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Inhibition of β-carbonic anhydrases with ureido-substituted benzenesulfonamides

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ABSTRACT

A series of sulfonamides was prepared by reaction of sulfanilamide with aryl/alkyl isocyanates. The ureido-substituted benzenesulfonamides showed a very interesting profile for the inhibition of several carbonic anhydrases (CAs, EC 4.2.1.1) such as the human hCA II and three β-CAs from pathogenic fungal or bacterial species. The *Candida albicans* enzyme was inhibited with potencies in the range of 3.4–3970 nM, whereas the *Mycobacterium tuberculosis* enzymes Rv1284 and Rv3273 were inhibited with K_is in the range of 4.8–6500 nM and of 6.4–6850 nM, respectively. The structure–activity relationship for this class of inhibitors is rather complex, but the main features associated with effective inhibition of both α- and β-CAs investigated here have been delineated. The nature of the moiety substituting the second ureido nitrogen is the determining factor in controlling the inhibitory power, probably due to the flexibility of the ureido linker and the possibility of this moiety to orientate in different subpockets of the active site cavities of these enzymes.

The carbonic anhydrases (CAs, EC 4.2.1.1) enzymes are widely distributed throughout the phylogenetic tree, with five genetically unrelated classes (α-, β-, γ-, δ- and ζ-) known to date.[1] These proteins which catalyze the interconversion between carbon dioxide and bicarbonate, with release of a proton, are not only involved in pH homeostasis and regulation, but also in biosynthetic reactions, such as gluconeogenesis and ureagenesis among others (in animals), CO_2 fixation (in plants and algae), electrolyte secretion in a variety of tissues/organs, with many of the 16 mammalian CA isozymes being established drug targets for the design of diuretics, antiglaucoma, antiepileptic, antiobesity and/or anticancer agents.[1–3] While the α-CA family is mainly present (but not exclusive to) in mammals and it has been thoroughly investigated from the drug design viewpoint,[1–3] only recently CAs belonging to the β- and γ-CA families, which are widespread in bacteria and fungi (the β-CAs) and *Archaea* (the β- and γ-CAs), respectively, started to be considered for such a purpose. Thus, a β-CA present in the gastric pathogen *Helicobacter pylori* was recently shown to be a possible target for gastric drugs,[4,5] with several low nanomolar inhibitors detected, which effectively inhibited the in vitro and in vivo growth of the pathogen,[4,5] whereas another enzyme from *Brucella suis* was also shown to be inhibited strongly by some sulfonamide derivatives, leading to impairment of bacterial growth.[6] The fungal β-CAs from *Candida albicans*[7] and *Cryptococcus neoformans*[8,9] (as well as the related enzyme present in *Saccharomyces cerevisiae*)[10,11] were also characterized, being shown to be susceptible to inhibition with the main classes of CA inhibitors (CAIs), that is, the inorganic anions and the sulfonamides and their bioisosteres.[6–11] Two other β-CAs from *Mycobacterium tuberculosis* were successfully cloned and crystallized by Suarez Covarrubias et al.[12,13] being denominated Rv1284 and Rv3588c. Our group identified, cloned and characterized the third such enzyme, encoded by the gene Rv3273.[14] The catalytic activity and inhibition with sulfonamides/sulfamates of all these β-class enzymes from bacterial and fungal parasites have been reported, but very few low nanomolar inhibitors were detected so far for most of them.[11,14,15] Sulfonamide/sulfamate CAIs targeting various mammalian α-CAs, such as acetazolamide **1**, dichlorophenamide **2** or sulfanilamide **3**, have been in clinical use for more than 50 years,[1] but such compounds generally show much less effective inhibition of the β-class enzymes.[11] Furthermore, they generally do not possess the appropriate pharmacological properties (e.g., good penetration through bacterial walls/membranes) to effectively impair the growth of many such pathogens (or they show weak such properties, as shown by work on *H. pylori* and *B. suis* enzymes mentioned above).[5,6] Thus, in order to understand whether β-CAs may constitute drug targets for developing antiinfective agents, it is essential to design compounds with different affinities and pharmacological properties compared to the classical CAIs of types **1–3**.

Here we report the synthesis and inhibition studies of several β-CAs from fungal or bacterial pathogens with a large series of ureido-substituted sulfonamides obtained from sulfanilamide **3** as lead molecule. A large series of 4-ureido-substituted benzenesulfonamides **4–30** was prepared by reaction of sulfanilamide **3**

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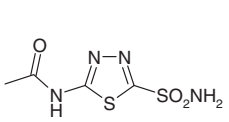

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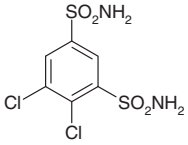

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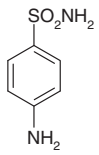

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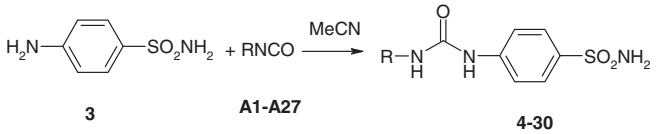
with aryl/alkyl-isocyanates **A1-A27** (Scheme 1).[16] The chemical diversity of these derivatives was generated by varying the nature of the starting isocyanate **A1-A27**, since, as shown by a recent X-ray crystallographic work on five of these derivatives for which the structure in complex with the human isoforms hCA II has been reported,[17] was determined that it is the nature of the R group that greatly influences the binding to the enzyme. For compounds **7, 13, 16, 25** and **28**[17] in adduct with hCA II, it has been observed that the benzenesulfonamide fragment binds in the usual manner, coordinating to the zinc ion as SO_2NH^- moiety. The phenylsulfamoyl part of the inhibitor was superimposable between all these adducts and bound in the usual way,[18] filling the middle of the active site cavity.[17,18]

However the different R moieties present in these compounds were observed to bind towards the external part of the enzyme active site, occupying various subpockets, and none of them was superimposable with each other. This phenomenon is probably due to the rather flexible nature of the ureido linker connecting the benzenesulfonamide with the R group in this series of compounds, and may also explain a fact reported earlier by our group,[19] that such ureido-substituted sulfonamides (different of the compounds investigated here) may show selective inhibition of isoform hCA I over the dominant one hCA II. Normally, it is hCA II having higher affinity for sulfonamides compared to hCA I, at least for most of the clinically used derivatives.[1–3]

A large variety of aromatic and aliphatic R moieties have been incorporated in the new CAIs **4–30** reported here, in order to expand the structure–activity relationship (SAR) insight regarding their interactions with various CA isoforms belonging to the β-class. The synthesis of such compounds is a one-step process, generally occurring in high yield, being possible to apply it for the preparation of a large number of derivatives.[16] It should be noted that most of the CAIs of sulfonamide type investigated earlier[1–3] contained CONH or SO_2NH linkers between the sulfonamide head moiety and the R group, instead of the ureido one present in **4–30**. These different linkers allow less flexibility for the inhibitor scaffold, and this is probably the reason why most such sulfonamides bind in the cannonical hydrophobic pocket of hCA II and also generally do not show isoform selectivity.[18,20] We have investigated the inhibition of the dominant, human isoform hCA II (offtarget) as well as three β-CAs from pathogenic organisms (fungi and bacteria) with derivatives **4–30** reported here (Table 1).[21,22] The following SAR could be observed from data of Table 1:

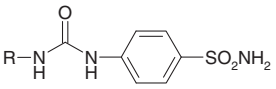
(i) the offtarget isoform, the cytosolic hCA II, was inhibited with potencies ranging from the low nanomolar to the micromolar by ureidosulfonamides **4–30**. Compounds **16, 19, 22, 24** and **30** were very potent hCA II inhibitors, with K_is in the range of 2.1–9.7 nM. These derivatives incorporate 2- or 4-substituted phenyl moieties or the indane ring. It is interesting to note that **16** and **19** contain in their molecule the rather bulky, lipophilic *i*-Pr, *n*-BuO or biphenyl moieties, which might be considered too bulky to fit well within the hCA II active site. However as shown here and in the crystallographic, preliminary communication,[17] the *i*-Pr moiety of **16** makes favorable hydrophobic contacts in a patch within the enzyme active site never seen earlier to accommodate inhibitors, also participating to a Π stacking with Phe131. This particular binding mode explains the excellent hCA II inhibitory properties of **16** (K_i of 3.3 nM). However, four other derivatives of the series investigated here, that is, **7**, **13**, **25** and **28**, exploited different binding pockets within the hCA II active site,[17] and also possessed diverse inhibitory power compared to **16**, with inhibition constants in the range of 15–226 nM (Table 1). Another series of investigated sulfonamides showed medium potency hCA II inhibition, with K_is in the range of 15–96 nM. These compounds (**7, 12, 13, 21, 23** and **25**) also incorporate aromatic R groups, such as 4-fluorophenyl, 3,5-di(trifluoromethyl)phenyl, pentafluorophenyl, 4-cyanophenyl, 4-phenoxyphenyl or 3-nitrophenyl. The remaining derivatives in this series showed lower hCA II inhibitory properties, with K_is in the range of 226–9600 nM. The least effective hCA II inhibitor was the derivative incorporating the long 4-*n*-octylphenyl moiety (**20**). It should be also noted that sulfanilamide **3** was a medium potency hCA II inhibitor (K_i of 240 nM) whereas acetazolamide **1** a potent one (K_i of 12 nM).



3 A1-A27 4-30

Scheme 1. Preparation of ureidosulfonamides **4**–**30** from sulfanilamide **3**.

Table 1
Inhibition of the β-CAs from *C. albicans*, the mycobacterial enzymes Rv1284 and Rv3273, and human hCA II with ureido-sulfonamides **4**–**30**, sulfanilamide **3** and acetazolamide **AAZ**, by a stopped-flow CO_2 hydrase assay[21]



4-30

No.	R	K_i[a] (nM)			
		hCA II	*C. albicans*	Rv1284	Rv3273
3	–(Sulfanilamide)	240	1086	9230	6240
4	Ph	3730	395	356	758
5	$PhCH_2$	2200	547	440	717
6	Ph_2CH	3725	3970	4330	6380
7	4-FC_6H_4	96	42.1	6.4	53.0
8	Cl-FC_6H_4	781	62.0	4.8	63.1
9	4-BrC_6H_4	1290	45.0	7.5	87.1
10	4-IC_6H_4	2634	42.5	6.8	56.4
11	4-$CF_3C_6H_4$	1150	61.0	69.3	533
12	3,5-$(CF_3)_2C_6H_3$	75	58.5	65.7	481
13	C_6F_5	50	38.0	5.0	6.4
14	2-$MeOC_6H_4$	4070	702	473	603
15	4-AcC_6H_4	1060	29.1	470	74.6
16	2-*i*-PrC_6H_4	3.3	3450	5610	6850
17	4-*i*-PrC_6H_4	5005	355	5690	57.5
18	4-*n*-BuC_6H_4	2485	49.9	4760	63.7
19	4-*n*-$BuOC_6H_4$	2.1	50.6	35.2	63.0
20	4-*n*-octyl-C_6H_4	9600	45.9	6500	81.2
21	4-NCC_6H_4	64.7	53.5	50.2	64.4
22	2-NCC_6H_4	2.4	326	534	463
23	4-$PhOC_6H_4$	85	3.4	560	818
24	2-PhC_6H_4	9.7	297	5590	748
25	3-$O_2NC_6H_4$	15	40.1	67.1	6.5
26	4-MeO-2-MeC_6H_3	3310	53.2	49.1	70.3
27	9H-Fluoren-2-yl	908	573	728	370
28	Cyclopentyl	226	565	509	733
29	3,5-$Me_2C_6H_3$	1765	65.9	315	768
30	Indan-5-yl	8.9	71.8	486	632
–	AAZ	12	132	481	104

[a] Errors were in the range of ±10% of the reported data, from three different assays.

(ii) the β-CA from *C. albicans* was inhibited by the ureido-sulfonamides **4–30** with a variable potency, with inhibition constants in the range of 3.4–3970 nM, compared to sulfanilamide **3** which was a weak inhibitor (K_i of 1086 nM) and acetazoalmide **1** which was a medium potency inhibitor (K_i of 132 nM). Thus, strong inhibition has been observed for derivatives **7–13, 15, 18–21, 23, 25, 26, 29** and **30**, which possessed inhibition constants in the range of 3.4–71.8 nM. These compounds incorporate 3- or 4-substituted phenyl moieties as R groups, such as 4-halogenophenyl, 4-trifluoromethylphenyl, 4-acetylphenyl-; 4-*n*-butyl/butoxyphenyl, 4-cyanophenyl-; 4-phenoxyphenyl-, 3-nitrophenyl-, as well as 3,5- or 2,4-disubstituted-phenyl moieties (except **30** which possesses an indane moiety and **13** which possesses the pentafluorophenyl moiety). It is obvious that a rather large number of substitution patterns lead to effective *C. albicans* β-CA inhibitors. The best such inhibitor was **23**, incorporating the elongated 4-phenoxyphenylureido moiety which, with an inhibition constant of 3.4 nM, is the best inhibitor of this enzyme ever reported in the literature.[8] Other substitution patterns present in these compounds led to less effective inhibitors. For example, derivatives **4–6, 14, 16, 17, 27** and **28**, having aryl, aralkyl or cycloalkyl R moieties, are weak *C. albicans* CAIs, with inhibition constants in the range of 297–3970 nM (Table 1). As for hCA II; small variations in the nature of the R moiety leads to very different inhibitory properties for this series of compounds, with highly effective, medium potency and ineffective CAIs detected in this congeneric series of sulfonamides.

(iii) the mycobacterial enzyme Rv1284 was very weakly inhibited by sulfanilamide **3** and weakly by acetazolamide **1**, with inhibition constants in the range of 481–9230 nM (Table 1). However the ureido-benzenesulfonamides **4–30** reported here generally showed a better inhibitory action against this enzyme, with K_is in the range of 4.8–6500 nM. Thus, a number of derivatives, such as **7–10**, and **13**, were the best Rv1284 inhibitors in this series, with K_is in the range of 4.8–7.5 nM. All of them incorporate 4-halogenosubstituted phenyl or pentafluorophenyl R moieties. Another subseries of these sulfonamides, such as **11, 12, 19, 21, 25** and **26**, showed slightly less effective inhibitory activity, with K_is in the range of 35.2–69.3 nM. They incorporate mono- or disubstituted phenyl R moieties containing trifluoromethyl, nitro, cyano, and alkoxy groups. Medium potency inhibition has been observed with compounds such as **4, 5, 14, 15, 22, 23, 27–30**, which incorporate aryl, aralkyl or cycloalkyl R moieties. The least effective inhibitors were **6, 16–18, 20** and **23**, most of them incorporating rather bulky R groups (diphenylmethyl, 2-*i*-Pr-phenyl, 4-*n*-Bu-phenyl, 4-*n*-octylphenyl, etc.), which showed micromolar affinity for this enzyme (K_is in the range of 4.33–6.50 μM).

(iv) the mycobacterial enzyme Rv3273 was inhibited by the new compounds **4–30** reported here with K_is in the range of 6.4–6850 nM, whereas the lead **3** and **AAZ** were ineffective and medium potency inhibitors, respectively (K_i of 6.24 μM for **3** and of 104 nM for **1**). The most effective CAIs against this enzyme were **13** and **25**, with K_is in the range of 6.4–6.5 nM. The incorporate the pentafluorophenyl and 3-nitrophenyl R moieties, respectively. A rather large groups of compounds showed medium potency inhibitory activity against Rv3273, with K_is in the range of 53.0–87.1 nM, and they include the halogenophenyl-substituted derivatives **7–10**, the 4-acetylphenyl group of **15**, and the alkyl-/alkoxyphenyl or cyanophenyl moieties present in **17–21**. The disubstituted compound **26** also belong to this category. Again a rather large number of R moieties lead to highly enhanced β-CA inhibitory properties compared to the lead **3**, which was a very ineffective inhibitor of this enzyme (Table 1). Several of the new derivatives were medium potency (**4, 5, 11, 12, 14, 22–24** and **27–30**) or ineffective (**6** and **16**) inhibitors of the Rv3273 enzyme. As for the other α- or β-CAs investigated here, it may be observed that all types of activities were detected against Rv3273 for the sulfonamides synthesized in this work.

(v) the selectivity ratios of the new sulfonamides investigated here, for the inhibition of the target versus offtarget CAs is a rather complex issue, due to the particular SAR discussed above for each particular enzyme. However, there are interesting aspects that may be evidenced. For example, several compounds such as **16, 22** and **24** showed low nanomolar hCA II inhibitory activity but were much less effective as β-CA inhibitors and can be considered as selective for the inhibition of the α- versus β-CAs. Sulfonamide **23** on the other hand was a *C. albicans* CA selective inhibitor, with selectivity ratios for inhibiting the fungal enzyme over the mammalian one hCA II of 25, and for inhibiting the fungal over mycobacterial enzymes of 164.7 and of 240.5, respectively. An inhibitor which was selective for the Rv1284 enzyme has also been discovered, **8**, which had selectivity ratios for inhibiting Rv1284 over hCA II of 162.7, for inhibiting Rv1284 over the fungal enzyme of 12.9 and for inhibiting Rv1284 over Rv3273 of 13.1. For Rv3273 the most selective inhibitor was **25**, with selectivity ratios of 2.3 over hCA II, of 6.1 over the *C. albicans* enzyme, and of 10.3 over Rv1284. These selectivity ratios are less high than for the other two investigate β-CAs, but these are the first ever reported CAIs showing this interesting profile and may be useful for understanding in greater detail the physiological role of some of these enzymes.

In conclusion, we report here a series of sulfonamides prepared by reaction of sulfanilamide with aryl/alkyl isocyanates. The ureido-substituted benzenesulfonamides showed a very interesting profile for the inhibition of hCAII and three β-CAs from pathogenic fungal or bacterial species. The *C. albicans* enzyme was inhibited with potencies in the range of 3.4–3970 nM, whereas the mycobacterial ones Rv1284 and Rv3273 with K_is in the range of 4.8–6500 nM and of 6.4–6850 nM, respectively. The structure–activity relationship for this class of inhibitors is rather complex but the main features associated with effective inhibition of both α- and each β-CAs investigated here have been delineated. The nature of the R moiety substituting the second ureido nitrogen is the determining factor in controlling the inhibitory power, probably due to the flexibility of the ureido linker and the possibility of the R group to orientate in different subpockets of the active site cavity of these enzymes.

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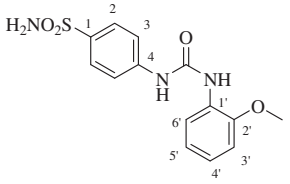
16. General procedure for the preparation of compounds **4**–**30**. Sulfanilamide **3** (2.90 mmols) was dissolved in acetonitrile (20–30 mL) and then treated with a stoichiometric amount of commercially available isocyanate **A1–A27**. The mixture was stirred at rt or heated at 50 °C for 2 h, until completion (TLC monitoring). The heavy precipitate formed was filtered-off, washed with diethyl ether (100 ml), dried under vacuo, and recrystallized. For example **8** was obtained by reaction of sulfanilamide **3** (0.50 g; 2.90 mmols) with 2-methoxyphenyl isocyanate (0.43 g; 2.90 mmols). The reaction was stirred at rt overnight, treated as described above, to give **8** as a white solid in 40.4% yield.



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4-{[(2′-Methoxyphenyl)amino]carbonyl)}aminobenzenesulfonamide (**8**): mp 234–236 °C; silica gel TLC R_f 0.47 (ethyl acetate/petroleum ether 33%); ν_{max} (KBr) cm^{-1}, 3362 (N–H urea), 2838 (C–H aliphatic), 1684 (C=O urea), 1592 (aromatic); δ_H (400 MHz, DMSO-d_6) 3.92 (3H, s, CH_3), 6.94 (1H, ddd, *J* 8.2 7.4 1.4, 4′-H), 7.01 (1H, ddd, *J* 8.0 7.4 1.6, 5′-H), 7.07 (1H, dd, *J* 8.2 1.2, 3′-H), 7.23 (2H, s, SO_2NH_2), 7.64 (2H, d, *J* 8.8, 2 × 2-H), 7.77 (2H, d, *J* 8.8, 2 × 3-H), 8.16 (1H, dd, *J* 8.0 1.6, 6′-H), 8.38 (1H, s, N*H*), 9.73 (1H, s, N*H*); δ_C (100 MHz, DMSO-d_6) 153.0 (C=O, urea), 148.7, 143.8, 137.7, 129.2, 127.8, 123.2, 121.5, 119.4, 118.1, 111.7, 56.7 (CH_3).

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21. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. 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 10–20 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-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier,[14,15] and represent the mean from at least three different determinations.
22. The α- and β-CAs used in this work were recombinant enzymes obtained as reported earlier.[7,8,14,15]