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ORIGINAL ARTICLE

Sulfonamides incorporating fluorine and 1,3,5-triazine moieties are effective inhibitors of three β-class carbonic anhydrases from *Mycobacterium tuberculosis*

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Abstract

A new series of fluorine containing 1,3,5-triazinyl sulfonamide derivatives obtained from cyanuric fluoride, sulfanilamide/4-aminoethylbenzenesulfonamide followed and incorporating also amin0, amino alcohol and amino acid moieties have been investigated as inhibitors of three β-carbonic anhydrases (CAs, EC 4.2.1.1) from the bacterial pathogen *Mycobacterium tuberculosis*, mtCA1 (Rv1284), mtCA 2 (Rv3588c) and mtCA 3 (Rv3273). All three enzymes were efficiently inhibited by these sulfonamides with K_I values in the nanomolar or submicromolar range, depending on the substitution of one or both fluorine atoms at the 1,3,5-triazine ring. As some of these enzymes are crucial for the life cycle of this bacterium, the class of β-CA inhibitors reported in this study may lead to antimycobacterial agents with a different mechanism of action compared to the clinically used such drugs for which the pathogen developed extensive drug resistance.

Keywords

β-Carbonic anhydrases, 1,3,5-triazine, carbonic anhydrase, fluoride, *Mycobacterium tuberculosis*, sulfonamide

History

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Introduction

The genome of the human pathogen *Mycobacterium tuberculosis* contains at least three carbonic anhydrases (CAs, EC 4.21.1) belonging to the β-class, which have been cloned, purified and characterized in the past. These enzymes, so called mtCA 1, 2 and 3 and encoded, respectively, by the genes *Rv1284*, *Rv3588c* and *Rv3273*[1–6]. Indeed, the CA superfamily of enzymes comprises the α-, β-, γ-, δ- and ζ-CA classes, all of which are generally efficient catalysts for the reversible interconversion between carbon dioxide and bicarbonate ($CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$)[7].

Their catalytic activity and inhibition profiles with many sulfonamides (the main class of CA inhibitors; CAIs) have recently been investigated[3–6]. The three mycobacterial CAs were thus shown to be active CAs, with appreciable catalytic activity for the physiological, and to be efficiently inhibited by sulfonamides and related compounds[7–11], with K_I in the range of low nanomolar or subnanomolar[3–6].

Due to an increase of anti-mycobacterial drugs resistance, which continuously reduces the susceptibility of mycobacteria to the clinically used agents, the inhibition of the β-CAs from this pathogen may represent a new approach for controlling the infections, through a new mechanism of action, as some of these enzymes were shown to be essential for the life cycle of the pathogen[12–16]. Indeed, many strains of Gram-negative/-positive bacteria (such as among others *Staphylococcus aureus*, *M. tuberculosis*, *Helicobacter pylori*, *Brucella suis*, *Streptococcus pneumoniae*, etc.) no longer respond to various classes of antibiotics[12–16]. Many of these new proteins have been recently explored, revealing CAs very interesting drug targets[11,17]. Indeed, it has been reported that sulfonamide derivates are potent inhibitors of bacterial CAs from *H. pylori*, *B. suis*, *S. pneumoniae* and several other similar pathogens, by inhibiting the growth of these bacteria through a mechanism of action not entirely investigated at this moment[11,17–21].

The primary sulfonamides, RSO_2NH_2, are considered the classical CAIs. They have been widely clinically used for more than 50 years as diuretic or systemically acting antiglaucoma drugs[7,8,22,23]. However, all these drugs target all mammalian CAs (16 such isoforms are known to date in vertebrates), and as thus, they show undesired side effects[7,8,22]. Only recently, a large of number of such derivates started to be investigated as anti-infectives, which target bacteria/fungal CAs (generally belonging to β-CA class), but this new field significantly collided with resistance from the scientific community in accepting these enzymes as anti-infective targets[7,11].

However, CAs belonging to the β-CA class are present in pathogenic organism such as fungi and bacteria and they lack from mammals, in which only the α-CAs (as 16 different isoforms, as mentioned above) are ubiquitous[7]. Therefore, these enzymes started to be seriously considered as possible drug targets for obtaining antibacterial agents devoid of the resistance problems mentioned above, which affect most classes of antibiotics in clinical use[12–15].

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The 1,3,5-triazine scaffold containing chloride atoms has been used in previous studies from our group[24] to design and synthesised potent sulfonamides CAIs. In this study, we intend to investigate the inhibition profile of 1,3,5-triazine sulphonamide derivatives in which both chlorine atoms have been replaced with fluoride atoms against three β-CAs (mtCA 1–3). We report hereby the inhibition studies against mtCA 1–3 from the bacterial pathogen *M. tuberculosis*, with a new series of fluorine containing 1,3,5-triazinyl sulfonamides **1**–**7**.

Materials and methods

Chemistry

Compounds **1**–**7** investigated in this study were reported in a previous study from our group[25].

Enzymology

mtCA1–mtCA 3 were recombinant enzymes obtained in-house as described earlier[1–6,26].

CA catalytic and inhibition assay

An Applied Photophysics (Oxford, UK) stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity[28]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 8.4) and 20 mM $NaBF_4$ (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 enzyme-inhibitor (E-I) complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier[3–6,26,29,30], and represent the mean from at least three different determinations.

Results and discussion

Chemistry

Reported here are novel β-CAI sulfonamide derivates obtained considering chloride containing 1,3,5-triazine scaffold previously studied from our group[24] in which the chlorine atoms were substituted by fluorine. The compounds were obtained by reaction of cyanuric fluoride with sulfanilamide and 4-(2-aminoethyl)-benzenesulfonamide affording, the key intermediates **1** and **2**, which were further enacted with one or two equivalents of nitrogen nucleophiles, such as aminoalcohols, α-amino acids and their esters, as well as methylamine (Scheme 1). The compounds were reported and characterized recently[25].

The inhibition studies against all mammalian CA isoforms of this new series of triazinyl-substituted benzenesulfonamides containing fluoride (**1**–**7**) have been recently investigated by our group[25]. In this study, we extend our earlier inhibition investigations of these triazinyl-containing sulfonamides against the three β-CAs from *M. tuberculosis*, mtCA1 (Rv1284), mtCA 2 (Rv3588c) and mtCA 3 (Rv3273).

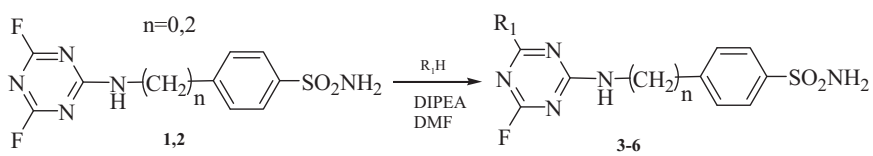
CA inhibition

The inhibition of three β-CAs from the bacterial pathogen *M. tuberculosis*, i.e. mtCA 1 (Rv1284), mtCA 2 (Rv3588c) and mtCA 3 (Rv3273) has been investigated with derivatives **1**–**7** reported here, and their inhibition potency compared to those of some sulfonamide inhibitors of mycobacterial CAs such as acetazolamide (**AAZ**), benzolamide (**BZA**) (an orphan drug[7]) and dichlorophenamide, reported earlier by our group[3–6,26].

Data for the inhibition of two of the dominant human CA isoforms (hCA I and II) with these compounds are also included in Table 1[25] for comparison reasons.

The following structure–activity relationship (SAR) can be observed from data of Table 1 for the inhibition of the β-CAs investigated here with the new group of triazinyl sulfonamides **1**–**7**:

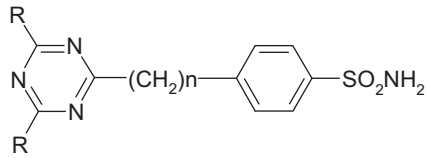
(i) All of the triazinyl-substituted benzenesulfonamides **1**–**7** behaved as very efficient inhibitors against mtCA I, with inhibition constants in the range of 0.042–0.57 μM, similar or slightly better to those of the best sulfonamide β-CAIs such as **AAZ** and **BZA**, which have, respectively, K_I values of 0.481 and 0.81 μM (Table 1). The best mtCA 1 inhibitor was the difluorotriazine derivative of 4-aminoethyl benzenesulfonamide **2** (K_I of 42 nM), which was also a very strong inhibitor of hCA II with K_I of 7.1 nM and a moderate inhibitor of the cytosolic isoform hCA I. The difluoro derivative of sulfanilamide (**1**) was 13.1-fold less effective as an mtCA 1 inhibitor compared to its longer congener (**2**). The ethanolamine derivative of the sulfanilamide (**3**) was 1.6 fold more effective mtCA 1 inhibitor compared to its difluoro derivative (**1**), whereas all the remaining compounds were quite highly effective mtCA 1 inhibitors, with similar inhibition constants in a narrow range of 0.46–0.61 μM. Therefore, SAR is almost impossible to delineate



Nº	*n*	R_1	R_2
1	0	F	F
2	2	F	F
3	0	$NH(CH_2)_2OH$	F
4	0	$NHCH_3$	F
5	2	$NHCH_2COOMe$	F
6	2	$NHCH_2COOH$	F
7	0	$NHCH_3$	$NHCH_3$

Scheme 1. Preparation of sulfonamides **3–7** by reaction of the difluoro-triazinyl-benzene sulfonamides **1**, **2** with nucleophiles (R_1H, R_2H) in presence of diisopropylethylamine (DIPEA), in DMF.

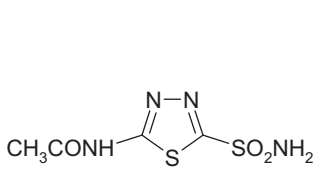
Table 1. Inhibition data of mycobacterial β-CA isoforms mtCA 1–3 with sulfonamides 1–7 reported here, and the standard sulfonamide inhibitors acetazolamide (AAZ), benzolamide (BZA) and dichlorophenamide (DCP) by a stopped flow CO_2 hydrase assay[27].



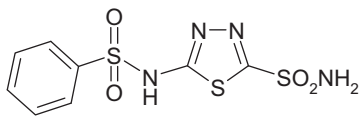
1-7

				K_I[a]				
No	*n*	R_1	R_2	hCA I (nM)	hCA II (nM)	mtCA 1 (μM)	mtCA 2 (μM)	mtCA 3 (μM)
1	0	F	F	88	4.9	0.55	>0.01	0.15
2	2	F	F	203	7.1	0.042	0.0086	0.0021
3	0	$NH(CH_2)_2OH$	F	238	5.5	0.35	>0.01	0.21
4	0	$NHCH_3$	F	90	5.4	0.58	0.0096	0.022
5	2	$NHCH_2COOMe$	F	81.5	5.6	0.46	>0.01	0.13
6	2	$NHCH_2COOH$	F	250	5.0	0.61	0.0081	>1
7	0	$NHCH_3$	$NHCH_3$	83[b]	23[b]	0.57	>0.01	0.21
AAZ	–	–	–	250	12	0.481	0.009	0.104
BZA				15	9	0.81	0.46	0.34
DCP				1200	38	0.87	2.01	0.611

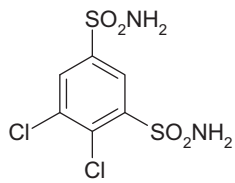
[a]Mean from three different assays, by a stopped flow technique (errors were in the range of ±5–10% of the reported values).
[b]From work by Suarez Covarrubias et al.[1]



AAZ



BZA



DCP

as all substitution patterns lead to highly effective inhibitors of this bacterial CA, mtCA 1.

(ii) Against the β-CA mtCA 2, Table 1 shows that most of these triazine-substituted compounds were weak inhibitors ($K_I > 0.01$ μM). Only derivates **2**, **4** and **6** behaved as very strong mtCA 2 inhibitors with K_Is in the range of 8.1–9.6 nM, very similar compared to that of **AAZ** with K_I of 9 nM. However, they revealed to be better mtCA 2 inhibitors with the K_I values in nanomolar range compared to **BZA** with K_I value only in submicromolar range. Although the active compounds inhibited efficiently mtCA 2, no significant change of K_I values has been observed. Therefore, a very flat SAR was noticed in this case.

(iii) The third bacterial CA investigated here, mtCA 3, was also efficiently inhibited by almost all derivates of this series of triazinyl-substituted benzenesulfonamides **1**–**7**. Indeed, these derivates showed K_Is in the range of 2.1 nM–0.21 μM. The most efficient mtCA 3 inhibitor was the difluorotriazine derivative of 4-aminoethylbenzenesulfonamide **2** (K_I of 2.1 nM), which was also a strong inhibitor of mtCA 2, revealing to be 71.4-fold more potent than its shorter congener **1** (difluoro derivative of sulfanilamide) with K_I of 0.15 μM. Substitution of one fluorine atom of the difluoro derivate of sulfanilamide **1** with a small moiety such as a methyl amine (**4**) led to a significant increase (16.8-fold) of the inhibitory potency, whereas substitution of one F atom with more bulky moiety such as ethanolamine (**3**) or of both F atoms (**7**) does not significantly change the inhibitory potency as mtCA 3 inhibitor (both with K_Is of 0.21 μM).

The mono-glycine methyl ester derivative of 4-aminoethylbenzenesulfonamide (**5**) (K_I of 0.13 μM) was roughly an order of magnitude less inhibitory compared to its difluoro 4-aminoethylbenzenesulfonamide derivative **2** (K_I of 2.1 nM). It is also interesting to notice as a small change of the side chain of this mono-glycine methyl ester derivative from an ester to a carboxylic group allows the glycine derivative of 4-aminoethylbenzenesulfonamide (**6**) to be the least effective mtCA 3 inhibitor of this series of compounds ($K_I > 1$ μM).

(iv) An overview of the current inhibition studies shows that among all the three bacterial CAs, mtCA 2 was the most prone to be inhibited by most of these triazinyl-substituted benzenesulfonamides **1**–**7** with K_I in the nanomolar range, followed by mtCA 3, whereas mtCA 1 was the least inhibited bacterial isoform. This inhibition profile is quite similar compared to that of the clinically used drug **AAZ**. However, it appeared slightly different from the inhibition profile of **BZA**. For this compound, in fact, the best inhibition has been shown against mtCA 3, followed by mtCA 2 and the least inhibited isoform was mtCA1 like in the series here investigated.

(v) Although the new series of compounds here investigated revealed to be potent bacterial β-CAIs showing a potential for developing new anti-infective drug, the dominant human carbonic anhydrase isoforms (hCA I and II) were also inhibited by compounds **1**–**7**. Indeed, the slow cytosolic

isoform hCA I was moderately inhibited by these compounds with inhibition constants in the range of 81.5–250 nM. On the other hand, against the physiologically dominant isoform hCA II, derivatives **1**–**7** behaved as very strong inhibitors with K_Is in the range of 4.9–23 nM.

Therefore, one of the main problems with these mtCA inhibitors investigated so far is that no selectivity for only bacterial CAs was observed since the hCA I and II were also inhibited.

Conclusions

A series of triazinyl-substituted benzenesulfonamides incorporating fluorine and sulfanilamide or 4-aminoethylbenzenesulfonamide scaffolds has been investigated for the inhibition of the three β-CAs from the bacterial pathogen *M. tuberculosis*, mtCA1 (Rv1284), mtCA 2 (Rv3588c) and mtCA 3 (Rv3273). Some compounds were very efficient inhibitors of mtCA 2 with K_I of nanomolar range, also showing excellent inhibition of mtCA 3 and rather efficient inhibitory potency against mtCA 1.

Some of these β-CAIs may have the potential for developing antimycobacterial agents with a different mechanism of action compared to the clinically used drugs for which many strains exhibit multi-drug resistance.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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