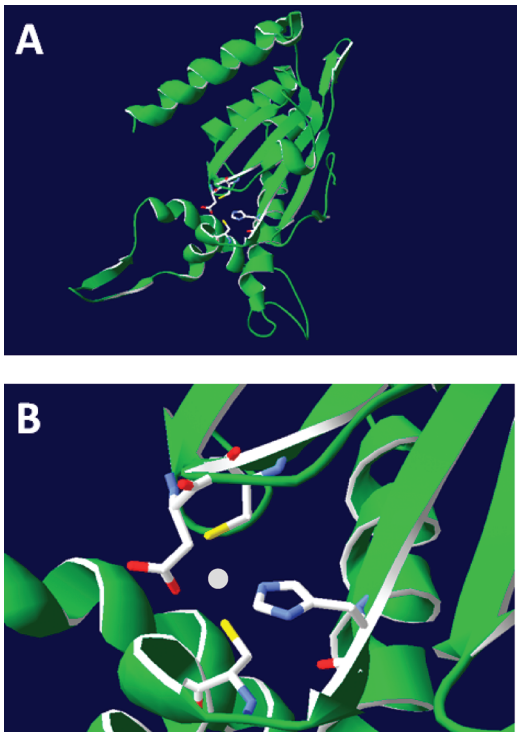

Figure 3. (A) Homology model of the *Malassezia globosa* CA based on its sequence and structure alignment with *E. coli* CA II (PDB code 1i6o) (done with UniProt). (B) Conserved active site Zn binding residues Cys47, Asp49, His103, and Cys106 (*Malassezia globosa* carbonic anhydrase numbering). The metal ion is the white sphere, and amino acid residues are shown in CPK colors.

In Vitro MG-CA Inhibition with Sulfonamides, Sulfamates, Sulfamides. Table 2 shows the MG-CA

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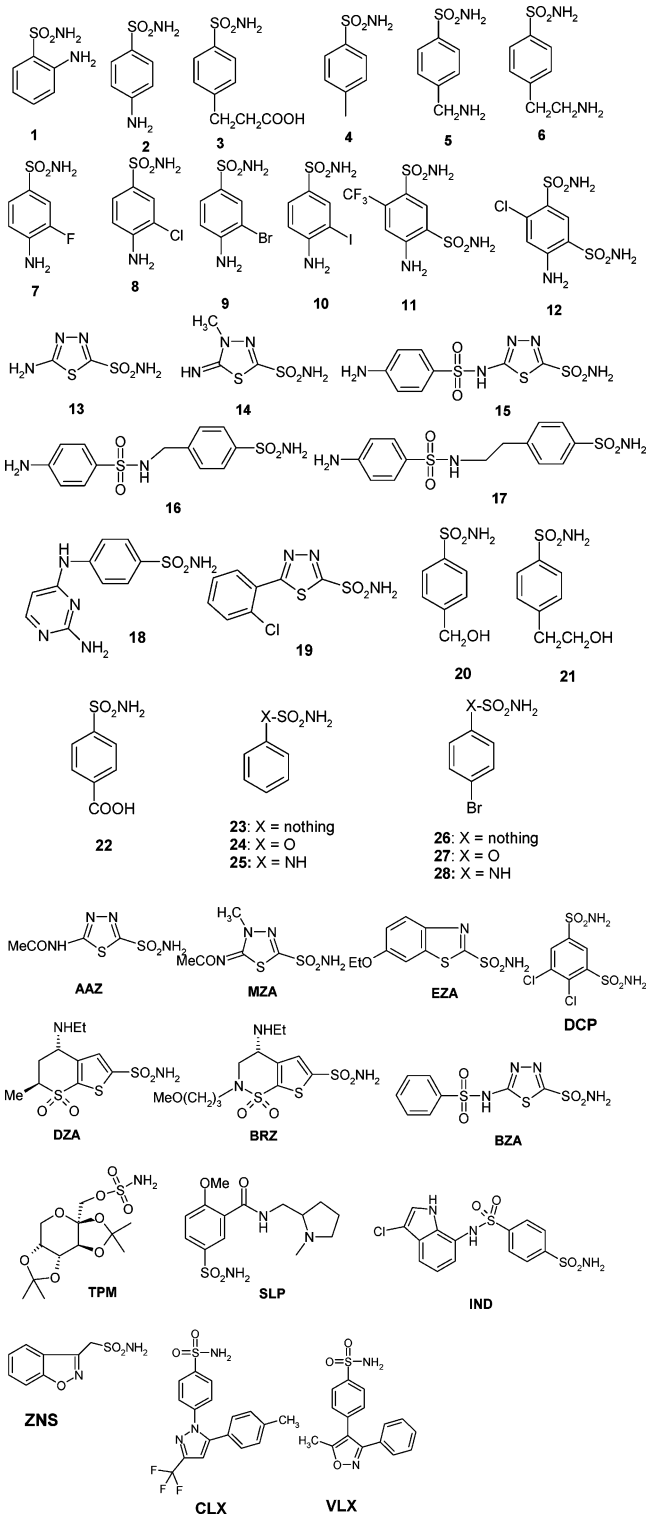

inhibition data with a panel of sulfonamides, sulfamates, and sulfamides (obtained for the CO_2 hydration reaction catalyzed by the CAs).[17] Some of these compounds are clinically used drugs,[4] such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA** (an orphan drug), topiramate **TPM**, sulpiride **SLP**, indisulam **IND**, zonisamide **ZNS**, celecoxib **CLX**, and valdecoxib **VLX**. In fact the sulfonamides and their bioisosteres represent the most investigated class of CA inhibitors (CAIs).[4] The simpler derivatives **1**–**28** were also included in the study, as they

Table 2. Inhibition Data of Human (h) and Fungal CAs with Sulfonamides, Sulfamates, Sulfamides, and Clinically Used Derivatives by a Stopped-Flow CO_2 Hydrase Assay[17]

	K_I[a] (nM)			
inhibitor	hCA II	Can2	CaNce103	MG-CA
1	295	379	1086	9800
2	240	765	7627	245
3	495	440	1277	152
4	320	1150	1204	6740
5	170	18490	1109	174
6	160	1394	3195	79
7	60	809	1240	116
8	110	605	1345	121
9	40	977	408	349
10	70	711	719	543
11	63	968	6115	90
12	75	300	874	92
13	60	791	832	79000
14	19	815	1368	85000
15	2	42	1092	236
16	46	971	1310	104
17	50	624	727	63
18	33	3887	1108	68
19	12	379	1293	35000
20	80	623	383	234
21	125	878	1209	118
22	133	484	861	94
23	305	746	2360	4530
24	1.3	703	1975	2560
25	13	731	1913	3100
26	81	612	894	650
27	1.5	345	715	374
28	23	410	763	413
AAZ	12	10.5	132	76000
MZA	14	63	484	74550
EZA	8	87	1070	38000
DCP	38	1203	909	346
DZA	9	8347	887	79000
BRZ	3	87	1095	84000
BZA	9	23	1510	482
TPM	10	367	1108	1460
SLP	40	812	760	320
IND	15	963	1090	113
ZNS	35	971	942	7650
CLX	21	3056	1017	34800
VLX	43	704	699	31500

[a]Mean from three assays. Errors were in the range of 5–10% of the reported value (data not shown).

contain the scaffolds most extensively used to design potent or isoform-selective CAIs.[4,19] Data for the inhibition of the dominant human isoforms hCA II[19] as well as the fungal β-class enzymes from *C. albicans*[20] and *C. neoformans*[6] with these compounds are also included in Table 2 for comparison reasons. The following structure–activity relationship (SAR) can be observed from data of Table 2:

(i) Many of the investigated compounds, such as **1**, **4**, **13**, **14**, **19**, **23**–**25**, **AAZ**, **MZA**, **EZA**, **DZA**, **BRZ**, **TPM**, **CLX**, and **VLX** showed very weak MG-CA inhibitory activity, with inhibition constants in the high micromolar range (K_I of 2.56–76 μM). This is a surprising result, considering that most of the clinically used inhibitors

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described above are usually highly effective inhibitors of α-CAs (such as hCA II; see Table 2) and of some fungal β-class CAs, for example, Can2 or CaNce103. Our data show that most of the heterocyclic sulfonamide derivatives investigated here (**13**, **14**, **19**, and the clinically used compounds mentioned above) show weak or very weak inhibitory activity against MG-CA. The same behavior was observed for orthanilamide **1**, tosylamide **4**, and the simple, unsubstituted benzenesulfonamide, -sulfamate, and -sulfamide (compounds **23**–**25**). We investigated these quite simple derivatives in order to understand whether the nature of the zinc-binding group influences potency. The sulfamate **24** was slightly more active than the sulfamide **25**, which in turn was more active than the benzenesulfonamide **23** (Table 2), but all these compounds show only micromolar inhibition of MG-CA, being more effective as hCA II and Can2 inhibitors.

(ii) Most of the investigated derivatives showed in vitro inhibitory activity in the submicromolar range, with K_I of 104–650 nM (Table 2). They include the 4-substituted benzenesulfonamides **2**, **3**, **5**, **20**, and **21**, the halogenosulfanilamides **7**–**10**, aminobenzolamide **15** and its benzo analogue **16**, the 4-bromosubstituted benzenesulfonamide, -sulfamate, and sulfamide **26**–**28**, as well as four clinically used/preclinical derivatives, **DCP**, **BZA**, **SLP**, and **IND**. Only benzolamide **BZA** and aminobenzolamide **15** among the heterocyclic sulfonamides showed efficient MG-CA inhibition; all other compounds were benzenesulfonamides (or the corresponding sulfamates/sulfamides). Again, the inhibition profile of other α- and β-CAs with these compounds is quite distinct compared to MG-CA.

(iii) Several of the investigated compounds showed inhibition constants of <100 nM, and they include **6** (4-aminoethylbenzensulfonamide), the benzene-1,3-disulfonamides **11** and **12**, the elongated molecules incorporating a benzenesulfonamide head **17** and **18**, and 4-carboxybenzenesulfonamide **22**. These compounds had K_I in the range of 63–94 nM. It may be observed that they are chemically heterogeneous except that all incorporate the benzenesulfonamide or 1,3-disulfonamide scaffolds.

(iv) The inhibition profile of MG-CA is highly distinct from those of hCA II and other pathogenic fungal β-CAs, such as the enzymes from *C. albicans* and *C. neoformans* (Table 2).

MICs against *Malassezia* spp. Although efficient in vitro inhibitors of some β-CAs have been detected, for example, the three enzymes from *M. tuberculosis*, this inhibition has not always been reproduced in vivo, mostly because of cell permeability issues.[21] Thus, we performed MIC determinations with a wide potency range of the described inhibitors (Table 3). Following compound testing, MIC values as low as 10 μg/mL were observed (for compound **2**), with growth inhibition apparent against all the *Malassezia* strains screened (Table 3). Although the MIC values for a number of the compounds were high (640 μg/mL), this may be a reflection of low cell permeability, a frequent problem with cell based assays and described above (Table 3).[21]

Murine Model of *M. pachydermatis* Skin Infection. Compound **6**, possessing a 79 nM MG-CA inhibitory activity in vitro (Table 2) and medium MIC efficacy (Table 3), was chosen for use in a mouse model of dandruff/*Malassezia* infection. Following infection with *Malassezia* and subsequent treatment with 5% 4-(2-aminoethyl)benzenesulfonamide **6**, 67% (4/6) of the mice demonstrated clinical improvement (Table 4). As a comparison, after treatment with the clinically used ketoconazole **B**, all the animals showed an improvement in skin appearance. No treatment was also used as a control (Table 4). Treatment with 4-(2-aminoethyl)-benzenesulfonamide caused damage to the *Malassezia*, resulting in hypha fragmentation, which is indicative of compound effect on fungal growth (Table 4).

Table 3. MICs of Selected Sulfonamides against Various *Malassezia* Species

	MIC (μg/mL)			
compd	*M. furfur*	*M. dermatis*	*M. pachydermatis*	*M. furfur*
1	640	640	>640	640
2	80	160	10	80
4	640	320	320	160
5	320	160	160	160
6	640	640	640	320
11	>640	>640	>640	320
12	640	640	640	640
22	>640	>640	640	640
23	160	>640	160	320
26	320	320	320	320
AAZ	640	320	640	320
DCP	>640	>640	>640	320

CONCLUSIONS

We have cloned a β-CA from the fungal pathogen *Malassezia globosa* and characterized this new enzyme (MG-CA) kinetically. We also investigated its inhibition with sulfonamides, sulfamates, and sulfamides. MG-CA has a significant catalytic activity in the CO_2 hydration reaction and was inhibited by sulfonamides, sulfamates, and sulfamides with K_I in the nanomolar to micromolar range. Several sulfonamides were also investigated for the inhibition of growth of *M. globosa*, *M. dermatis*, *M. pachydermatic*, and *M. furfur* cultures, whereas a mouse model of the disease showed that treatment with sulfonamides led to fragmented fungal hyphae and clinical improvement. These data prompt us to propose MG-CA as a new antidandruff drug target.

EXPERIMENTAL PROTOCOLS

Chemistry. Compounds **1**–**22** and **AAZ** – **SAC** either are commercially available (Sigma-Aldrich) or were prepared as described earlier.[19]

Cloning and Preparation of Recombinant MG-CA. *M. globosa* strain ATCC 96807/CBS 7966[22] has been used for the experiments (UniProtKB/Swiss-Prot entry A8PZS4, EIF3F_MALGO). It has been annotated that MGL_1814 hypothetical protein K01673 is a β-CA. We substituted the codons of the initial three amino acid residues with *E. coli*-favoring codons, which are most frequently used in *E. coli*: Gly (GGT:GGC), Phe (TTC:TTT), and Lys (AAG:AAA). Addition of *Eco*RI recognition sequence (underlined in the following sequence) resulted in the forward primer sequence: 5′-CGGAATTCCCATGGGCTTTAAAGGCCCG-3′. The 3′-primer sequence including the *Sal*I recognition site (underlined in the following sequence) was 5′-CGGTCGACCTAGGGCGTGAC-3′. The PCR reaction was hot-started with incubation for 5 min at 94 °C and consisted of 40 cycles of 30 s at 94 °C, 30 s at 57 °C, and 90 s at 72 °C and terminated with

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Table 4. Results for Skin Appearance and Culture Experiments for Three Treatment Options of Mice Infected with *Malassezia*: Sulfonamide CAI Treatment, Ketoconazole Treatment, and Control Experiment (No Treatment)

	4-(2-aminoethyl)benzenesulfonamide		ketoconazole		control	
compd	skin appearance	skin and hair culture	skin appearance	skin and hair culture	skin appearance	skin and hair culture
1	recovered	hyphae fragmented	recovered	uninfected	looked infected	moderate infection
2	recovered	hyphae fragmented	recovered	moderate infection	looked infected	moderate infection
3	recovered	moderate infection	recovered	hyphae fragmented	looked infected	moderate infection
4	recovered	heavy infection	recovered	moderate infection	not infected	mild infection
5	looked infected	hyphae fragmented	recovered	moderate infection	looked infected	uninfected
6	looked infected	hyphae fragmented	recovered	moderate infection	looked infected	mild infection

incubation for 10 min at 72 °C. The PCR products were cleaved with *Eco*RI and *Sal*I and then ligated in-frame into the pGEX-4T2 vector (Amersham, U.K.). The proper DNA sequences of the MG-CA insert subcloned into the vector were reconfirmed by DNA sequencing. The constructs were then transfected into *E. coli* strain BL21 for production of the GST-MG-CA fusion protein as previously reported.[8−10] Following induction of the protein expression by addition of 1 mM isopropyl-γ-D-thiogalactopyranoside, the bacteria were harvested and sonicated in PBS. The sonicated cell extracts were further homogenized twice with a Polytron (Brinkmann) for 30 s each at 4 °C. Centrifugation at 30000*g* for 30 min afforded the supernatant containing the soluble proteins. The obtained supernatants were then applied to prepacked glutathione Sepharose 4B columns (Amersham, U.K.). The columns were extensively washed with buffer, and then the GST- MG-CA fusion protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. Finally, the GST part of the fusion protein was cleaved with thrombin. The obtained MG-CA recombinant protein was further purified by sulfonamide affinity chromatography and the amount of enzyme was determined spectrophotometrically as reported for similar β-CAs[8−10] (an SDS page is shown in the Supporting Information, Figure 1).

CA Catalytic Activity and Inhibition. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[17] Phenol red (at 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10−20 mM Hepes (pH 7.5, for α-CAs) or Tris (pH 8.3 for β-CAs) as buffers and 20 mM Na_2SO_4 (for α-CAs) or 20 mM NaCl (for β-CAs) (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10−100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5−10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled−deionized water, and dilutions up to 0.01 nM were done thereafter with distilled−deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E−I complex. The inhibition constants were obtained by nonlinear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes were from Lineweaver−Burk plots, as reported earlier,[10] and represent the mean from at least three different determinations.

Homology Modeling of MG-CA. The program UniProt[22] has been used to build a homology model of the new enzyme, based on its sequence and the alignment with *E. coli* β-CA, for which the X-ray crystal structure (PDB file 1i6o) is available.[16]

MIC Determinations against *Malassezia* sp. In order to evaluate the effect of the identified MG-CA inhibitors on *Malassezia* growth, susceptibility tests were performed on *Malassezia dermatis* CBS 9145, *Malassezia furfur* CBS 9569, *Malassezia pachydermatis* CBS 6536, and *Malassezia globosa* CBS 7966. *Malassezia* strains were cultured in ambient air at 30 °C on modified Leeming Notman agar for up to 120 h before testing, to ensure their purity. The inoculum for each strain was prepared by picking distinct colonies from the culture flasks and suspending them in 3 mL of sterile water. The turbidity was then adjusted to McFarland standard 5.0. The inoculum was completely resuspended by vigorous shaking on a vortex mixer for 15 s. Any remaining particulates were then disrupted by passing the inoculum through a fine 27G sterile needle. The inocula were then adjusted by diluting 1:10 in sterile water.

All assays were performed in Christensen's urea medium (supplemented with 0.2% Tween 80 and 1.0% Tween 60), solidified using 1% agar to aid growth. Stock solutions of each of the compounds were prepared at 2.5 g/L in either DMSO or sterile water. All stock samples were then further diluted in modified double strength Christensen's urea medium to give a starting concentration of 640 mg/L. In a 96-well plate, column 1 was filled with 200 μL of the appropriate test compound, and 100 μL amounts were taken from wells in column 1 and diluted 2-fold by transferring them to column 2 with a multichannel pipet (±2% coefficient of variation). Samples (100 μL) were then removed from column 2 and transferred to column 3 and so on through to column 10. *Malassezia* inoculum suspension (100 μL) in sterile water was added to the appropriate well, producing a well containing 200 μL final volume (made up of 100 μL of diluted compound and 100 μL of inoculum). All plates were incubated at 30 °C in air within a darkened incubator for up to 7 days. Plates were read visually with the end point taken as the lowest concentration of drug that inhibited growth by more than 50% compared to the drug free control. MIC values were determined for a number of the MG-CA inhibitors used in the enzymatic studies (against purified MG-CA) together with additional compounds.[23]

Murine Model of *M. pachydermatis* Skin Infection. Mice used in this study, male CD1 mice, were supplied by Charles River (Margate, U.K.) and were specific pathogen free. Mice were housed in individual ventilated cages supplied with HEPA filtered air. Sterile Aspen chip bedding was supplied in preautoclaved boxes. Sterile water was provided ad libitum using disposable pouches in addition to standard mouse chow. Mice experienced a 12 h light−dark cycle at 22 ± 1 °C, 55−60% relative humidity, and background noise of <60 dB. For the study, animals were treated in groups of six mice per treatment group. Cortisone acetate was prepared as a 12.5 mg/mL suspension in PBS containing 0.05% Tween 80. All animals were immunosuppressed with a dose of 125 mg/kg cortisone acetate subcutaneously 5 days before infection and reimmunosuppressed at the same dose 24 h before infection. On the day of infection, the mice were lightly anesthetized with 2.5% isofluorane. Both flanks were then shaved (2 cm × 2 cm), and just prior to infection the shaved flanks were lightly scarified with a scrubbing brush. Following surface abrasion, flanks were coated with a suspension of *Malassezia pachydermatis* containing 2×10^7 blastoconidia/mlL. Following infection all mice were observed as regularly as their clinical conditions required. Animals exceeding the severity band of the experiment (mild) would have been humanely euthanized. Mice were treated 48 h postinfection (just before the appearance skin lesions), once daily for 6 days by liberally spreading the relevant cream or vehicle control onto the infected flank using cotton tipped swabs. The skin of the shaved flanks was examined daily by investigators that were blinded to the treatment mice received or the infecting organism. Following euthanasia, approximately 10 hairs were removed from each flank of the mouse and were cultured onto Leeming Notman agar and incubated at 30 °C for up to 20 days. Skin

biopsies were also removed from the flanks of the mice and cultured as indicated. The compound chosen for the in vivo studies was 4-(2-aminoethyl)benzenesulfonamide, which was the most potent molecule from the enzymatic studies with a K_I of 79 nM. A 5% cream was prepared by dissolving 100 mg of 4-(2-aminoethyl)-benzenesulfonamide powder in 200 μL of DMSO and then mixing this solution with 2 g of E45 cream and vortexing for 2 min to disperse. A 2% Nizoral (ketoconazole) cream was used as a positive control. The vehicle control was 5% E45 cream alone.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Complete amino acid sequences of the new protein and of all other CAs used in the alignment and an SDS−PAGE of the purified MG-CA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): The cloning, purification, and inhibition data from the paper were patented by two of the authors, K.S.H. and C.T.S. (K. S. Hewitson, C. T. Supuran. Carbonic anhydrase inhibitors for treating dandruff and related disorders. WO 2010/061185).

■ ABBREVIATIONS USED

CA, carbonic anhydrase; CAI, carbonic anhydrase inhibitor; MG-CA, *Malassezia globosa* carbonic anhydrase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis

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