Discovery of Low Nanomolar and Subnanomolar Inhibitors of the Mycobacterial β-Carbonic Anhydrases Rv1284 and Rv3273

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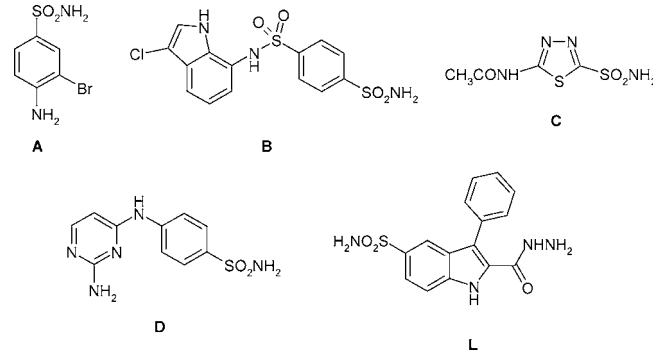
A series of 2-(hydrazinocarbonyl)-3-aryl-1*H*-indole-5-sulfonamides has been derivatized by reaction with 2,4,6-trimethylpyrylium perchlorate, leading to pyridinium derivatives. The new sulfonamides were evaluated as inhibitors of two β-carbonic anhydrases (CAs, EC 4.2.1.1) from *Mycobaterium tuberculosis*, Rv1284 and Rv3273. The whole series showed excellent nanomolar inhibitory activity, with several subnanomolar inhibitors being detected. Rv1284 and Rv3273 have potential for developing antimycobacterial agents with an alternate mechanism of action.

Introduction

Recently, we have shown that two of the three carbonic anhydrases (CAs,[a] EC 4.2.1.1) present in *Mycobacterium tuberculosis*[1,2] may be targeted by inhibitors belonging to the sulfonamide class. Indeed, the β-CAs encoded by the Rv1284[1,3] and Rv3273[2] genes of this widespread human pathogen[4] were shown to be catalytically active enzymes for the physiologic reaction catalyzed by these proteins, i.e., CO_2 hydration to bicarbonate and protons,[5] and to be susceptible to inhibition with a wide range of aromatic and heteroaromatic sulfonamides as well as the bioisosteric sulfamates.[1,2] Indeed, *M. tuberculosis* infection affects a large number of the world population, with more than 9 million new cases diagnosed each year, of which many are lethal, with multidrug resistant and extensively multidrug resistant tuberculosis (TB) being more and more widespread.[6,7] The therapy used to treat nondrug resistant TB is based on agents developed 30−40 years ago, with no new drugs launched ultimately.[8] Our preliminary work on mycobacterial CA inhibition mentioned above,[1,2] as well as molecular biology data showing these enzymes to be essential for the growth/virulence of *M. tuberculosis* (based on mutagenesis studies in strain H37Rv[9] and up-regulation of the encoding genes under the starvation conditions used to model persistent bacteria),[10] suggest that inhibition of mycobacterial β-CAs may be used for drug design campaigns aiming to find antimycobacterial agents possessing a new mechanism of action.

Although we showed that Rv1284[1] and Rv3273[2] may be inhibited by many types of sulfonamides or sulfamates, the best inhibitors detected so far showed only medium-low potency, with the best Rv1284 inhibitors possessing K_Is in the range of 100−200 nM and the best Rv3273 inhibitors having K_Is in the range of 90−500 nM. Furthemore, the best such compounds possessed simple scaffolds (e.g., 3-bromosulfanilamide **A** and indisulam **B** were the most effective Rv1284 inhibitors,[1] whereas acetazolamide **C** and 2-amino-pyrimidin-4-yl-sulfanilamide **D** were the best Rv3273 inhibitors),[2] which are not easily amenable to derivatization. Thus, we decided to explore different scaffolds incorporating the sulfamoyl zinc-binding groups (ZBGs) for the design of β-CA inhibitors targeting these mycobacterial enzymes.



Recently, we have reported the excellent inhibitory activity against various mammalian CA isozymes (belonging to the α-CA genetic family)[5] of a series of of 2-(hydrazinocarbonyl)-3-aryl-1*H*-indole-5-sulfonamides.[11] The X-ray crystal structure for the adduct of one of these compounds (the lead molecule **L**) with the physiologically relevant isoform human (h) hCA II has also been reported,[11a] evidencing a large number of favorable interactions between the active site of the enzyme and this inhibitor (Figure 1).

Considering this X-ray crystal structure of the hCA II - **L** adduct as starting point[11a] and the good inhibitory activity of this class of sulfonamides against many α-CA isoforms,[11,12] we decided to investigate whether this scaffold may also lead to effective β-CA inhibitors targeting the mycobacterial enzymes. We report here the synthesis and inhibitory properties against the mycobacterial CAs of a series of sulfonamides incorporating this new scaffold.

Results and Discussion

Chemistry. The reaction between pyrylium salts and amines, leading to pyridinium derivatives, constitutes a versatile method for the preparation of a large number of positively charged derivatives, not readily accessible by other synthetic proce-

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[a] Abbreviations: CA, carbonic anhydrase; CAI, CA inhibitor; hCA, human CA; GST, glutathione-*S*-transferase; mtCA, *Mycobacterium tuberculosis* CA; TB, tuberculosis.

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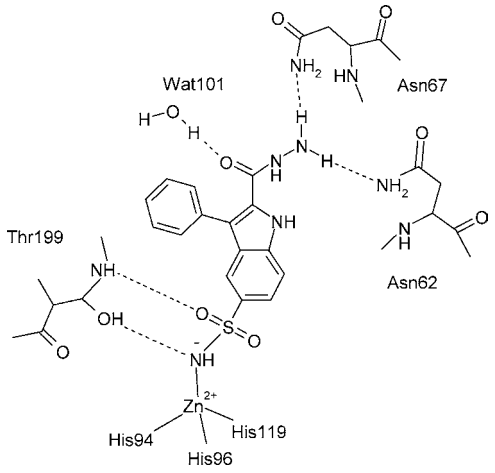


Figure 1. Schematic interactions to which **L** participates when bound within the hCA II active site, as determined by X-ray crystallography (PDB code 3B4F).[11a] Hydrogen bonds in which several moieties of the inhibitor participate with amino acid residues Asn62, Asn67, and Thr199 from the enzyme active site and a water molecule (Wat101) are shown as dotted lines. The trimethypyridinium group of the newly dsigned compounds **3**−**17** reported here presumably lies in the hydrophilic half of the CA active site, between Asn62 and Asn67.

dures.[13] Many biologically active compounds have been obtained in this way,[13−15] among which also different classes of sulfonamide CAIs.[14,15] The apparently simple reaction between a pyrylium salt and an amine, leading to pyridinium salts, is in reality a complicated process, as established by detailed spectroscopic and kinetic data from Balaban's and Katritzky's groups.[16−18] Thus, the nucleophilic attack of a primary amine RNH_2 on pyrylium cations generally occurs in the α position, with the formation of intermediates that are deprotonated in the presence of bases leading to 2-amino-tetradehydropyran derivatives and in the end to pyridinium salts.[16,17] A supplementary complication appears when the moiety substituting the 2- and/or 6-position(s) of the pyrylium ring is a methyl, a case in which a concurrent cyclization with formation of anilines in addition to the pyridinium salts may also take place.[16−18] These concurrent reactions mentioned above are generally important when the amine to be converted into pyridinium salt possesses weak nucleophilicity or basicity, as it happens to be the case of our sulfonamides **1**,[11] used as key intermediates for preparing the new compounds reported here (Scheme 1).[16−18]

The Bayer−Piccard synthesis[13−18] was successfully applied to sulfonamides **1**,[15] possessing the nucleophilic amino moiety incorporated in the carbohydrazide functionality. These compounds were reacted with 2,4,6-trimethylpyrylium perchlorate **2**, leading to a series of new pyridinium salts of type **3**−**17** (Scheme 1). Yields were in the range of 41−79%, and no complications for the synthesis and purification of these new derivatives were encountered. The new compounds **3**−**17** were characterized by standard physicochemical procedures, which confirmed their structures (see Experimental Section and Supporting Information for details) and possess a purity of >99.5%.

Mycobacterial CA Inhibition Studies. Inhibition data of the two β-CAs of *M. tuberculosis*, encoded by the genes Rv1284 and Rv3273, are shown in Table 1. Data of Table 1 also present the inhibitory activity against two physiologically relevant host CA isozymes (hCA I and II, which are cytosolic, ubiquitous isoforms)[5] of the new compounds **3**−**17** reported here, as the search of pathogen-selective CAIs is an important aspect in the design of new applications for this class of pharmacological agents. Inhibition data with the best mycobacterial CAIs detected so far,[1,2] i.e., compounds **A**−**D** are also shown in Table 1 for comparison reasons.

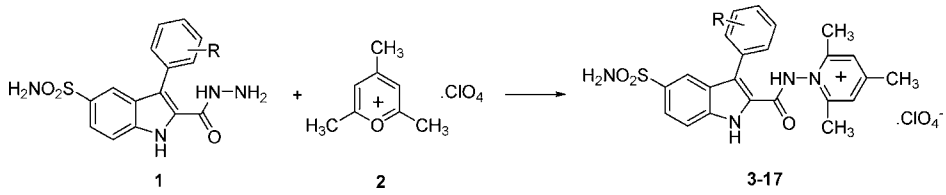
The following should be noted regarding the inhibition data of Table 1:

(i) Rv1284 was effectively inhibited by the lead molecule **L** possessing a new indolesulfonamide scaffold, with a K_I of 48 nM. **L** is thus already two times a better Rv1284 CAI as compared to the best such compound detected so far, indisulam **B** (K_I of 97 nM).[1] Probably this new scaffold fits better within the Rv1284 active site compared to the scaffolds of derivatives investigated earlier for the inhibition of this enzyme. However, the pyridinium derivatives obtained by reaction of **L** with 2,4,6-trimethylpyrylium salts, of types **3**−**17**, were much more effective CAIs as compared to **L** or any other sulfonamide/sulfamate investigated earlier.[1] Thus, the new class of sulfonamides reported here showed inhibition constants in the range of 0.92−35.3 nM against Rv1284. The least effective derivatives were **7**−**10**, which with K_Is in the range of 20.9−35.3 nM were anyhow much more effective Rv1284 CAIs compared to compounds **A** and **B** investigated previously (K_Is of 97−186 nM). These least effective CAIs incorporate 2-, 3-, 4-chloro- and 2-bromophenyl moieties in position 3 of the indolesulfonamide scaffold. Another group of the new derivatives, among which **3**−**6**, **11**, **12**, and **14**, showed an enhanced inhibitory activity toward Rv1284, with K_Is in the range of 1.5−9.7 nM. These compounds incorporate the following substitution patterns at the 3-phenyl moiety of the indolesulfonamide scaffold: unsubstituted phenyl; 2-, 3-, and 4-fluorophenyl, 3-bromo- and 4-bromophenyl and 3-tolyl. The remaining derivatives **13** and **15**−**17** were subnanomolar inhibitors of Rv1284, with K_Is of 0.92−0.98 nM. They incorporate 2- and 4-tolyl, 3-methoxyphenyl, and perfluorophenyl moieties in the 3 position of the indolesulfonamide scaffold. Thus, not only the 2-(hydrazinocarbonyl)-3-substituted-phenyl-1*H*-indole-5-sulfonamide derivatives lead to highly effective Rv1284 CAIs but the pyridinium derivatives obtained from this lead molecule show a very interesting SAR, with the nature of the group substituting the 3-phenyl ring strongly influencing the enzyme inhibitory activity. Many such favorable substitution patterns are reported here, such as among others the perfluorophenyl, tolyl, or 3-methoxyphenyl ones.

(ii) Rv3273 was also highly inhibited by **L** and its pyridinium derivatives **3**−**17** investigated here, with K_Is in the range of 0.88−31 nM (Table 1). **L** was the least effective such CAI (K_I of 31 nM) although being at least a 3 times better inhibitor compared to compounds **C** and **D** investigated earlier[2] and found to be the most effective inhibitors of this mycobacterial CA. All substitution patterns present in the pyridinium derivatives **3**−**17** were highly effective in inducing excellent Rv3273 inhibitory properties, as the entire class of compounds showed a compact behavior of very potent CAIs, with inhibition constants <8 nM. Thus, the unsubstituted compound **3**, its 2-, 3-, 4-fluoro, 2- and 3-chloro, 2-, 3-, and 4-methyl, as well as 3-methoxy derivatives, showed K_Is of 1.8−8.0 nM, whereas the remaining compounds were even better CAIs, with inhibition constants in the range of 0.88−1.01 nM (Table 1). These last derivatives incorporate 4-chlorophenyl, 2-, 3-, and 4-bromophenyl, and perfluorophenyl moieties in position 3 of the indolesulfonamide scaffold. It should be noted that the simple sulfonamides **A**−**D** investigated earlier,[2] as Rv3273 inhibitors are several orders of magnitude weaker CAIs as compared to the compounds investigated here.

(iii) Compounds **3**−**17** were also investigated as inhibitors of two human CAs, the cytosolic isoforms hCA I and II. Against

Scheme 1. Preparation of 1-(([5-(Aminosulfonyl)-3-substituted-phenyl-1*H*-indol-2-yl]carbonyl)amino)-2,4,6-trimethylpyridinium Perchlorates **3**−**17**[a]



[a] Reagents and conditions: MeOH (reflux), 6% $HClO_4$.

Table 1. Inhibition of CA Human Isoforms hCA I, II, and Mycobacterial Enzymes Rv1284 and Rv3273 with Sulfonamides **3**−**17**, **L**, as well as Simple Sulfonamides or Clinically Used CA Inhibitors **A**−**D** as Standards[a]

		K_I[b] (nM)			
inhibitor	R	hCA I[c]	hCA II[c]	Rv1284[d]	Rv3273[d]
L		7.5	7.2	48	31
3	H	9.0	71	6.5	8.0
4	2-F	8.5	91	9.3	6.9
5	3-F	11.3	3380	7.6	6.0
6	4-F	7.6	65	9.7	6.5
7	2-Cl	25.1	100	35.3	6.7
8	3-Cl	113	1800	31.8	6.6
9	4-Cl	3.2	77	20.9	**0.96**
10	2-Br	43.4	38	25.1	1.01
11	3-Br	30.8	74	3.2	**0.97**
12	4-Br	12.3	85	5.2	**0.96**
13	2-Me	10.5	106	**0.98**	6.8
14	3-Me	110	104	1.5	7.8
15	4-Me	5.1	68	**0.97**	3.6
16	3-OMe	8.6	2840	**0.92**	1.8
17	F_5	9.7	0.93	**0.93**	**0.88**
A		6500	40	186	7320
B		31	15	97	7840
C		250	12	481	104
D		109	33	750	91

[a] Subnanomolar inhibitors are highlighted in bold characters. [b] Errors in the range of 5−10% of the shown data, from three different assays, by a CO_2 hydration stopped-flow assay.[16] [c] Human, recombinant isozymes, pH 7.5, 20 mM TRIS·HCl buffer. [d] Bacterial recombinant enzyme, at 20 °C, pH 8.3 in 20 mM TRIS·HCl buffer and 20 mM NaCl.

the cytosolic isoform hCA I, the new pyridinium sulfonamides **3**−**17** generally showed good inhibitory activity (Table 1), with K_Is in the range of 3.2−113 nM, being thus more active than the clinically used sulfonamide acetazolamide **C** and having a similar activity to indisulam **C**, a compound in clinical development as an anticancer agent.[5] Most of the new derivatives **3**−**17**, similarly to the lead **L**, were in fact low nanomolar hCA I inhibitors (K_Is in the range 3.2−30.8 nM), except for **8**, **10**, and **14**, which were less active (K_Is in the range 43.4−113 nM). SAR was thus rather flat except for the three less active compounds mentioned earlier, proving that most of the substitution patterns present in the phenyl ring in position 3 are beneficial for the hCA I inhibitory properties of these compounds.

(iv) Although the lead **L** showed excellent hCA II inhibitory activity (K_I of 7.2 nM), the derivatives **3**−**17** reported here were generally much less effective inhibitors of this ubiquitous isoform, with K_Is in the range of 38−3380 nM, except for the pentafluorophenyl derivative **17**, which was a subnanomolar hCA II inhibitor (K_I of 0.93 nM). These data are indeed very interesting, as they prove that the substitution pattern of the phenyl moiety in the pyridinium salts **3**−**17** is crucial for their hCA II inhibitory activity. Thus, a very active compound has been detected (**17**), together with moderate inhibitors (such as **3**, **4**, **6**, **7**, **9**−**15**, K_Is in the range 38−106 nM), as well as three very ineffective inhibitors (**5**, **8**, and **16**, possessing K_Is in the range 1800−3380 nM). It should be noted that all these ineffective hCA II inhibitors have the substituent of the phenyl moiety in the meta-position, probably provoking a clash with some amino acid residues present in the hCA II active site, as already documented by us earlier.[19] However, in the absence of detailed structural data for this class of inhibitors this remains a hypothesis to be checked. The pentafluorophenyl derivative **17** on the other hand, is 7.7 times more effective as a hCA II inhibitor as compared to the lead **L**, and it would be also of great interest to resolve its high resolution X-ray crystal structure in adduct with hCA II for understanding the elements leading to this excellent inhibitory activity.

(v) Many of the new sulfonamides reported here showed a much better inhibition of the mycobacterial β-CAs Rv1284 and Rv3273 than for the host enzymes hCA I and II belonging to the α-CA genetic family. Furthermore, many of these compounds showed appreciable inhibition of only hCA I, an enzyme whose physiologic function is not well understood but seems to be marginal,[5] whereas the physiologically dominant hCA II showed a weak inhibition with these compounds (except one of them, **17**, which behaved as a very strong hCA II inhibitor). Thus, our finding is important not only for detecting low nanomolar and subnanomolar inhibitors of the two mycobacterial CAs but also because these compounds showed a much higher affinity for these β-CAs than for hCA II. Thus, the selectivity ratios for the inhibition of the pathogen over the host enzymes are indeed very favorable for the potential use of these compounds for in vivo antimycobacterial studies. Furthermore, two of the three mycobacterial CAs known so far are both highly inhibited by this class of sulfonamides investigated here.

Conclusions

The β-CAs encoded by the genes Rv1284 and Rv3273 of *Mycobacterium tuberculosis*, which show appreciable catalytic activity for the physiological reaction, CO_2 hydration to bicarbonate, and a proton, play an important role in the pathogen life cycle and are inhibited by sulfonamides. We report here a series of 2-(hydrazinocarbonyl)-3-aryl-1*H*-indole-5-sulfonamides possessing various 2-, 3-, or 4-substituted phenyl groups with methyl-, halogeno-, and methoxy- functionalities as well as a perfluorophenyl moieties in their molecule. They were derivatized by reaction with 2,4,6-trimethylpyrylium perchlorate, leading to pyridinium derivatives. These compounds were highly effective, low nanomolar or subnanomolar inhibitors of the two mycobacterial enzymes, having a good affinity for the host enzyme hCA I but much lower inhibitory properties against the major, physiologically dominant isoform hCA II. These new compounds are several orders of magnitude better mycobacterial CAIs compared to sulfonamides/sulfamates investigated earlier. Rv1284 and Rv3273 have thus potential for developing antimycobacterial agents with an alternate mechanism of action, considering that many *M. tuberculosis* strains exhibit multidrug

resistance and extensive multidrug resistance to the existing therapeutics.

Experimental Section

Materials and Methods. Sulfonamides **1** used in the synthesis were reported earlier.[11] 2,4,6-Trimethylpyrylium perchlorate **2** and standard sulfonamides were commercially available compounds (from Sigma-Aldrich, Milan, Italy). The CA isozymes used in the experiments were recombinant ones obtained and purified as reported earlier by this group.[1,2] ^{1}H, DEPT, NOESY, COSY, HMQC, and HMBC spectra were recorded using a Bruker Advance III 300 MHz spectrometer. The chemical shifts are reported in parts per million (ppm). Melting points (mp) were measured in open capillary tubes using a Büchi melting point B-540 melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} aluminum backed plates. Elution of the plates was carried out using MeOH/DCM or MeOH/$CHCl_3$ systems. Visualization was achieved with UV light at 254 nm. Elemental analysis was done in house by combustion. Electron ionization mass spectra (30 eV) were recorded in positive or negative mode on a Water MicroMass ZQ. The purity has been determined by means of analytical HPLC, performed on a reversed-phase C_{18} Bondapack column, with a Beckman EM-1760 instrument. Both conbustion and HPLC confirmed a purity of >99.5% for the new compounds reported here.

General Procedure for the Preparation of Compounds 3−17. 2,4,6-Trimethylpyrylium perchlorate **2** (1.5 mM) was dissolved in 20 mL of methanol. After addition of 2-(hydrazinocarbonyl)-3-substituted-phenyl-1*H*-indole-5-sulfonamide derivatives **1** (3 mM), the solution was refluxed overnight. The cold mixture was treated with 200 mL of 6% perchloric acid to precipitate the pyridinium salts. The obtained products were recrystallized from water with 6% perchloric acid.

1-(([5-(Aminosulfonyl)-3-phenyl-1*H*-indol-2-yl]carbonyl)amino)-2,4,6-trimethylpyridinium Perchlorate 3. Yield 79%; mp > 300 °C (dec). ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 2.61 (9H, s, pyridinium 2,4,6-$(CH_3)_3$), 7.24 (2H, s, SO_2NH_2), 7.42−7.68 (6H, m, Ar−H), 7.70−7.84 (2H, m, Ar−H), 7.90 (2H, s, Ar−H), 8.05 (1H, s, CONH), 12.50 (1H, s, indole NH). LC/MS: *m*/*z* 436 $(M + H)^+$. Elem anal. (C, H, N, S).

All other compounds (**4−17**) have been characterized as **3** (see Supporting Information for details).

CA Catalytic Activity and Inhibition. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity[20] as reported earlier.[1,2]

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Supporting Information Available: The complete characterization of compunds **3−17** is described in detail. This material is available free of charge via the Internet at http://pubs.acs.org.

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