Molecular Cloning, Characterization, and Inhibition Studies of the Rv1284 β-Carbonic Anhydrase from *Mycobacterium tuberculosis* with Sulfonamides and a Sulfamate

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The β-carbonic anhydrase (CA, EC 4.2.1.1) encoded by the gene Rv1284 (mtCA 1) of *Mycobacterium tuberculosis* shows appreciable catalytic activity for CO_2 hydration, with a k_{cat} of 3.9×10^5 s^{-1} and a k_{cat}/K_m of 3.7×10^7 M^{-1} s^{-1}. A panel of 36 sulfonamides and one sulfamate, some of which are used clinically, were assayed for their effect on mtCA 1 catalytic activity. Most sulfonamides exhibited K_I values in the range of 1−10 μM, but several derivatives, including sulfanilyl-sulfonamides acetazolamide, methazolamide, dichlorophenamide, dorzolamide, brinzolamide, benzolamide, and the sulfamate topiramate, exhibited submicromolar inhibition (K_I values of 0.481−0.905 μM). The best inhibitors were 3-bromosulfanilamide and indisulam (K_I values of 97−186 nM). This study demonstrates that mtCA 1 can be inhibited by sulfonamides and sulfamates and thus has potential for developing antimycobacterial agents with an alternate mechanism of action. This is an important finding to explore further, as many strains exhibit multidrug resistance and extensive multidrug resistance to existing therapeutics.

Introduction

Infection with *Mycobacterium tuberculosis* and related *Mycobacteria* affects much of the world population, with an estimated 9.2 million new cases each year, of which many lead to death.[1−4] Furthermore, multidrug resistant and extensively multidrug resistant tuberculosis (TB)[a] is present in 50 countries posing serious concern to the global healthcare system.[1] Indeed, the combination therapy used to treat TB is based on agents developed in the 1960−1980s, with no new drugs launched for the past 30 years.[2−4] At present, there is a huge need for anti-TB drugs with a novel mechanism of action, and several agents belonging to the fluoroquinolone, oxazolidinone, diarylquinoline, and nitroimidazo-oxazole/-oxazine classes are in various stages of development.[2−4] The complete sequencing of the *M. tuberculosis* genome in 1998[5] greatly facilitated the identification of possible new drug targets which may lead to the development of compounds possessing a new mechanism of action and thus resolve the drug resistance problem mentioned above. Among the new proteins identified after the *M. tuberculosis* genome was published, there are also two carbonic anhydrases (CAs, EC 4.2.1.1) belonging to the β-class, which have been cloned, purified, and characterized by means of X-ray crystallography by Suarez Covarrubias et al.[6]

CAs are widespread enzymes all over the phylogenetic tree, with five genetically unrelated classes (α, β, γ, δ, and ζ) known to date.[7] These enzymes catalyze the interconversion between carbon dioxide and bicarbonate, with release of a proton, and are involved not only in pH homeostasis and regulation but also in biosynthetic reactions, such as gluconeogenesis and ureagenesis among others (in animals), CO_2 fixation (in plants and algae), and electrolyte secretion in a variety of tissues/organs, with many of the 16 mammalian CA isozymes being established drug targets for the design of diuretics, antiglaucoma, antiepileptic, antiobesity, and/or anticancer agents.[7,8] Whereas the α-CA family (mainly present in but not exclusive to mammals) has been thoroughly investigated from the drug design viewpoint,[7,8] only more recently have CAs belonging to the β- and γ-families [widespread in bacteria and fungi (β-CAs) and *Archaea* (the β- and γ-CAs)] been studied for this purpose. Thus, a β-CA present in the gastric pathogen *Helicobacter pylori* was recently shown to be a possible target for gastric drugs,[9] with several low nanomolar inhibitors detected, which effectively inhibited the in vitro and in vivo growth of the pathogen,[10] whereas the fungal β-CAs from *Candida albicans*[11] and *Cryptococcus neoformans*[12] (as well as the related enzyme present in *Saccharomyces cerevisiae*[13]) were also shown to be susceptible to inhibition with the main classes of CA inhibitors (CAIs), i.e., the inorganic anions and the sulfonamides and their bioisosteres.[11−13] Sulfonamide/sulfamate CAIs targeting various mammalian α-CAs have been in clinical use for more than 50 years.[7]

Suarez Covarrubias et al.[6] have previously cloned and crystallized two β-CA enzymes from *M. tuberculosis*, reporting their X-ray crystal structures at a resolution of 1.75−2.00 Å; however, no enzymatic activity (for the CO_2 hydration reaction) could be measured for one such enzyme (Rv1284), whereas for the second one (Rv3588c), such an activity has been detected (but no kinetic parameters have been reported). As a consequence, inhibitors of these β-CAs (Rv1284, named mtCA 1 hereafter, and Rv3588c, named mtCA 2 hereafter) are also unknown at this moment. Since β-CAs were proved to be virulence or survival factors for many bacterial[9,10] or fungal pathogens,[11,12] their inhibition may constitute a means of controlling these infections through a new mechanism of action, devoid of drug resistance problems associated with classical antibiotics/antifungals. We thus decided to reinvestigate *M. tuberculosis* β-CAs as possible drug targets. We report here the molecular cloning, biochemical characterization, and inhibition studies of one of these enzymes, i.e., mtCA 1 encoded by the

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[a] Abbreviations: CA, carbonic anhydrase; Cab, β-CA from *Methanobacterium thermoautotrophicum*; Can2, β-CA from *Cryptococcus neoformans*; hCA, human CA; GST, glutathione *S*-transferase; mtCA, *M. tuberculosis* CA; Nce103, β-CA from *Candida albicans*; TB, tuberculosis.

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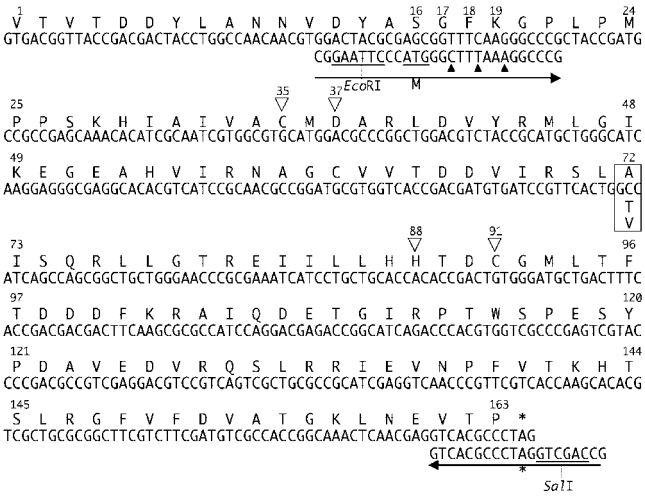


Figure 1. Amino acid and oligonucleotide sequences of mtCA 1. Polymorphism at amino acid position 72 is shown by a box, together with the amino acid residues involved in the catalytic cycle (triangles).

Rv1284 gene. We were unable to clone the second enzyme from *M. tuberculosis*, i.e., Rv3588c, until now.

Results and Discussion

mtCA 1 Cloning and Purification. The amino acid sequence of a truncated form of mtCA 1, lacking an N-terminal 15-amino acid polypeptide (Figure 1), showed one nucleotide substitution as compared to that of Rv1284 deposited in the database (GenBank entry NC000962),[5] resulting in one amino acid substitution at position 72, i.e., ^{72}Ala(GCC) → Val(GTC). There was no difference in the nucleotide sequence between the clone used in this study and the originally reported Rv1284.[5] As the nucleotide sequence of the only other mtCA 1 clone known to date, obtained from *M. tuberculosis* strain CDC1551 and deposited in GenBank as entry 924749, locus tag MT1322, was shown to be identical to that of the originally reported RV1284,[5] the substitution of ^{72}Ala with Ala represents a unique polymorphism of the *M. tuberculosis* strain infecting the Japanese patient from which the bacterium used in this study had been isolated (see Experimental Protocols for details). A GST−mtCA 1 fusion protein was then obtained and purified as described earlier by this group[9] for the *β*-CA from *H. pylori* (see Experimental Protocols for details). In fact, we have shown earlier that these GST fusion constructs generally lead to a high level of protein expression in *E. coli* and that the protein is properly folded, being obtained in high yield and with a good catalytic activity.[9,10]

mtCA 1 Catalytic Activity. We obtained purified mtCA 1 using GST fusion proteins for expression and purification, whereas the previous investigators[6] used His-tagged protein for the same purpose. The enzyme obtained previously[6] was reported to contain only 0.3 equiv of zinc and 0.18 equiv of nickel which placed the type and role of the active site metal ion in question and probably also explains the lack of catalytic activity. The enzyme (obtained by a different cloning and purification procedure) contains 1 equiv of Zn(II) per polypeptide chain (data not shown) and possesses good CO_2 hydration activity (Table 1), which is inhibited by a sulfonamide compound, proving that this enzyme is suitable for drug screening and drug design. With purified enzyme available, we next performed a kinetic investigation and compared the kinetic parameters (k_{cat} and k_{cat}/K_m) of mtCA 1 with those of thoroughly investigated CAs, including the cytosolic, ubiquitous human isozymes hCA I−III, the mitochondrial ones hCA VA and VB (α-class CAs), and Cab, the best-characterized *β*-CA from the archaeon *Methanobacterium thermoautotrophicum*[14,15] (Table 1).

Table 1. Kinetic Parameters for the CO_2 Hydration Reaction Catalyzed by Human Cytosolic α-hCA Isozymes I−III and Mitochondrial Isozymes hCA VA and VB, at 20 °C and pH 7.5 in 10 mM HEPES Buffer and Their Inhibition Data with Acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), a Clinically Used Drug[7a]

isozyme	activity level	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	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
hCA III	very low	1.0×10^4	3.0×10^5	300000
hCA VA	low	2.9×10^5	2.9×10^7	63
hCA VB	high	9.5×10^5	9.8×10^7	54
Cab	low	3.1×10^4	1.8×10^6	12100
mtCA 1	moderate	3.9×10^5	3.7×10^7	480

[a] The activity or inhibition of *M. tuberculosis* enzyme Rv1284 (mtCA 1) and *M. thermoautotrophicum* enzyme Cab (*β*-CAs) were measured at 20 °C and pH 8.3 in 20 mM Tris-HCl buffer in the presence of 20 mM NaCl.

Data from Table 1 show that like other CAs belonging to the α- or *β*-class, mycobacterial enzyme mtCA 1 possesses appreciable CO_2 hydrase activity, with a k_{cat} of 3.9×10^5 s^{-1} and a k_{cat}/K_m of 3.7×10^7 M^{-1} s^{-1}. Thus, mtCA 1 is 100 times more active than the least active hCA isoform (hCA III), possessing similar activity with hCA I or hCA VA, established drug targets in the α-CA family.[7] mtCA 1 is also a more active carbon dioxide hydrase than Cab.[14,15] Data from Table 1 also show that these enzymes (except Cab)[14] are inhibited appreciably by the clinically used sulfonamide acetazolamide **AAZ** (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), with inhibition constants in the range of 12−480 nM (see the discussion later in the text). Thus, our data confirm that mtCA 1 is an active CA, possessing a moderate catalytic activity for the physiologic reaction, on the same order of magnitude as widely spread mammalian isoforms hCA I and hCA VA.

Like Cab[14] or Can2[12b] (the fungal *β*-CA from *C. neoformans*), mtCA 1 possesses an "open" active site (Figure 2), according to the nomenclature of Suarez Covarrubias et al.[6] Thus, unlike mtCA 2 (encoded by the gene Rv3588c) in which the Zn(II) ion from the active site is coordinated by two Cys ligands, one His ligand, and one Asp ligand, with no water directly bound to the metal ion,[6] mtCA 1 has the Zn(II) ion coordinated by Cys35, His88, and Cys91 (Figure 1), the fourth metal ligand being a water molecule/hydroxide ion, which is the nucleophile attacking CO_2 and transforming it to bicarbonate.[7,12,13] However, there is another amino acid dyad involved in the catalytic cycle of open-active site *β*-CAs, such as Cab, mtCA 1, Can2, or Nce103.[11−15] Indeed, as shown by the valuable crystallographic work of Suarez Covarrubias et al.[6] a conserved (in all cloned *β*-CAs known to date)[6,12−15] Asp-Arg dyad is involved in the zinc water activation mechanism, by means of hydrogen bonds and salt-like interactions, which can favor the release of the proton and generation of the nucleophilic species of the enzyme, with hydroxide as the fourth zinc ligand (Figure 2). In the case of mtCA 1, this dyad is formed of Asp37 and Arg39 (Figures 1 and 2). As for the well-documented α-CAs,[7,16−23] inhibitors substitute the fourth zinc ligand, leading to the inhibition mechanism depicted in Figure 2. The proposed inhibition mechanism has been confirmed by the recent X-ray crystal structure of the adduct of Can2 with acetate.[12b]

Figure 3 shows an alignment of the amino acid sequences of bacterial *β*-CAs investigated in detail up until now (by means of X-ray crystallography and kinetic methods), such as the *Escherichia coli* T2 and T enzymes,[20] the CA from *Haemophilus influenzae*,[21] and the two mycobacterial enzymes encoded by

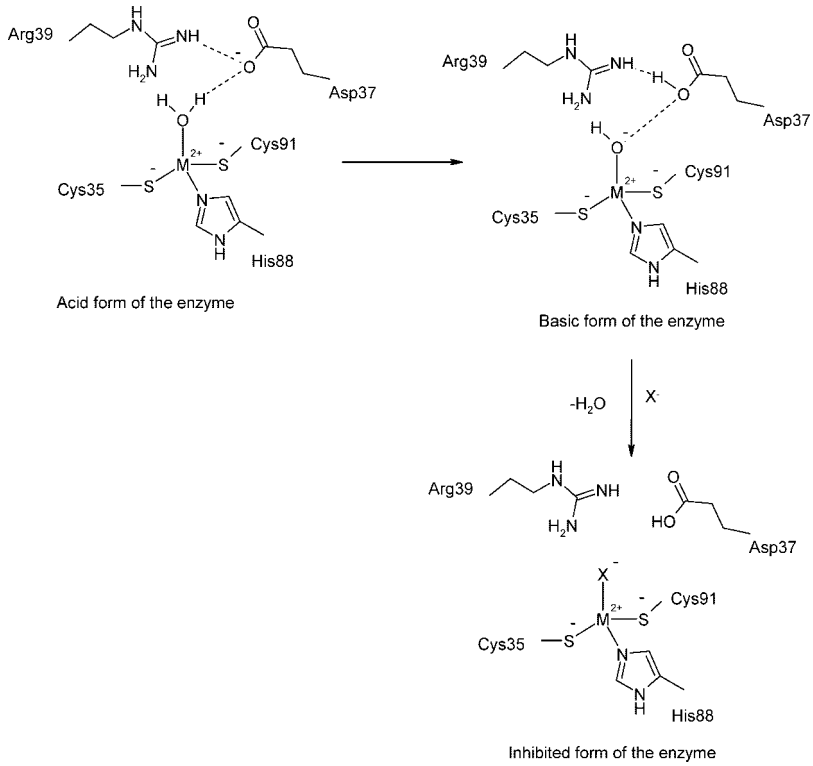


Figure 2. Proposed zinc water activation and inhibition mechanisms of β-CAs [Rv1284 numbering of amino acid residues (see Figure 1)]. The transfer of the proton from the acidic form of the enzyme (with water coordinated to the zinc ion) is assisted by the conserved Asp37-Arg39 amino acid dyad, which leads to the catalytically active, nucleophilic species (with hydroxide coordinated to zinc). Inhibitors (X^- may be the nitrogen-deprotonated form of a sulfonamide/sulfamate) may display either the hydroxide (as depicted above) or the zinc-bound water, leading to the tetrahedral Zn(II), inhibited form of the enzyme. The mechanism is supported by the recent X-ray crystal structure of the adduct of Can2 with acetate, in which acetate is bound as depicted for X^- above, as the fourth zinc ligand.[12b]

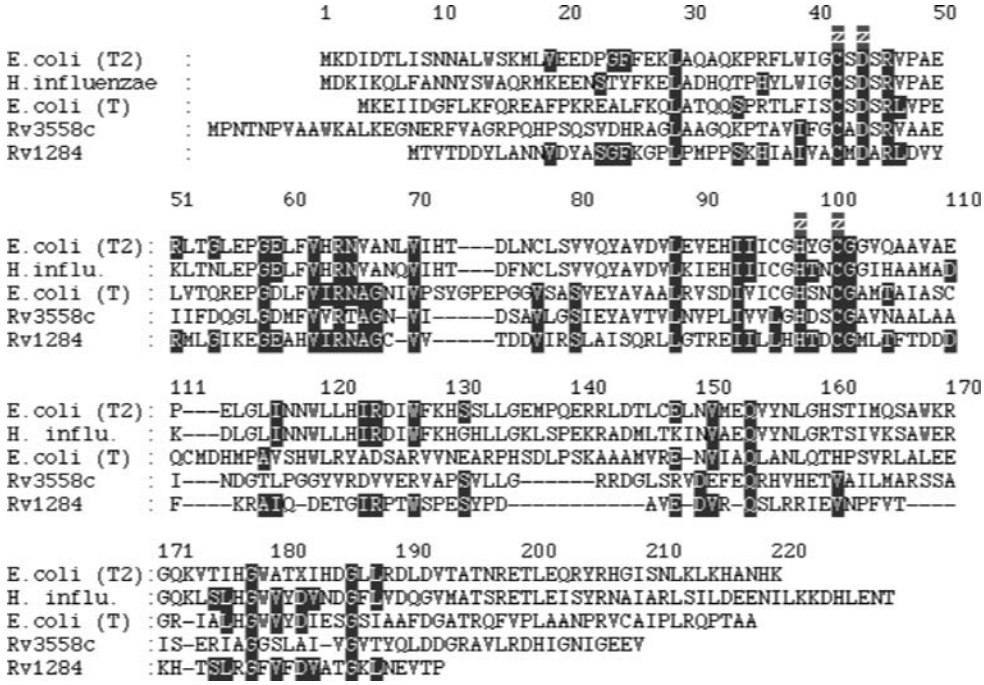


Figure 3. Alignment of amino acid sequences of bacterial β-CAs from *E. coli* (enzymes encoded by the genes T2 and T),[20] *H. influenzae*,[21] and *M. tuberculosis* (Rv3558c and Rv1284).[6] Conserved amino acid residues are highlighted in black, whereas the Zn(II) ligand is denoted by the letter z above the corresponding amino acids. Residue numbers are based on the *E. coli* T2 numbering system.[20]

the genes Rv3558c (mtCA 2) and Rv1284 (mtCA 1).[6] It may be observed that like all these β-CAs, mtCA 1 possesses all the amino acids involved in the catalytic cycle of this class of enzymes, i.e., the Zn(II) binding residues Cys35, His88, and Cys91 as well as the Asp-Arg dyad mentioned above (in this case constituted by Asp37 and Arg39). However, unlike the other CAs shown in Figure 3, mtCA 1 is a smaller enzyme, especially when the other mycobacterial CA discussed here, mtCA 2, is considered. Another main difference between mtCA 1 and mtCA 2 is the fact that the last enzyme has an additional

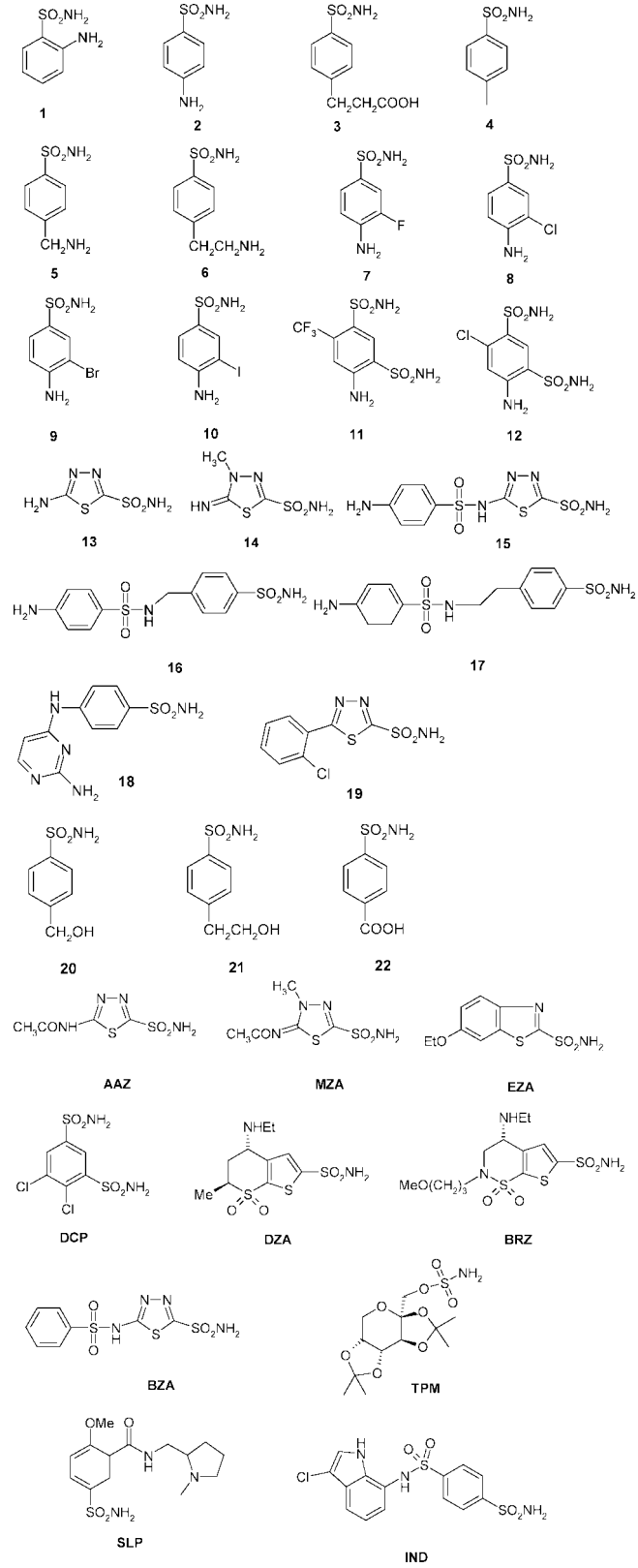
Table 2. hCA I, II, and *Mycobacterium* Rv1284 CA (mtCA 1) Inhibition Data with Sulfonamides **1**−**22** and 15 Clinically Used Derivatives, **AAZ-SAC** (inhibition data of isoforms I and II from ref 18)

	K_I[a] (nM)		
inhibitor	hCA I[b] (nM)	hCA II[b] (nM)	mtCA 1[c] (nM)
1	45400	295	9230
2	25000	240	9840
3	6690	495	7930
4	78500	320	4920
5	25000	170	8690
6	21000	160	9560
7	8300	60	8740
8	9800	110	7520
9	6500	40	186
10	6000	70	7710
11	5800	63	8100
12	8400	75	1720
13	8600	60	11540
14	9300	19	12650
15	6	2	905
16	164	46	612
17	185	50	853
18	109	33	750
19	690	12	7480
20	55	80	9560
21	21000	125	5510
22	23000	133	8210
AAZ	250	12	481
MZA	50	14	781
EZA	25	8	1030
DCP	1200	38	872
DZA	50000	9	744
BRZ	45000	3	839
BZA	15	9	810
TPM	250	10	612
SLP	12000	40	2300
IND	31	15	97
ZNS	56	35	28680
CLX	50000	21	10350
VLX	54000	43	12970
SLT	374	9	5160
SAC	18540	5950	7960

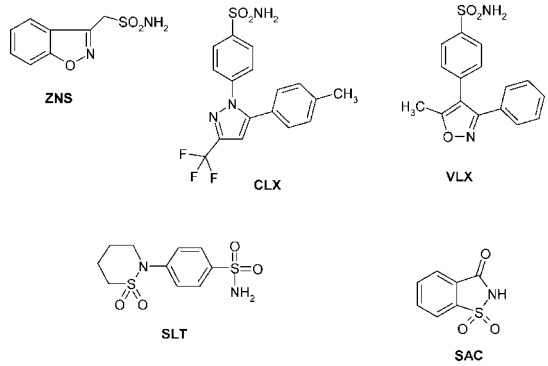
[a] Errors in the range of 5−10% of the shown data, from three different assays. [b] Human recombinant isozymes, stopped-flow CO_2 hydrase assay method, pH 7.5, 10 mM HEPES buffer.[17] [c] Bacterial recombinant enzyme, at 20 °C and pH 8.3, in 20 mM Tris-HCl buffer and 20 mM NaCl.[17]

aspartate residue coordinated to the Zn(II) ion, possessing the so-called "closed" active site center,[6,22] a situation also observed in some algal β-CAs (from *Porphyridium purpureum*).[22] It may also be observed from data depicted in Figure 3 that many amino acid residues of mtCA 1 are different as compared to those of mtCA 2 or other β-CAs (residue numbers are based on the *E. coli* T2 numbering system),[20] which clearly explains the differences in catalytic activity and affinity for inhibitors of this particular enzyme.

mtCA 1 Inhibition with Sulfonamides and Sulfamates. Table 2 shows the mtCA 1 inhibition data with a panel of 36 sulfonamides and one sulfamate (obtained for the CO_2 hydration reaction catalyzed by CAs),[17] some of which are clinically used drugs,[7] such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA** (an orphan drug),[7] topiramate **TPM**, sulpiride **SLP**, indisulam **IND**, zonisamide **ZNS**, celecoxib **CLX**, valdecoxib **VLX**, sulthiame **SLT**, and saccharin **SAC**. The simpler derivatives **1**−**22** were also included in the study as they were the scaffolds most extensively used to design potent or isoform-selective CAIs.[7,18] Data for the inhibition of the dominant human isoforms hCA I and II[18] with these compounds are also included in Table 2, for the sake of comparison. The following SAR can be observed from data of Table 2.



(i) Several of the investigated compounds, such as **13**, **14**, **ZNS**, **CLX**, and **VLX** exhibit very weak mtCA 1 inhibitory activity, with inhibition constants in the range of 11.54−28.68 μM. Whereas **13** and **14** are heterocyclic sulfonamides (precursors of acetazolamide and methazolamide, respectively), the



three last drugs possess a more complicated scaffold. Undoubtedly, these scaffolds are not very appropriate ones for obtaining effective inhibitors of this enzyme, although these sulfonamides act as quite efficient, low nanomolar hCA II (and much less efficient hCA I) inhibitors (Table 2).

(ii) Medium-potency inhibition of mtCA 1 has been observed with a large number of investigated sulfonamides, such as **1**−**8**, **10**−**12**, **19**−**22**, **EZA**, **SLP**, **SLT**, and **SAC**. These derivatives showed K_I values in the range of 1.03−9.84 μM. It is clear that they belong to rather heterogeneous classes of compounds, some of them (**1**−**6** and **20**−**22**) possessing rather simple scaffolds based on the benzenesulfonamide motif, possibly substituted in ortho or para with simple groups (amino, methyl, alkylamino, carboxyalkyl, carboxyl, hydroxyalkyl, etc.), whereas others are more complicated, such as the clinically used **EZA**, **SLP**, **SLT**, etc. Some of these derivatives (e.g., **12** and **EZA**) exhibit inhibition constants close to 1−2 μM, making it possible to consider them as promising leads for obtaining better mtCA 1 inhibitors. Many of these compounds act as efficient hCA II and less efficient hCA I inhibitors (Table 2).

(iii) Another subseries of derivatives, including **15**−**18**, **AAZ**, **MZA**, **DCP**, **DZA**, **BRZ**, **BZA**, and **TPM**, showed effective, submicromolar inhibition of mtCA 1, with K_I values in the range of 0.481−0.905 μM. Again, the SAR is complicated, as some of these derivatives (**15**−**17**) are sulfanilyl-sulfonamides possessing an elongated molecule, a shape which can be ascribed also to the pyrimidinyl-sulfanilamide derivative **18**, whereas the clinically used compounds are (except **TPM**) heterocyclic five-member ring or bicyclic derivatives incorporating various ring systems. **DCP** is an aromatic benzene-disulfonamide, whereas **TPM** is a sugar sulfamate. These data clearly show that a large variety of chemotypes lead to effective, submicromolar inhibitors of mtCA 1 and that both the sulfonamide and sulfamate can be used as zinc-binding groups[19] to generate effective inhibitors.

(iv) Two of the investigated compounds, **9** and **IND**, exhibited quite effective mtCA 1 inhibitory properties, with inhibition constants of 97−186 nM (Table 1). The bromosulfanilamide derivative **9** and the rather complicated scaffold of **IND** have little in common except the benzenesulfonamide part, and it is difficult to explain their efficacy for inhibiting mtCA 1; however, this result is important for the future design of compounds with enhanced efficacy eventually targeting the mycobacterial enzyme.

(v) All compounds investigated here were better inhibitors of the ubiquitous host enzyme hCA II than for the parasite one, mtCA 1, whereas their efficacy for inhibiting hCA I was quite diverse, with many of these sulfonamides being weak or very weak hCA I inhibitors; few of them (e.g., **15**, **EZA**, **BZA**, and **IND**) were quite effective, low nanomolar inhibitors.

Here we prove that mtCA 1 is a catalytically active enzyme for the CO_2 hydration reaction and that it can be inhibited by a large number of sulfonamides and sulfamates. As the enzyme was reported to be essential for the growth of *M. tuberculosis* (based on mutagenesis studies in strain H37Rv[23] and upregulation of the encoding gene under the starvation conditions used to model persistent bacteria[24]), our data may represent the starting point for designing new antimycobacterial agents possessing a completely new mechanism of action.

Conclusions

The β-CA encoded by the Rv1284 gene of *M. tuberculosis*, mtCA 1, shows appreciable catalytic activity for the physiological reaction, CO_2 hydration to bicarbonate and a proton, with a k_{cat} of 3.9×10^5 s^{-1} and a k_{cat}/K_m of 3.7×10^7 M^{-1} s^{-1}. A panel of 36 sulfonamides and one sulfamate were assayed for their interaction with mtCA 1. Most simple sulfonamide derivatives exhibited K_I values in the range of 1−10 μM, but several compounds, including sulfanilyl-sulfonamides, acetazolamide, methazolamide, dichlorophenamide, dorzolamide, brinzolamide, benzolamide, and topiramate, exhibited effective, submicromolar inhibition, with K_I values in the range of 0.481−0.905 μM. The best inhibitors were 3-bromosulfanilamide and indisulam with K_I values of 97−186 nM. This study demonstrates that it is possible to inhibit mtCA 1 activity and indicates this enzyme has potential as a druggable target for the development of new antimycobacterial agents operating with a novel mechanism of action.

Experimental Protocols

Chemistry. Compounds **AAZ-SAC** are commercially available (Sigma-Aldrich), whereas **1**−**22** were prepared as described previously.[13,18]

***M. tuberculosis* Strain.** *M. tuberculosis* was obtained from a Japanese patient with pneumonia. Sputum discharged from the patient was fully mixed with 2−4 volumes of a 4% NaOH solution and immediately spread onto a 2% Ogawa culture plate (Japan BCG, Tokyo, Japan). Following incubation at 37 °C for approximately 10 days, DNAs were extracted from the colonized bacteria by using a DNeasy kit (Qiagen, Hilden, Germany) and stored at −20 °C until later use.

Preparation of Recombinant mtCA 1. Initially, we tried to obtain a full-length mtCA 1 based on the sequence deposited in GenBank (entry NC000962, locus tag Rv1284),[5] which was obtained from *M. tuberculosis* strain H37Rv, but failed to obtain a clone in the pGEX expression vector. Subsequently, we obtained a truncated form of mtCA 1 lacking the 15-amino acid N-terminal polypeptide, since we have previously shown that the CA catalytic activity of the *H. pylori* β-CA which lacked the N-terminal polypeptide was the same as that of the full-length enzyme.[9] As seen in Figure 1, the ^{16}Ser residue (AGC) was substituted with Met (ATG), resulting in Kozac sequence (ATGG, double underlined in the sequence described later). In this study, we further substituted the codons of the three initial amino acid residues with *E. coli*-favoring codons, which are most frequently used in *E. coli*: ^{17}Gly (GGT:GGC), ^{18}Phe (TTC:TTT), and ^{19}Lys (AAG:AAA). Addition of the *Eco*RI recognition sequence (underlined in the following sequence) resulted in the forward primer sequence 5′-CGGAATTCCCATGGGCTTTAAAGGCCCG-3′ (see Figure 1). The 3′-primer sequence including the *Sal*I recognition site (underlined in the following sequence) was 5′-CGGTCGACCTAGGGCGTGAC-3′. The polymerase chain reaction (PCR) was hot-started with incubation for 5 min at 94 °C, consisted of 40 cycles of 30 s at 94 °C, 30 s at 57 °C, and 90 s at 72 °C, and terminated with 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, Tokyo, Japan). The proper DNA sequences of the mtCA 1 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−CA fusion protein as previously reported

for the corresponding β-CA of *H. pylori*.[9,10] Following induction of the expression of proteins 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). The columns were extensively washed with buffer, and then the GST−mtCA 1 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 mtCA 1 recombinant protein was further purified by sulfonamide affinity chromatography, and the amount of enzyme was determined spectrophotometrically as reported for similar β-CAs.[9,10]

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 a concentration of 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 a constant 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 achieved thereafter with distilled−deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to the assay, to allow 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 from Lineweaver−Burk plots, as reported previously,[18] represent the means from at least three different determinations.

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