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SHORT COMMUNICATION

Dihalogenated sulfanilamides and benzolamides are effective inhibitors of the three β-class carbonic anhydrases from *Mycobacterium tuberculosis*

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Abstract

A series of halogenated sulfanilamides and halogenated benzolamide derivatives have been investigated as inhibitors of three β-carbonic anhydrases (CAs, EC 4.2.1.1) from the bacterial pathogen *Mycobacterium tuberculosis*, mtCA 1 (Rv1284), mtCA 2 (Rv3588c) and mtCA 3 (Rv3273). All three enzymes were inhibited with efficacies between the submicromolar to the micromolar one, depending on the substitution pattern at the sulfanilamide moiety/fragment of the molecule. Best inhibitors were the halogenated benzolamides (K_Is in the range of 0.12–0.45 μM) whereas the halogenated sulfanilamides were slightly less inhibitory (K_Is in the range of 0.41–4.74 μM). This class of β-CA inhibitors may have the potential for developing antimycobacterial agents with a diverse mechanism of action compared to the clinically used drugs for which many strains exhibit multi-drug/extensive multi-drug resistance.

Keywords: Carbonic anhydrase, β-carbonic anhydrase, sulfonamide, dihalogenated sulfanilamide/benzolamide, enzyme inhibitor, *Mycobacterium tuberculosis*

Introduction

The genome of the human pathogen *Mycobacterium tuberculosis* contains at least three β-carbonic anhydrases (CAs, EC 4.2.1.1), called mtCA 1, 2 and 3, and encoded by the genes Rv1284, Rv3588c Rv3273[1–6]. These enzymes have been cloned and their catalytic activity and inhibition profiles with sulfonamides recently investigated[3–6]. As most CAs described to date, these enzymes are inhibited by sulfonamides and related compounds[8–12]. Indeed, interesting, low nanomolar or subnanomolar sulfonamide inhibitors targeting these CAs have already been detected[3–6]; it is not yet clear whether the *in vivo* inhibition of these enzymes has an antimycobacterial effect[7,12].

Resistance to antibiotics belonging to several different classes is escalating and represents a worldwide problem[13–17]. Many strains of Gram-negative/positive bacteria (such as among others *Staphylococcus aureus*, *M. tuberculosis*, *Helycobacter pylori*, *Brucella suis, Streptococcus pneumoniae*, etc.) no longer respond to various classes of antibiotics[13–17]. Cloning of the genomes of bacterial pathogens offers however the possibility to explore alternative pathways for inhibiting virulence factors or proteins essential for the pathogens life cycle. Among the many new such proteins recently explored, CAs emerged as interesting drug targets[12,18]. Indeed, potent sulfonamide inhibitors of the bacterial CAs from *H. pylori, B. suis, S. pneumoniae* and several other such pathogens were shown to inhibit the growth of the pathogen, by a mechanism of action not entirely understood at this moment[12,18–22].

The classical CA inhibitors (CAIs) are the primary sulfonamides, RSO_2NH_2, which are used clinical for more than 50 years as diuretics or systemically acting antiglaucoma drugs[8,9,23,24]. Around 30 clinically used drugs (or agents in clinical development) belonging to the sulfonamide or sulfamate class, show significant CA inhibitory activity and are used for the management of a variety of disorders[8,9,23]. However, all these

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drugs target all mammalian CAs, of which 16 different isoforms are known so far, and as thus, they may show undesired side effects[8,9,23]. Ultimately, a large number of such derivatives started to be investigated as anti-infectives, which target bacterial/fungal CAs (generally belonging to the β-CA class), but this field is still a very new one, with non-irrelevant resistance from the scientific community in accepting these enzymes as anti-infective targets[8,12].

Bacteria predominantly encode for β-class CAs, which are not present in vertebrates, and these enzymes were started to be seriously considered as possible drug targets for obtaining antibacterials devoid of the resistance problems mentioned above, which affect most classes of antibiotics in clinical use[13–16].

Thus, exploring various chemotypes among the sulfonamides may lead to the discovery of β-CA inhibitors with good affinity for enzymes from pathogenic species. Here, we report that the sulfonamides incorporating halogenated benzolamide/sulfanilamide moieties, a class of α-CAIs reported by our group[25], also act as highly effective inhibitors of the three β-CAs from *M. tuberculosis*, i.e. mtCA 1–mtCA 3.

Materials and methods

Chemistry

Enzymology

mtCA 1–mtCA 3 were recombinant enzymes obtained as described earlier[1–7].

CA catalytic activity and inhibition assay

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity[26]. 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. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-square methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver-Burk plots, as reported earlier[3–7,27,28], and represent the mean from at least three different determinations.

Results and discussions

We report here the investigation of a series of sulfonamides of type **1–10** for their interaction with three β-CAs from the bacterial pathogen *M. tuberculosis*, i.e. mtCA 1 (Rv1284), mtCA 2 (Rv3588c) and mtCA 3 (Rv3273). The rationale for investigating these compounds as inhibitors of these enzymes is based on the fact that benzolamide **BZA**, an orphan drug belonging to the CAIs[8], is one of the best (submicromolar) sulfonamide inhibitor of the mycobacterial CAs, as reported earlier by our group[3–7].

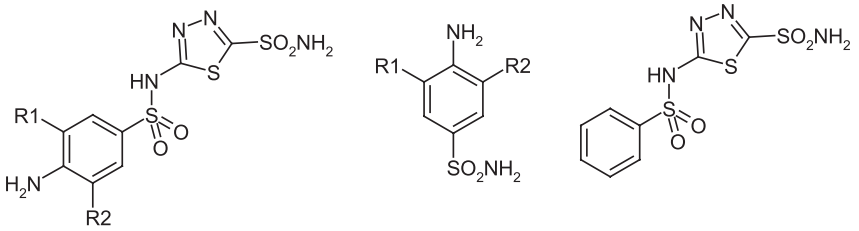
The following structure-activity relationship (SAR) can be observed from data of Table 1:

(i) Against mtCA 1 the sulfonamides **1–10** behaved as efficient inhibitors, with inhibition constants in the range of 0.12–1.27 μM, comparable (but slightly better in many cases) to that of the lead molecule sulfonamide, **BZA** which has a K_I of 0.81 μM (Table 1). A quite straightforward SAR may be observed for the inhibition of this enzyme with the sulfonamides **1–10** investigated here. Thus, the best mtCA 1 inhibitors were the halogenated benzolamides **1–3**, for which efficiency increased with the atomic weight of the halogen atom present in the molecule. Indeed, the iodo-fluoro-substituted compound **3** was the best inhibitor detected here (a 6.75-fold increase of activity compared to **BZA**), followed by the bromo-fluoro-substituted benzolamide **2** and the monochloro-derivative **1**. For the halogenated sulfanilamides **4–10**, activity was also good, with the best inhibitors being the bromo-chloro-derivative **8** and the dibromosulfanilamide **10** (K_Is of 0.41–0.58 μM). It may be observed that the nature of the halogen atoms substituting the sulfanilamide ring or the benzenesulfonamide fragment of the molecule (in the **BZA** derivatives), is the main factor influencing activity.

(ii) mtCA 2 was also inhibited efficiently by sulfonamides **1–10**, with inhibition constants in the range of 0.41–4.74 μM. As **BZA**, its halogenated derivatives **1–3** were the best mtCA 2 inhibitors, with K_Is in the range of 0.41–0.46 μM. Thus, for this enzyme, the presence of the halogeno substituents in the **BZA** scaffold does not significantly increase potency as mtCA 2 inhibitors (whereas, as discussed above, for mtCA 1 a significant such increase has been observed). The halogenated sulfanilamides **4–10** were roughly an order of magnitude less inhibitory compared to the benzolamides **1–3** and **BZA**, with K_Is in the range of 4.14–4.74 μM. Thus, a very flat SAR is observed in this case.

(iii) The third bacterial CA investigated here, mtCA 3, was also sensitive to inhibition with sulfonamides **1–10**, these derivatives show K_Is in the

Table 1. Inhibition data of mycobacterial β-CA isoforms mtCA 1–3 with sulfonamides **1–10** and benzolamide **BZA** by a stopped-flow, CO_2 hydrase assay[25].



1-3 **4-10** **BZA**

			K_I (μM)*		
Compound	R1	R2	mtCA 1	mtCA 2	mtCA 3
1	H	Cl	0.29	0.45	0.17
2	F	Br	0.14	0.43	0.27
3	F	I	0.12	0.41	0.3
4	F	Cl	1.17	4.56	3.02
5	F	Br	0.94	4.31	3.19
6	F	I	1.22	4.49	2.79
7	Cl	Cl	1.26	4.14	2.66
8	Cl	Br	0.58	4.62	2.69
9	Cl	I	1.27	4.74	2.67
10	Br	Br	0.41	4.52	2.03
–	BZA (benzolamide)		0.81	0.46	0.34

*Errors in the range of ± 10 % of the reported values, by a CO_2 hydrase assay method (from three different measurements).

range of 0.17–3.19 μM. The lead molecule **BZA** was also an effective mtCA 3 inhibitor, with K_I of 0.34 μM (Table 1). Again the best inhibitors detected in this study were the benzolamides. Substitution of one or two hydrogen atoms from the lead **BZA** with halogens led to a slight increase (2-fold) of the inhibitory power, the most effective compound being the monochloro-derivative **1** (K_I of 0.17 μM). The presence of additional halogens or the increase of their atomic weight, as in **2** and **3**, leads to a slight decrease of potency but the compounds remain active, similar to the lead **BZA**. The halogenated sulfanilamides were again around one order of magnitude less inhibitory compared to the benzolamides, with K_Is in the range of 2.03–3.19 μM (Table 1). The best substitution pattern seems to be the one with two bromine atoms (compound 10) which with a K_I of 2.03 μM is the most effective in this series.

(iv) mtCA 1 was the most prone to be inhibited by sulfonamides **1–10**, followed by mtCA 3 whereas mtCA 2 was the least inhibited isoform. This is quite different from the inhibition profile of the lead **BZA**. Indeed, for this compound, the best inhibition was seen against mtCA 3, followed by mtCA 2 and the least inhibited isoform was mtCA 1.

One of the main problems with mtCA inhibitors investigated so far is that no inhibition of the bacterial growth *in vivo* has been observed to date (unpublished results from this laboratory and ref. 21), probably due to the very hydrophilic character of sulfonamides and their difficulty to cross the thick mycobacteria cell wall[21]. The rationale of this study was just to increase the lipophilicity of the sulfonamide inhibitors which may allow a better penetrability profile to such compounds. Further studies are thus warranted to obtain highly effective and lipophilic mtCA inhibitors and prove their efficacy *in vivo*.

Conclusion

We evaluated a series of sulfonamide as inhibitors of three β-CAs from the bacterial pathogen *M. tuberculosis*, mtCA 1 (Rv1284), mtCA 2(Rv3588c) and mtCA 3 (Rv3273). These enzymes were inhibited with efficacies between the submicromolar to the micromolar one, depending on the substitution pattern at the sulfanilamide moiety/fragment of the molecule. Best inhibitors were the halogenated benzolamides (K_Is in the range of 0.12–0.45 μM) whereas the halogenated sulfanilamides were slightly less inhibitory (K_Is in the range of 0.41–4.74 μM) against all isoforms. This class of β-CA inhibitors may have the potential for developing antimycobacterial agents with a diverse mechanism of action compared to the clinically used drugs for which many strains exhibit multi-drug/extensive multi-drug resistance.

Declaration of interest

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