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Carbonic anhydrase inhibitors: Cloning and sulfonamide inhibition studies of a carboxyterminal truncated α-carbonic anhydrase from *Helicobacter pylori*

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Abstract—A library of sulfonamides/sulfamates has been investigated for the inhibition of the carboxyterminal truncated form of the α-carbonic anhydrase (CA, EC 4.2.1.1) isolated from the gastric pathogen *Helicobacter pylori* (hpCA). This enzyme, incorporating 202 amino acid residues, showed a catalytic activity similar to that of the full length hpCA, with k_{cat} of 2.35×10^5 s^{-1} and k_{cat}/K_M of 1.56×10^7 M^{-1} s^{-1} at 25 °C and pH of 8.9, for the CO_2 hydration reaction. All types of activity for inhibition of the bacterial enzyme have been detected. Dorzolamide and simple 4-substituted benzenesulfonamides were weak hpCA inhibitors (inhibition constants, K_Is, in the range of 830–4310 nM). Sulfanilamide, orthanilamide, some of their derivatives, and indisulam showed better activity (K_Is in the range of 310–562 nM), whereas most of the clinically used CA inhibitors, such as methazolamide, ethoxzolamide, dichlorophenamide, brinzolamide, topiramate, zonisamide, etc., acted as medium potency hpCA inhibitors (K_Is in the range of 124–287 nM). Some potent hpCA inhibitors were detected too (K_Is in the range of 20–96 nM) such as acetazolamide, 4-amino-6-chloro-1,3-benzenedisulfonamide, 4-sulfanilyl-aminoethyl-benzenesulfonamide, and 4-(2-amino-pyrimidin-4-yl)-benzenesulfonamide. Most of the investigated derivatives acted as better inhibitors of the human isoform hCA II than as hpCA inhibitors. Since hpCA is essential for the survival of the pathogen in acid, its inhibition by compounds such as those investigated here might be used as a new pharmacologic tool in the management of drug resistant *H. pylori*.

The α-carbonic anhydrases (CAs, EC 4.2.1.1) are widespread metalloenzymes in all life kingdoms.[1–5] In higher vertebrates, including humans, 16 isoforms have been described up to now,[6–8] of which at least CA II, IV, VA, VB, VII, IX, XII, XIII, and XIV constitute valid targets for the development of novel antiglaucoma, antitumor, antiobesity or anticonvulsant drugs.[9–13] Indeed, by catalyzing a simple but fundamental reaction, the reversible hydration of CO_2 to bicarbonate and a proton, these metalloenzymes are involved in a multitude of physiological and pathological processes,[1–5] and their inhibition leads to responses that may be exploited therapeutically. Presently, other three genetically distinct classes of CAs have been described in various organisms, the β-CA-δ-CA families, proving that such a critical catalyst for life processes has been invented by nature at least in 4 different occasions. Among them, very recently representatives of the α- or β-CA class have been cloned and characterized in *Plasmodium falciparum*,[14] *Mycobacterium tuberculosis*,[15] *Cryptococcus neoformans*[16] or *Candida spp.*,[17] some of them being also investigated from the inhibition point of view,[14,16] as it has been proved that these enzymes are critical for the growth or virulence of these pathogens.[14–17] Since many of these organisms are highly pathogenic, and present different degrees of resistance to the currently available drugs targeting them, inhibition of their CAs may constitute novel approaches to fighting such diseases.[14–17]

Helicobacter pylori, a Gram-negative neutralophile discovered by Warren and Marshall in the early 1980s,[18] was shown to be associated with chronic gastritis, peptic

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ulcers and, more recently, gastric cancer, the second most common tumor in humans.[19] *H. pylori* is a globally spread pathogen with roughly 50% of the human population being contaminated, causing sometimes severe gastrointestinal diseases that lead to a significant morbidity and mortality.[20] Although there is an effective treatment for peptic ulcer disease caused by *H. pylori*, usually consisting of a triple therapy using two antibiotics (amoxicillin and clarithromycin) and a proton pump inhibitor (PPI), such first-line treatment/eradication regimens are constantly being compromised by an increase in the prevalence of antibiotic resistance.[21–23] After failure of the eradication by the first-line treatment, an empirical quadruple regimen (PPI, bismuth, tetracycline, and metronidazole) has generally been used as the second-line therapy. However, several studies have demonstrated that even two consecutive regimens failed to eradicate *H. pylori* in some patients.[21–23] Thus, there is a real need for the development of alternative therapies, eventually exploiting novel targets, that should be devoid of the problems araising with currently available drugs.[23]

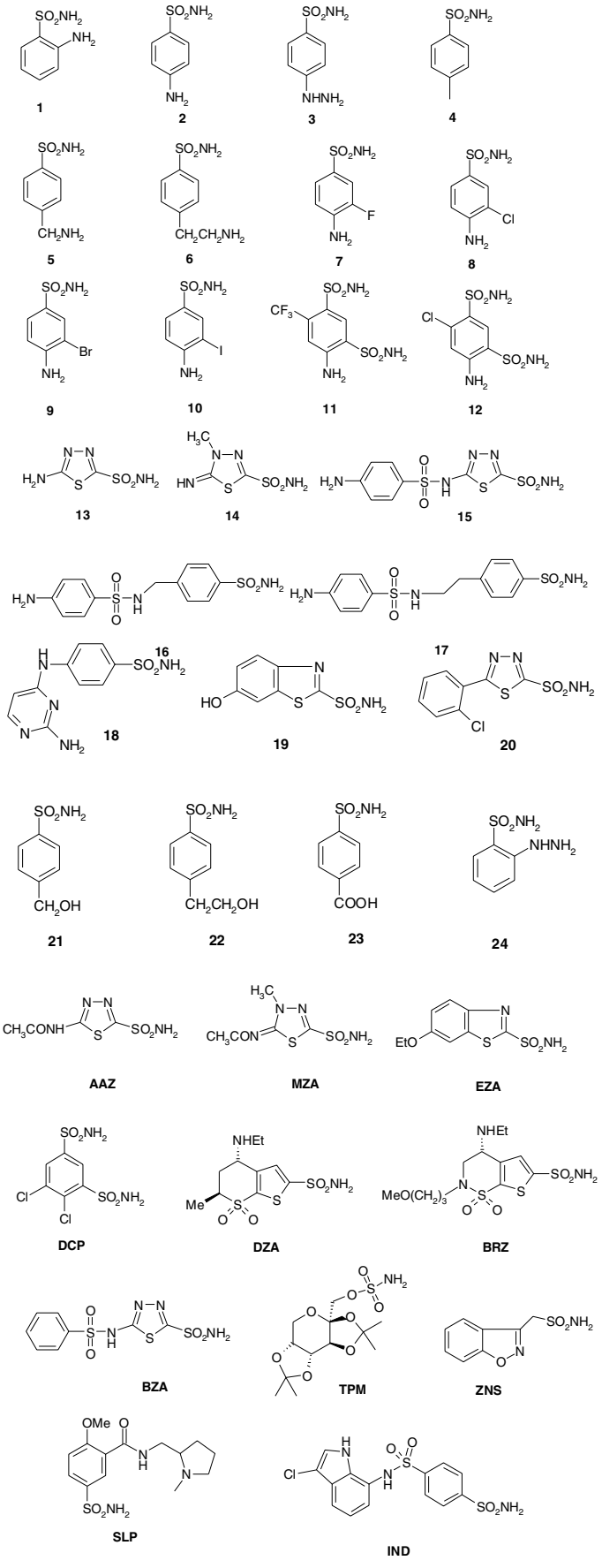
Helicobacter pylori has the unique ability among bacteria to grow and multiply in the stomach, in the harsh and highly acidic conditions at pH values as low as 1.4.[24] Therefore, the pathogen has evolved in specialized processes that maintain the cytoplasmic pH around 6.4 for survival and growth. Basically, at least two enzymes are involved in these processes: urease[24] in the cytoplasm, and an α-CA (from now on designated as hpCA) in the periplasm[24,25] which separates an outer membrane and an inner membrane of this bacterium. However, a β-CA has also been found in the cytoplasm of *H. pylori*, where it seems to play an important role in the urea and bicarbonate metabolism, as well as acid resistance of the pathogen.[26]

hpCA was cloned and purified by Lindskog's group,[27] which showed that the enzyme contains one polypeptide chain of 247 amino acid residues, has a catalytic activity similar to that of the human slow isoform hCA I (highly abundant in red blood cells and the gastro-intestinal tract)[1–3] and that the enzyme is susceptible to inhibition by sulfonamides (and thiocyanate). However, no quantitative data were provided for acetazolamide inhibition of hpCA in this study.[27] Recently, Sachs' group[24,25] has proved that hpCA is essential for the acid acclimation and survival of the pathogen. In an elegant study,[25] using CA deletion mutants of *H. pylori* as well as the potent, clinically used sulfonamide inhibitor acetazolamide **AAZ**, it has been shown that the urease generation of NH_3 has a major role in regulation of the periplasmic pH and inner membrane potential under acidic conditions, allowing adequate bioenergetics necessary for survival and growth of the pathogen. In addition to urease, interestingly, hpCA was also shown to be crucial to these processes. Thus, Western analysis confirmed that hpCA was bound to the inner membrane, being present only within the periplasm. Furthermore, in the deletion mutant (i.e., *H. pylori* lacking the hpCA) or in the wild-type organism in the presence of acetazolamide, there was an approximate 3 log10 decrease in acid survival of the pathogen. Thus, buffering of the periplasm to a pH consistent with viability depends not only on the ammonia efflux from the cytoplasm (and thus, urease) but also on the conversion of CO_2 (produced by urease) to bicarbonate by the periplasmic hpCA.[25] This excellent study[25] is in fact a proof-of-concept that hpCA may be an attractive drug target for developing anti-*Helicobacter pylori* agents, provided that potent (and hopefully specific) inhibitors can be found.

In the present study, we cloned and isolated a truncated form of hpCA containing 202 amino acid residues, based on the sequence deposited in the GenBank by the TIGR sequencing center [http://www.tigr.org].[28] Furthermore, we evaluated the inhibitory effects of a panel of sulfonamides/sulfamates (known inhibitors of other α-CAs)[29] against this enzyme, showing that effective inhibitors targeting this bacterial CA can be detected/designed.

A large number of derivatives were investigated for the inhibition of the bacterial enzymes hpCA. Simple aromatic and heteroaromatic sulfonamides of types **1**–**24** are among them. Derivatives **AAZ-IND** are clinically used drugs: acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, and dichlorophenamide **DCP** are the classical, systemically acting CA inhibitors (CAIs).[1–3] Dorzolamide **DZA** and brinzolamide **BRZ** are topically acting antiglaucoma agents,[1,4] benzolamide **BZA** is an orphan drug belonging to this class of pharmacological agents, whereas topiramate **TPM** and zonisamide **ZNS** are widely used antiepileptic drugs.[11b,30] Sulpiride **SLP**[31] and indisulam **IND**[32] were recently shown by this group to belong to this class of pharmacological agents. Compounds **1**, **2**, **4**–**6**, **11**, **12**, **18**–**20**, **23**, and **AAZ-SLP** are commercially available, whereas **3**, **7**–**10**,[33] **13**–**17**,[34,35] **21**, **22**[36], and **24**[37] were prepared as reported earlier by this group.

Two different length-polypeptides (202 and 247 residues) were previously reported for hpCA.[27,28] In the present study, a recombinant form of hpCA lacking the last 45 carboxyterminal amino acid residues was synthesized based on the sequence deposited in the GenBank by TIGR sequencing center.[28] The hpCA preparations were obtained in this study by means of a GST-fusion protein technique,[38] previously reported for other α-CAs by our group.[9,11,12] The enzyme showed a catalytic activity very similar to that of the enzyme reported earlier by Lindskog's group (k_{cat} of $2.4 \times 10^5\ s^{-1}$ and k_{cat}/K_M of 1.5×10^7 for the CO_2 hydration reaction),[27] with a k_{cat} of $2.35 \times 10^5\ s^{-1}$ and k_{cat}/K_M of $1.56 \times 10^7\ M^{-1}\ s^{-1}$ at 25 °C and pH of 8.9. Our data clearly show that the short, carboxyterminal truncated form and the full length form of hpCA are identical from the catalytic point of view, which is in fact a logical result, since the carboxyterminal peptide is generally not involved in the formation of the active site in other α-CAs.[1–5,27] On the other hand, hpCA is quite similar to hCA I (k_{cat} of 2.0×10^5 and k_{cat}/K_M of 5×10^7 in the same conditions),[9a] as already noted by Chirica et al.[27] However, in contrast to the previous preparation method,[27] the GST-fusion protein technique allows the facile preparation of high amounts of recombinant hpCA needed for the inhibitor screening studies.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Sulfonamides and sulfamates are well known for their high affinity for many α-CA isozymes, acting as potent inhibitors with clinical applications as antiglaucoma, diuretic, antiobesity or antitumor drugs.[1–5,29] Various isoforms are responsible for specific physiological functions, and drugs with such a diversity of actions target in fact quite different isozymes.[1–5,29] In all of them, the sulfonamide/sulfamate drug binds in deprotonated form to the catalytically critical Zn(II) ion, also participating in extensive hydrogen bond and van der Waals interactions with amino acid residues both in the hydrophobic and hydrophilic halves of the enzyme active site, as shown by X-ray crystallographic work of enzyme-inhibitor complexes.[10,11,13,34]

In the early 1970s, it has been reported in the literature that acetazolamide, **AAZ**, the CA inhibitor par excellence, is also effective in the therapy of gastric and duodenal ulcers.[42] The antiulcer effects of this potent CAI (developed in the 1950s as the first nonmercurial diuretic)[43] were assigned as being due to the inhibition of CA isoforms present in gastric mucosa, mainly CA I and II, which were considered to be involved in gastric acid secretion due to H^+ ions generated by hydration of carbon dioxide.[42,43] Interestingly, although the treatment of ulcers with CAIs has not been widely used except by Puscas,[42] this approach was quite successful, since the healing rate after 30 days of **AAZ** was 94% (as compared to 48% for antacid-treated patients), and the relapse after 2 years was only 6.2% after **AAZ** treatment as compared to a relapse 34% for the antacid-treated patients.[42] In light of the recent findings of Sachs' group[24,25] that hpCA is essential for the life cycle of *H. pylori* (its absence in knockout bacteria, or due to inhibition with **AAZ** leading to a 3 log 10 decrease of the pathogen survival in acid), and considering that **AAZ** is probably a potent hpCA inhibitor, we may reinterpret Puscas' data[42] as another proof-of-concept 'experiment' that hpCA inhibitors can successfully be used for the management of gastric diseases. Thus, we decided to investigate a library of sulfonamides/sulfamates (of types **1**–**24** and **AAZ**–**IND**) for their interaction with hpCA, in order to detect potent inhibitors with potential use as gastric drugs. Inhibition data of these sulfonamides/sulfamates against the host isozymes hCA I, hCA II (highly abundant in the blood and gastrointestinal tract)[1–3] and the bacterial enzyme hpCA are shown in Table 1.[41]

Data of Table 1 show that all 35 compounds investigated here act as inhibitors of the bacterial enzyme hpCA, at the same time being inhibitory against the host α-CA isoforms hCA I and II, but with a quite different behavior against the three targets (data for hCA I and II of compounds **1**–**24** and **AAZ**–**IND** have previously been published[4,11–13] but are included for discussing the selectivity issue of these CAIs for the bacterial versus the host enzymes, see later in the text). The hCA I **ZNS** data are new, together with the hpCA inhibition data of all derivatives.

The following SAR can be drawn from data of Table 1 for the library of investigated sulfonamides/sulfamates:

Table 1. Inhibition of *Hlicobacter pylori* CA (hpCA) and of the human isoforms hCA I and hCA II, with compounds **1**–**24** and the 11 clinically used sulfonamides/sulfamates **AAZ**–**IND**

Inhibitor	K_I* (nM)			Selectivity ratio
	hCA I[a]	hCA II[a]	hpCA[b]	hCA II/hpCA
1	45,400	295	465	0.63
2	25,000	240	431	0.55
3	28,000	300	334	0.89
4	78,500	320	473	0.67
5	25,000	170	830	0.20
6	21,000	160	1035	0.15
7	8,300	60	1310	0.04
8	9,800	110	395	0.27
9	6,500	40	463	0.08
10	6,000	70	504	0.13
11	5,800	63	402	0.15
12	8,400	75	41	1.83
13	8,600	60	310	0.19
14	9,300	19	494	0.03
15	6	2	213	0.009
16	164	46	124	0.37
17	185	50	96	0.52
18	109	33	72	0.45
19	95	30	174	0.17
20	690	12	84	0.14
21	55	80	870	0.09
22	21,000	125	1157	0.10
23	23,000	133	1083	0.12
24	24,000	125	562	0.22
AAZ	250	12	20	0.60
MZA	50	14	171	0.08
EZA	25	8	155	0.05
DCP	1,200	38	363	0.10
DZA	50,000	9	4310	0.002
BRZ	45,000	3	246	0.01
BZA	15	9	287	0.03
TPM	250	10	205	0.04
ZNS	56	35	218	0.16
SLP	1,200	40	179	0.22
IND	31	15	434	0.03

[a] Human recombinant isozymes, stopped flow CO_2 hydrase assay method.[41]

[b] Recombinant, carboxyterminal truncated *Helicobacter pylori* enzyme lacking the last 45 amino acid residues, stopped flow CO_2 hydrase assay method.[41]

* Errors in the range of 5–10% of the shown data, from three different assays.

(i) a group of derivatives, including **5**–**7**, **21**–**23**, and **DZA**, acted as weak hpCA inhibitors, with inhibition constants in the range of 830–4310 nM. Except for the heterocyclic sulfonamide **DZA**, all these compounds are benzenesulfonamide derivatives possessing moieties substituting the benzene ring in the *para* position with respect to the sulfamoyl group, of the aminomethyl/ethyl-; hydroxymethyl/ethyl- or carboxy type (**7** is the fluorinated derivative of sulfanilamide). It may also be noted that all these derivatives act as much better hCA II and as weaker hCA I inhibitors, as compared to their activity on hpCA; (ii) derivatives **1**–**4**, **8**–**11**, **13**, **14**, **24**, **DCP**, and **IND** were better hpCA inhibitors as compared to the previously mentioned sulfonamides, with inhibition constants in the range of 310–562 nM. From the structural point of view, these sulfonamides belong to a rather heterogeneous group of derivatives, being

either orthanilamides (**1**, **24**), 4-substituted benzenesulfonamides (**2**–**4**, **IND**), halogenated sulfanilamides (**8**–**10**) or the heterocyclic compounds **13** and **14**, and the benzene-1,3-disulfonamide derivatives **11** and **DCP**. As the previously discussed sulfonamides, also these derivatives are much better hCA II inhibitors and much weaker hCA I inhibitors, as compared to their action on the bacterial enzyme. It should be noted the equipotency of sulfanilamide **2** with its halogenated derivatives **8**, **9**, and **10**, whereas the fluoroderivative **7** is a much weaker inhibitor; (iii) a group of derivatives, including **15**, **16**, **19**, **MZA**, **EZA**, **BRZ**, **BZA**, **TPM**, **ZNS**, and **SLP**, showed medium potency as hpCA inhibitors, with K_Is in the range of 124–287 nM. Again a rather large structural heterogeneity was noted, with some derivatives being (3),4-(di)substituted benzene-sulfonamides/halogenated sulfanilamides (**16** and **SLP**); but most of them belonging to the heteroaromatic class of sulfonamides, mainly 1,3,4-thiadiazole-2-sulfonamide (**15**, **MZA**, **BZA**) and benzothiazole-2-sulfonamide derivatives (**19**, **EZA**). The clinically used antiglaucoma heterocyclic sulfonamide **BRZ**, the antiepileptic sulfamate **TPM** as well as the aliphatic sulfonamide **ZNS** also belong to this class of medium potency hpCA inhibitors. It should be noted the tremendous difference of activity between **DZA** and **BRZ**, although the two compounds are structurally similar. Thus, **DZA** is approximately 17.5 times less effective as a hpCA inhibitor as compared to **BRZ** (these two compounds are also very weak hCA I inhibitors but very potent hCA II inhibitors). On the other hand, all these sulfonamides/sulfamates act as very potent hCA II inhibitors (Table 1), whereas their activity on hCA I is much more variable, as some of them are strong inhibitors (**15**, **MZA**, **EZA**, **BZA**, **ZNS**, and **IND**), others are medium potency inhibitors, whereas others, as mentioned above, are quite weak hCA I inhibitors (**DZA**, **BRZ**); (iv) a last group of four derivatives (**12**, **17**, **18**, and **AAZ**) showed potent hpCA inhibitory activity, with inhibition constants in the range of 20–96 nM. Several findings should be noted here. A large difference of activity against hpCA has been found for the two structurally related benzene-1,3-disulfonamides **11** and **12**, with the last one being 9.80 times a better inhibitor as compared to **11**. Thus, the bulkier trifluoromethyl group in the 6 position of the benzene ring is detrimental to activity on hpCA as compared to a chlorine atom in the same position. We also confirm the strong inhibitory activity of **AAZ** against hpCA, as mentioned by Chirica et al.[27] (but with no quantitative data published in their work), which possesses a K_I of 20 nM, being the most potent inhibitor detected in our study. Interesting activity was also shown by the bicyclic derivatives **17** and **18**, which may be considered as good leads for the design of better inhibitors. It may also be observed by comparing the activity of derivatives **16** and **17**, that an increase in the chain connecting the two rings is beneficial for increasing the hpCA inhibitory efficacy; (v) a main issue regarding the CAIs is their selectivity for the target isozyme, considering the fact that the ubiquitous human isoform hCA II (which is also a target for many types of CAI-based drugs)[1–5] has a high affinity for sulfonamides/sulfamates.[1–5] Indeed, as observed from data of Table 1, most of the investigated compounds from this study are better hCA II than hpCA inhibitors, with selectivity ratios under the unity. Only one of the investigated compounds, **12**, showed a selectivity ratio of 1.83, being thus a better inhibitor of the bacterial over the host enzyme. Acetazolamide **AAZ**, the best hpCA inhibitor detected here, shows a selectivity ratio of 0.60, being thus a better hCA II than hpCA inhibitor. Work is in progress in our laboratories to detect even more selective and potent hpCA inhibitors belonging to other classes of compounds.

In conclusion, a library of sulfonamides/sulfamates has been investigated for the inhibition of the carboxyterminal truncated form of hpCA incorporating 202 amino acid residues. This enzyme showed a catalytic activity similar to that of the full length hpCA, with k_{cat} of $2.35 \times 10^5\ s^{-1}$ and k_{cat}/K_M of $1.56 \times 10^7\ M^{-1}\ s^{-1}$ at 25 °C and pH of 8.9, for the CO_2 hydration reaction. All types of activity for inhibition of the bacterial enzyme have been detected. Dorzolamide and simple 4-substituted benzenesulfonamides were weak hpCA inhibitors (K_Is in the range of 830–4310 nM). Sulfanilamide, orthanilamide, some of their derivatives, and indisulam showed better activity (K_Is in the range of 310–562 nM), whereas most of the clinically used CA inhibitors, such as methazolamide, ethoxzolamide, dichlorophenamide, brinzolamide, topiramate, zonisamide, etc., acted as medium potency hpCA inhibitors (K_Is in the range of 124–287 nM). Some potent hpCA inhibitors were detected too (K_Is in the range of 20–96 nM) such as acetazolamide, 4-amino-6-chloro-1,3-benzenedisulfonamide, 4-sulfanilylaminoethyl-benzenesulfonamide, and 4-(2-amino-pyrimidin-4-yl)-benzenesulfonamide. Most of the investigated derivatives acted as better hCA II than hpCA inhibitors. Since hpCA is essential for the survival of the pathogen in acid, its inhibition by compounds as those reported here might be used as a new pharmacologic tool in the management of drug resistant *H. pylori*.

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38. For hpCA DNA cloning, *H. pylori* was isolated from the stomach of a healthy volunteer (44-year-old, male, Japanese) and cultured as previously reported.[39] Following incubation under a microaerophilic condition using the Campy-Pouch system (Becton–Dickinson, Cockeysville, MD), DNA was extracted from cultured cells by using a DNeasy kit (Qiagen, Hilden, Germany). The DNA fragment encoding the open-reading frame of *H. pylori* α-CA was amplified by PCR. The primer sequences were based on the sequence obtained from the *H. pylori* strain 26695 (HP1186, Accession No. AE000511) and deposited in the GenBank by TIGR sequencing center.[28] For subcloning into an expression vector, adopter primers including *Bam*HI and *Eco*RI recognition sequences (underlined in the following sequences, respectively) were synthesized: 5′-CG GGATCCATGGACACCAAATGGGATTATAAGAAT AAAGAA-3′ and 5′-CGGAATTCTCACAAACCATGC CACCCCCTCTGTGC-3′. The PCR was hot-started with incubation for 1 min at 94 °C and consisted of 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 90 s at 72 °C. The PCR products were cleaved with *Bam*HI and *Eco*RI, and ligated in-frame into the pGEX-4T2 vector (Amersham, Tokyo, Japan). The proper DNA sequence of the hpCA insert included in the vector was reconfirmed by DNA sequencing. The constructed vector was transfected into *E. coli* strain BL21 for production of a GST-α-CA fusion protein as previously reported.[9a] Following induction of the protein expression by adding 1 mM isopropyl-β-D-thiogalactopyranoside, the bacteria were harvested and sonicated in PBS. The cell homogenate was incubated at room temperature for 15 min and homogenized twice with a Polytron (Brinkmann) for 30 s each at 4 °C. Centrifugation at 30,000*g* for 30 min afforded the supernatant containing the soluble

proteins. The obtained supernatant was applied to a prepacked glutathione–Sepharose 4B column (Amersham), which was extensively washed with buffer and then the GST-α-CA fusion protein eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. Finally the GST part of the fusion protein was cleaved with thrombin. The obtained hpCA recombinant protein was further purified by sulfonamide affinity chromatography,[40] the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with CO_2 as substrate.[41]

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41. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561, An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed 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 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction. 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 (1 mM) were prepared in distilled–deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations) 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, from Lineweaver–Burk plots, as reported earlier, and represent the mean from at least three different determinations.[9–11].
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