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Carbonic anhydrase inhibitors. Inhibition of the Rv1284 and Rv3273 β-carbonic anhydrases from *Mycobacterium tuberculosis* with diazenylbenzenesulfonamides

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ABSTRACT

A series of diazenylbenzenesulfonamides obtained from sulfanilamide or metanilamide by diazotization followed by coupling with phenols or amines, was tested for the inhibition of the β-carbonic anhydrases (CAs, EC 4.2.1.1) encoded by the genes Rv1284 and Rv3273 of *Mycobacterium tuberculosis*. Several low micromolar inhibitors of the two enzymes were detected, with prontosil being the best inhibitor (K_Is of 126–148 nM). Inhibition of pathogenic β-CAs may lead to the development of antiinfectives with a new mechanism of action, devoid of resistance problems encountered with classical antibiotics.

Mycobacterium tuberculosis and related *Mycobacteria* infections (e.g., *Mycobacterium avium*) affect a large number of the world population, with an estimated 9.2 million new cases each year, many of which leading to deaths.[1–4] Multi-drug resistant and extensively multi-drug resistant tuberculosis (TB) worsens even more the situation, as such strains are now present in more than 50 countries, posing serious concern to the global healthcare system, as this disease is largely unresponsible to the presently available drugs.[1] Indeed, the combination therapy used to treat TB is based on agents developed in the 60–80s, with no new drugs launched for the last 30 years.[2–4] There is thus a huge interest for novel anti-TB drugs, possessing alternative mechanisms of action compared to the clinically used antibiotics. The complete sequencing of *M. tuberculosis* genome some years ago[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 such proteins identified after the *M. tuberculosis* genome was published, there are also several carbonic anhydrases (CAs, EC 4.2.1.1) belonging to the β-class.[6–9] *M. tuberculosis* contains three β-CA genes in its genome, that is, Rv1284 (encoding for a protein we named mtCA 1), Rv3588c (encoding for mtCA 2), and Rv3273 (encoding for a third enzyme, mtCA 3).[6,7] The catalytic activity and inhibition studies with a range of sulfonamides and one sulfamate of two these enzymes, that is, mtCA 1 and mtCA 3 have been recently reported,[7–9] whereas Covarrubias et al. reported the X-ray crystal structure of mtCA 1 and mtCA2.[6] CAs belonging to the β-class[10] are found in many pathogenic organisms such as fungi (*Candida albicans, C. glabrata*, and *Cryptococcus neoformans* among others)[10–12] and bacteria (*Helicobacter pylori*, *Arthrobacter aurescens*, *Leptospira borgpetersenii*, *Legionella pneumophila*, and *Haemophilus influenzae*)[13,14] but they lack from mammals, in which only α-CAs (under the form of 16 different isoforms) are present.[10] Thus, inhibition of such β-CAs started to be considered as a new possible approach for designing antiinfectives (antifungal or antibacterial agents) possessing a different mechanism of action compared to the classical pharmacological agents in clinical use for a long period, for which pathogenic fungi and bacteria developed various degrees of resistance.[15,16] The drug resistance problem with presently used antifungals and antibiotics represents a serious medical problem worldwide.[17]

In previous work from our group[7–9] we have investigated the inhibition of mtCA 1 and mtCA 3 with the classical sulfonamides/sulfamates in clinical use as well as with simple, aromatic derivatives used as building blocks in the design of CA inhibitors (CAIs) targeting the mammalian, α-class enzymes (of which at least 16 isoforms are presently known and well characterized).[10,18] Several of the clinically used sulfonamides, such as acetazolamide **AAZ**, methazolamide **MZA**, dorzolamide **DZA** or brinzolamide **BRZ**

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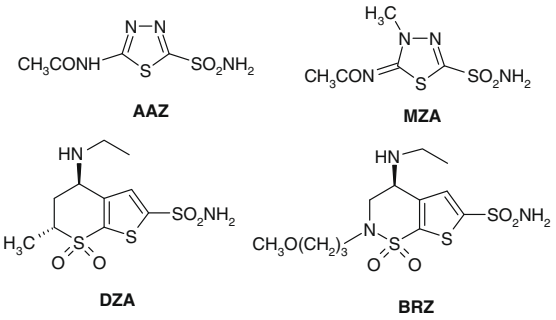
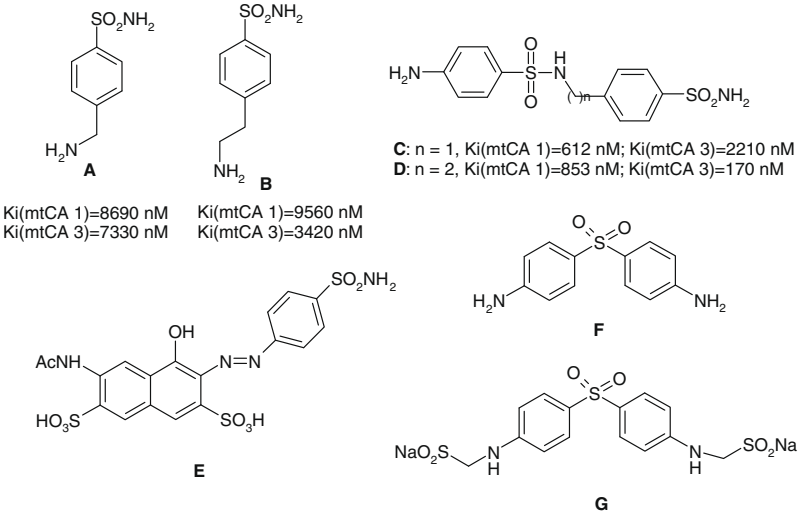


showed interesting inhibitory activity against mtCA 1 and mtCA 3, with inhibition constants in the submicromolar ranges (K_Is of 481–839 nM against mtCA 1[8] and of 104–562 nM against mtCA 3[7]), but all these compounds were much more potent inhibitors of the host, human isoforms (such as the ubiquitous CA II).[10]

Screening analysis for genes specifically required for the mycobacterial growth recently showed that some genes encoding β-CAs (e.g., Rv3588c) are required whereas others (e.g., Rv3273) are not essential for the bacterial growth in vivo.[19] However, such findings cannot conclusively exclude the biological significance of these enzymes in the survival and/or pathogenicity of *M. tuberculosis*. For example, an elegant study by Miltner et al.[20] identified six invasion-related genes in *M. avium*. Constitutive expression of proteins encoded by these genes, among which an orthologue of Rv3273, showed significantly increased ability of bacterial invasion into HEp-2 and HT-29 intestinal epithelial cells. These findings indicate that the mycobacterial CAs plays a crucial role in the infection process, which is however poorly understood at the present time. Considering the small number of inhibitors investigated to date for their interaction with the mycobacterial CAs,[7–9] and the fact that potent such inhibitors may be relevant for developing novel drugs, we decided to investigate other classes of sulfonamides as possible mtCA 1 and mtCA 3 inhibitors. In this Letter we report inhibition studies of these enzymes with a series of diazenylbenzenesulfonamides and two simple sulfonates. Actually sulfonates were not investigated up to now for their interaction with β-CAs.

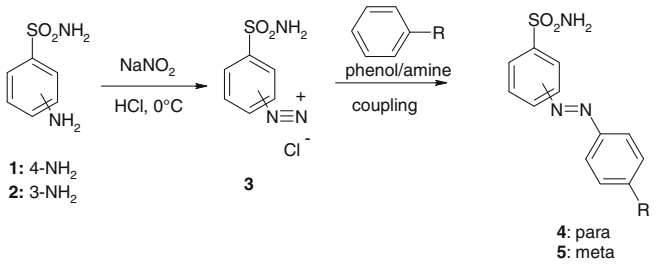
constants of 170 nM–2.21 μM. We have thus decided to explore whether compounds with an elongated molecule may have better mycobacterial CA inhibitory activity, and the diazenylsulfonamides appeared to us as interesting candidates, also because we have recently investigated two such compounds (**4a** and **4b**) as human (h) CA II inhibitors, observing that their hCA II inhibitory activity is not very good[21] (which may be a positive feature for a compound that should target the pathogenic, bacterial β-CAs and not the mammalian, α-class enzymes). It should be noted that azo-derivatives incorporating sulfonamide moieties are highly important from the drug design point of view, since the antibacterial sulfa drugs still widely used in therapy[22a] were discovered considering prontosil **E** as a lead molecule. Indeed, prontosil was the first chemotherapeutic agent to show effective antibacterial effects in animals and humans with bacterial infections untreatable before the advent of this drug, being subsequently shown that the active metabolite of **E** is sulfanilamide **1**.[22b–d] Although sulfones such as dapsone **F** and its methylsulfinate sodium salt sulfoxone sodium **G** do not possess a sulfonamide moiety, their mechanism of antibacterial activity is similar to that of the antibacterial sulfonamides (such as **E**, **1**, etc.), that is, these clinically used antileprosy agents[23] (targeting *Mycobacterium leprae*, a pathogen related to *M. tuberculosis*) block the bacterial folate biosynthesis by antagonizing 4-amino-benzoic acid reaction with pteridine, and acting thus as dihydropteroate synthase inhibitors.[22,23] It should be noted that **G** incorporates alkane-sulfinic acid moieties in its molecule, which may be zinc binders in metalloenzymes such as CAs.[18] Thus, we have used compounds **A**–**G** as lead molecules in the drug design of antimyobacterial CAs mtCA 1 and mtCA 3 reported here.

We prepared a series of *para*- and *meta*-substituted diazenylbenzenesulfonamides **4** and **5**, starting from sulfanilamide **1** or metanilamide **2**, which have been converted to the corresponding diazonium salts **3** by treatment with sodium nitrite and hydrochloric acid at low temperatures (Scheme 1). The diazonium salts **3** were then coupled with phenols or amines, leading to the target compounds **4** and **5** (Scheme 1), which are obviously similar to prontosil **E** as lead molecule.[24] The R moieties present in compounds **4** and **5** have been chosen in such a way as to provide structure–activity relationship (SAR) insights regarding the inhibi-



In the initial work[7,8] for discovering mtCA 1/3 inhibitors we observed that the simple aminosulfonamides **A** and **B** show rather modest inhibitory activity against these enzymes, with K_Is in the range of 3.42–9.56 μM, whereas compounds **C** and **D**, incorporating an additional sulfanilyl moiety and thus possessing an elongated molecule, were more effective mycobacterial CAIs, with inhibition

tion of mtCAs with this class of compounds. Thus, both hydroxy-, amino, methylamino and dimethylamino such moieties were incorporated in the molecules of sulfonamides **4** and **5** reported here. Since water solubility is not one of the principal feature of sulfonamides, we have also prepared the sulfonate derivatives **4e**, **4f** and **5e**, **5f**, which incorporate a water solubilizing moiety



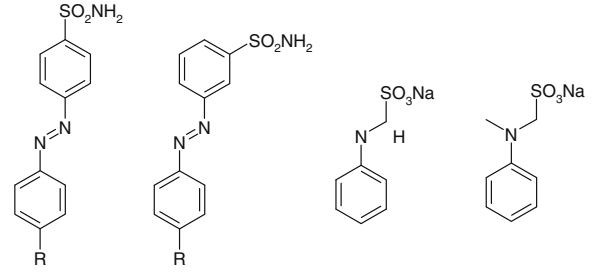
Scheme 1. Preparation of diazenylbenzenesulfonamides **4** and **5** from sulfanilamide **1** or metanilamide **2**, by diazotization followed by coupling of the diazonium salts **3** with phenols/amines.

(as sodium salts) and confer increased hydrosolubility to these sulfonamides, similar to the sulfinate sodium salt moieties present in the antileprosy agent **G** mentioned above. The intermediate sulfonates **6** and **7** used to prepare these hydrosoluble sulfonamides (**4e**, **4f** and **5e**, **5f**) were also tested for their CA inhibitory properties, since this class of derivatives was scarcely investigated on these enzymes (very few studies for the inhibition of human, α-CAs are available,[25] but no β-CA were investigated up until now for their interaction with such compounds, possessing a SO_3^- instead of a SO_2NH^- zinc-binding group—ZBG).

Inhibition data of the two mycobacterial enzymes mtCA 1 and 3 with compounds **4**–**7** and prontosil **E** are reported in Table 1.[26] Inhibition data for the host enzyme hCA II (the dominant CA isoform in humans)[10] are also provided for comparison in Table 1. The following SAR is to be noted from data of Table 1:

(i) The mycobacterial enzyme mtCA 1 was inhibited by all diazenylbenzenesulfonamides **4** and **5** reported here, with inhibition constants in the range of 6.78–63 μM. Only one compound was a weak inhibitor (**5f**, K_I of 63 μM) whereas all the other investigated sulfonamides showed a compact behavior of effective, micromolar inhibitors (K_Is of 6.78–9.27 μM). Thus, most of the substitution patterns present in **4** and **5** lead to efficient mtCA 1 inhibitors. The only exception is the metanilamide derivative possessing the rather bulky aminomethylsulfonate moiety found in **5f**. It is interesting to note that the corresponding desmethyl derivative **5e** is an effective mtCA 1inhibitor, similarly to the corresponding *para*-substituted compound **4f**, the two sulfonamides having K_Is of 7.51–8.71 μM. There were no other notable differences between the sulfanilamide and metanilamide derivatives, except **5f** mentioned above. These compounds were on the other hand less effective mtCA 1 inhibitors compared to the clinically used drugs **AAZ**–**BRZ** or the leads **C** and **D**, but were anyhow more active than the parent compounds **A**, **B** or sulfanilamide (data not shown). Thus, our working hypothesis that compounds with elongated molecule will act as better inhibitors, was not entirely confirmed by the obtained data, but the new compounds show anyhow an interesting SAR and probably better activity may be obtained by changing the R groups present in derivatives **4** and **5**. However, a very interesting result emerged regarding the inhibition data with the simple sulfonates **6** and **7**, which showed comparable inhibition data with sulfonamides **4** and **5**, with K_Is of 7.86–8.67 μM (Table 1), and with prontosil **E**. Thus, the sulfonate moiety may be an alternative ZBG or the design of novel β-CA inhibitors. This result is even more significant considering that these sulfonates are an order of magnitude weaker CA inhibitors against the major human isoform, hCA II, which was inhibited with K_Is in the range of 58.3–63.6 μM (see discussion later in the text too). On the other hand, the bulkier (then **4** and **5**) azo derivative prontosil **E** was the best mtCA 1 inhibitor detected here, with an inhibition constant of 126 nM. It is thus obvious that diazenylbenzenesulfonamides incorporating bulkier moieties to the second aromatic ring (not the one with the sulfamoyl moiety), such as prontosil E, may lead to quite potent mtCA 1 inhibitors, this compound being a more effective inhibitor than the clinically used sulfonamides **AAZ**, **MZA**, **DZA**, and **BRZ**. In fact prontosil is among the best mtCA 1 inhibitors detected so far.

Table 1
Inhibition of CAs of human (hCA II) or mycobacterial origin mtCA 1 and mtCA 3 with sulfonamides **4** and **5**, the sulfonates **6** and **7**. Standard sulfonamide inhibitors data are also provided for comparison[26]



4 **5** **6** **7**

No.	R	K_I (μM)[#]		
		hCA II[a]	mtCA 1[b]	mtCA 3[b]
4a	OH	0.665	9.27	12.40
4b	NH_2	0.106	7.20	8.78
4c	NHMe	0.093	7.69	9.18
4d	NMe_2	0.638	6.86	30.7
4e	$NHCH_2SO_3Na$	0.105	6.78	8.90
4f	$N(Me)CH_2SO_3Na$	0.104	8.71	9.03
5a	OH	0.106	8.97	9.23
5b	NH_2	0.088	7.00	8.68
5d	NMe_2	0.105	7.54	9.36
5e	$NHCH_2SO_3Na$	0.107	7.51	9.45
5f	$N(Me)CH_2SO_3Na$	0.109	63	7.40
6	—	58.3	8.67	8.90
7	—	63.6	7.86	9.11
E		13	0.126	0.148
AAZ*	—	0.012	0.481	0.104
MZA*		0.014	0.781	0.562
DZA*	—	0.009	0.744	0.137
BRZ*		0.003	0.839	0.201

[#] Mean value from at least three different measurements.[26] Errors were in the range of ±5% of the obtained value (data not shown).
[a] h = human cloned full length enzyme.
[b] Mycobacterial, recombinant enzyme.
* From Refs. 7,8.

(ii) mtCA 3 showed an inhibition profile with compounds **4**–**7** and **E** rather similar to that of mtCA 1. Thus, all sulfonamides **4** and **5** were medium potency inhibitors, with K_Is in the range of 7.40–30.7 μM. The least effective inhibitor was **4d** (K_I of 30.7 μM) whereas all remaining sulfonamides, together with the sulfamates **6** and **7** had K_Is of 7.40–12.40 μM. SAR is thus rather simple here too, with the metanilamide derivatives **5** being slightly more effective mtCA 3 inhibitor compared to the corresponding sulfanilamide derivatives **4**. Again the clinically used compounds were more effective inhibitors compared to the sulfonamides reported here, except prontosil **E**. Indeed, this compound showed very effective mtCA 3 inhibitory activity, with a K_I of 148 nM (Table 1).

(iii) The host, hCA II isoform was more effectively inhibited by the diazenebenzenesulfonamides **4** and **5**, with inhibition constants in the range of 88–665 nM, but were very weakly inhibited by the sulfonates **6** and **7** (K_Is of 58.3–63.6 μM) (Table 1). Thus, we confirmed the earlier observation that these azo dyes are not very good hCA II inhibitors (compared to the clinically used compounds of Table 1, which have K_Is in the low nanomolar range, of 3–14 nM) but they interact better with the α-class enzyme than with the two β-CA investigated here). Prontosil E was also a very effective hCA II inhibitor (K_I of 13 nM).

(iv) Selectivity issues: except for the sulfonates **6** and **7** which are much better β-CA than α-CA inhibitors, all sulfonamides inhibited much better the host (α-class) over the pathogenic (β-class enzyme). Thus, the main finding of this study, except for the medium-weak mtCA 1/3 inhibitors belonging to the sulfonamide class that we evidenced, is that sulfonates may be better binding and also selectively inhibit the pathogenic over the host CAs. Another important aspect is related to the good inhibitory activity of prontosil against mtCA 1 and 3, although this compound is also not a selective CAI for the pathogenic over the host enzymes. Work is warranted to confirm these finding on different classes of sulfonates and to design compounds based on the prontosil scaffold as lead.

In conclusion, we report here a series of diazenylbenzenesulfonamides which have been obtained from sulfanilamide or metanilamide by diazotization followed by coupling with phenols or amines. The compounds were tested for the inhibition of the β-CAs encoded by the genes Rv1284 and Rv3273 of *M. tuberculosis*. Several low micromolar inhibitors of the two enzymes were detected with the best inhibitor being prontosil (K_Is of 126–148 nM). Some sulfonates tested for the same interaction showed excellent mtCA inhibitory activity and selectivity for the inhibition of the pathogenic over host enzymes. Inhibition of pathogenic β-CAs may thus lead to the development of antiinfectives with a new mechanism of action, devoid of resistance problems encountered with classical antibiotics

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24. Aminonenzenesulfonamide (sulfanilamide **1** or metanilamide **2**) (0.20 g, 1.0 equiv) was dissolved in a freshly prepared 40% solution of concentrated hydrochloric acid in deionised water (3.0 ml) and then cooled down to −5 °C. A 2.3 M aqueous solution of $NaNO_2$ (1.2 equiv) was added dropwise and the mixture was kept stirring at the same temperature until a persistent pale yellow solution, for sulfanilamide, or pale-orange solution, for metanilamide, was formed (5–10 min). The solution was used freshly prepared for the coupling reactions. For example, a solution of diazotized sulfanilamide/metanilamide **3** was added dropwise to a solution of *N*,*N*-dimethylaminobenzene (0.14 g, 0.15 ml, 1.0 equiv) in a saturated aqueous solution of AcONa (3.0 ml) at −5 °C. The solution turned bright orange immediately and a precipitate was formed. The mixture was stirred at the same temperature for 15 min, then warmed at room temperature and the pH adjusted to 7. The solid was collected by filtration, washed with a minimum amount of H_2O, dried under vacuo and purified by silica gel column chromatography eluting with 5% MeOH in dichloromethane to give **4d** as an orange solid in 79% yield. 4-(4′-Dimethylaminophenyl)diazenylbenzenesulfonamide **4d**: mp260–261 °C silica gel TLC R_f 0.38 (MeOH/DCM 5%); ν_{max} (KBr) cm^{-1}, 1604 (Aromatic), 1520 (N=N), 1370 (SO_2–N); δ_H (400 MHz, DMSO-d_6) 3.13 (6H, s, 2 × CH_3), 6.90 (2H, d, *J* 9.2, 2 × 3′-H), 7.48 (2H, s, SO_2NH_2, exchange with D_2O), 7.87 (2H, d, *J* 9.2, 2 × 2′-H), 7.93 (2H, d, *J* 7.8, 2 × 2-H), 7.99 (2H, d, *J* 7.8, 2 × 3-H); δ_C (100 MHz, DMSO-d_6) 155.9 (ipso), 154.8 (ipso), 145.0 (ipso), 144.0 (ipso), 128.5 (C-2), 127.0 (C-3), 123.6, 113.2, 30.8 (2 × CH_3).
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26. Khalifah, R. G.. *J. Biol. Chem.* **1971**, *246*, 2561. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. 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 constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. 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 with 5–10% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.1 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 non-linear least-squares methods using PRISM 3, and represent the mean from at least three different determinations. CA isozymes were recombinant ones obtained as reported earlier.[7–9]