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Carbonic anhydrase inhibitors. Identification of selective inhibitors of the human mitochondrial isozymes VA and VB over the cytosolic isozymes I and II from a natural product-based phenolic library

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ABSTRACT

We have investigated the enzyme inhibition characteristics of a natural product (NP)-based phenolic library against a panel of human carbonic anhydrases (hCAs, EC 4.2.1.1) which included hCAs I and II (cytosolic) and hCA VA/VB (mitochondrial isoforms). Most of these compounds were weak, micromolar inhibitors of the two cytosolic hCAs (K_Is >10 μM) but showed good hCA VA/VB inhibitory activity with inhibition constants in the range of 70–125 nM. The selectivity ratios for inhibiting the mitochondrial over the cytosolic isoforms for these phenol derivatives were in the range of 120–3800, making them the most isoform-selective compounds for inhibiting hCA VA/VB known to date. The CA VA/VB enzymes are involved in biosynthetic processes such as gluconeogenesis, lipogenesis and ureagenesis, and no pharmacological inhibitors with good selectivity are currently available. Thus the NP inhibitors identified during these studies are excellent leads for obtaining even more effective compounds that selectively target mitochondrial hCAs, and also have the potential to be used as tools for understanding the physiological processes that are regulated by the two mitochondrial CA isoforms.

1. Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) are thoroughly investigated metalloenzymes due to their involvement in many physiologic and pathologic processes.[1,2] The 16 different isoforms described so far in mammals, including *Homo sapiens*, are involved in pH and CO_2 homeostasis, respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (e.g., gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity, and many other processes.[1–5] The Zn(II) ion of CAs is critical for the catalytic cycle and inhibition of these enzymes, being coordinated by three His residues and a water molecule/hydroxide ion, which acts as a nucleophile in the conversion of carbon dioxide to bicarbonate and a proton, the physiological reaction catalyzed by these enzymes.[1–3] Inhibition of hCAs has been used in clinical medicine for more than 55 years,[2] with the main class of inhibitors being sulfonamide derivatives and their bioisosteres, such as the sulfamates, sulfamides, etc.[1–5] Sulfonamides and sulfamates bind to the Zn(II) ion within the CA active site, displacing the water molecule/hydroxide ion.[1–3]

Sulfanilamide **SA**, is a simple sulfonamide used as lead compound for obtaining many sulfonamide pharmacological agents with a variety of uses, such as antibacterials, diuretics, protease inhibitors, anticancer drugs, etc.[1–3] Sulfonamide CA inhibitors (CAIs) are used as diuretics, antiglaucoma, anticonvulsant, antiobesity and antitumor drugs/diagnostic agents, and different isoforms are targeted for diverse applications.[1–3] Examples of CAIs that are currently used in the clinic include, acetazolamide **AAZ**, zonisamide **ZNS** and the sulfamate topiramate **TPM**.[3] However, the lack of selectivity for the target isozyme constitutes a main problem with the sulfonamide/sulfamate CAIs,[2] and this led to the search of different chemotypes which may show potent enzyme inhibitory activity and a more selective inhibition profile. Indeed, we recently reported a novel class of CAIs belonging to a new chemotype, the coumarins, which act as suicide inhibitors, being converted to 2-hydroxycinnamic acid derivatives within the enzyme active site, and deciphered in detail the inhibition mechanism of these compounds.[6]

However, an interesting and different chemotype, scarcely investigated for its interaction with CAs, is constituted by the phenols. Christianson's group reported the X-ray structure of the simple phenol, PhOH with hCA II,[7] showing that this compound binds in an unprecedented way within the enzyme active site (Fig. 1), being anchored by means of the phenolic OH moiety to the zinc-bound water molecule/hydroxide ion, through a hydrogen bond.

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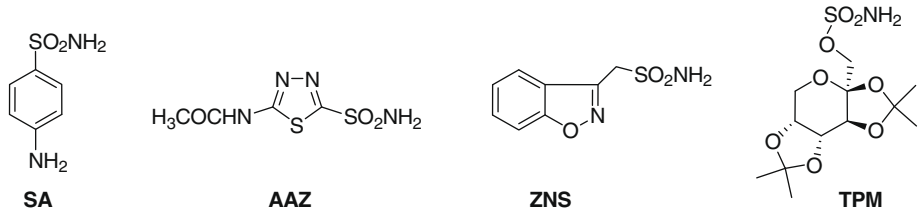

A second hydrogen bond is formed between the same OH moiety of the inhibitor and the NH amide of Thr199, an amino acid residue critical for the catalysis and inhibition of CAs.[1,2,6,7] More recently, our group explored various classes of simple phenols, substituted derivatives of PhOH or naphthols, for their interactions with all mammalian CA isoforms, CA I–XV, and detected some compounds showing low micromolar or submicromolar affinity for some of these enzymes.[8] The inhibition profile of phenols against various CA isozymes differ significantly from those of sulfonamides and their bioisosteres (sulfamates, sulfamides, etc), which most of the time are promiscuous inhibitors,[1] hence identifying that phenols have desirable properties for putative pharmacological applications. However, few phenols, especially those produced by nature have been investigated in detail up to now. Due to the unique chemical diversity associated with natural products,[18] and the under investigation of phenol-based secondary metabolites, we chose to study the interaction of a small natural product inspired phenolic library with the ubiquitous, cytosolic isoforms CA I and II (off-targets) as well as with the mitochondrial isozymes CA VA and CA VB. The latter enzymes have been recognised as potential targets for designing antiobesity agents with a novel mechanism of action.[19]

2. Results and discussion

2.1. Chemistry

As part of the on-going collaborative research effort between our two groups, a library of phenolic NPs and their semi-synthetic derivatives (**1–26**) were considered for CA inhibitory investigations. The common structural feature of compounds **1–23** is the presence of at least one phenolic moiety, which has the potential to act as a CA active site anchoring group, either directly to the zinc or indirectly through the zinc-bound water molecule, similarly to the unsubstituted phenol described above.[7] Compounds **24–26** on the other hand are simple NP carboxylic acids or their derivatives, and they were included in the study because they may show the same binding pattern as the coumarins, recently discovered CAIs,[6] which bind as 2-hydroxy-cinnamic acids (coumarin hydrolysis products) at the entrance of the CA II active site, blocking thus the access of the substrate to the critically important Zn(I) ion, situated at the bottom of the active site cavity.[6] Compounds **1–26** were either purified from endophytic fungi fermentations and plant extracts or were obtained via total synthesis and combinatorial chemistry studies.[9–17] Specifically the library included a series of simple mono- or disubstituted phenols **1–6**,[9,10] eight semi-synthetic amide derivatives **7–14**,[10] (−)-xylariamide A **15**[12] and its synthetic enantiomer (+)-xylariamide A **16**,[12] polyandrocarpamines A **17** and B **18**,[13] xanthones **19** and **20**,[14] endiandrins A **21**,[15] and B **22**,[16] and (−)-dihydroguaiaretic acid **23**.[15] Synthetic amides **7–14** are based on the NP scaffold present in the fungal metabolite, 4-hydroxy-3-chlorophenylacetamide **5**.[10] The synthesis[17] and bovine CA II (bCA II) inhibitory data[11] for **7–14** have been previously reported, and compound **11** was identified as a potent inhibitor of bCA II with a K_I of 77.4 nM. None of the other compounds (**1–6, 15–26**) have been screened for CA inhibitory effects up until now. It is thus of significant interest to investigate these NP-based phenols for their interactions with various α-CA isozymes with medicinal chemistry applications. Specifically, we investigated this library against the mitochondrial isoforms hCA VA and VB (involved in gluconeogenesis, ureagenesis and lipogenesis)[1,2,19,20] as well as the ubiquitous cytosolic isoforms hCA I and II (possible off-targets) (see Fig. 2).

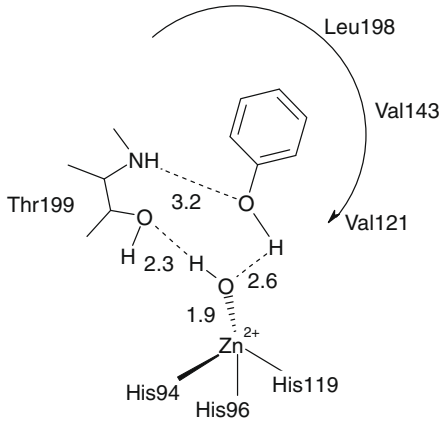

Figure 1. The binding of phenol to the hCA II active site. Figures represent distances (in Å), as reported by Nair et al.[7]

2.2. CA inhibition

Inhibition data against human CA isozymes hCA I, II (cytosolic), hCA VA and VB (mitochondrial) with compounds **1–26** are reported in Table 1 and were obtained using a stopped-flow, CO_2 hydrase assay,[21] monitoring the physiologic reaction in which these enzymes participate. The following should be noted regarding the inhibition data of Table 1.

2.2.1. hCA I inhibition

Compounds **7–10, 13, 14** and **17** inhibited hCA I similarly to phenol (K_I = 10.1 μM) with K_Is in the range of 9.6–11.9 μM. Other NPs were weaker inhibitors with K_Is in the range of 158–430 μM. Compound **11**, with a submicromolar K_I of 0.70 μM, was a notable exception, albeit consistent with the presence of a sulfonamide moiety in this semi-synthetic NP derivative, the sulfonamide being a proven effective zinc binding function in α-CAs. Interestingly, compound **11** shows better hCA I inhibitory activity than **SA** (K_I of 25 μM), but is a slightly weaker inhibitor than **AAZ**, **TPM** and **ZNS** (K_Is of 0.056–0.25 μM). Carboxylates **24–26** were very weak hCA I inhibitors (K_Is of 244–395 μM), whereas the sulfonamide/sulfamate drugs showed more potent activity (K_Is of 56–250 nM).

2.2.2. hCA II inhibition

hCA II was generally better inhibited by compounds **1–26** than hCA I, with activity in the low micromolar range for many compounds and similar to that of phenol (K_I of 5.5 μM). NPs **5** and **23**, whilst also better hCA II than hCA I inhibitors, showed

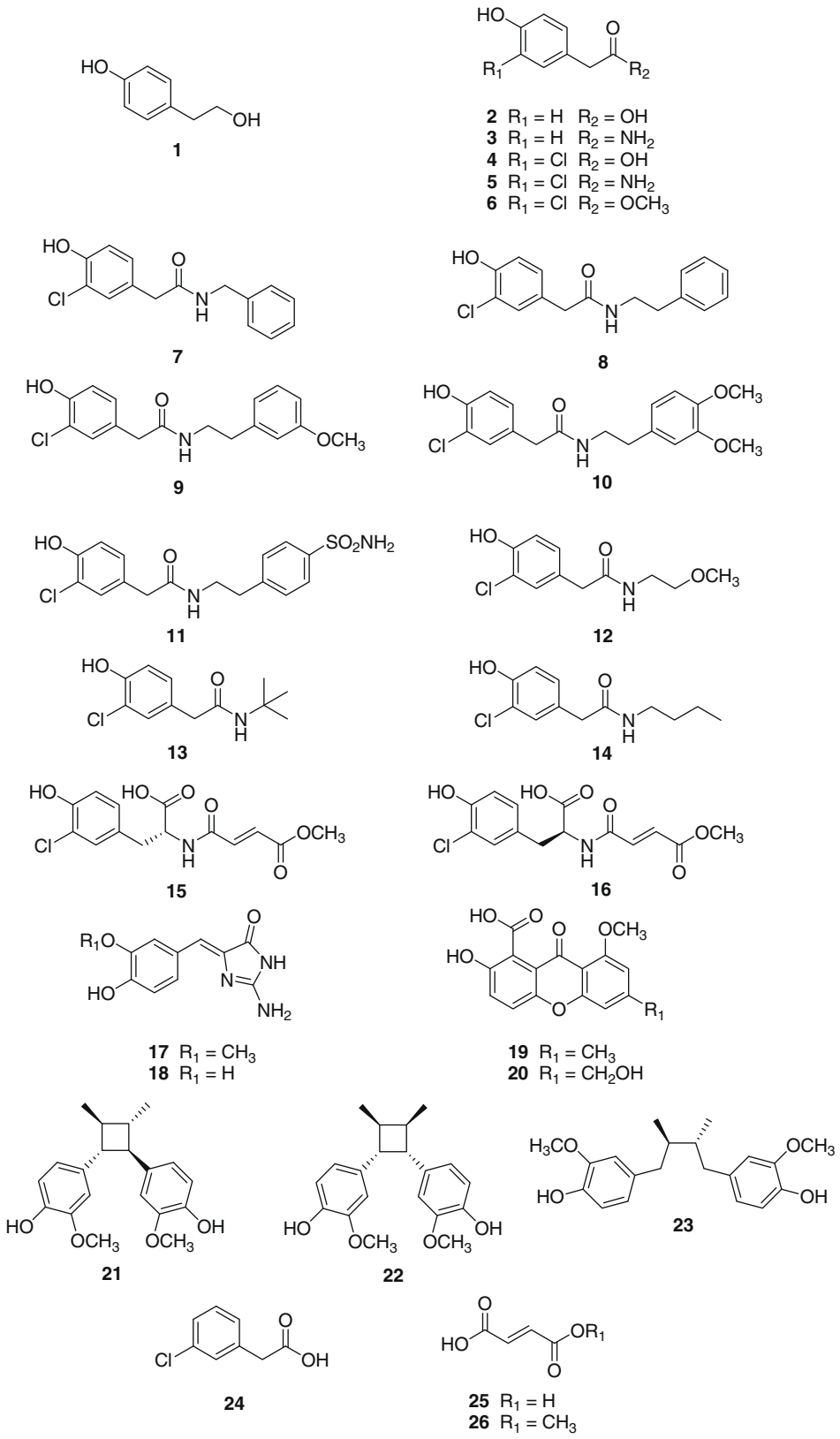

Figure 2. Library of NP-based phenols.

only weak hCA II inhibitory activity (K_Is of 131 and 230 μM, respectively) compared to the remainder of the NP-based library members. It is interesting to note that minimal structural changes can lead to significant differences of CA inhibitory properties. For example, **4** and its methyl ester **6**, while structurally related to amide **5**, were much stronger hCA II inhibitors than **5** (15- and 12-fold, respectively) indicating a potential avenue for further SAR exploration. Also of note is that the more flexible

Table 1
Inhibition data of hCA isozymes I, II (cytosolic), hCA VA and VB (mitochondrial) with compounds **1–26** and standard inhibitors

Compound	K_I*			
	hCA I (μM)	hCA II (μM)	hCA VA (nM)	hCA VB (nM)
1	430 ± 21	8.7 ± 0.6	101 ± 4	105 ± 3
2	309 ± 11	10.3 ± 0.8	99 ± 6	107 ± 6
3	309 ± 14	11.2 ± 1	92 ± 5	81 ± 2
4	265 ± 8	8.6 ± 0.7	100 ± 6	118 ± 5
5	237 ± 9	131 ± 5	110 ± 4	106 ± 7
6	369 ± 16	10.7 ± 0.9	109 ± 3	125 ± 8
7	10.5 ± 0.2	11.4 ± 1	96 ± 6	85 ± 1
8	9.6 ± 0.4	9.8 ± 0.6	101 ± 7	91 ± 4
9	11.2 ± 0.9	10.8 ± 0.9	103 ± 4	87 ± 6
10	11.9 ± 1.0	11.5 ± 1	94 ± 2	79 ± 3
11	0.70 ± 0.05	0.018 ± 0.002	93 ± 5	69 ± 2
12	158 ± 8	10.4 ± 0.9	102 ± 4	84 ± 5
13	11.4 ± 0.9	10.8 ± 0.6	105 ± 6	89 ± 7
14	10.7 ± 0.6	9.4 ± 0.6	108 ± 8	81 ± 1
15	239 ± 11	8.3 ± 0.4	95 ± 3	114 ± 4
16	231 ± 10	8.0 ± 0.5	108 ± 7	102 ± 2
17	10.5 ± 0.4	9.6 ± 0.2	99 ± 3	70 ± 4
18	355 ± 15	13.1 ± 1.5	101 ± 5	76 ± 2
19	201 ± 7	8.4 ± 0.5	93 ± 4	103 ± 3
20	374 ± 18	9.2 ± 0.4	94 ± 6	102 ± 5
21	368 ± 13	11.7 ± 0.8	91 ± 2	69 ± 1
22	354 ± 14	12.1 ± 1	98 ± 5	79 ± 5
23	307 ± 9	230 ± 17	85 ± 3	71 ± 4
24	244 ± 8	10.1 ± 1	96 ± 6	85 ± 7
25	395 ± 18	10.5 ± 0.7	97 ± 4	90 ± 6
26	284 ± 8	7.5 ± 0.3	103 ± 1	98 ± 7
PhOH[a]	10.2 ± 0.3	5.5 ± 0.2	55,100 ± 350	4200 ± 27
SA[b]	25.0 ± 1.5	0.24 ± 0.03	32,000 ± 500	3650 ± 20
AAZ[b]	0.25 ± 0.01	0.012 ± 0.001	63 ± 5	54 ± 3
TPM[c]	0.25 ± 0.03	0.010 ± 0.002	63 ± 4	30 ± 0.7
ZNS[d]	0.056 ± 0.005	0.035 ± 0.004	20 ± 0.9	6033 ± 41

[a] From Ref. 8b.
[b] From Ref. 22.
[c] From Ref. 23.
[d] From Ref. 24.
* Human recombinant proteins obtained in-house. Mean ± standard error (n = 3).

lignan, (−)-dihydroguaiaretic acid **23**, showed 19-fold weaker hCA II inhibitory activity compared to the more rigid cyclobutane lignans, endiandrins A **21** and B **22**. As for hCA I, the most active hCA II inhibitor is the sulfonamide **11** with a K_I of 18 nM, this compound has inhibition comparable to the clinically used compounds **AAZ** and **TPM** (K_Is of 10 and 12 nM, respectively). Compound **11** has been shown earlier by one of our groups[21] to also act as an efficient inhibitor of the bovine enzyme ortholog to hCA II, namely bCA II. Carboxylic acid derivatives **24–26** showed similar activity with phenols **1–23**, with K_Is in the range of 7.5–10.5 μM (Table 1).

2.2.3. hCA VA inhibition

The simple phenol PhOH is an extremely weak hCA VA inhibitor (K_I of 55.1 μM)[8b] whereas all NP derivatives **1–23** investigated here, surprisingly show orders of magnitude better inhibitory activity, with K_Is in the range of 85–108 nM. No clear-cut SAR can be evidenced except for this excellent trend of inhibition of the mitochondrial enzyme with all these NPs, which show a very compact behavior. As only an X-ray crystal structure of a truncated form of hCA VA is available, lacking the first 54 amino acid residues which form a substantial part of the active site,[1] it is impossible to rationalize the obtained inhibition data with these phenols. Furthermore, no hCA VA—inhibitor adducts have been reported up to now.[1b] It is also interesting to note that the carboxylates **24–26** showed comparable hCA VA inhibitory activity with phenols **1–23**. Sulfanilamide **SA** was a very weak hCA VA inhibitor, whereas the sulfonamide/sulfamate clinically used compounds (**AAZ, TPM** and **ZNS**) acted as much stronger inhibitors (K_Is of 20–63 nM).[22–24]

2.2.4. hCA VB inhibition

The second mitochondrial isoform, hCA VB[22] was again weakly inhibited by PhOH (K_I of 4.2 μM) but much more susceptible to inhibition with the NP derivatives **1–26**, which showed K_Is in the range of 69–125 nM (Table 1). Thus, again the SAR is rather flat, as for hCA VA. The best hCA VB inhibitors were the sulfonamide **11** and the diphenol **21**, which both displayed K_I values of 69 nM, although it would be predicted that these two compounds would possess different inhibition mechanisms. Indeed, presumably the sulfonamide binds to the Zn(II) ion within the enzyme active site as the fourth ligand,[1] whereas the phenol **21** is probably bound to the Zn(II)-coordinated water molecule within the enzyme active site cavity (similarly to the simple phenol).[8] These data prove thus that it is possible to obtain equipotent CAIs possessing different inhibition mechanism, as the compounds **11** and **21** showed inhibitory activity similar to the clinically used drugs **AAZ** and **TPM** (K_Is of 30–54 nM).

2.2.5. Selectivity issues for the inhibition of the mitochondrial over the cytosolic isozymes

As seen from data of Table 1, the NP-based library **1–26** generally inhibits the mitochondrial isozymes hCA VA and hCA VB with K_Is in the range of 70–100 nM, whereas the cytotolic isoform hCA I is inhiibted with K_Is in the range of 10–350 μM (except the sulfonamide **11**) and hCA II K_Is in the range of 7.5–230 μM (except the sulfonamide **11**). Thus, it is clear that many of the investigated phenols show a promising selectivity ratio for the inhibition of the mitochondrial over cytosolic isoforms. These data could be further exploited in the development of more selective and potent hCA VA/VB small molecules. Specific hCA VA/VB inhibitors have not been reported to date,[2] however their availability to researchers could potentially assisted in the better understanding of the physiological role of these two mitochondrial CAs. For example, compound **3** has a selectivity ratio of 3358 for the inhibition of hCA VA over hCA I, of 121 for the inhibition of hCA VA over hCA II, of 3814 for the inhibition of hCA VB over hCA I and of 138 for the inhibition of hCA VB over hCA II, respectively. This selectivity data identifys **3** as one of the most hCA VA/VB-selective inhibitors described so far.[25] The same can be observed for many other compounds investigated here, thus providing an entire series of interesting leads for future mitochondrial hCA inhibition research.

3. Conclusions

We have investigated the enzyme inhibition characteristics of a phenolic-based NP library (compounds **1–26**) against a panel of CAs encompassing the human hCAs I and II (cytosolic enzymes) and hCA VA/VB (mitochondrial isoforms). Most of these comnpounds were weak, micromolar inhibitors of the two cytosolic isoforms but showed good hCA VA/VB inhibitory activity with inhibition constants in the range of 70–125 nM. The selectivity ratios for inhibiting the mitochondrial over the cytosolic isoforms for these phenol NPs were thus in the range of 120–3800, making them the most isoform-selective compounds for inhibiting hCA VA/VB. These particular enzymes are known to be involved in biosynthetic processes such as gluconeogenesis, lipogenesis and ureagenesis, and no small molecule inhibitors with good selectivity have been reported up until now. The bioactive and selective natural products reported in this paper are thus excellent leads that could be further exploited in the development of more selective and potent hCA VA/VB agents.

4. Experimental part

4.1. Chemistry

The isolation or synthesis of compounds **1–26** have been described earlier.[10–17] All compounds were analyzed for purity by C_{18} μPLC and shown to be >95%. This microfluidic purity analysis methodology has been previously reported.[17] Sulfonamides used as standards in the enzymatic assay were from Sigma–Aldrich (Milan, Italy).

4.2. CA inhibition

The assay of the CA catalysed CO_2 hydration activity and inhibition has been done by the Khalifah method[21] as described earlier.[1,6,7]

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