Bioorganic & Medicinal Chemistry Letters 26 (2016) 1821–1826

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

# Sulfonamide inhibition studies of the β-carbonic anhydrase from the newly discovered bacterium *Enterobacter* sp. *B13*

Ayşenur Eminoğlu [a], Daniela Vullo [b], Aycan Aşık [c], Dilşat Nigar Çolak [d], Sabriye Çanakçı [e], Ali Osman Beldüz [e,*], Claudiu T. Supuran [b,f,*]

[a] Recep Tayyip Erdogan University, Faculty of Art and Science, Department of Biology, Molecular Biology Research Laboratories, Rize, Turkey
[b] Università degli Studi di Firenze, Dipaertimento di Chimica, Via della Lastruccia 3, 50019 Sesto Fiorentino (Firenze), Italy
[c] Selcuk University, Faculty of Medicine, Medical Biology Department, Konya, Turkey
[d] Giresun University, Bulancak School of Applied Sciences, Giresun, Turkey
[e] Karadeniz Technical University, Faculty of Science, Department of Biology, Trabzon, Turkey
[f] Università degli Studi di Firenze, Dipartimento Neurofarba, Sezione di Scienze Farmaceutiche e Nutraceutiche, Via U. Schiff 6, 50019 Sesto Fiorentino, Florence, Italy

ARTICLE INFO

*Article history:*
Received 8 January 2016
Revised 10 February 2016
Accepted 12 February 2016
Available online 16 February 2016

*Keywords:*
Carbonic anhydrase
Metalloenzymes
Inhibitors
Sulfonamide
Pathogenic bacteria
*Enterobacter* spp. *B13*

ABSTRACT

The genome of the newly identified bacterium *Enterobacter* sp. *B13* encodes for a β-class carbonic anhydrases (CAs, EC 4.2.1.1), EspCA. This enzyme was recently cloned, and characterized kinetically by this group (*J. Enzyme Inhib. Med. Chem.* **2016**, *31*). Here we report an inhibition study with sulfonamides and sulfamates of this enzyme. The best EspCA inhibitors were some sulfanylated sulfonamides with elongated molecules, metanilamide, 4-aminoalkyl-benzenesulfonamides, acetazolamide, and deacetylated methazolamide ($K_I$s in the range of 58.7–96.5 nM). Clinically used agents such as methazolamide, ethoxzolamide, dorzolamide, brinzolamide, benzolamide, zonisamide, sulthiame, sulpiride, topiramate and valdecoxib were slightly less effective inhibitors ($K_I$s in the range of 103–138 nM). Saccharin, celecoxib, dichlorophenamide and many simple benzenesulfonamides were even less effective as EspCA inhibitors, with $K_I$s in the range of 384–938 nM. Identification of effective inhibitors of this bacterial enzyme may lead to pharmacological tools useful for understanding the physiological role(s) of the β-class CAs in bacterial pathogenicity/virulence.

© 2016 Elsevier Ltd. All rights reserved.

The upsurge of antimicrobial resistance to commonly used antibiotics is a phenomenon which reached dramatic levels all over the world.[1–3] The current antibiotics were developed decades ago and lost effectiveness due to resistance phenomena and their inappropriate use, whereas few new such drugs reached the market ultimately.[1–4] Especially Gram-negative pathogens, among which *Enterococcus faecium*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., and the Gram-positive *Staphylococcus aureus* (the so-called ESKAPE pathogens)[4] are poorly responsive to most clinically used antibiotics. Furthermore, some of these bacteria are able to produce biofilms which are non-responsive to therapy. All of them are common pathogens, also provoking nosocomial infections difficult to eradicate, and representing thus a major threat to the public health worldwide.[4]

Annotation of the genomes of many Gram-positive and Gram-negative pathogenic bacteria gives the opportunity to discover diverse pathways for inhibiting their vital functions and/or to block their virulence.[4] In recent years, some metabolically important enzymes became an interesting research topic for the discovery of alternative, druggable anti-infective targets. Among them, the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1)[5–7] revealed to be an interesting novel drug target in fighting infections provoked by pathogenic protozoa,[8] fungi[9] and some bacteria.[10] This is due to the essential role that CAs play in carbon dioxide hydration to bicarbonate and protons (the physiologic reaction that they catalyze), which is used by the pathogens in essential functions such as pH regulation, chemosensing, or biosynthetic reactions.[11,12]

The CAs are present in many pathogenic bacteria, suggesting a pivotal role in microbial virulence.[6,10] These enzymes were classified into several classes, which include the α-, β-, γ-, δ-, ζ- and η-CAs, of which only α-, β-, and γ-ones are present in bacteria. The metal ion from the enzyme active site is crucial in the catalytic process, being coordinated by three His residues in the α-, γ- and δ-classes, by one His, and two Cys residues in β- and ζ-CAs or by two His and one Gln residues in η-class with the fourth ligand being a water molecule/hydroxide ion acting as nucleophile in

* Corresponding authors. Tel./fax: +90 4623772522 (A.O.B.), +39 055 4573729 (C.T.S.).
*E-mail addresses:* belduz@ktu.edu.tr (A.O. Beldüz), claudiu.supuran@unifi.it (C.T. Supuran).

http://dx.doi.org/10.1016/j.bmcl.2016.02.032
0960-894X/© 2016 Elsevier Ltd. All rights reserved.

the enzyme catalytic cycle.[5–8] The distribution pattern of these enzymes in bacteria is very intriguing as some of them encode CAs belonging just to one class (either α- or β), others from two (α- and β-CAs) whereas there are bacteria which contain all three different genetic classes identified in these organisms so far.[6,10] Furthermore, there are several species which seem not to encode for CAs, such as the Gram-negative bacteria belonging the genera *Buchnera* and *Rickettsia*,[6e] but it is not improbable that they may contain CAs from a yet unidentified novel genetic family.

Investigations of CA in the bacteria domain may undoubtedly reveal novel aspects of microbial virulence. For example, bacteria lacking the bicarbonate transporter system, such as *Vibrio cholerae*, a Gram-negative bacterium responsible of the human disease cholera, can increase cytosolic bicarbonate levels through the action of CAs, which convert into bicarbonate the metabolic $CO_2$ and/or atmospheric $CO_2$ entered into the cell by simple diffusion.[10] Furthermore, the sulfonamide ethoxzolamide, a potent CA inhibitor (CAI, see later in the text), with a low nanomolar affinity for the CAs from this bacterium,[10f,13] inhibits the bicarbonate-mediated virulence, suggesting that conversion of $CO_2$ into bicarbonate by the *V. cholerae* CAs plays a role in virulence induction, and that inhibition of these enzymes may modulate infectivity.[10,13] In the gastric pathogen *Helicobacter pylori*, which encodes CAs belonging to the α- and β-classes investigated in some detail,[14] it has been demonstrated that inhibition of the two bacterial enzymes with sulfonamides such as acetazolamide, a low-nanomolar *H. pylori* CA inhibitor, is lethal for the pathogen,[14,15] which explains why sulfonamide CAIs (acetazolamide and ethoxzolamide) were clinically efficient as anti-ulcer drugs.[15b] Thus, the proposal of bacterial CA inhibitors as anti-infective agents,[5a,10] eventually in combination with other drug classes, is an idea supported by preclinical and clinical data,[10,14,15] and prompts us to investigate such proteins in various pathogenic species, as well as to screen various classes of CAIs[16–18] for their in vitro and in vivo effects against such bacteria.

Recently, our group cloned and purified a recombinant β-CA from a newly identified bacterium belonging to the genus *Enterobacter*, more precisely *Enterobacter* spp. *B13*, which appeared to be similar to *Enterobacter asburiae*.[19] The new enzyme, denominated from now on as EspCA, was shown to possess a good catalytic activity for the reaction that converts $CO_2$ to bicarbonate and protons, with a $k_{cat}$ of $4.8 \times 10^5$ $s^{-1}$ and a $k_{cat}/K_m$ of $5.6 \times 10^7$ $M^{-1} \times s^{-1}$, at pH 8.3 and 20 °C.[19] No inhibition studies were reported so far for this enzyme belonging to the pathogenic CAs from the ESKAPE group of bacteria mentioned above.[4,19] Identification of potent and possibly selective EspCA inhibitors may lead to pharmacological tools useful for understanding the physiological role(s) of these under-investigated enzymes in pathogenic bacteria. Here we report the first inhibition study of EspCA with a series of sulfonamides and one sulfamate, the main class of CAIs.

Although few pathogenic β-CAs were characterized so far by means of X-ray crystallography (among them are those from *Escherichia coli*,[20] *Mycobacterium tuberculosis*,[21] *Haemophilus influenzae*[22] and *V. cholerae*)[13] it appears that sulfonamides/sulfamates possess the same inhibition mechanism against these enzymes as for the α-CAs, that is, the inhibitor coordinates to the catalytically crucial metal ion, impairing catalysis by substituting the metal-bound nucleophile.[7] This is the reason why such classical CAIs were included in this study. A library of 40 compounds, comprising **39** sulfonamides and one sulfamate were assayed for EspCA inhibition.[23] Derivatives **1**–**24** and **AAZ**–**HCT** are either simple aromatic/heterocyclic sulfonamides widely used as building blocks for obtaining new families of such pharmacologic agents, or they are clinically used agents, among which acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA** and dichlorophenamide **DCP**, are the classical, systemically acting antiglaucoma CAIs.[24] Dorzolamide **DZA** and brinzolamide **BRZ** are topically-acting antiglaucoma agents,[5] benzolamide **BZA** is an orphan drug belonging to this class of pharmacological agents, whereas topiramate **TPM**, zonisamide **ZNS** and sulthiame **SLT** are antiepileptic drugs.[25] Sulpiride **SLP** and indisulam **IND** were also shown to belong to this class of pharmacological agents, together with the COX2 'selective' inhibitors celecoxib **CLX** and valdecoxib **VLX**.[5] Saccharin and the diuretic hydrochlorothiazide **HCT** are also known to act as CAIs.[5]

Data of Table 1 show the inhibition data of EspCA with the 40 derivatives mentioned above, as obtained by a stopped-flow $CO_2$ hydrase assay monitoring the physiologic reaction catalyzed by CAs.[23] Inhibition data of the human (h), possibly off-target isoforms hCA I and II, and of the β-CA (VchCA β) from another bacterial pathogen, *V. cholerae*, recently crystallized and investigated by us,[13] are also presented in Table 1, for comparison reasons (Chart 1).

The following structure–activity relationship (SAR) can be drawn from the inhibition data of Table 1:

(i) Weak EspCA inhibition was observed for three sulfonamides investigated here, **10**–**12** which were micromolar inhibitors: **10** and **11** with $K_I$s of 8.42–8.95 μM, and **12** with a $K_I$ of >10 μM. It should be noted that **11** and **12** are the only benzene-1,3-disulfonamide derivatives (together with **DCP**,

**Table 1**
Inhibition data of hCA I, hCA II, VchCAβ and EspCA with compounds **1**–**HCT**[23]

| Compound | hCA I | hCA II $K_I$ (nM)* | VchCAβ | EspCA |
|---|---|---|---|---|
| **1** | 45,400 | 295 | 463 | 73.9 |
| **2** | 25,000 | 240 | 447 | 583 |
| **3** | 28,000 | 300 | 785 | 812 |
| **4** | 78,500 | 320 | >10,000 | 384 |
| **5** | 25,000 | 170 | >10,000 | 93.6 |
| **6** | 21,000 | 160 | 463 | 90.2 |
| **7** | 8300 | 60 | >10,000 | 700 |
| **8** | 9800 | 110 | 9120 | 879 |
| **9** | 6500 | 40 | >10,000 | 87.8 |
| **10** | 6000 | 70 | >10,000 | 8420 |
| **11** | 5800 | 63 | 879 | 8950 |
| **12** | 8400 | 75 | 4450 | >10,000 |
| **13** | 8600 | 60 | 68.1 | 523 |
| **14** | 9300 | 19 | 82.3 | 83.7 |
| **15** | 6 | 2 | 349 | 720 |
| **16** | 164 | 46 | 304 | 88.1 |
| **17** | 185 | 50 | 3530 | 674 |
| **18** | 109 | 33 | 515 | 856 |
| **19** | 95 | 30 | 2218 | 775 |
| **20** | 690 | 12 | 859 | 453 |
| **21** | 55 | 80 | 4430 | 647 |
| **22** | 21,000 | 125 | 757 | 485 |
| **23** | 23,000 | 133 | 817 | 96.5 |
| **24** | 24,000 | 125 | 361 | 58.7 |
| **AAZ** | 250 | 12 | 4512 | 78.9 |
| **MZA** | 50 | 14 | 6260 | 137 |
| **EZA** | 25 | 8 | 6450 | 132 |
| **DCP** | 1200 | 38 | 2352 | 938 |
| **DZA** | 50,000 | 9 | 4728 | 103 |
| **BRZ** | 45,000 | 3 | 845 | 107 |
| **BZA** | 15 | 9 | 846 | 120 |
| **TPM** | 250 | 10 | 874 | 131 |
| **ZNS** | 56 | 35 | 8570 | 128 |
| **SLP** | 1200 | 40 | 6245 | 132 |
| **IND** | 31 | 15 | 7700 | 513 |
| **VLX** | 54,000 | 43 | 8200 | 133 |
| **CLX** | 50,000 | 21 | 4165 | 788 |
| **SLT** | 374 | 9 | 455 | 138 |
| **SAC** | 18,540 | 5959 | 275 | 801 |
| **HCT** | 328 | 290 | 87.0 | 133 |

* Mean from 3 different assay. Errors in the range of ±10% of the reported values (data not shown).

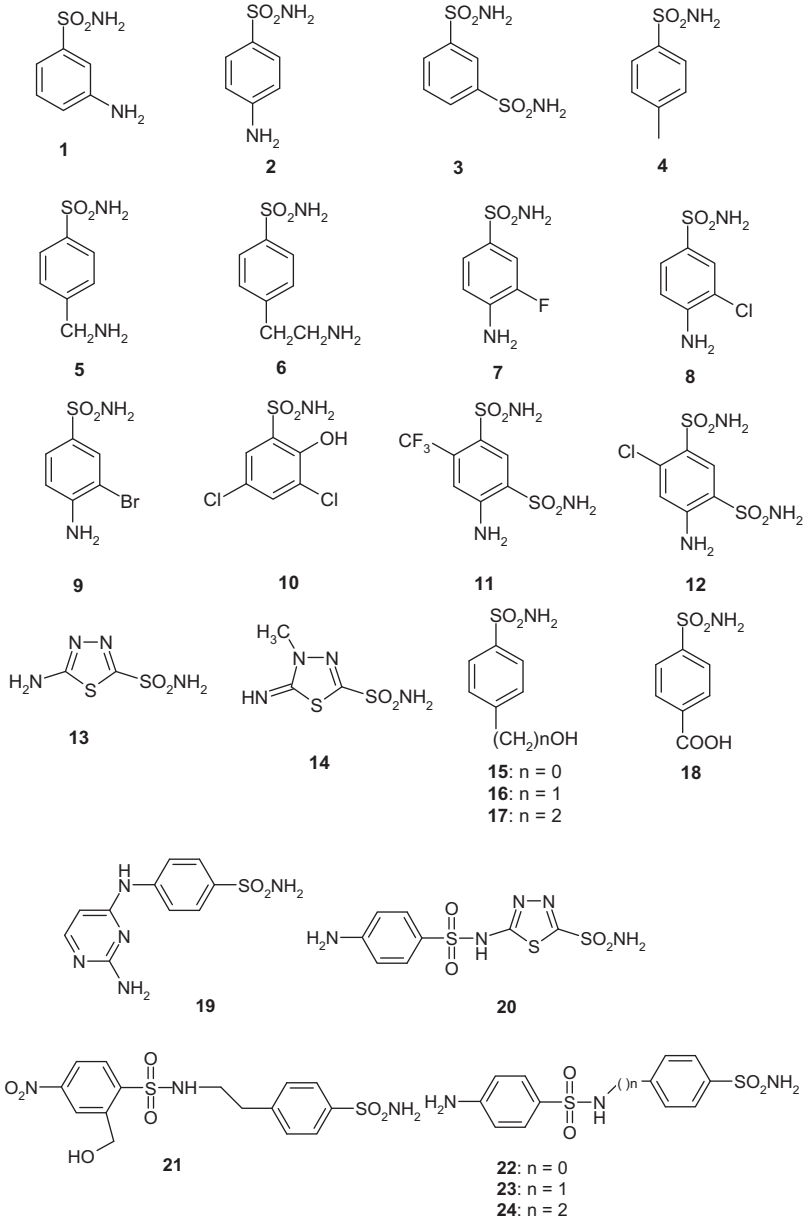


**Chart 1.** Structure of sulfonamides/sulfamates investigated in the Letter.

which is also not a quite effective inhibitor, with a $K_I$ of 938 nM) investigated here, whereas **10** is also one of the few tetra-substituted benzene derivatives in the series. This prompts us to conclude that bulky, poly-substituted compounds at the aromatic ring are associated with poor inhibitory activity against this enzyme (Table 1).

(ii) Many of the simple, aromatic sulfonamides with one or two different substituents at the benzene ring, such as **2**–**4**, **7**, **8**, **17**–**22**, the clinically used agents with a more complex scaffold based on the benzenesulfonamide motif **DCP**, **IND**, **CLX** and the heterocyclic sulfonamide **13** (deacetylated acetazolamide **AAZ**), were weak—medium potency EspCA inhibitors with $K_I$s in the range of 384–938 nM (Table 1). Again the SAR is rather obvious, as these compounds are mono- or disubstituted benzenesulfonamides possessing compact moieties in the *meta*- or *para*-positions with respect to the sulfonamide group (**2**–**4**, **17**, **18**) or they incorporate bulkier moieties in the 4-position, as in the case of **19**, **21** and **22**. Compound **20** is a benzolamide (**BZA**) derivative, and **BZA** itself is a rather effective inhibitor ($K_I$ of 120 nM). The additional 4-amino moiety present in **20** leads to a four-fold loss of activity compared to the parent compound. Another type of compounds in this category is represented by some halogenated sulfanilamides, such as **7** and **8**. However the replacement of the F or Cl atoms from **7** and **8** by a Br (in **9**) leads to a 10-time increase in the inhibitory power, with the bromo-derivative **9** being an effective EspCA inhibitor ($K_I$ of 87.8 nM). Thus minimal variations in the structure lead to dramatic changes in the enzyme inhibitory power of these sulfonamides.

(iii) Many of the clinically used sulfonamide/sulfamate drugs, such as **MZA**, **EZA**, **DZA**, **BRZ**, **TPM**, **ZNS**, **SLP**, **VLX**, **SLT** and **HCT** showed rather efficient EspCA inhibitory properties, with $K_I$s in the range of 103–138 nM (Table 1). Although these compounds possess quite diverse scaffolds (mono- or bicyclic aromatic/heterocyclic moieties; protected sugar

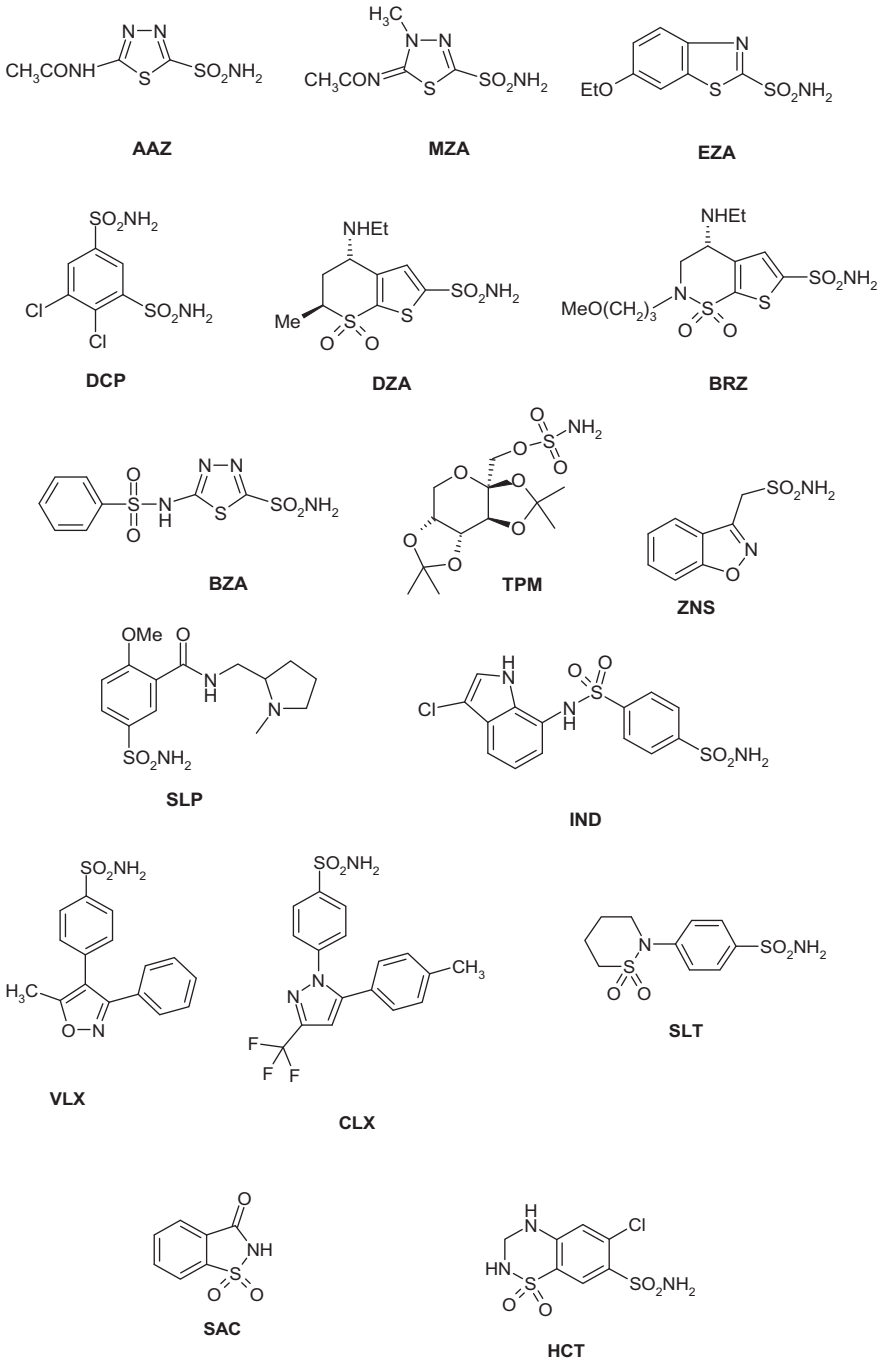


**Chart 1.** (*continued*)

moiety with the sulfamate zinc-binding group in **TPM**, aliphatic sulfonamide with a heterocyclic ring attached to it in **ZNS**, etc.), their inhibition profiles against this enzyme are quite similar, proving thus that a rather large number of diverse chemotypes belonging to the sulfonamide/sulfamate classes can effectively bind within the enzyme active site.

(iv) The most effective EspCA inhibitors were **1**, **5**, **6**, **9**, **14**, **16**, **23**, **24** and **AAZ**, which had $K_I$s in the range of 58.7–96.5 nM (Table 1). They include the simple benzenesulfonamides with *meta* (metanilamide **1**) or *para* (**5**, **6** and **15**) groups, of the amino, aminoalkyl or hydroxyalkyl type. It is interesting to note again that small structural changes (e.g., the position of the amino moiety in **1** and **2**) lead to drastic changes of activity. Metanilamide **1** is 7.96 times a better EspCA inhibitor compared to its isomer, sulfanilamide **2**. Comparing the aminoalkyl series (compounds **2**, **5**, **6**, considering the linker of 0 carbon atoms for **2**) with the hydroxyalkyl one (derivatives **15**–**17**), the differences of inhibitory profiles are again quite different. For the aminoalkyl series the inhibitory activity increased with the length of the linker between the sulfamoyl-phenyl ring and the amino moiety (from 0 to 2) whereas for the hydroxyalkyl series, the behavior was quite irregular, with **15** ($n = 0$) and **17** ($n = 2$) behaving as weak inhiibtors and **16** ($n = 1$) as a potent one ($K_I$ of 88.1 nM)—Table 1. Comparing the aminoethyl derivative **6**

with the corresponding hydroxyethyl one **17**, again the difference of inhibitory power is important, with **6** being 7.5-times a better inhibitor compared to **17**. Another effective chemotype as EspCA inhibitor is represented by the imino derivative **14** (de-acetylated methazolamide) which is a better inhibitor compared to **MZA** and especially compared to the structurally related de-acetylated **AAZ**, **13**, which is 6.2 times a weaker inhibitor compared to **14**. For the sulfanilyl-sulfonamides subseries (**22**–**24**) activity increased with the length of the linker from 0 to 2, with **22** behaving as a weak inhibitor, whereas **23** and **24** as effective ones. In fact **24** was the most effective EspCA inhibitors discovered so far.

(v) The inhibition profile of EspCA is quite different from that of the β-class enzyme from *V. cholerae*, VchCAβ or the human isoforms I and II (belonging to the α-class). This is an interesting observation as it prompts us to hypothesize that it might be possible to design EspCA-selective CAIs.

In conclusion, we investigated the inhibition with sulfonamides of the newly identified CA from the bacterium *Enterobacter* sp. *B13*, EspCA. The best EspCA inhibitors were some sulfanylated sulfonamides with elongated molecules, metanilamide, 4-amino-alkyl-benzenesulfonamides, acetazolamide, and deacetylated methazolamide ($K_I$s in the range of 58.7–96.5 nM), whereas the clinically used agents such as methazolamide, ethoxzolamide, dorzolamide, brinzolamide, benzolamide, zonisamide, sulthiame, sulpiride, topiramate and valdecoxib were slightly less effective ($K_I$s in the range of 103–138 nM). Saccharin, celecoxib, dichlorophenamide and many simple benzenesulfonamides were less effective as EspCA inhibitors, with $K_I$s in the range of 384–938 nM. Identification of effective inhibitors of this bacterial enzyme may lead to pharmacological tools useful for understanding the physiological role(s) of the β-class CAs in bacterial pathogenicity/virulence.

## Acknowledgment

This work was supported in part by an FP7 European Union Project (Gums & Joints, Grant agreement Number HEALTH-F2-2010-261460).

## References and notes


1. (a) Cooper, M. A.; Shlaes, D. *Nature* **2011**, *472*, 32; (b) Furtado, G. H.; Nicolau, D. P. *Expert Opin. Ther. Pat.* **2010**, *2010*, 1273; (c) Darriba, S.; Iglesias, D.; Ruiz, M.; Rodriguez, R.; Lopez, C. *J. Invertebr. Pathol.* **2010**, *104*, 23.
2. (a) Maurer, K.; Reyes-Robles, T.; Alonzo, F., 3rd; Durbin, J.; Torres, V. J.; Cadwell, K. *Cell Host Microbe* **2015**, *17*, 429; (b) Chung, S. H.; Rosa, C.; Scully, E. D.; Peiffer, M.; Tooker, J. F.; Hoover, K.; Luthe, D. S.; Felton, G. W. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 15728; (c) Mostowy, S.; Cossart, P. *Trends Cell Biol.* **2012**, *22*, 283.
3. (a) Sabharwal, V.; Figueira, M.; Pelton, S. I.; Pettigrew, M. M. *Microbes Infect.* **2012**, *14*, 712; (b) Wangdi, T.; Winter, S. E.; Baumler, A. J. *Gut Microbes* **2012**, *3*, 88; (c) Lanotte, P.; Watt, S.; Mereghetti, L.; Dartiguelongue, N.; Rastegar-Lari, A.; Goudeau, A.; Quentin, R. *J. Med. Microbiol.* **2004**, *53*, 73; (d) Marshall, B. J. *J. Gastroenterol. Hepatol.* **1991**, *6*, 121.
4. (a) Titz, A. *Top. Med. Chem.* **2014**, *12*, 169; (b) Rice, L. B. *J. Infect. Dis.* **2008**, *197*, 1079; (c) Peleg, A. Y.; Hooper, D. C. *New Engl. J. Med.* **1804**, *2010*, 362.
5. (a) Supuran, C. T. *Nat. Rev. Drug Disc.* **2008**, *7*, 168; (b) Supuran, C. T. *Future Med. Chem.* **2011**, *3*, 1165; (c) De Simone, G.; Supuran, C. T. *Biochim. Biophys. Acta* **2010**, *1804*, 404; (d) Neri, D.; Supuran, C. T. *Nat. Rev. Drug Disc.* **2011**, *10*, 767; (e) Zolfaghari Emameh, R.; Kuuslahti, M.; Vullo, D.; Barker, H. R.; Supuran, C. T.; Parkkila, S. *Parasit. Vectors* **2015**, *8*, 479.
6. (a) Supuran, C. T.; Capasso, C. *Expert Opin. Ther. Targets* **2015**, *19*, 551; (b) Capasso, C.; Supuran, C. T. *Expert Opin. Ther. Targets* **2015**, *19*, 1689; (c) Capasso, C.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2014**, *29*, 379; (d) Capasso, C.; Supuran, C. T. *Expert Opin. Ther. Pat.* **2013**, *23*, 693; (e) Capasso, C.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2015**, *30*, 325.
7. (a) Alterio, V.; Di Fiore, A.; D'Ambrosio, K.; Supuran, C. T.; De Simone, G. *Chem. Rev.* **2012**, *112*, 4421; (b) Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2016**. http://dx.doi.org/10.3109/14756366.2015.1122001; (c) Supuran, C. T. *Expert Opin. Ther. Pat.* **2013**, *23*, 677; (d) Nishimori, I.; Vullo, D.; Innocenti, A.; Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *J. Med. Chem.* **2005**, *48*, 7860; (e) Supuran, C. T.; Scozzafava, A.; Mastrolorenzo, A. *Expert Opin. Ther. Pat.* **2001**, *11*, 221.
8. (a) De Simone, G.; Di Fiore, A.; Capasso, C.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 1385; (b) Del Prete, S.; Vullo, D.; Fisher, G. M.; Andrews, K. T.; Poulsen, S. A.; Capasso, C.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 4389; (c) Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2012**, *27*, 759; (b) Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2013**, *28*, 229; (d) Ozensoy Guler, O.; Capasso, C.; Supuran, C. T. *Med. Chem.* **2016**, *31*. in press; (e) Ceruso, M.; Del Prete, S.; Alothman, Z.; Capasso, C.; Supuran, C. T. *ACS Med. Chem. Lett.* **2014**, *5*, 826; (f) Alafeefy, A. M.; Ceruso, M.; Al-Tamimi, A. M.; Del Prete, S.; Capasso, C.; Supuran, C. T. *Bioorg. Med. Chem.* **2014**, *22*, 5133.
9. (a) Carta, F.; Osman, S. M.; Vullo, D.; AlOthman, Z.; Del Prete, S.; Capasso, C.; Supuran, C. T. *Bioorg. Med. Chem.* **2015**, *23*, 6794; (b) Lehneck, R.; Neumann, P.; Vullo, D.; Elleuche, S.; Supuran, C. T.; Ficner, R.; Poggeler, S. *FEBS J.* **2014**, *281*, 1759; (c) Hewitson, K.; Vullo, D.; Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *J. Med. Chem.* **2012**, *55*, 3513; (d) Del Prete, S.; De Luca, V.; Vullo, D.; Osman, S. M.; AlOthman, Z.; Carginale, V.; Supuran, C. T.; Capasso, C. *J. Enzyme Inhib. Med. Chem.* **2016**, *31*, 23. http://dx.doi.org/10.3109/14756366.2015.1102137. in press 2015; (e) Del Prete, S.; Vullo, D.; Osman, S. M.; AlOthman, Z.; Capasso, C.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 5194.
10. (a) Capasso, C.; Supuran, C. T. *Curr. Med. Chem.* **2015**, *22*, 2130; (b) Del Prete, S.; De Luca, V.; Iandolo, E.; Supuran, C. T.; Capasso, C. *Bioorg. Med. Chem.* **2015**, *23*, 3747; (c) De Luca, V.; Del Prete, S.; Carginale, V.; Vullo, D.; Supuran, C. T.; Capasso, C. *J. Enzyme Inhib. Med. Chem.* **2015**, *30*, 989; (d) Del Prete, S.; Vullo, D.; De Luca, V.; AlOthman, Z.; Osman, S. M.; Supuran, C. T.; Capasso, C. *J. Enzyme Inhib. Med. Chem.* **2015**, *30*, 366; (e) Del Prete, S.; De Luca, V.; Scozzafava, A.; Carginale, V.; Supuran, C. T.; Capasso, C. *J. Enzyme Inhib. Med. Chem.* **2014**, *29*, 23; (f) Del Prete, S.; Isik, S.; Vullo, D.; De Luca, V.; Carginale, V.; Scozzafava, A.; Supuran, C. T.; Capasso, C. *J. Med. Chem.* **2012**, *55*, 10742; (g) Del Prete, S.; De Luca, V.; Supuran, C. T.; Capasso, C. *J. Enzyme Inhib. Med. Chem.* **2015**, *30*, 920; (h) Vullo, D.; Nishimori, I.; Minakuchi, T.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3591.
11. (a) Schlicker, C.; Hall, R. A.; Vullo, D.; Middelhaufe, S.; Gertz, M.; Supuran, C. T.; Muhlschlegel, F. A.; Steegborn, C. *J. Mol. Biol.* **2009**, *385*, 1207; (b) Hall, R. A.; Mühlschlegel, F. A. Fungal and Nematode Carbonic Anhydrases: Their Inhibition in Drug Design. In *Drug Design of Zinc-Enzyme Inhibitors: Functional, Structural, and Disease Applications*; Supuran, C. T., Winum, J. Y., Eds.; John Wiley & Sons: Hoboken, 2009; pp 301–322; (c) Klengel, T.; Liang, W. J.; Chaloupka, J.; Ruoff, C.; Schropel, K.; Naglik, J. R.; Eckert, S. E.; Mogensen, E. G.; Haynes, K.; Tuite, M. F.; Levin, L. R.; Buck, J.; Mühlschlegel, F. A. *Curr. Biol.* **2005**, *15*, 2021; (d) Bahn, Y. S.; Cox, G. M.; Perfect, J. R.; Heitman, J. *Curr. Biol.* **2013**, *2005*, 15.
12. (a) Mogensen, E. G.; Janbon, G.; Chaloupka, J.; Steegborn, C.; Fu, M. S.; Moyrand, F.; Klengel, T.; Pearson, D. S.; Geeves, M. A.; Buck, J.; Levin, L. R.; Mühlschlegel, F. A. *Eukaryot. Cell* **2006**, *5*, 103; (b) Bahn, Y. S.; Mühlschlegel, F. A. *Curr. Opin. Microbiol.* **2006**, *9*, 572; (c) Supuran, C. T. *Front. Pharmacol.* **2011**, *2*, 34; (d) Tripp, B. C.; Smith, K. S.; Ferry, J. G. *J. Biol. Chem.* **2001**, *276*, 48615; (b) Smith, K. S.; Ferry, J. G. *FEMS Microbiol. Rev.* **2000**, *24*, 335.
13. Ferraroni, M.; Del Prete, S.; Vullo, D.; Capasso, C.; Supuran, C. T. *Acta Crystallogr. D Biol. Crystallogr.* **2015**, *71*, 2449.
14. (a) Nishimori, I.; Minakuchi, T.; Kohsaki, T.; Onishi, S.; Takeuchi, H.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3585; (b) Nishimori, I.; Onishi, S.; Takeuchi, H.; Supuran, C. T. *Curr. Pharm. Des.* **2008**, *14*, 622; (c) Nishimori, I.; Minakuchi, T.; Morimoto, K.; Sano, S.; Onishi, S.; Takeuchi, H.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2006**, *49*, 2117.
15. (a) Modak, J. K.; Liu, Y. C.; Machuca, M. A.; Supuran, C. T.; Roujeinikova, A. *PLoS ONE* **2015**, *10*, e0127149; (b) Buzás, G. M.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2016**, *31*. http://dx.doi.org/10.3109/14756366.2015.1051042. in press.
16. (a) Şentürk, M.; Gülçin, I.; Daştan, A.; Küfrevioğlu, Ö. I.; Supuran, C. T. *Bioorg. Med. Chem.* **2009**, *17*, 3207; (b) Supuran, C. T.; Casini, A.; Mastrolorenzo, A.; Scozzafava, A. *Mini. Rev. Med. Chem.* **2004**, *4*, 625; (c) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146; (d) Supuran, C. T. *Curr. Med. Chem.* **2012**, *19*, 831; (e) Scozzafava, A.; Briganti, F.; Mincione, G.; Menabuoni, L.; Mincione, F.; Supuran, C. T. *J. Med. Chem.* **1999**, *42*, 3690.
17. (a) Winum, J.-Y.; Temperini, C.; El Cheikh, K.; Innocenti, A.; Vullo, D.; Ciattini, S.; Montero, J.-L.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2006**, *49*, 7024; (b) Abbate, F.; Winum, J. Y.; Potter, B. V. L.; Casini, A.; Montero, J. L.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 231; (c) Nishimori, I.; Minakuchi, T.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem.* **2011**, *19*, 5023; (d) Touisni, N.; Maresca, A.; McDonald, P. C.; Lou, Y.; Scozzafava, A.; Dedhar, S.; Winum, J. Y.; Supuran, C. T. *J. Med. Chem.* **2011**, *54*, 8271.
18. (a) Dubois, L.; Douma, K.; Supuran, C. T.; Chiu, R. K.; van Zandvoort, M. A. M.; Pastoreková, S.; Scozzafava, A.; Wouters, B. G.; Lambin, P. *Radiother. Oncol.* **2007**, *83*, 367; (b) Scozzafava, A.; Supuran, C. T. *Subcell. Biochem.* **2014**, *75*, 349; (c) Carta, F.; Supuran, C. T.; Scozzafava, A. *Future Med. Chem.* **2014**, *6*, 1149; (d) Bonneau, A.; Maresca, A.; Winum, J. Y.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2013**, *28*, 397; (e) Sharma, A.; Tiwari, M.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2014**, *29*, 292.
19. Eminoglu, A.; Vullo, D.; Aşık, A.; Çolak, D. N.; Supuran, C. T.; Çanakçı, S.; Beldüz, A. O. *J. Enzyme Inhib. Med. Chem.* **2016**, *31*. http://dx.doi.org/10.3109/14756366.2015.1100176. in press.
20. (a) Cronk, J. D.; Endrizzi, J. A.; Cronk, M. R.; O'Neill, J. W.; Zhang, K. Y. *Protein Sci.* **2001**, *10*, 911; (b) Cronk, J. D.; O'Neill, J. W.; Cronk, M. R.; Endrizzi, J. A.; Zhang, K. Y. *Acta Crystallogr. D Biol. Crystallogr.* **2000**, *56*, 1176; (c) Hoffmann, K. M.; Million-Perez, H. R.; Merkhofer, R.; Nicholson, H.; Rowlett, R. S. *Biochemistry* **2015**, *54*, 598.

21. Covarrubias, A. S.; Bergfors, T.; Jones, T. A.; Hogbom, M. *J. Biol. Chem.* **2006**, *281*, 4993.
22. Rowlett, R. S.; Tu, C.; Lee, J.; Herman, A. G.; Chapnick, D. A.; Shah, S. H.; Gareiss, P. C. *Biochemistry* **2009**, *48*, 6146.
23. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. A stopped-flow $CO_2$ hydration assay with an applied photophysics instrument has been used for measuring catalytic activity and inhibition of the enzymes investigated here. 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 buffers, and 20 mM $NaClO_4$ (for maintaining constant the ionic strength). The initial rates of the CA-catalyzed $CO_2$ hydration reaction were followed for a period of 10–100 s. The concentrations of substrate ($CO_2$) ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants, with at least six traces of the initial 5–10% of the reaction being used for determining the initial velocity, for each inhibitor. The uncatalyzed rates were subtracted from the total observed rates. Stock solutions of inhibitors (10 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done with the assay buffer. Enzyme and inhibitor solutions were pre-incubated 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 the Cheng–Prusoff equation as reported earlier by our groups.[24,25] The kinetic parameters for the uninhibited enzymes were derived from Lineweaver–Burk plots, as reported earlier,[24,25] and represent the mean from at least three different determinations. EspCA was obtained as described in Ref. 19 and its concentration in the assay system was of 14.6 nM.
24. (a) Dathan, N. A.; Alterio, V.; Troiano, E.; Vullo, D.; Ludwig, M.; De Simone, G.; Supuran, C. T.; Monti, S. M. *J. Enzyme Inhib. Med. Chem.* **2014**, *29*, 500; (b) Durdagi, S.; Scozzafava, G.; Vullo, D.; Sahin, H.; Kolayli, S.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2014**, *29*, 469; (c) Chohan, Z. H.; Scozzafava, A.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 259.
25. (a) Del Prete, S.; De Luca, V.; Vullo, D.; Scozzafava, A.; Carginale, V.; Supuran, C. T.; Capasso, C. *J. Enzyme Inhib. Med. Chem.* **2014**, *29*, 532; (b) Ceruso, M.; Vullo, D.; Scozzafava, A.; Supuran, C. T. *J. Enzyme Inhib. Med. Chem.* **2014**, *29*, 686; (c) Del Prete, S.; Vullo, D.; De Luca, V.; Supuran, C. T.; Capasso, C. *J. Enzyme Inhib. Med. Chem.* **2014**, *29*, 906; (d) Bayram, E.; Senturk, M.; Kufrevioglu, O. I.; Supuran, C. T. *Bioorg. Med. Chem.* **2008**, *16*, 9101.