Bioorganic & Medicinal Chemistry Letters 18 (2008) 3593–3596

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Carbonic anhydrase inhibitors: Inhibition of the new membrane-associated isoform XV with phenols

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ARTICLE INFO

Article history:
Received 8 April 2008
Revised 30 April 2008
Accepted 30 April 2008
Available online 4 May 2008

Keywords:
Carbonic anhydrase
Isoform XV
Phenol
Enzyme inhibitor
Paracetamol
Salicylic acid

ABSTRACT

Inhibition of the newest isoform of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), CA XV, with a series of phenols was investigated. Murine CA XV showed an inhibition profile by phenols distinct of those of the cytosolic human isoforms CA I and II. Phenol and some of its 2-, 3-, and 4-substituted derivatives incorporating hydroxy, fluoro, carboxy, and acetamido moieties were effective CA XV inhibitors, with inhibition constants in the range of 7.20–11.30 μM, whereas compounds incorporating 4-amino-, 4-cyano, or 3-hydroxy groups were less effective (K_Is of 335–434 μM). The best phenol inhibitor was clioquinol (K_I of 2.33 μM). Phenols show a different inhibition mechanism as compared to sulfonamides and their isosteres, and may lead to the design of compounds with selectivity for inhibiting different CA isozymes with medicinal chemistry applications.

Phenol was discovered to be a carbonic anhydrase (CA, EC 4.2.1.1) inhibitor (CAI) by Lindskog who investigated its interaction with the predominant cytosolic, human isoform II (hCA II).[1] In a very elegant study, Christianson's group then reported the X-ray crystal structure for the adduct of this isozyme with phenol,[2] showing the inhibitor to bind in a completely unprecedented manner as compared to other classes of CAIs, such as the inorganic anions or the sulfonamides and their isosteres,[3–9] with the phenol OH moiety hydrogen-bonded to the zinc-bound water/hydroxide ion of the enzyme as well as to the NH amide of Thr199 (an amino acid conserved in all α-CAs).[3,4] Furthermore, the phenyl moiety of phenol was found to lay in the hydrophobic part of the hCA II active site, where presumably the substrate of these enzymes, CO_2, also binds in a precatalytic complex (Fig. 1), explaining thus the behavior of unique CO_2 competitive inhibitor of this compound.[1,2] Very recently, we investigated[10] the inhibition of all active mammalian α-CA isoforms, that is, CA I–CA XIV with phenol and two structurally related derivatives (3,5-difluorophenol and the clinically used 8-hydroxyquinoline derivative clioquinol), evidencing micromolar or submicromolar inhibition for some of these CA isozymes with the three investigated compounds. Considering that many CAs are targets for the drug design of ophthalmic, diuretic, anticonvulsant, antitumor, or antiinfective agents,[3,4] inhibition with phenols may represent a new approach to design CAIs different of the classical sulfonamides, sulfamates, or sulfamides. Indeed, all these compounds, some of which are clinically used for decades,[3,4] possess the SO_2NH_2 zinc binding group, which coordinates in deprotonated state to the Zn(II) ion within the CA active site.[11,12] On the contrary, as mentioned above, phenols show a different inhibition mechanism, as they bind to the zinc-bound water and one of the

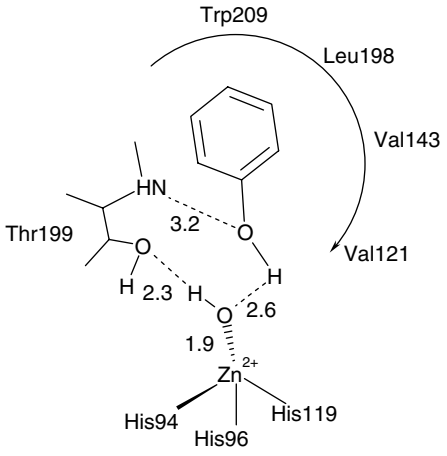

Figure 1. Schematic representation for binding of phenol to the hCA II active site as determined by X-ray crystallography[2] (figures represent distances in Å; hydrogen bonds are represented as dashed lines).

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doi:10.1016/j.bmcl.2008.04.077

gate-keeper residues (Thr199) of the enzyme, as illustrated schematically in Figure 1.

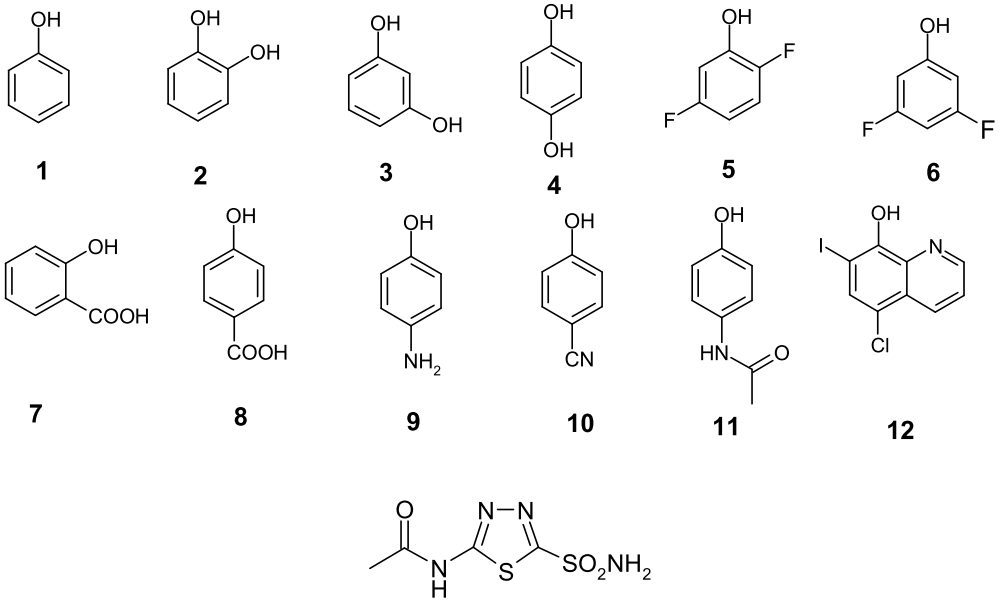
Isoform XV is the last mammalian CA to be discovered in 2005 by Hilvo et al.[13] who showed it to be a membrane-associated isoform, with an extracellular active site, similarly to CA IV. Unlike other isozymes, CA XV appears to be a unique member of the CA family because the gene encoding it has become a non-processed pseudogene in humans and chimpanzees.[13] However, several other vertebrates possess an active gene coding for CA XV. Sequence analysis revealed that at least the dog (*Canis familiaris*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), chicken (*Gallus gallus*), frog (*Xenopus tropicalis*) as well as three fish species (*Danio rerio*, *Fugu rubripes*, and *Tetraodon nigroviridis*) possess an active CA XV. Thus, CA XV has been conserved throughout evolution but has become an inactive gene quite recently in terms of the evolutionary timescale, being absent only in primates.[13,14] Recombinant mouse CA XV (mCA XV) was recently produced in the baculovirus expression system in order to study its kinetic properties.[14] For the physiological, CO_2 hydration reaction, mCA XV showed a k_{cat} of 4.7×10^5 s^{-1}, K_M of 14.2 mM, and k_{cat}/K_M of 3.3×10^7 M^{-1} s^{-1} (at pH 7.5 and 20 °C).[14] The activity of mCA XV is thus comparable to those of other extracellular human isoforms, such as CA XII and XIV.[3,14] No inhibition studies of this isoform are available with any class of CAI, except for the acetazolamide data reported by us earlier (acetazolamide, the classical CAI *par excellence*, showed a K_I of 72 nM against mCA XV).[14] Here we report an inhibition study of mCA XV with a series of phenols. Considering the fact that we recently reported[10] the phenol inhibition data with all other mammalian isoforms (CA I–XIV) it appeared of interest to explore the behavior of isoform CA XV with this class of relatively uninvestigated modulators of enzyme activity. We included in addition to simple phenol **1** some of its 2-, 3- and 4-substituted derivatives (diphenols **2**–**4**, as well as compounds incorporating carboxy-, amino-, acetamido-, and cyano- moieties), and 2,5- and 3,5-difluoro-substituted compounds (**5** and **6**). Clioquinol **12**, investigated for its interactions with other isoforms earlier,[10] was the only bicyclic phenol included in our study. Some of the investigated derivatives such as salicyclic acid **7**, paracetamol **11**, and clioquinol **12** are clinically used drugs.[15,16]

Table 1
Inhibition of CA isoforms I, II, and XV (of human, h; and murine, m origin) with phenols **1**–**12** and acetazolamide **13**[17]

Compound	K_I[#] (μM)		
	hCA I[*]	hCA II[*]	mCA XV[*]
1	10.2[a]	5.5[a]	10.5
2	4003	9.91	10.2
3	795	7.70	385
4	10.7	0.090	10.6
5	134	870	212
6	38.8[a]	33.9[a]	11.3
7	9.92	7.12	7.20
8	9.80	10.6	9.32
9	159	752	335
10	131	0.108	434
11	10.0	6.20	9.23
12	6.6[a]	6.5[a]	2.33
13	0.25	0.012	0.072

[a] From Ref. 10.
[*] h, human; m, murine isozyme.
[#] Errors in the range of ±5% of the reported data from three different assays.

Inhibition data against CA isozymes I, II, and XV with phenols **1**–**12** as well as acetazolamide **13**, as standard, for comparison, are shown in Table 1.[17] The hCA I and II inhibition data with these phenols are also provided, in order to compare the inhibition of the new isoform (CA XV) with those of the cytosolic, widespread members of this family, hCA I and II.[3] Most of these inhibition data are also new, as we reported earlier the inhibition of CA I and II only with phenols **1**, **6**, and **12**.[10] The following should be observed from data of Table 1: (i) against isozyme hCA I phenols **2**, **3**, **5**, **9**, and **10** showed a behavior of weak inhibitors, with K_Is in the range of 131–4003 μM. The remaining compounds showed a rather compact behavior of much better inhibitors, with K_Is in the range of 6.6–10.7 μM, except for 3,5-difluorophenol **6**, which was a weaker inhibitor (K_I of 38.8 μM). It is thus clear that quite minor structural changes in the molecule of a phenol lead to drastic changes in its CA I inhibition properties. For example, an additional *ortho* OH moiety, as in pyrocatechol **2**, leads to a dramatic loss of inhibitory power as compared to phenol **1** (**2** is 392.4 times less inhibitory than **1**).



13 acetazolamide

When the additional phenolic moiety is placed in the *meta*-position, as in resorcinol **3**, the loss of activity is only of 77.9 times as compared to **1**, whereas hydroquinone **4** with the second OH moiety in *para* has basically the same activity as phenol **1**. Although another *ortho*-substituted compound (i.e., **5**) showed a weak CA I inhibitory activity, salicyclic acid **7** was equipotent to phenol **1** or to its *para*-substituted isomer **8**. However, a *p*-amino group, as in **9**, leads to a 14.8 times weaker inhibitor as compared to the corresponding *p*-hydroxy substituted compound **4**. On the other hand its acetylation, as in paracetamol **11**, restored activity, leading to an effective CA I inhibitor. Indeed, this drug possesses equivalent potency as CA I inhibitor with phenol **1** or hydroquinone **4**. However, the 4-cyano-substituted derivative **10** was again a less effective CA I inhibitor. The best CA I inhibitor in this series of compounds was clioquinol **12** (K_I of 6.6 μM), anyhow weaker than acetazolamide **13**, which showed an inhibition constant of 0.25 μM. It is thus clear that this new class of CAIs, the phenols, shows a rather complicated structure–activity relationship (SAR), with even small structural changes leading to a great variation in the biological activity; (ii) weak inhibitory activity against the ubiquitous, clinically relevant isoform hCA II was observed with phenols **5** and **9** (K_Is of 752–870 μM), whereas the other difluorosubstituted compound, **6**, was a medium potency inhibitor (K_I of 33.9 μM). Most of the investigated phenols (i.e., **1**–**3**, **7**, **8**, **11**, and **12**) were efficient, micromolar CA II inhibitors, with inhibition constants in the range of 5.5–10.6 μM, but two compounds, hydroquinone **4** and 4-cyanophenol **10**, showed much better inhibitory activity, with K_Is in the range of 90–108 nM (Table 1). Acetazolamide **13** remains the best CA II inhibitor among the investigated derivatives (K_I of 12 nM), but it was only 7.5 times more effective than hydroquinone **4**, the best phenol CA II inhibitor detected so far. Thus, a 4-substituent of the OH or CN type on the phenyl moiety is quite effective in inducing robust CA II inhibitory properties to the phenol class of CAIs, an issue which is being investigated in our laboratory by means of X-ray crystallography in order to understand the molecular features responsible for this good activity. As for the discussion above on the inhibition of hCA I, SAR is very sensitive to minor structural changes in the scaffold of the investigated phenols. However, the differences between the *ortho*- and *meta*-substituted diphenols **2** and **3** are not so large as for the CA I inhibition (as compared to the parent derivative **1**), whereas the *para*-disubstituted phenol **4** was the best CA II inhibitor, as stressed above, with a 61-fold gain in potency against hCA II over the simple derivative **1**. It is also interesting to note that 4-cyanophenol **10** was quite ineffective as a hCA I inhibitor but showed good inhibition for hCA II, with a difference of potency of 1212 times between the two isozymes. Correlated to the fact that **10** is also a quite weak CA XV inhibitor (see discussion later in the text), 4-cyanophenol can be considered a very selective, medium potency CA II inhibitor (such an inhibition profile was not evidenced so far for any other class of CAIs).[3,4] Clioquinol **12**, paracetamol **11**, and salicyclic acid **7**, all clinically used derivatives, showed effective, micromolar affinity for this ubiquitous isoform (K_Is in the range of 6.2–7.7 μM). No literature data are available so far regarding a possible influence of CA II binding on the pharmacology of these derivatives, but such studies are warranted, considering the wide use of salicylates and paracetamol as painkillers or in the management of fever[16]; (iii) similarly to the cytosolic isozymes I and II, mCA XV was also inhibited by all phenols **1**–**12** investigated here. Derivatives **3**, **5**, **9**, and **10** showed weak binding to this enzyme, with inhibition constants in the range of 212–434 μM, whereas the remaining compounds were better inhibitors, with K_Is in the range of 2.33–11.3 μM (Table 1). The inhibition profile of this isozyme with phenols **1**–**12** and the SAR are quite distinct of those discussed above for isoforms I and II. Thus, phenol **1** has an inhibition constant against mCA XV very similar to that shown against hCA I, of around 10 μM. Furthermore, pyrocatechol **2** and hydroquinone **4** showed very similar inhibitory activities with the parent compound **1**, whereas resorcinol **3** was much less effective as a CA XV inhibitor. This is in marked contrast with the behavior of these three diphenols against both CA I and II (Table 1). Again the 4-amino-substituted compound **9** was among the least effective CA XV inhibitors (as for CA I and II), whereas its acetylation led to a much better inhibitor, **11**. Compound **6** is interesting as it showed good CA XV inhibitory power (K_I of 11.3 μM) and less effective binding to CA I and II (K_Is in the range of 33.9–38.8 μM). Clioquinol was the best CA XV inhibitor among the investigated phenols (as for CA I), but acetazolamide showed a K_I of 72 nM, proving that the sulfonamide zinc binding group is more effective for generating nanomolar CA XV inhibitors as compared to the phenol OH moiety. However, the low micromolar inhibition observed with many of the investigated simple phenols is very promising for eventually designing compounds with a more complicated scaffold, which may lead to low nanomolar CA XV inhibitors. Indeed, the bicyclic clioquinol is already 4.5 times more effective as CA XV inhibitor as compared to the simplest phenol, compound **1**.

In conclusion, we investigated the binding of some phenols to the least investigated mammalian CA isoform, CA XV. mCA XV has an inhibition profile by phenols distinct of those of the cytosolic isoforms CA I and II. Phenol and some of its 2-, 3-, and 4-substituted derivatives incorporating hydroxy, fluoro, carboxy, and acetamido moieties were effective CA XV inhibitors, with inhibition constants in the range of 7.20–11.30 μM, whereas compounds incorporating 4-amino-, 4-cyano, or 3-hydroxy groups were less effective (K_Is of 335–434 μM). The best phenol inhibitor was clioquinol (K_I of 2.33 μM).

Acknowledgments

This research was financed in part by two grants of the 6th Framework Programme of the European Union (EUROXY and DeZnIT projects).

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