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RESEARCH PAPER

Sulphonamide inhibition studies of the β-carbonic anhydrase from the bacterial pathogen *Clostridium perfringens*

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ABSTRACT

The β-carbonic anhydrases (CAs, EC 4.2.1.1) from the pathogenic bacterium *Clostridium perfringens* (CpeCA) was recently characterised kinetically and for its anion inhibition profile. In the search of effective CpeCA inhibitors, possibly useful to inhibit the growth/pathogenicity of this bacterium, we report here an inhibition study of this enzyme with a panel of aromatic, heterocyclic and sugar sulphonamides/sulphamates. Some sulphonamides, such as acetazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, sulthiame and 4-(2-hydroxymethyl-4-nitrophenyl-sulphonamido)ethylbenzenesulphonamide were effective CpeCA inhibitors, with K_Is in the range of 37.4–71.6 nM. Zonisamide and saccharin were the least effective such inhibitors, whereas many other aromatic and heterocyclic sulphonamides were moderate – weak inhibitors with K_Is ranging between 113 and 8755 nM. Thus, this study provides the basis for developing better clostridial enzyme inhibitors with potential as antiinfectives with a new mechanism of action.

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1. Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes present in all three of life's phylogenetic domains (*Bacteria*, *Archaea* and *Eukarya*) and various isoforms present in most organisms investigated so far[1–12]. By converting metabolism-generated CO_2 to soluble products, bicarbonate and protons, these enzymes are crucial in a multitude of physiologic processes connected among others with pH homoeostasis, biosynthetic reactions in which CO_2/bicarbonate are involved, electrolyte secretion, photosynthesis, etc.[1–12]. The α-class CAs present in vertebrates, including humans[1–6], are drug targets for obtaining antiglaucoma agents[13,14], anticonvulsants[15], drugs for the treatment of idiopathic intracranial hypertension and other neurologic disorders[15,16], antiobesity agents[17] and diuretics[18–20]. Most of these clinically used CA inhibitors (CAIs) belong to the sulphonamide class, as they possess the primary sulphonamide (or its isosteres, sulphamate and sulphamide moieties) as the zinc-binding function[1–6,21]. Indeed, these compounds bind (in deprotonated, anionic form) to the CA active-site zinc ion, which is crucial for the catalytic activity[21]. Representatives of this class of pharmacological agents have multiple therapeutic applications for decades, although many of these first-/second-generation agents do show side effects connected with the inhibition of off-target isoforms, due to the fact that in humans there are 15 CAs which do not differ significantly in their active site architecture[3–5,22] and most of them show high affinity for this class of CAI[1–6]. However, in the last decade, a large number of different classes of CAIs and diverse inhibition mechanisms were reported, with a range of new chemotypes such as the coumarins[23–26], sulphocoumarins[27,28], polyamines[29], dithiocarbamates[30,31] and carboxylates[32,33] among others. Many of these novel types of CAIs show significant isoform-selective inhibition profiles, making this class of drugs much more attractive as candidates for the development of new generation pharmacological agents[4,23–33].

Bacteria encode CAs belonging to three classes, the α-, β- and γ-CAs[7,34–40]. These enzymes seem to be involved in crucial metabolisms, which probably explains both their wide distribution in Gram-negative and Gram-positive bacteria, as well as their generally very effective catalytic properties for the hydration of CO_2 to bicarbonate and protons[34–42]. Thus, ultimately, inhibition of bacterial CAs has been proposed as an alternative approach for obtaining antibiotics with an alternative mechanism of action compared to the classical drugs that interfere with bacterial cell wall biosynthesis, DNA-gyrase or similar such targets, which led to an extensive drug resistance phenomenon[7,12,34,36].

In previous work from our groups, we have reported the cloning and characterisation of a new β-CA from the bacterial pathogen *Clostridium perfringens*, CpeCA [41] that has also been investigated for its interaction with anions and other small molecules known to interact with metalloenzymes such as CA[42]. We previously observed that most anions are millimolar CpeCA inhibitors, whereas sulphamate, sulphamide, phenylboronic acid and phenylarsonic acid are the most effective inhibitors, with K_Is in the range of 7–75 μM. Thus, no highly effective CpeCA inhibitors were detected so far and this is the reason why we investigated the interaction of this enzyme with sulphonamides and sulphamates, the class of CAIs which usually leads to effective antimicrobial agents.

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2. Materials and methods

2.1. Chemistry

Compounds **1**–**24** and **AAZ**-**HCT** were commercially, highest purity available derivatives from Sigma-Aldrich (Milan, Italy) and were used without further purification or were prepared as reported earlier by our group[43–51].

2.2. Carbonic anhydrase assay

An applied photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity [52]. 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 TRIS (pH 8.3) as buffer, and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalysed 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 uncatalysed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionised water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were pre-incubated together for 15 min at room temperature prior to assay, in order to allow for

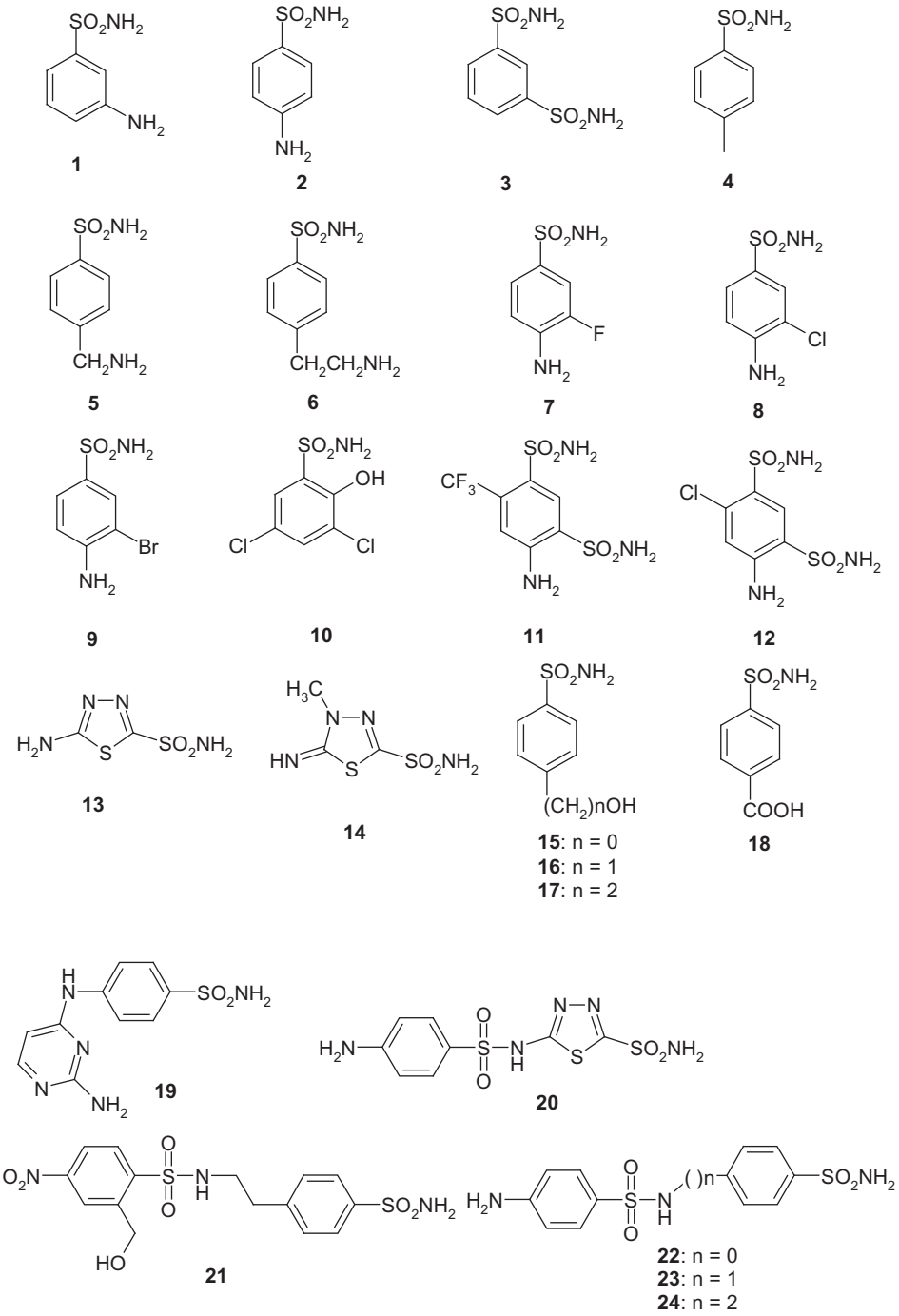

Figure 1. Structure of sulphonamides investigated as CAIs in this work.

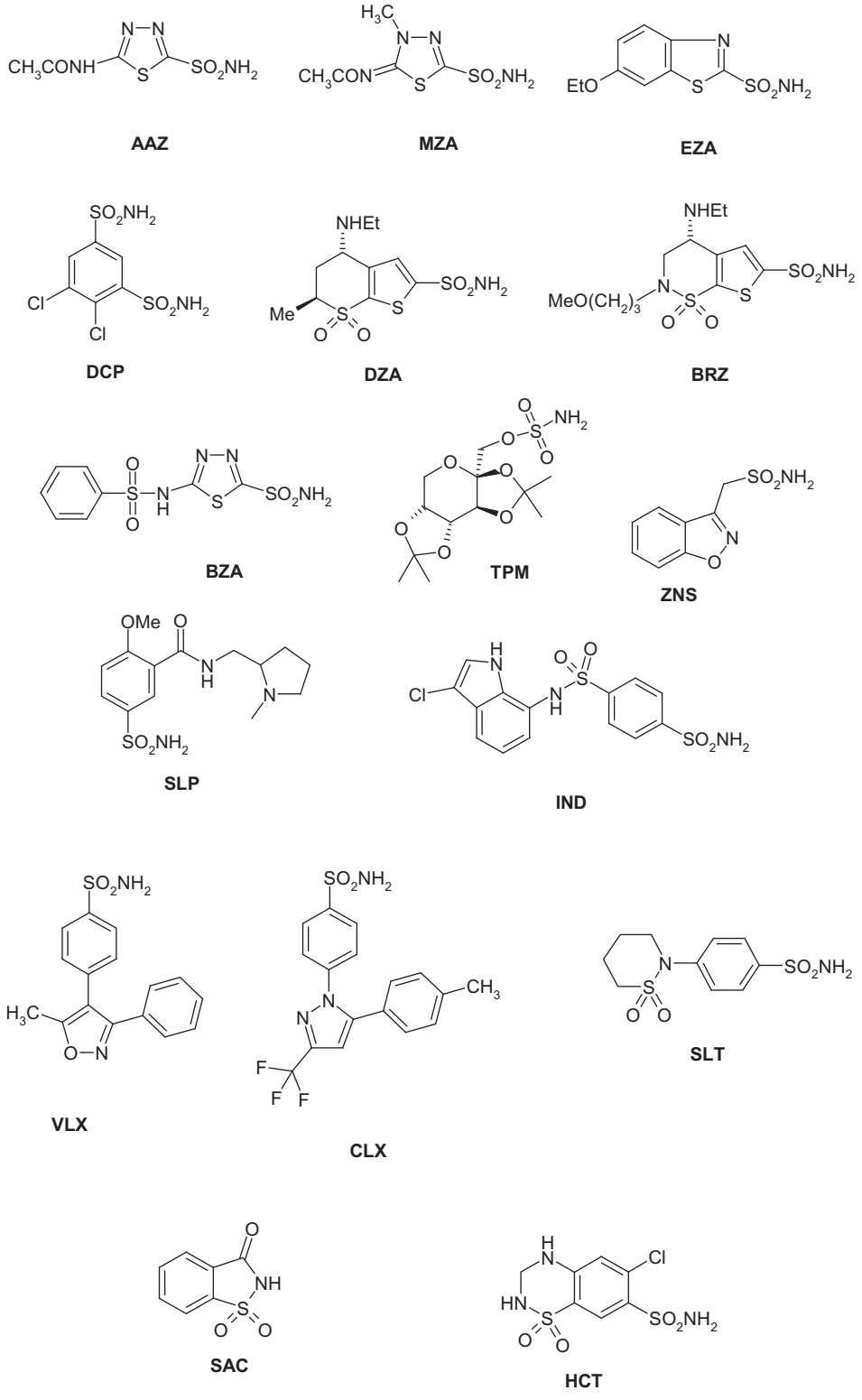

Figure 1. Continued.

the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng–Prusoff equation, as reported earlier[23–27] and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in-house as reported earlier[41,42].

3. Results and discussion

We investigated the inhibition of CpeCA with a panel of sulphonamides of type **1–24**, which include both aromatic and heterocyclic derivatives, employed extensively for the design of various classes of CAIs with interesting physicochemical properties[43–51] (Figure 1).

The clinically used agents acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA**, topiramate **TPM**, zonisamide **ZNS**, sulpiride **SLP**, indisulam **IND**, valdecoxib and **VLX**, celecoxib **CLX**, sulthiame **SLT**, saccharin **SAC** and hydrochlorothiazide **HCT** [9] were also included in this study as they incorporate the sulphonamide/sulphamate zinc-binding function and act as potent CAIs against many β-CAs investigated earlier [7]. The inhibition observed with these derivatives against CpeCA and the human (h) off-target α-class enzymes hCA I and II, are shown in Table 1.

The following structure–activity relationship (SAR) can be drawn from the data of Table 1 regarding CpeCA inhibition with these compounds

i. The least effective CpeCA inhibitors were zonisamide and saccharin, which did not affect the enzyme activity up to 100 μM (Table 1). **ZNS** is in fact the only aliphatic sulphonamide, whereas **SAC** the only secondary, acylated sulphonamide among the investigated compounds.
ii. Moderate–weak inhibitory action, in the micromolar range, was observed for the following sulphonamides: **5, 6, 10–12** and **18**, which had K_Is in the range of 1.268–8.755 μM. These compounds belong to the aminoalkyl–benzenesulphonamide (**5** and **6**) and tetrasubstituted benzenesulphonamide/disulphonamide (**10**–**12**) series. Probably, the large number of substituents on the phenyl ring for the last type of derivatives is detrimental to their efficient binding to the enzyme.
iii. More effective but moderate CpeCA inhibitors were the following compounds: **1**–**4, 7**–**9, 13, 14, 17, 19, 21**–**23, BZA, TPM, SLP**–**CLX** and **HCT**, which had K_Is in the range of 160–713 nM. It is obvious that these derivatives belong to a variety of different classes, with both aromatic, heterocyclic and sugar derivatives among them. Thus, a real SAR is difficult to draw, but it is important to note that many structural variations in the scaffold of aromatic/heterocyclic sulphonamides are tolerated without a significant loss of the CpeCA inhibitory action.
iv. The most effective CpeCA inhibitors were **15, 16, 20, 24, AAZ, MZA, EZA, DCP, DZA, BRZ** and **SLT**, which showed K_Is in the range of 37.4–145 nM (Table 1). Again many different chemotypes led to quite effective CAIs, among which the most notable are dorzolamide, a rather bulky bicyclic sulphonamide (the best inhibitor with a K_I of 37.4 nM), acetazolamide (the second best inhibitor with a K_I of 49.1 nM) as well as the aromatic compound 4–(2-hydroxymethyl-4-nitrophenyl-sulphonamido)ethylbenzenesulphonamide **24**, with a K_I of 51.2 nM (Table 1). All of them are highly different structurally, which is of extreme importance for the possible design of even better CpeCA inhibitors belonging to the sulphonamide class.
v. The off-target isoforms hCA I and II have a very different inhibition profile with the compounds investigated here (Table 1), whereas hCA I has generally a lower affinity for most of these inhibitors, hCA II is highly inhibited by most of them, usually in the low nanomolar range, which makes it quite difficult to obtain CpeCA-selective inhibitors form this class of agents.

4. Conclusions

Species belonging to the genus Clostridium, such as *Clostridium tetani*, *C. botulinum*, *C. barati*, *C. butirycum*, *C. hystolyticum* and *C. perfringens* among others, are strictly anaerobic pathogens that provoke serious human disease, such as tetanus, botulism, gas gangrene, bacterial corneal keratitis and other infections[53,54]. Although some progress has been achieved ultimately for designing pharmacological agents effective against these diseases, such as for example protease inhibitors targeting various metalloproteases essential for the life cycle of these pathogens, there is a constant search for novel drug targets that may lead to new classes of such agents, considering the serious antibiotic drug resistance problems emerging worldwide with the clinically used drugs[53,54]. In the search of effective compounds interfering with the metabolism of these pathogens, in this paper, we investigated potential CpeCA inhibitors, possibly useful to inhibit the growth/pathogenicity of this bacterium. A panel of aromatic, heterocyclic and sugar sulphonamides/sulphamates were employed for the inhibition of this bacterial β-class enzyme. Some sulphonamides, such as acetazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, sulthiame and 4-(2-hydroxymethyl-4-nitrophenyl-sulphonamido)ethylbenzenesulphonamide were effective CpeCA inhibitors,

Table 1. Inhibition of human isoforms hCA I and hCA II (off-target enzymes), as well as the bacterial enzyme from *C. perfringens* (CpeCA) with sulphonamides **1–24** and the clinically used drugs **AAZ–HCT**, by a stopped-flow, CO_2 hydrase assay [52].

	K_I* (nM)		
Inhibitor/ Enzyme class	hCA I[a] α	hCA II[a] α	CpeCA[b] β
1	28,000	300	451
2	25,000	240	402
3	79	8	210
4	78,500	320	379
5	25,000	170	3690
6	21,000	160	2430
7	8300	60	443
8	9800	110	320
9	6500	40	713
10	7300	54	4670
11	5800	63	8755
12	8400	75	7635
13	8600	60	297
14	9300	19	460
15	5500	80	131
16	9500	94	145
17	21,000	125	314
18	164	46	1268
19	109	33	179
20	6	2	105
21	69	11	697
22	164	46	513
23	109	33	312
24	95	30	51.2
AAZ	250	12	49.1
MZA	50	14	113
EZA	25	8	71.6
DCP	1200	38	68.0
DZA	50,000	9	37.4
BRZ	45,000	3	121
BZA	15	9	497
TPM	250	10	204
ZNS	56	35	>100,000
SLP	1200	40	389
IND	31	15	173
VLX	54,000	43	160
CLX	50,000	21	312
SLT	374	9	63.1
SAC	18,540	5959	>100,000
HCT	328	290	219

*Errors in the range of 5–10% of the shown data, from three different assays.
[a]Human recombinant isozymes, stopped flow CO_2 hydrase assay method, from reference [3–5].
[b]Recombinant bacterial enzyme, stopped flow CO_2 hydrase assay method, this work.

with K_Is in the range of 37.4–71.6 nM. Zonisamide and saccharin were the least effective inhibitors, whereas many other aromatic and heterocyclic sulphonamides were moderate–weak inhibitors with K_Is ranging between 113 and 8755 nM. This study thus provides the basis for developing better clostridial enzyme inhibitors with potential as antiinfectives with a new mechanism of action.

Disclosure statement

No potential conflict of interest was reported by the authors.

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