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SHORT COMMUNICATION



Inhibition of pathogenic bacterial carbonic anhydrases by monothiocarbamates

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ABSTRACT

Carbonic anhydrases (CAs) from the pathogenic bacteria *Nisseria gonorrhoeae* and vancomycin-resistant enterococci (VRE) have recently been validated as antibacterial drug targets. Here we explored the inhibition of the α -CA from *N. gonorrhoeae* (α -NgCA), of α - and γ -class enzymes from *Enterococcus faecium* (α -EfCA and γ -EfCA) with a panel of aliphatic, heterocyclic and aryl-alkyl primary/secondary monothiocarbamates (MTCs). α -NgCA was inhibited *in vitro* with K_s ranging from 0.367 to 0.919 μ M. The compounds inhibited the α -EfCA and γ -EfCA with K_i ranges of 0.195–0.959 μ M and of 0.149–1.90 μ M, respectively. Some MTCs were also investigated for their inhibitory effects on the growth of clinically-relevant *N. gonorrhoeae* and VRE strains. No inhibitory effects on the growth of VRE were noted for all MTCs, whereas one compound (**13**) inhibited the growth *N. gonorrhoeae* strains at concentrations ranging from 16 to 64 μ g/mL. This suggests that compound **13** may be a potential antibacterial agent against *N. gonorrhoeae*.

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Introduction

The emergence of drug resistance to most antibacterials used clinically over the last decades^{1–6}, coupled with the climate change which exacerbates this problem^{7,8}, makes the finding of novel approaches to fight bacterial infections as well as agents with lower rates of resistance a crucial task for medicinal chemists and microbiologists^{1,2,9}. Inhibition of bacterial carbonic anhydrases (CAs, EC 4.2.1.1) is one such approach, already proposed more than a decade ago¹⁰. CAs play crucial roles in bacterial metabolism and pH regulation^{10–14}. Many bacterial CAs have been investigated over the last decade, and they have been validated as a bacterial target^{11–14}, for several bacteria such as *Neisseria gonorrhoeae*¹⁵, vancomycin-resistant enterococci (VRE)¹⁶, *Helicobacter pylori*¹⁷ and *Vibrio cholerae*¹⁸. Most such studies have been performed with sulphonamide CA inhibitors (CAIs), one of the most investigated class of inhibitors for these enzymes. However, other classes of CAIs have recently been investigated for their inhibitory potential against bacterial CAs, such as coumarins¹⁹, dithiocarbamates²⁰ and phenols²¹. Monothiocarbamates (MTCs) represent another class of zinc-binding CAIs²², which have not yet been investigated for their interaction with bacterial CAs. In this work, we report the *in vitro* inhibitory activity of a panel of MTCs against CAs of *N. gonorrhoeae* and VRE, which were reported before to be inhibited by CAIs. We do this in the search for non-sulphonamide leads which might possess antibacterial activity.

Materials and methods

Chemistry

MTCs **1–15** were obtained as reported earlier²², whereas acetazolamide **AAZ** (as standard CAI) and buffers were of >99% purity, commercially available from Sigma-Aldrich (Milan, Italy). Antibiotics were purchased commercially: Azithromycin (TCI America, OR, USA), linezolid (Chem-Impex, IL, USA), and vancomycin (GoldBio, MO, USA).

Enzymology and CA activity and inhibition measurements

CO₂ hydration activity of CAs from *N. gonorrhoeae* (α -NgCA) and *E. faecium* (α -EfCA and γ -EfCA) as well as their inhibition in the presence of MTC inhibitors has been assessed by a stopped-flow method reported by Khalifah²³. The experiments were performed at the pH of 7.4 for the α -class enzymes, and the pH of 8.3 for the γ -CA, as reported earlier^{15,16}. The bacterial enzymes were recombinant proteins obtained as reported earlier by our group^{15,16}.

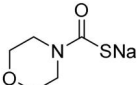
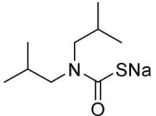
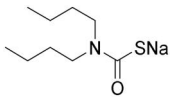
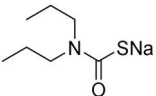
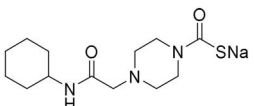
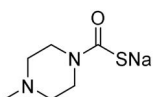
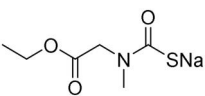
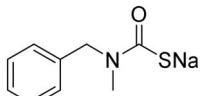
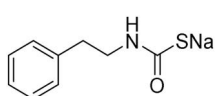
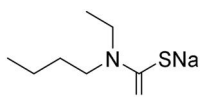
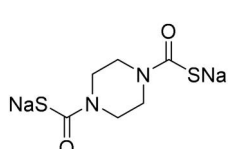
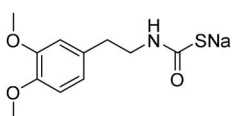
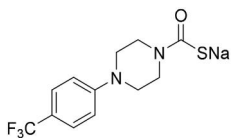
The minimum inhibitory concentrations (MICs) determination

The MICs were assessed using the broth microdilution method against clinically-relevant *N. gonorrhoeae* and VRE strains, as described in previous reports^{15,16,24–26}. Briefly, serial dilutions of the test agent were incubated with bacteria at 37 °C either aerobically or in the presence of 5% CO₂. MICs were determined as the

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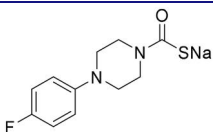
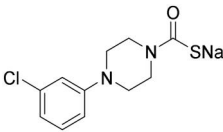
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Table 1. Inhibition data of hCA I and II and bacterial α -NgCA, α -EfCA and γ -EfCA, using **AAZ** as a standard drug, by a stopped-flow CO₂ hydase assay²².

Name	Structure	K _i (μ M)a				
		hCA I	hCA II	α -NgCA	α -EfCA	γ -EfCA
1		0.569	>2	0.761	0.740	1.03
2		0.681	0.043	0.890	0.665	0.811
3		0.909	>2	0.825	0.517	0.498
4		>2	0.046	0.799	0.550	0.733
5		0.949	0.045	0.395	0.290	0.417
6		>2	0.035	0.729	0.959	1.90
7		0.827	0.044	0.919	0.788	0.279
8		>2	>2	0.843	0.686	0.611
9		>2	0.043	0.648	0.428	0.642
10		0.700	>2	0.766	0.806	0.971
11		0.876	0.022	0.630	0.915	0.695
12		0.891	0.026	0.512	0.357	0.358
13		>2	0.043	0.394	0.477	0.334

(continued)

Table 1. Continued.

Name	Structure	K_i (μ M) ^a				
		hCA I	hCA II	α -NgCA	α -EfCA	γ -EfCA
14		0.895	0.046	0.417	0.195	0.149
15		0.686	>2	0.367	0.300	0.214
AAZ	–	0.250	0.012	0.075	0.056	0.322

^aMean from 3 different assays, by a stopped-flow technique (errors were in the range of \pm 5–10% of the reported values).

Table 2. MICs (μ g/mL) of MTCs **1**, **6**, **9**, **12** and **13** against *Neisseria gonorrhoeae* clinical isolates.

Test agents/Control antibiotics	<i>Neisseria gonorrhoeae</i> strains					
	CDC-178		CDC-181		CDC-194	
	5% CO ₂	Ambient air	5% CO ₂	Ambient air	5% CO ₂	Ambient air
1	>64	>64	>64	>64	>64	>64
6	>64	>64	>64	64	>64	32
9	>64	>64	>64	>64	>64	>64
12	>64	>64	64	>64	>64	>64
13	>64	64	32	16	64	32
AAZ	>64	2	>64	2	>64	1
Azithromycin	2	2	>64	>64	1	0.5

lowest concentration that completely inhibited bacterial growth as observed visually.

Results and discussion

The inhibitory effects of MTC derivatives **1–15** on three bacterial CAs isoforms, α -NgCA from *N. gonorrhoeae*, α -EfCA and γ -EfCA from *E. faecium*, were investigated (Table 1). The inhibition data of these compounds against the human offtarget isoforms hCA I and II are also given in the Table, for comparison reasons. The K_i values of MTCs ranged from the high nanomolar to the low micromolar ranges. The K_i values of MTC compounds were in the range of 0.367–0.919 μ M against α -NgCA, of 0.195–0.959 μ M against α -EfCA, and of 0.149–1.90 μ M against γ -EfCA. In the case of α -NgCA, MTCs **1–4** and **6–11** were poorly effective inhibitors, showing K_i values in the high nanomolar range (0.630–0.919 μ M), whereas the piperazine derivatives **5** and **13–15** exhibited better inhibitory effects, displaying K_i values of 0.367–0.417 μ M. The γ -EfCA was the most effectively inhibited isoform among the bacterial CAs investigated. For instance, MTCs **7** and **12–15** inhibited γ -EfCA with K_i values of 0.149–0.357 μ M, which were comparable with the K_i value of the standard drug **AAZ** (K_i = 0.322 μ M). Among them, compound **14** (K_i = 149 nM), bearing a 4-fluorophenyl tail, resulted in 2-fold better potency than the reference drug, **AAZ**. On the other hand, the simple piperazine-tailed MTC **6** was the weakest inhibitor of γ -EfCA, with a K_i value in the low micromolar range (1.90 μ M). In the case of α -EfCA, the piperazine derivative **11** proved to be one of the weakest inhibitors showing a K_i value of 0.914 μ M, whereas MTCs **5**, **12**, **14** and **15** showed better inhibition constants of 290 nM, 357 nM, 195 nM and 300 nM, respectively. However, compound **14**, which was superior to **AAZ** in inhibiting the γ -EfCA, was at least 3-fold less potent than **AAZ**

(K_i = 56 nM) in inhibiting the α -EfCA. Only the piperazine derivative **15** (NgCA α : K_i = 367 nM; EfCA α : K_i = 300 nM; EfCA γ : K_i = 214 nM) demonstrated a good selectivity against the discussed bacterial isozymes over the human widely expressed hCA II isoform (hCA II: K_i > 2 μ M).

Next, the antibacterial activity of selected MTCs (**1**, **6**, **9**, **12** and **13**) was evaluated against a panel of multidrug-resistant strains of *N. gonorrhoeae* and VRE. Compound **13**, which has both a lipophilic trifluoromethyl-phenyl fragment and hydrophilic moieties (piperazine and monothiocarbamate functionality), displayed a modest activity against *N. gonorrhoeae* in addition to its inhibition of the α -NgCA. The compound inhibited *N. gonorrhoeae* strains with MIC values ranging between 16 and 64 μ g/mL under ambient air conditions, while showing limited activity in the presence of 5% CO₂ (Table 2). This suggests that the antigonococcal activity of **13** could be mediated by CA inhibition. Although other compounds, such as **12**, **14** and **15** showed similar *in vitro* α -NgCA inhibitory properties to **13**, only the last compound was antibacterial *in vivo*, presumably due to its enhanced lipophilicity due to the presence of the trifluoromethyl moiety. All the tested MTCs were inactive against the VRE strains tested (MICs >64 μ g/mL) (Table 3).

Since the DTCs investigated earlier²⁰ showed an intermediate behaviour between the ineffective MTCs and the quite effective sulphonamides^{15,16}, we can speculate that the highly hydrophilic nature of the MTCs may interfere with their uptake by the bacterial cells. Thus, effective compounds targeting bacterial CAs should not only be lipophilic enough to cross the bacterial cell wall or traverse the water-filled porins, but also they should incorporate zinc-binding groups that allow a potent coordination to the active site metal ion, which is crucial both for catalysis and inhibition of these enzymes²⁷.

Table 3. MICs (μg/mL) of MTCs 1, 6, 9, 12 and 13 against VRE strains.

Test agents/Control antibiotics	VRE strains					
	<i>E. faecalis</i> NR-31971		<i>E. faecalis</i> NR-31972		<i>E. faecium</i> HM-965	
	5% CO ₂	Ambient air	5% CO ₂	Ambient air	5% CO ₂	Ambient air
1	>64	>64	>64	>64	>64	>64
6	>64	>64	>64	>64	>64	>64
9	>64	>64	>64	>64	>64	>64
12	>64	>64	>64	>64	>64	>64
13	>64	>64	>64	>64	>64	>64
AAZ	>64	2	>64	2	>64	1
Linezolid	1	1	1	1	1	1
Vancomycin	>64	>64	>64	>64	>64	>64

Conclusion

We report here the first inhibition study of bacterial CAs with MTCs, a class of CAIs that is less investigated as compared to the most well-known classes of CAIs, sulphonamides and their isosteres. CAs of *N. gonorrhoeae* (α-NgCA) and *E. faecium* (α-EfCA and γ-EfCA) were inhibited by the panel of 15 MTCs with K_s in the medium-high nanomolar range, with some compounds showing similar activity to the clinically used sulphonamide CAI, acetazolamide. However, the activity of these compounds in inhibiting the growth of these bacteria *in vitro* was lower as compared to the sulfonamides. Thus, the MTCs seem to be less effective as bacterial CAIs, but the investigated series of compounds is too small for concluding that such compounds should not be investigated in the future.

Disclosure statement

CT Supuran is Editor-in-Chief of the Journal of Enzyme Inhibition and Medicinal Chemistry. He was not involved in the assessment, peer review, or decision-making process of this paper. The authors have no relevant affiliations of financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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