



BRIEF REPORT



Inhibition studies of bacterial α -carbonic anhydrases with phenols

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ABSTRACT

The α -class carbonic anhydrases (CAs, EC 4.2.1.1) from the bacterial pathogens *Neisseria gonorrhoeae* (NgCA α) and *Vibrio cholerae* (VchCA α) were investigated for their inhibition by a panel of phenols and phenolic acids. Mono-, di- and tri-substituted phenols incorporating additional hydroxyl/hydroxymethyl, amino, acetamido, carboxyl, halogeno and carboxyethenyl moieties were included in the study. The best NgCA α inhibitors were phenol, 3-aminophenol, 4-hydroxy-benzylalcohol, 3-amino-4-chlorophenol and paracetamol, with K_i values of 0.6–1.7 μ M. The most effective VchCA α inhibitors were phenol, 3-amino-4-chlorophenol and 4-hydroxy-benzyl-alcohol, with K_i values of 0.7–1.2 μ M. Small changes in the phenol scaffold led to drastic effects on the bacterial CA inhibitory activity. This class of underinvestigated bacterial CA inhibitors may thus lead to effective compounds for fighting drug resistant bacteria.

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1. Introduction

Phenol (PhOH) was first investigated for its interaction with the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) by Koenig's group in 1980¹, and few years later Lindskog's group² demonstrated that this compound is one of the few competitive inhibitors (with CO₂ as substrate) of these enzymes, more precisely of the human (h) isoform hCA II. However, only in 1994 Christianson's group³ elegantly demonstrated by using X-ray crystallography that phenol shows a new inhibition mechanism against this enzyme: it is anchored to the zinc-coordinated water molecule through hydrogen bonds involving the phenolic OH group, without displacing the zinc-bound water as other classes of inhibitors known at that time³. Subsequently, a large number of simple, synthetic and natural product phenols and polyphenols, incorporating various ring systems and possessing a range of substitution patterns were investigated for their inhibitory effects against all mammalian isoforms (CA I–XV) as well as for their interactions with pathogenic bacterial or fungal such enzymes^{4–10}. For example dodoneine (Figure 1), a dihydropyranone phenolic compound isolated from the African mistletoe *Agelanthus dodoneifolius*¹¹ was demonstrated to possess significant CA inhibitory effects and to induce vasorelaxation through interference with calcium channels blockade and CA inhibition in the vascular smooth muscle cells¹¹.

Such results prompted an intense research in the synthesis of phenolic derivatives incorporating diverse scaffolds (e.g. sugars, steroids, Mannich bases, etc.)^{12,13}, which have been tested for the inhibition of all hCAs as well as of enzymes belonging to non- α -CA classes, such as β -, γ -, δ -, η -CAs from bacteria, fungi, algae, diatoms and protozoans^{14–16}. Recent X-ray crystallographic of

aspirin (hydrolyzed to salicylic acid) or caffeic acid bound to hCA II¹⁷ also allowed a better understanding of the binding mode of such compound to the enzyme (Figure 2) and to rationalise their inhibition mechanism with useful hints for the drug design of novel classes of CA inhibitors (CAIs)^{18,19}. It may be observed that salicylic acid binds with its carboxylic acid moiety to the zinc-coordinated water molecule through a network of hydrogen bonds, with two water molecules being observed coordinated to the metal ion^{17a}. Caffeic acid anchors with its catechol moiety to the zinc-coordinated water and to the deep water from the CA active site^{17b} – Figure 2.

Such data prompted us to investigate the interactions of a series of simple phenols and some of their derivatives with bacterial CAs which have recently been proposed^{20,21} as novel drug targets for fighting the emergence of drug resistant bacteria, which no longer respond to clinically used antibiotics²². We included in the study NgCA α from *Neisseria gonorrhoeae* and VchCA α from *Vibrio cholerae*.

2. Materials and methods

2.1. Enzymology and CA activity and inhibition measurements

An Applied Photophysics stopped-flow instrument was used to assay the CA-catalysed CO₂ hydration activity²³. Phenol red (0.2 mM) was used as a pH indicator, working at the absorbance maximum of 557 nm, with 10 mM HEPES (pH 7.4) as a buffer, and in the presence of 10 mM NaClO₄ to maintain constant ionic strength, in order to follow the initial rates of the CA-catalysed CO₂ hydration reaction for a period of 10–100 s. The CO₂ 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 were used to determine the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitors (10–20 mM) were prepared in distilled-deionized water, and dilutions up to 10 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min prior to the 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 previously^{21,22}, and represent the mean from at least three different determinations. The NgCA α concentration in the assay system was 7.1 nM whereas the VchCA α was 10.3 nM. The used enzymes were recombinant proteins obtained in-house, as described earlier^{22,24}.

2.2. Chemistry

Compounds **1–22**, buffers, acetazolamide **AAZ** and other reagents were of > 99% purity and were commercially available from Sigma-Aldrich (Milan, Italy).

3. Results and discussion

Bacterial CAs were investigated in the last decade for their inhibition with the several types of classical CAIs, among which the sulphonamides and their isosteres, the metal complexing anions and more recently also the coumarins^{18–22}. However, no detailed inhibition data with many other classes of inhibitors, including phenols, are available so far in the literature for many of these enzymes, among which NgCA α and VchCA α belong^{22,24}. The first of this enzyme was recently validated²¹ as a potential drug target for

developing antibiotics able to reverse or at least to alleviate the extensive drug resistance phenomenon that has emerged for this *N. gonorrhoeae*²⁵. Although phenols usually act as weaker CAIs compared to other classes such as the sulphonamides or the sulfamates, we decided to investigate here the susceptibility of these enzymes to inhibition by a series of simple phenols and phenolic acids, of types **1–22** (Table 1) investigated earlier for their interaction with the human CA isoforms^{4,6}.

The following structure activity relationship (SAR) can be evidenced from the inhibition data presented in Table 1:

- For NgCA α , the compounds **1–22** investigated here showed inhibitory activity, with K_i -s in the range of 0.6–76.0 μ M. The most effective, submicromolar inhibitors were **1**, **6**, **12**, **13**, and paracetamol **8**, with K_i values of 0.6–1.7 μ M. These compounds are the simple phenol, its 4-hydroxymethyl derivative (**12**) as well as 3-amino-phenol or 4-acetamido moiety in derivatives (**6**, **8** and **13**). The presence of a second/third H-bond donating moiety such as NH₂ or OH lead to slight loss of inhibitory activity K_i values in the range of 1.7–6.0 μ M (Table 1) for derivatives **2–7**. Among this group, moving of the amino group to the *meta*-position in **6** provided the most potent analog at 1.7 μ M. The same range of activity is observed also for 4-hydroxy-benzoic acid **11**, the 3,5-difluoro-phenol **16**, 3,4-dihydroxybenzoic acid **18** and derivative **21** (K_i values in the range of 3.7–10.0 μ M). However, salicylic acid **10**, 4-cyanophenol **9**, the di-substituted phenols **14** and **15** and caffeic acid **22**, were much less effective inhibitors, with K_i values in the range of 24.2–76.0 μ M. Thus, relatively small changes in the scaffold of the phenolic compound lead to drastic changes in the inhibitory activity, with one of the best examples being the pair **21/22**, with the introduction off a second OH moiety in the scaffold of **21** leading to a consistent loss of inhibitory activity. Indeed, caffeic acid **22** is 7.6 times less effective as NgCA α inhibitor compared to **21**.
- VchCA α was inhibited by the investigated compounds with K_i -s in the range of 0.7–81.6 μ M. The most effective inhibitors were phenol **1**, 3-amino-4-chlorophenol **13** and 4-hydroxybenzyl-alcohol **12** (K_i -s of 0.7–1.2 μ M). Several other phenols, such as derivatives **3–8**, **11**, **16** and **20** showed effective micromolar inhibition, with K_i values in the range of 3.5–10.6 μ M, whereas **2**, **9**, **10**, **14**, **15**, **17–19** and **21**, **22** were less effective as VchCA α inhibitors (K_i values in the range of

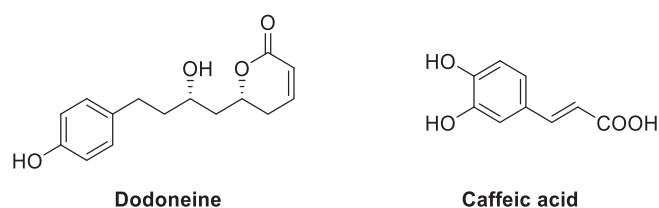


Figure 1. Phenolic derivatives dodoneine isolated from *Agelanthus dodoneifolius*, and caffeic acid, a widespread phenolic acid in many plants.

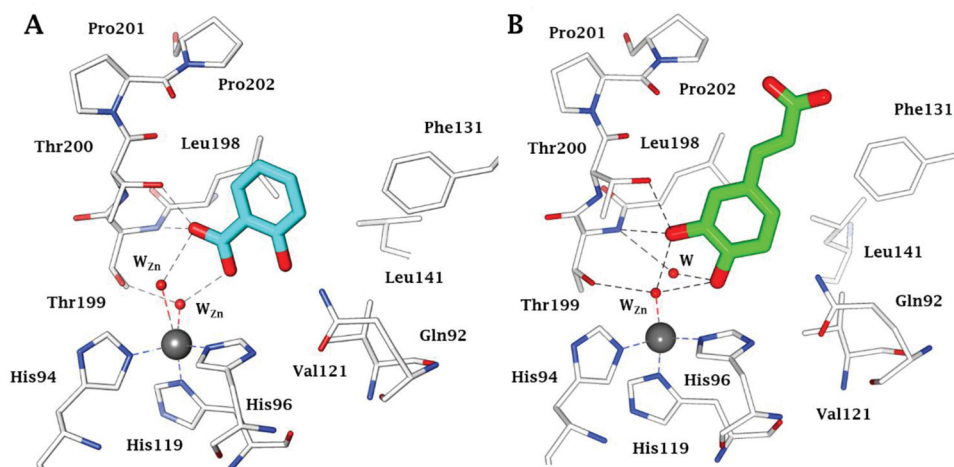
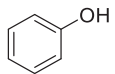
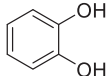
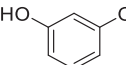
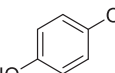
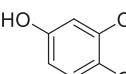
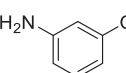
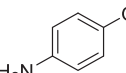
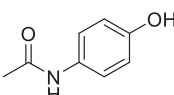
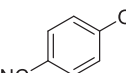
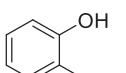
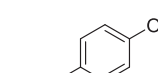
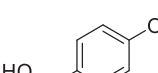
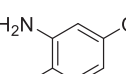
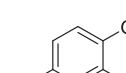
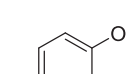


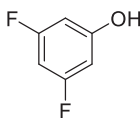
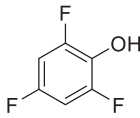
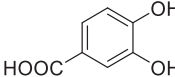
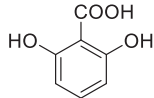
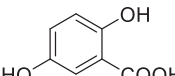
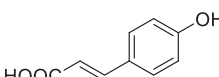
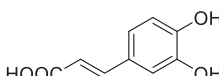
Figure 2. Active site view of hCA II in adduct with A) deacetylated aspirin (PDB 6UX1) and B) caffeic acid (PDB 6YRI). H-bonds are represented as black dashed lines. The active site zinc ion is shown as a grey sphere, and the water molecules as red spheres. Amino acid residues coordinating the metal ion or involved in inhibitor binding are also evidenced.

Table 1. Inhibition data of human CA isoforms I and II and bacterial NgCA α and VchCA α using AAZ as standard drug by a stopped-flow CO $_2$ hydrase assay method²³.

Name	Structure	K $_i$ (μ M) ^a			
		hCA I	hCA II	NgCA α	VchCA α
1		10.2	5.5	0.9	0.8
2		>100	5.5	5.3	20.3
3		>100	9.4	2.2	8.6
4		10.7	0.1	4.7	8.4
5		>100	>100	3.9	9.4
6		4.9	4.7	1.7	3.9
7		>100	>100	6.0	10.6
8		10.0	6.2	1.5	7.0
9		>100	0.1	24.2	17.2
10		9.9	7.1	43.8	39.1
11		9.8	10.6	8.7	3.5
12		68.9	95.3	0.6	1.2
13		6.3	4.9	0.8	0.7
14		57.8	57.5	71.5	81.6
15		>100	>100	35.9	36.6

(continued)

Table 1. Continued.

Name	Structure	K $_i$ (μ M) ^a			
		hCA I	hCA II	NgCA α	VchCA α
16		38.8	33.9	3.7	4.9
17		>100	>100	63.1	57.3
18		1.1	0.5	9.3	13.8
19		5.7	5.2	33.1	49.6
20		4.2	4.1	16.3	7.3
21		1.1	1.3	10.0	40.5
22		2.4	1.6	76.0	78.1
AAZ	–	0.25	0.01	0.075	0.0068

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

13.8–81.6 μ M, Table 21. Again, as for the enzyme discussed above, small structural changes in the molecule of the inhibitor lead to significant differences of the inhibitory action (for example compare the three difluorinated phenols **15**, **16** and **17**, with one isomer, **16** being an effective inhibitor – K $_i$ of 4.9 μ M – and the remaining two acting as much weaker inhibitors, with K $_i$ -s of 36.6 and 57.3 μ M, respectively).

iii. The inhibition pattern of the two bacterial enzymes was generally rather distinct, although some compounds, such as **1**, **12**, **13** and **16** showed effective inhibition for both of them. However, in most cases, the NgCA α was more susceptible to phenol containing analogs than VchCA α (compare **2**, **8**, **19** or **21** for their K $_i$ s). These data points suggest that presumably it may be possible to obtain both phenols that show a wide action against various bacterial CAs, but also selective bacterial CA inhibitors belonging to the phenol and phenolic acid classes of derivatives.

iv. There were relevant differences of inhibitory activity of the investigated phenols towards the human over the bacterial enzymes (Table 1). Few of the investigated derivatives (e.g. **18**, **21**, **22**) showed effective hCA I inhibitory activity while moderately inhibiting the bacterial enzymes. Most derivatives were on the other hand poor or ineffective hCA I inhibitors (e.g. **2**, **3**, **5**, **9**, **12**, **14**, **15**–**17**). hCA II was effectively inhibited by **4**, **9**, **18** and **20**–**22**, with few compounds being low activity or inactive as inhibitors (**5**, **7**, **12**, **14**–**17**) and most of

them being moderate, micromolar inhibitors. However, even on this small panel of tested phenols, some compounds showed selectivity for inhibiting the bacterial over the human isoforms, such as for example **12**, and to a lower extent **13**. In fact 4-hydroxy-benzylalcohol has a selectivity index for inhibiting NgCA α over hCA I of 114.8 and over hCA II of 158.8. For VchCA α , the selectivity ratios are of 57.4 (bacterial enzyme over hCA I) and of 79.4 (bacterial enzyme over hCA II). It should be mentioned that benzylalcohol, a compound structurally similar to **12**, was crystallised in adduct with hCA II, and it binds to the enzyme in a similar manner to phenol, by anchoring with the OH group to the zinc-coordinated water molecule²⁶.

4. Conclusions

We report a study of bacterial CA inhibition with simple phenols and few phenolic acids. Two α -class enzymes from bacterial pathogens which developed drug resistance to classical antibiotics, i.e. NgCA α from *Neisseria gonorrhoeae* and VchCA α from *Vibrio cholerae* were included in the study. The panel of 22 phenols and phenolic acids inhibited both enzymes with K_i values in the range of 0.6–76.0 μ M for NgCA α , and of 0.6–76.0 μ M for VchCA α . The best NgCA α inhibitors were phenol, 3-aminophenol, 4-hydroxy-benzylalcohol, 3-amino-4-chlorophenol and paracetamol, with K_i s of 0.6–1.7 μ M. The most effective VchCA α inhibitors were phenol, 3-amino-4-chlorophenol and 4-hydroxy-benzylalcohol, with K_i s of 0.7–1.2 μ M. Small changes in the phenol scaffold led to drastic effects on the bacterial CA inhibitory activity. This class of underinvestigated bacterial CA inhibitors may thus lead to effective compounds for fighting drug resistant bacteria.

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