Carbonic Anhydrase Inhibitors. Cloning, Characterization, and Inhibition Studies of a New β-Carbonic Anhydrase from *Mycobacterium tuberculosis*

Isao Nishimori,[†] Tomoko Minakuchi,[†] Daniela Vullo,[‡] Andrea Scozzafava,[‡] Alessio Innocenti,[‡] and Claudiu T. Supuran*[,‡]

Department of Gastroenterology, Kochi Medical School, Nankoku, Kochi 783-8505, Japan, Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica, Rm. 188, Via della Lastruccia 3, I-50019 Sesto Fiorentino (Firenze), Italy

Received January 28, 2009

The Rv3273 gene product of *Mycobacterium tuberculosis*, a β-carbonic anhydrase (CA, EC 4.2.1.1), mtCA 3, shows appreciable catalytic activity for CO_2 hydration (k_{cat} of 4.3×10^5 s^{-1}, and k_{cat}/K_m of 4.0×10^7 $M^{-1} \cdot s^{-1}$). A series of sulfonamides/sulfamates was assayed for their interaction with mtCA 3. Sulfanilylsulfonamides, acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, benzolamide, and zonisamide, showed effective, submicromolar inhibition (K_Is of 104−611 nM), the best inhibitor being 2-amino-pyrimidin-4-yl-sulfanilamide (K_I of 91 nM).

Introduction

The genome of the human pathogen *Mycobacterium tuberculosis* contains at least two carbonic anhydrases (CAs,[a] EC 4.2.1.1).[1,2] These enzymes, encoded by the genes Rv1284 and Rv3588c, belong to the β-class,[3] and similarly to those encoded by α-, γ-, δ-, and ζ-CA families (widespread all over the phylogenetic tree),[3] are catalysts for the reversible interconversion between carbon dioxide and bicarbonate ($CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$). β-CAs are widespread in various human infecting agents, such as the fungal pathogens *Candida albicans* and *Cryptococcus neoformans*,[4−6] as well as bacteria, such as *Helicobacter pylori*,[7] *Heamophilus influenzae*,[8] *Escherichia coli*,[9] etc., and it has recently been shown, mainly by this group, that they are druggable targets.[3,5,7,10,11] In a recent work,[12] we have also cloned and characterized kinetically one of the *Mycobacterium tuberculosis* β-CAs, more precisely Rv1284, named mtCA 1, showing that it has appreciable catalytic activity for the physiological, CO_2 hydration reaction, with a k_{cat} of 3.9×10^5 s^{-1}, and a k_{cat}/K_m of 3.7×10^7 $M^{-1} \cdot s^{-1}$.[12] Furthermore, some clinically used sulfonamides/sulfamates investigated by us as CA inhibitors (CAIs), targeting mammalian α-CAs,[3] showed promising in vitro inhibitory activity against mtCA1, with the potential for developing antimycobacterial agents with a diverse mechanism of action compared to the clinically used drugs for which many strains exhibit multidrug resistance and extensive multidrug resistance.[12] Indeed, *M. tuberculosis* infection affects a large number of the world population, with an estimated 9.2 million new cases each year, of which many are lethal.[13,14] The therapy used to treat TB is based on agents developed 30−40 years ago, with no new drugs launched ultimately.[14−16] Presently, there is a huge interest for novel anti-TB drugs, with agents belonging to the fluoroquinolone, oxazolidinone, diarylquinoline, and nitroimidazo-oxazole/-oxazine classes in various stages of development.[14−16] The complete sequencing of the *M. tuberculosis* genome[17] facilitated the identification of possible new drug targets, but more than 60% of this genome encodes proteins whose functions are unknown at this moment.[1,2,17] Among these genes, Rv3273 is annotated as a transmembrane protein encoding for a sulfate transporter (at the amino-terminal region) and a putative β-CA (at the carboxy-terminal end of the protein).[1,2,17] Considering our interest in β-CAs as possible new drug targets, we report here the cloning, characterization, and inhibition studies with a panel of sulfonamides/sulfamates of the carboxy-terminal part of Rv3273, denominated here mtCA 3 (the isoform mtCA 2, encoded by the gene Rv3588c, has been reported and characterized crystallographically by Covarrubias et al.,[1,2] but its inhibition has not been investigated for the moment, whereas mtCA 1 has been investigated recently by us,[12] as mentioned above).

Results and Discussion

mtCA 3 Cloning and Purification. The Rv3273 gene was deposited from the genome analysis of *M. tuberculosis* strain H37Rv in 2002,[18] being suggested to encode for a transmembrane CA because of its hydrophobic N-terminal part. Its gene product was predicted to be a 764 amino acid residues long polypeptide, consisting of a bifunctional protein: a sulfate transporter domain (amino acids 121−414) and a β-CA domain in the C-terminal part (encoded by amino acid residues 571−741) as shown schematically in Figure 1.[17,18] The deduced domain organization of this protein seems to be similar to that of a number of bacterial proteins found in *Arthrobacter aurescens* (YP_949116), *Leptospira borgpetersenii* (YP_798800), *Legionella pneumophila* (YP_126096), and *Leptospira interrogans* (NP_710760), all of which are putative bifunctional such proteins, i.e., they contain a sulfate transporter and a β-CA (Figure 1A,B).[18] In this study, we report the cloning of the full β-CA domain encoded by amino acid residues 550−764 of Rv3273, which we denominated mtCA 3.

The deduced amino acid sequence of mtCA 3 was aligned with that of the other two β-CAs present in *M. tuberculosis* (i.e., mtCA 1, encoded by Rv1284,[1,12] and mtCA 2, encoded by Rv3538c)[1,2] and with those of several β-CAs from different bacteria, such as *E. coli* CynT2 (T2) and CynT (T), *H. influenzae*, etc.[19] (Figure 1C). The amino acid sequence of the DNA clone obtained in this study (see Experimental Protocols for details) was identical to that of Rv3273 reported in the literature,[17,18] except for an amino acid substitution, i.e., ^{606}Arg(CGC):Cys(TGC) (Figure 1). mtCA 3 conserved the 3 (or 4) zinc ligands present in the other two β-CAs of *M.*

* To whom correspondence should be addressed. Phone: +39-055-457 3005. Fax: +39-055-4573385. E-mail: claudiu.supuran@unifi.it.
[†] Department of Gastroenterology, Kochi Medical School.
[‡] Università degli Studi di Firenze, Laboratorio di Chimica Bioinorganica.
[a] Abbreviations: CA, carbonic anhydrase; CAI, CA inhibitor; hCA, human CA; GST, glutathione-*S*-transferase; mtCA, *Mycobacterium tuberculosis* CA; TB, tuberculosis.

10.1021/jm9003126 CCC: $40.75
Published on Web 04/01/2009

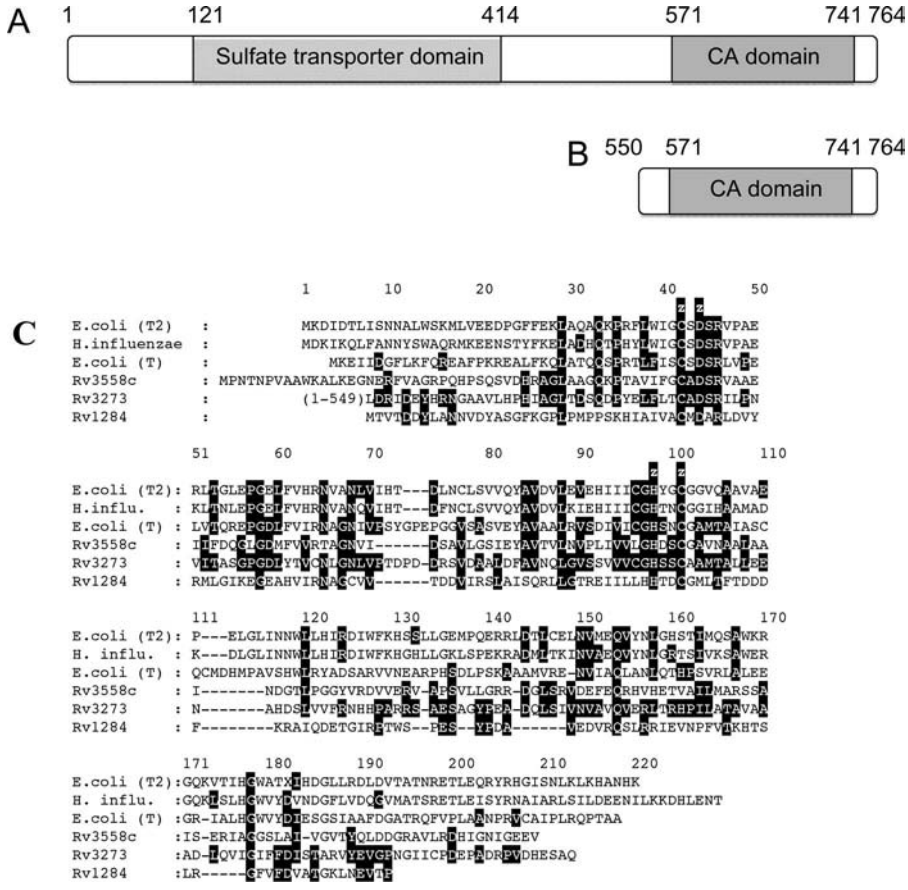


Figure 1. Schematic representation of the Rv3273 gene products (A), the recombinant mtCA 3 protein obtained in this study (B), and the amino acid sequences of some bacterial β-CAs, including Rv3273 (C). Conserved amino acid residues between Rv3273 and other β-CAs are indicated by a black box. The four zinc-binding residues, ^{42}Cys, ^{44}Asp, ^{98}His, and ^{101}Cys, are indicated by the "z" sign (residue numbers are based on the *E. coli* CynT2 numbering system).[8]

tuberculosis, mtCA 1 and mtCA 2,[1,2,12] which are Cys584, His642, Cys645, and probably Asp586 (Figure 1C, residue numbers based on the *E. coli* CynT2 numbering system).[8] The Zn(II) ion is coordinated by two cysteines, one histidine, and a water molecule in mtCA 1, and by these residues plus an aspartate in mtCA 2[1,2] However, to function as catalysts for CO_2 hydration, the active site of mtCA 2 undergoes a reorganization,[1,2] which leads to its "opening", i.e., the aspartate residue is replaced by a water molecule. Interestingly, all these four catalytically critical residues of mtCA 3 are present at the corresponding positions in mtCA1 and mtCA 2. On the basis of the present data, we cannot deduce the Zn(II) coordination of mtCA 3: it may be of the open active site type (as mtCA 1, i.e., with two Cys, one His, and a water molecule), or of the closed active site type (as mtCA 2, i.e., with two Cys, one His, and one Asp residues bound to the metal ion).

mtCA 3 Catalytic Activity. We performed a kinetic investigation of purified mtCA 3 comparing its kinetic parameters (k_{cat} and k_{cat}/K_m) with those of thoroughly investigated α-CAs, such as the cytosolic, ubiquitous human isozymes hCA I and II[3,20−23] (Table 1). As CAs are susceptible to be inhibited by sulfonamides,[3,24,25] data of Table 1 also present the inhibition constant of these enzymes with acetazolamide (**AAZ**), a clinically used drug.[3]

It may be observed that mtCA 3 shows appreciable catalytic activity for the physiologic reaction, with kinetic parameters in the same range as α- or β-CAs investigated earlier, such as hCA I and mtCA 1.[12,21−23] Indeed, mtCA 3 has a k_{cat} of 4.3×10^5 s^{-1}, and k_{cat}/K_m of 4.0×10^7 $M^{-1} \cdot s^{-1}$, being slightly more active than mtCA 1 investigated earlier.[12] Only hCA II was much more active a catalysts for CO_2 hydration as compared to mtCA 3 among the enzymes shown in Table 1. Furthermore, this catalytic activity is inhibited by the sulfonamide CAI par excellence, acetazolamide **AAZ**, with a K_I of 104 nM, lower than that of the related enzyme mtCA 1.

Table 1. Kinetic Parameters for the CO_2 Hydration Reaction[23] Catalyzed by the α-hCA Isozymes I, II at 20 °C and pH 7.5 in 10 mM HEPES Buffer and the *Mycobacterium tuberculosis* Enzymes Rv1284 (mtCA 1) and Rv3273 (mtCA 3) at 20 °C, pH 8.3, in 20 mM TRIS·HCl Buffer and 20 mM NaCl and Their Inhibition Data with Acetazolamide **AAZ**

isozyme	activity level (s^{-1})	k_{cat} ($M^{-1} \cdot s^{-1}$)	k_{cat}/K_m (nM)	K_I (acetazolamide) (nM)
hCA I	moderate	2.0×10^5	5.0×10^7	250
hCA II	very high	1.4×10^6	1.5×10^8	12
mtCA 1	moderate	3.9×10^5	3.7×10^7	480
mtCA 3	moderate	4.3×10^5	4.0×10^7	104

mtCA 3 Inhibition with Sulfonamides and Sulfamates. Table 2 shows mtCA 3 inhibition data with a panel of 36 sulfonamides and one sulfamate (obtained for the CO_2 hydration reaction catalyzed by CAs),[23] some of which are clinically used drugs,[3] such as acetazolamide (**AAZ**), methazolamide (**MZA**), ethoxzolamide (**EZA**), dichorophenamide (**DCP**), dorzolamide (**DZA**), brinzolamide (**BRZ**), benzolamide (**BZA**), topiramate (**TPM**), sulpiride (**SLP**), indisulam (**IND**), zonisamide (**ZNS**), celecoxib (**CLX**0, valdecoxib (**VLX**), sulthiame (**SLT**), and saccharin (**SAC**). The simpler derivatives **1**−**22** were also included in the study, as they represent the most extensively used scaffolds for designing potent or isoform-selective CAIs.[3,26]

Table 2. hCA I, II, *M. tuberculosis* CA Rv1284 (mtCA 1), and Rv3273 (mtCA 3) Inhibition Data with Sulfonamides **1**−**22** and 15 Clinically Used Derivatives **AAZ**−**SAC**[a]

	K_I*[b]			
inhibitor	hCA I[c] (nM)	hCA II[c] (nM)	mtCA 1[d] (μM)	mtCA 3[e] (μM)
1	45400	295	9.23	6.24
2	25000	240	9.84	7.11
3	6690	495	7.93	7.83
4	78500	320	4.92	7.02
5	25000	170	8.69	7.33
6	21000	160	9.56	3.42
7	8300	60	8.74	7.90
8	9800	110	7.52	1.51
9	6500	40	0.186	7.32
10	6000	70	7.71	5.81
11	5800	63	8.10	2.35
12	8400	75	1.72	21.7
13	8600	60	11.54	7.63
14	9300	19	12.65	7.92
15	6	2	0.905	3.10
16	164	46	0.612	2.21
17	185	50	0.853	0.170
18	109	33	0.750	0.091
19	690	12	7.48	7.60
20	55	80	9.56	7.82
21	21000	125	5.51	2.51
22	23000	133	8.21	7.40
AAZ	250	12	0.481	0.104
MZA	50	14	0.781	0.562
EZA	25	8	1.03	0.594
DCP	1200	38	0.872	0.611
DZA	50000	9	0.744	0.137
BRZ	45000	3	0.839	0.201
BZA	15	9	0.810	0.338
TPM	250	10	0.612	3.02
SLP	12000	40	2.30	7.92
IND	31	15	0.097	7.84
ZNS	56	35	28.68	0.208
CLX	50000	21	10.35	7.76
VLX	54000	43	12.97	7.81
SLT	374	9	5.16	6.72
SAC	18540	5950	7.96	7.15

[a] Data of isoforms I and II are from ref 22, whereas data of mtCA 1 are from ref 12. [b] Errors in the range of 5−10% of the shown data, from three different assays. [c] Human recombinant isozymes, stopped flow CO_2 hydrase assay method, pH 7.5, 20 mM TRIS·HCl buffer.[23] [d] Bacterial recombinant enzyme, at 20 °C, pH 8.3, in 20 mM TRIS·HCl buffer and 20 mM NaCl, from ref 12. [e] Bacterial recombinant enzyme, at 20 °C, pH 8.3, in 20 mM TRIS·HCl buffer and 20 mM NaCl, this work.

Data for the inhibition of the dominant human isoforms hCA I and II[3] as well as those of the other *M. tuberculosis* enzyme, mtCA 1,[12] with these compounds are also included in Table 2 for comparison reasons. The following SAR can be observed from data of Table 2:

(i) Medium-weak mtCA 3 inhibitory activity was observed for a rather large group of investigated derivatives, such as **1**−**5**, **7**, **9**, **10**, **12**−**14**, **19**, **20**, **22**, **SLP**, **IND**, **CLX**, **VLX**, **SLT**, and **SAC** (Chart 1). These sulfonamides showed inhibition constants in the range of 5.81−21.7 μM (Table 2). They belong to heterogeneous classes of compounds, some of them (**1**−**6**, **7**, **9**, **10**, **20**) possessing rather simple scaffolds based on the benzenesulfonamide motif, whereas others incorporate more complicated scaffolds, such as those present in the clinically used derivatives **CLX**, **VLX**, **SLT**, **SAC**, etc. It should be noted that many of them act as efficient hCA II and less efficient hCA I inhibitors (Table 2). It may be observed that quite small structural changes in the inhibitor scaffold lead to dramatic changes of enzyme inhibitory activity. This is obvious for the halogeno-substituted sulfanilamides **7**−**10**, with the fluoro-, bromo-, and iodine-substituted derivatives being medium-weak inhibitors, whereas the chloro-sulfanilamide **8** is a more potent inhibitor (K_I of 1.51 μM).

(ii) A second group of derivatives, including **6**, **8**, **11**, **15**, **16**, **21**, and **TPM**, show much more effective inhibitory properties against mtCA 3, with inhibition constants in the range of 1.51−3.42 μM (Table 2). Compounds **6**, **8**, **11**, and **21** are simple benzenesulfonamides, not very different structurally from many of the derivatives discussed above, whereas **15** and **16** (and the much more active **17**, which will be discussed shortly) belong to the class of sulfanylated sulfonamides,[27] possessing thus an elongated molecule with two benzene rings in it, which was previously shown to act as excellent inhibitors of many α-CA isoforms.[27] Topiramate (**TPM**), on the other hand, possesses a completely different scaffold of all other derivatives investigated here, being a sugar sulfamate. Thus, many efficient inhibitors (K_I < 3.5 μM) were evidenced, belonging to numerous structural classes.

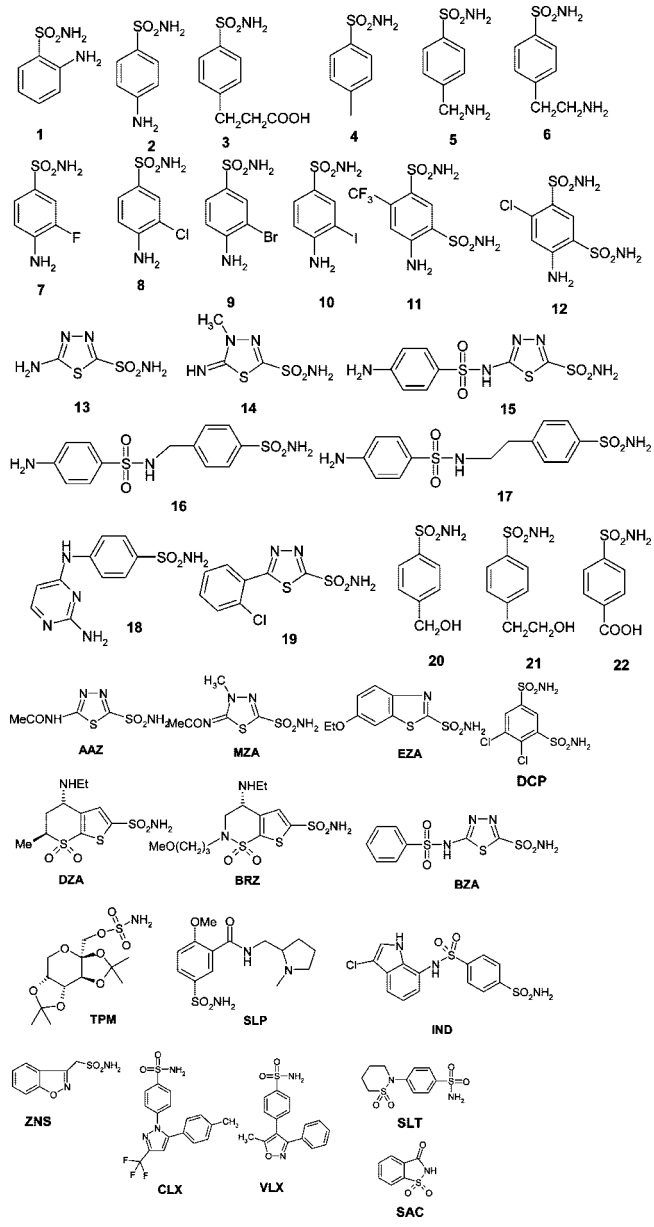
(iii) A significant number of derivatives, such as **17**, **18**, **AAZ**, **MZA**, **EZA**, **DCP**, **DZA**, **BRZ**, **BZA**, and **ZNS** showed submicromolar inhibition against mtCA 3, with K_Is in the range of 91−611 nM (Table 2). The least effective sulfonamides in this subgroup were **MZA**, **EZA**, **BZA**, and **DCP** (K_Is of 338−611 nM), whereas the remaining compounds showed quite effective inhibitory properties, with K_Is in the range of 91−208 nM. Again, there are many classes of compounds that may be considered as excellent leads to develop better mtCA 3 inhibitors. Among them, the sulfanylated sulfonamide **17** and the pyrimidinyl-substituted sulfanilamide **18** (the best mtCA 3 inhibitor detected so far) possess two ring systems in their molecule and a free amino group easily derivatizable. Zonisamide **ZNS** is also an interesting case, as it is the only aliphatic sulfonamide tested here, and its activity is also very promising (K_I of 208 nM).

Putative Biological Function of the Rv3273 Gene Product. Screening analysis for genes specifically required for the mycobacterial growth showed that Rv3588c is required but Rv3273 is not essential for the bacterial growth in vivo.[28,29] However, such findings cannot conclusively exclude the biological significance of Rv3273 in the survival and/or pathogenicity of *M. tuberculosis*. An elegant study by Miltner et al.[30] identified six invasion-related genes of these bacteria. Constitutive expression of the proteins encoded by these genes showed significantly increased ability of the bacterial invasion into HEp-2 and HT-29 intestinal epithelial cells. One of these genes is homologous to *M. tuberculosis* Rv3273, which showed 1.4−1.6 times increase of infected cell numbers by *M. avium* overexpressing its gene product. These findings indicate that Rv3273 plays a role in the bacterial infection process, which is however poorly understood at the present time. Rv3273 is predicted to code a bifunctional transmembrane protein that consists of an N-terminal sulfate transporter domain and a C-terminal CA domain, and we herein demonstrated the significant catalytic activity of this CA domain. Although it is uncertain how the CA activity of the Rv3273 gene product works in the bacterial infection process, it is not improbable that effective CAIs may prevent bacterial infection. In addition to confirm the sulfate transporter function of the amino-terminal end of Rv3273, further studies are warranted for understanding the biological role of this bifunctional transmembrane protein.

Conclusions

The β-CA encoded by the carboxy terminal part of the protein product of gene Rv3273 from *M. tuberculosis*, mtCA 3, was cloned and purified, being shown to possess an appreciable

Chart 1



catalytic activity for the physiological reaction, CO_2 hydration to bicarbonate and a proton, with a k_{cat} of 4.3×10^5 s^{-1} and a k_{cat}/K_m of 4.0×10^7 $M^{-1} \cdot s^{-1}$. A panel of sulfonamides/sulfamates CAIs were assayed for their interaction with mtCA 3. Many simple sulfonamide derivatives showed K_Is in the range of 5.81−21.7 μM, but several compounds, including sulfanilylsulfonamides, acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, brinzolamide, benzolamide, and zonisamide, showed effective, submicromolar inhibition, with K_Is in the range of 104−611 nM. The best inhibitor was 2-amino-pyrimidin-4-yl-sulfanilamide (K_I of 91 nM), which is an interesting lead for obtaining tighter-binding compounds. This study proves mtCA 3 to be a druggable target with potential for developing antimycobacterial agents with a diverse mechanism of action.

Experimental Protocols

Chemistry. Compounds **1**−**22** and **AAZ**−**SAC** are either commercially available (Sigma-Aldrich) or were prepared as described earlier.[7,24]

Preparation of Recombinant mtCA 3 Protein. To the full length mtCA 3 (encoded by amino acid residues 550−764 of the Rv3273 gene) we set a pair of adopter primers including *Bam*HI and *Eco*RI recognition sequences (underlined) and a start codon (bolded) as follows: 5′-CGGGATCCTCGGTT**ATG**CTCGACCG-CATC-3′ and 5′-CGGAATTCCTACTGCGCTGATTCGTGGTC-3′. Genomic DNA purified from the cultured strain of *M. tuberculosis* was subjected to PCR by using KOD DNA polymerase (Toyobo, Tokyo, Japan) with a strong 3′→5′ exonuclease activity. The PCR reaction was hot-started with incubation for 2 min at 94 °C and consisted of 35 cycles of 15 s at 94 °C, 30 s at 58 °C, and 60 s at 68 °C and terminated with incubation for 5 min at 68 °C. The PCR products were cleaved with *Bam*HI and *Eco*RI and then ligated in-frame into the pGEX-4T2 vector (Amersham, Tokyo, Japan). The PCR product was sequenced using an ABI PRISM dye termination cycle sequencing kit (Perkin-Elmer, Foster City, CA) and an ABI 370A DNA sequencer (ABI, Foster City, CA). The DNA constructs was then transfected into *E. coli* strain BL21 for production of the GST-CA fusion protein and in the end the full length mtCA 3, as previously reported for various α- and β-CAs by our group.[7,10−12]

CA Catalytic Activity and Inhibition. An Applied Photophysics stopped-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity[23] as reported earlier.[7,10−12]

Acknowledgment. This research was financed in part by a grant of the 6th Framework Programme of the European Union (DeZnIT project).

References

(1) Suarez Covarrubias, A.; Larsson, A. M.; Hogbom, M.; Lindberg, J.; Bergfors, T.; Bjorkelid, C.; Mowbray, S. L.; Unge, T.; Jones, T. A. Structure and function of carbonic anhydrases from *Mycobacterium tuberculosis*. *J. Biol. Chem.* **2005**, *280*, 18782–18789.

(2) Suarez Covarrubias, A.; Bergfors, T.; Jones, T. A.; Hogbom, M. Structural mechanics of the pH-dependent activity of the β-carbonic anhydrase from *Mycobacterium tuberculosis*. *J. Biol. Chem.* **2006**, *281*, 4993–4999.

(3) Supuran, C. T. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat. Rev. Drug Discovery* **2008**, *7*, 168–181.

(4) Mogensen, E. G.; Janbon, G.; Chaloupka, J.; Steegborn, C.; Fu, M. S.; Moyrand, F.; Klengel, T.; Pearson, D. S.; Geeves, M. A.; Buck, J.; Levin, L. R.; Mühlschlegel, F. A. Fungal adenylyl cyclase integrates CO_2 sensing with cAMP signaling and virulence. *Curr. Biol.* **2005**, *15*, 2021–2026.

(5) Innocenti, A.; Mühlschlegel, F. A.; Hall, R. A.; Steegborn, C.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors. Inhibition of the beta-class enzymes from the fungal pathogens *Candida albicans* and *Cryptococcus neoformans* with simple anions. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5066–5070.

(6) Schlicker, C.; Hall, R. A.; Vullo, D.; Middelhaufe, S.; Gertz, M.; Supuran, C. T.; Mühlschlegel, F. A.; Steegborn, C. Structure and inhibition of the CO_2-sensing carbonic anhydrase Can2 from the pathogenic fungus *Cryptococcus neoformans*. *J. Mol. Biol.* **2009**, *385*, 1207–1220.

(7) Nishimori, I.; Minakuchi, T.; Kohsaki, T.; Onishi, S.; Takeuchi, H.; Vullo, D.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors: the beta-carbonic anhydrase from *Helicobacter pylori* is a new target for sulfonamide and sulfamate inhibitors. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3585–3594.

(8) Cronk, J. D.; Rowlett, R. S.; Zhang, K. Y.; Tu, C.; Endrizzi, J. A.; Lee, J.; Gareiss, P. C.; Preiss, J. R. Identification of a novel noncatalytic bicarbonate binding site in eubacterial β-carbonic anhydrase. *Biochemistry* **2006**, *45*, 4351–4361.

(9) Cronk, J. D.; Endrizzi, J. A.; Cronk, M. R.; O'Neill, J. W.; Zhang, K. Y. Crystal structure of *E. coli* β-carbonic anhydrase, an enzyme with an unusual pH-dependent activity. *Protein Sci.* **2001**, *10*, 911–922.

(10) Nishimori, I.; Minakuchi, T.; Morimoto, K.; Sano, S.; Onishi, S.; Takeuchi, H.; Vullo, D.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors: DNA cloning and inhibition studies of the alpha-carbonic anhydrase from *Helicobacter pylori*, a new target for developing sulfonamide and sulfamate gastric drugs. *J. Med. Chem.* **2006**, *49*, 2117–2126.

(11) Nishimori, I.; Onishi, S.; Takeuchi, H.; Supuran, C. T. The alpha and beta classes carbonic anhydrases from *Helicobacter pylori* as novel drug targets. *Curr. Pharm. Des.* **2008**, *14*, 622–630.

(12) Minakuchi, T.; Nishimori, I.; Vullo, D.; Scozzafava, A.; Supuran, C. T. Molecular cloning, characterization and inhibition studies of the Rv1284 β-carbonic anhydrase from *Mycobacterium tuberculosis* with sulfonamides and a sulfamate. *J. Med. Chem.*, **2009**, *52*, 10.1021/jm9000488.

(13) Dye, C. Doomsday postponed? Preventing and reversing epidemics of drug-resistant tuberculosis. *Nat. Rev. Microbiol.* **2009**, *7*, 81–87.

(14) Ginsberg, A. M. Emerging drugs for active tuberculosis. *Semin. Respir. Crit. Care Med.* **2008**, *29*, 552–559.

(15) Showalter, H. D.; Denny, W. A. A roadmap for drug discovery and its translation to small molecule agents in clinical development for tuberculosis treatment. *Tuberculosis* **2008**, *88* (Suppl 1), S3–S17.

(16) Tomioka, H.; Tatano, Y.; Yasumoto, K.; Shimizu, T. Recent advances in anti-tuberculous drug development and novel drug targets. *Expert Rev. Resp. Med.* **2008**, *2*, 455–471.

(17) Cole, S. T.; Brosch, R.; Parkhill, J.; Garnier, T.; Churcher, C.; Harris, D.; Gordon, S. V.; Eiglmeier, K.; Gas, S.; Barry, C. E., III.; Tekaia, F.; Badcock, K.; Basham, D.; Brown, D.; Chillingworth, T.; Connor, R.; Davies, R.; Devlin, K.; Feltwell, T.; Gentles, S.; Hamlin, N.; Holroyd, S.; Hornsby, T.; Jagels, K.; Krogh, A.; McLean, J.; Moule, S.; Murphy, L.; Oliver, K.; Osborne, J.; Quail, M. A.; Rajandream, M. A.; Rogers, J.; Rutter, S.; Seeger, K.; Skelton, J.; Squares, R.; Squares, S.; Sulston, J. E.; Taylor, K.; Whitehead, S.; Barrell, B. G. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature (London)* **1998**, *393*, 537–544.

(18) Camus, J. C.; Pryor, M. J.; Médigue, C.; Cole, S. T. Re-annotation of the genome sequence of *Mycobacterium tuberculosis* H37Rv. *Microbiology* **2002**, *148*, 2967–2973.

(19) Smith, K. S.; Jakubzick, C.; Whittam, T. S.; Ferry, J. G. Carbonic anhydrase is an ancient enzyme widespread in prokaryotes. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 15184–15189.

(20) Nishimori, I.; Minakuchi, T.; Onishi, S.; Vullo, D.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors. DNA cloning, characterization and inhibition studies of the human secretory isoform VI, a new target for sulfonamide and sulfamate inhibitors. *J. Med. Chem.* **2007**, *50*, 381–388.

(21) Hilvo, M.; Baranauskiene, L.; Salzano, A. M.; Scaloni, A.; Matulis, D.; Innocenti, A.; Scozzafava, A.; Monti, S. M.; Di Fiore, A.; De Simone, G.; Lindfors, M.; Janis, J.; Valjakka, J.; Pastorekova, S.; Pastorek, J.; Kulomaa, M. S.; Nordlund, H. R.; Supuran, C. T.; Parkkila, S. Biochemical characterization of CA IX: one of the most active carbonic anhydrase isozymes. *J. Biol. Chem.* **2008**, *283*, 27799–27809.

(22) Vullo, D.; Innocenti, A.; Nishimori, I.; Pastorek, J.; Scozzafava, A.; Pastorekova, S.; Supuran, C. T. Carbonic anhydrase inhibitors. Inhibition of the transmembrane isozyme XII with sulfonamides—A new target for the design of antitumor and antiglaucoma drugs. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 963–969.

(23) Khalifah, R. G. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J. Biol. Chem.* **1971**, *246*, 2561–2573.

(24) (a) Supuran, C. T. Diuretics: from classical carbonic anhydrase inhibitors to novel applications of the sulfonamides. *Curr. Pharm. Des.* **2008**, *14*, 641–648. (b) Supuran, C. T.; Scozzafava, A.; Conway, J. *Carbonic Anhydrase—Its Inhibitors and Activators*; CRC Press: Boca Raton, New York, London, 2004; pp 1−363; (c) Stiti, M.; Cecchi, A.; Rami, M.; Abdaoui, M.; Barragan-Montero, V.; Scozzafava, A.; Guari, Y.; Winum, J. Y.; Supuran, C. T. Carbonic anhydrase inhibitor coated gold nanoparticles selectively inhibit the tumor-associated isoform IX over the cytosolic isozymes I and II. *J. Am. Chem. Soc.* **2008**, *130*, 16130–16131.

(25) Isik, S.; Kockar, F.; Aydin, M.; Arslan, O.; Ozensoy Guler, O.; Innocenti, A.; Supuran, C. T. Carbonic anhydrase inhibitors. Inhibition of the β-class enzyme from the yeast *Saccharomyces cerevisiae* with sulfonamides and sulfamates. *Bioorg. Med. Chem.* **2009**, *17*, 1158–1163.

(26) Alterio, V.; Vitale, R. M.; Monti, S. M.; Pedone, C.; Scozzafava, A.; Cecchi, A.; De Simone, G.; Supuran, C. T. Carbonic anhydrase inhibitors: X-ray and molecular modeling study for the interaction of a fluorescent antitumor sulfonamide with isozyme II and IX. *J. Am. Chem. Soc.* **2006**, *128*, 8329–8335.

(27) Supuran, C. T.; Clare, B. W. Carbonic anhydrase inhibitors. Part 57. Quantum chemical QSAR of a group of 1,3,4-thiadiazole and 1,3,4-thiadiazoline disulfonamides with carbonic anhydrase inhibitory properties. *Eur. J. Med. Chem.* **1999**, *34*, 41–50.

(28) Sassetti, C. M.; Boyd, D. H.; Rubin, E. J. Genes required for mycobacterial growth defined by high density mutagenesis. *Mol. Microbiol.* **2003**, *48*, 77–84.

(29) Betts, J. C.; Lukey, P. T.; Robb, L. C.; McAdam, R. A.; Duncan, K. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol. Microbiol.* **2002**, *43*, 717–731.

(30) Miltner, E.; Daroogheh, K.; Mehta, P. K.; Cirillo, S. L.; Cirillo, J. D.; Bermudez, L. E. Identification of *Mycobacterium avium* genes that affect invasion of the intestinal epithelium. *Infect. Immun.* **2005**, *73*, 4214–4221.

JM9003126