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Carbonic anhydrase inhibitors: Inhibition of mammalian isoforms I–XIV with a series of substituted phenols including paracetamol and salicylic acid

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ABSTRACT

Inhibition of 12 mammalian isoforms of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1), CA I–XIV, with a series of phenols was investigated. The inhibition profile by phenols of these CAs was distinct from those of the sulfonamides and their isosteres, the main class of clinically used inhibitors. Phenol and some of its 2-, 3- and 4-substituted derivatives incorporating hydroxy-, fluoro-, carboxy-, amino-, cyano- and acetamido-moieties were generally effective low micromolar CA inhibitors, with inhibition constants in the range of 9.8–4003 μM against CA I, of 0.090–870 μM against CA II, of 0.71–885 μM against CA III, of 9.5–809 μM against CA IV, of 8.7–867 μM against CA VA, of 4.2–649 μM against CA VB, of 11.4–658 μM against CA VI, of 9.1–1359 μM against CA VII, of 8.8–517 μM against CA IX, of 4.1–598 μM against CA XII, of 12.2–697 μM against CA XIII and of 10.1–49.8 μM against CA XIV, respectively. The different mechanisms of inhibition by phenols as compared to sulfonamides, and their diverse inhibition profile for these mammalian isozymes, makes this class of derivatives of great interest for the design of compounds with selectivity and/or specificity for some of the medicinal chemistry targets belonging to this enzyme family.

1. Introduction

In a recent preliminary work from this group,[1] we investigated the interaction between phenol and two of its substituted derivatives with the 12 catalytically active isoforms of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1).[2–4] Indeed, phenol was shown by Lindskog's group to be the only competitive inhibitor with CO_2 as substrate for the main isoform of CA, that is, human CA II (hCA II).[5] In a very elegant study, Christianson's group then reported the X-ray crystal structure for the adduct of hCA II with phenol,[6] showing indeed this inhibitor to bind to the enzyme by anchoring its OH moiety to the zinc-bound water/hydroxide ion of the enzyme through a hydrogen bond as well as to the NH amide of Thr199, an amino acid conserved in all α-CAs and critically important for the catalytic cycle of these enzymes.[2–4] Furthermore, the phenyl moiety of this inhibitor was found to lay in the hydrophobic part of the hCA II active site, where presumably CO_2, the physiologic substrate of the CAs, binds in the precatalytic complex, explaining thus the behaviour of phenol as a unique CO_2 competitive inhibitor.[5]

In fact, the two other major classes of CA inhibitors (CAIs), the metal-complexing anions, and the unsubstituted sulfonamides and their bioisosteres (i.e., the sulfamates, sulfamides and related compounds), bind to the Zn(II) ion of these enzymes either by substituting the non-protein zinc ligand to generate a tetrahedral adduct or by addition to the metal coordination sphere, generating trigonal–bipyramidal species, and are non-competitive inhibitors with CO_2 as substrate (Fig. 1A and B).[2–4] This is in net contrast to the binding mode of phenol which does not substitute the non-protein zinc ligand, but interacts with it by means of the two hydrogen bonds mentioned above (Fig. 1C). Such a particular binding mode offers the possibility to design CAIs possessing a different inhibition mechanism as compared to the classical sulfonamide or sulfamate inhibitors,[2–4] as we recently showed by investigating three simple phenols as inhibitors of the 12 catalytically active mammalian isoforms, that is, CA I–XIV.[1] In our preliminary study,[1] we observed inhibition in the low micromolar or submicromolar range for several CA isozymes with phenol, 3,5-difluorophenol and clioquinol (a clinically used compound),[1] as well as a very distinct inhibition profile of the various mammalian isozymes with this class of inhibitors.

Sulfonamide CAIs are clinically used drugs as diuretics, antiglaucoma, or anticonvulsant agents,[2–4,7,8] whereas more recent drug design studies evidenced some other classes of such derivatives as molecules of interest for developing novel therapies for obesity[9] and cancer[2,10–12] based on selective inhibition of CA isozymes involved in such pathologies. However, few isozyme-selective CAIs are known to date,[2–4] and it is of great relevance to investigate alternative classes of compounds which may selectively inhibit some CA isoforms among the 16 presently known α-CAs in vertebrates.[2–4,7–13] Thus, in the search of non-sulfonamide CAIs belonging to different classes of compounds, and possibly showing selectivity for isozymes of medicinal chemistry

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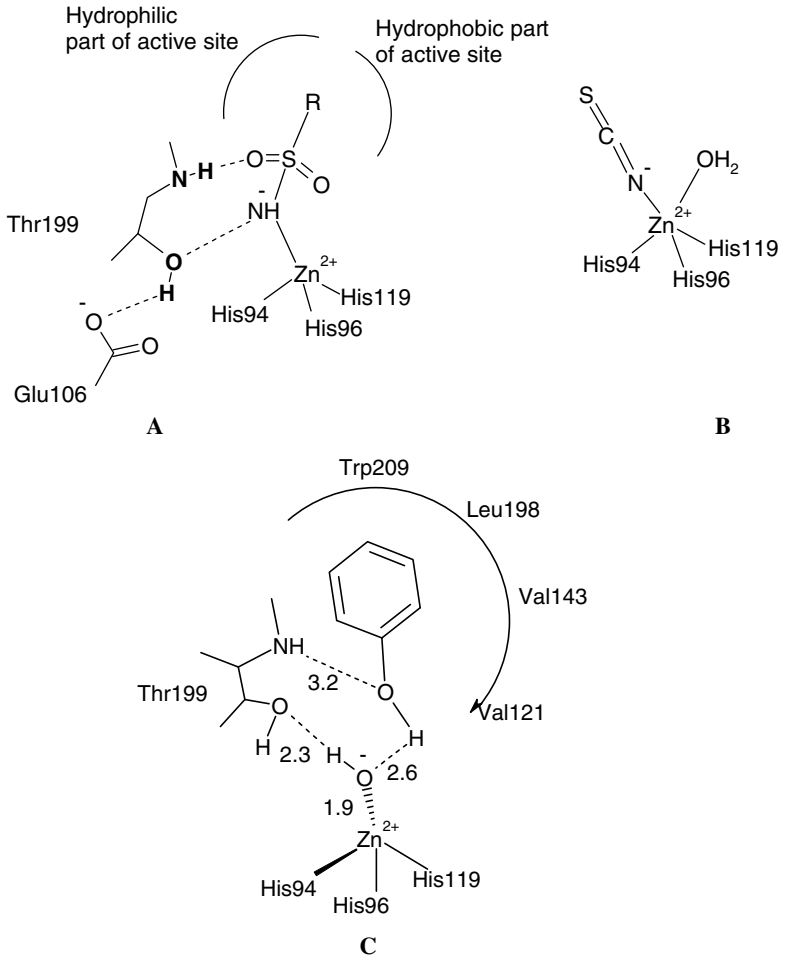


Figure 1. Schematic representation for interactions of the three main classes of CAIs with the enzyme active site: (A) Sulfonamide (and their isosteres, sulfamate and sulfamide) inhibitors. (B) Inorganic anion inhibitors (thiocyanate as an example). (C) Phenol **1**. The interactions in which phenol participates when bound to the hCA II active site are shown in detail (numbers close to hydrogen bonds in (C) represent distances in Å; hydrogen bonds are represented as dashed lines).

interest,[2–4] we report here an inhibition study of the 12 catalytically active mammalian CA isozymes with a series of substituted phenols, derivatives of the monocyclic, simple phenol **1**, incorporating various 2-, 3- and 4-substituents as well as some disubstituted such derivatives, of type **1**–**11**.

2. Results and discussion

2.1. Chemistry

Considering the very simple lead molecule, phenol **1**, which has been shown[6] to bind to CA II in a very interesting manner by means of X-ray crystallography (Fig. 1C), we decided to investigate some simple phenol derivatives incorporating various substituents, of types **2**–**11**, for their interaction with all the catalytically active mammalian CA isozymes, that is, CA I–XIV. The standard, clinically used sulfonamide inhibitor acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) **12** was also included in the study for comparison reasons.[2,3]

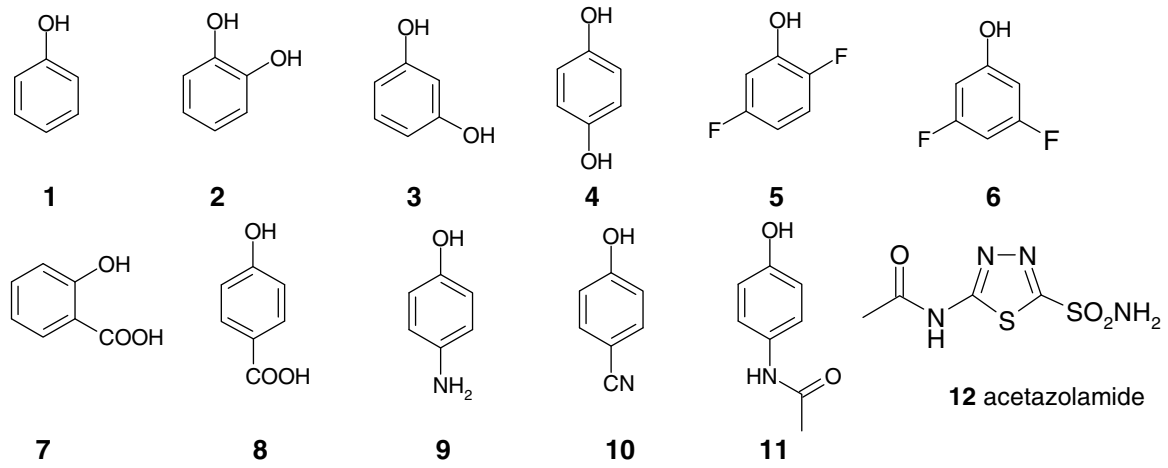
Compounds **2**–**11** are 2-, 3-, or 4-substituted derivatives of phenol **1**, such as the diphenols **2**–**4**, whereas other derivatives incorporate carboxy-, amino-, acetamido- and cyano- moieties, as well as 2,5- and 3,5-difluoro substitution patterns, as in compounds **5** and **6**. They were chosen in such a way as to have a range of substituents with different electronic and steric properties, in order to understand SAR for this class of less investigated CAIs. On the other hand, some of these moieties (such as the OH, COOH or NH_2 ones present in derivatives **2**–**4** and **7**–**9**) may be easily derivatized for generating libraries of compounds with different physico-chemical properties and presumably also CA inhibitory activity, in case these simple phenols prove to be interesting leads as non-sulfonamide CAIs.

2.2. CA inhibition studies

Inhibition data against all the catalytically active mammalian α-CA isozymes, that is, CA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII and XIV with compounds **1**–**12** are presented in Table 1, and they were obtained at pH 7.4 in 10 mM Hepes buffer, at 25 °C by a stopped-flow assay monitoring the CO_2 hydration reaction.[13–16]

The following should be noted regarding the inhibition of these mammalian α-CA isozymes with phenols **1**–**11**: (i) Against isozyme hCA I phenols **2**, **3**, **5**, **9** and **10** showed a behaviour of weak inhibitors, with K_Is in the range of 131–4003 μM. The remaining compounds showed a rather compact behaviour 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**). When the additional

Table 1
Inhibition of CA isozymes I–XV (of human = h, and murine = m origin) with phenols **1**–**11** and acetazolamide **12** as standard inhibitor[13]



Compound	K_I (μM)[#,*]											
	hCA I	hCA II	hCA III	hCA IV	hCA VA	hCA VB	hCA VI	hCA VII	hCA IX[**]	hCA XII[**]	mCA XIII	hCA XIV
1	10.2[a]	5.5[a]	2.7[a]	9.5[a]	218[a]	543[a]	208[a]	710[a]	8.8[a]	9.2[a]	697[a]	11.5[a]
2	4003	9.9	13.0	10.9	55.1	4.2	606	714	115	8.9	12.2	48.9
3	795	7.7	605	570	8.7	7.1	550	644	69.7	7.5	62.6	10.7
4	10.7	0.090	8.2	10.8	14.1	12.5	521	883	32.5	7.8	74.3	42.0
5	134	870	10.8	426	12.0	51.2	90.6	554	68.7	598	69.5	34.0
6	38.8[a]	33.9[a]	0.71[a]	10.7[a]	65.0[a]	98.4[a]	82.6[a]	163[a]	9.4[a]	70.2[a]	84.2[a]	10.6[a]
7	9.9	7.1	885	11.1	678	355	11.9	82.1	78.7	8.8	67	49.8
8	9.8	10.6	696	809	9.2	10.5	11.4	716	118	4.8	86	34.3
9	159	752	9.5	743	867	649	23.5	1359	517	479	657	10.1
10	131	0.108	6.7	634	520	557	13.7	777	56.0	9.2	45	29.1
11	10.0	6.2	7.1	11.4	802	296	658	9.1	70.7	4.1	30.3	10.6
12	0.25	0.012	200	0.074	0.063	0.054	0.011	0.0025	0.025	0.0057	0.017	0.041

[a] From Ref. 1.
[*] h = human; m = murine isozyme.
[**] Catalytic domain.
[#] Errors in the range of ±5% of the reported data from three different assays.

phenol OH 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 hydroquinol **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. It is thus clear that this new class of CAIs, the phenols, show 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** and **11**) were efficient, micromolar CA II inhibitors, with inhibition constants in the range of 5.5–10.6 μM, but two compounds, hydroquinol **4** and 4-cyanophenol **10**, showed much better activity, with K_Is in the range of 90–108 nM (Table 1). Acetazolamide **12** 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] Paracetamol **11** and salicyclic acid **7**, 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.[17]

(iii) Isozyme hCA III was weakly inhibited by phenols **3**, **7** and **8** (K_Is in the range of 605–885 μM), which similarly to the sulfonamide inhibitor acetazolamide **12** (K_I of 200 μM) may have steric clashes with Phe198, a bulky amino acid residue placed in the middle of the CA III active site,[2,3] explaining thus their inefficient inhibitory activity. However, the remaining phenols show a much better inhibitory activity against this isozyme, with inhibition constants in the low micromolar or submicromolar ramge (K_Is of 0.71–13.0 μM, Table 1). Indeed, both the simple phenol **1** and its substituted derivatives **2**, **4**, **5**, **6** and **9**–**11** showed this behaviour of efficient CA III inhibitors. The difluoro substituted derivative **6** is in fact the best hCA III inhibitor detected so far, since it was shown recently[16] that sulfonamides and sulfamates are rather ineffective inhibitors of this isoform, mainly due to the reason mentioned above, related to the presence of the bulky Phe198 residue in the middle of the active site cavity. Thus, phenols may constitute a class of compounds that might lead to much more effective CA III inhibitors than the presently available sulfonamides and sulfamates. This should be also helpful to understand in more detail the physiological role of this isoform, poorly understood at this moment.[2,16]

(iv) The membrane-anchored isoform hCA IV was poorly inhibited by five of the investigated phenols, that is, compounds **3**, **5**, **8**, **9** and **10**, which showed K_Is in the range of 426–809 μM. These derivatives incorporate either a 3-substituent in *meta* to the phenol OH moiety (such as resorcinol **3** and the difluorophenol **5**) or the 4-carboxy-, 4-amino- and 4-cyano moieties (in derivatives **8**–**10**). However, subtle and minor changes in the position of such groups (i.e., the *ortho*- and *para*-substituted diphenols **2** and **4**; the isomeric to **5** difluorophenol **6**, or their derivatization (by the transformation of the amino moiety of **9** to the corresponding acetamido one, present in **11**) lead to much more effective hCA IV inhibitors, these compounds showing similarly to the lead **1**, inhibition constants in the range of 9.5–11.4 μM. These phenols are anyhow much weaker inhibitors as compared to the sulfonamide **12** (K_I of 74 nM against hCA IV, Table 1).

(v) Phenol **1**, similarly to its derivatives **7**, **9**, **10** and **11**, act as quite weak inhibitors of the first mitochondrial isoform, hCA VA, with K_Is in the range of 218–867 μM. Two other compounds, pyrocatechol **2** and 3,5-difluorophenol **6**, are medium potency inhibitors (K_Is of 55.1–65 μM), whereas compounds **3**–**5** and **8** are much more effective hCA VA inhibitors, with inhibition constants in the low micromolar range, of 8.7–14.1 μM. Again SAR is very sensitive to minor changes in the scaffold of the investigated phenols: for example, pyrocatechol **2** is a 6.33 times weaker hCA VA inhibitor as compared to its isomer resorcinol **3**, whereas hydroquinol **4** with a K_I of 14.1 μM is around 3.9 times more efficient inhibitor, as compared to **2**. Furthermore, all these diphenols are 3.95–25 times better hCA VA inhibitors as compared to the simple lead **1**. The other isomeric compounds **7** and **8**, incorporating COOH moieties in the phenol scaffold are also very different in their behaviour towards hCA VA, with *p*-hydroxybenzoic acid **8** being a 73.7 times better inhibitor as compared to salicylic acid **7**. (vi) The second mitochondrial isoform, hCA VB has an inhibition profile with phenols quite different of that of the related isozyme hCA VA discussed above. Thus, phenol itself **1** as well as its derivatives **7**, and **9**–**11**, behave as weak inhibitors (K_Is in the range of 296–649 μM), whereas the difluorinated phenols **5** and **6** are medium potency inhibitors (K_Is of 51.2–98.4 μM). Much better inhibition was observed on the other hand with the diphenols **2**–**4** and *p*-hydoxybenzoic acid **8**, which inhibited hCA VB with K_Is of 4.2–10.5 μM. As for other discussed isoforms, minimal structural variations in the scaffold of the phenol lead to important changes in the hCA VB inhibitory activity. One should also note the important differences in the pattern of inhibition of the two mitochondrial isozymes (hCA VA abd hCA VB) by this class of compounds, in contrast to the sulfonamide **12** which is a strong inhibitor for both of them (K_Is of 54–63 nM).

(vii) Phenol **1** and some of its congeners such as **2**–**4** and **11** are also weak inhibitors of the secreted isozyme hCA VI, with K_Is of 208–658 μM. However, again the difluorinated phenols **5** and **6** are medium potency inhibitors (K_Is of 82.6–90.6 μM), and derivatives **7**–**10** show a higher affinity for this isozyme, with inhibition constants in the range of 11.4–23.5 μM (Table 1).

(viii) Isoform hCA VII was the least inhibitable one among the investigated CAs by this class of derivatives, with most of the investigated phenols acting as quite ineffective inhibitors. Indeed, compounds **1**–**6** and **8**–**10** showed K_Is in the range of 163–1359 μM, with only salicyclic acid behaving as a medium potency inhibitor (K_I of 82.1 μM) and paracetamol **11** acting as an effective hCA VII inhibitor, with a K_I of 9.10 μM. The inhibitory activity of this last compound (paracetamol **11**) and its deacetylated derivative **9** are indeed very different, with **11** being a 149.3 times more effective as a hCA VII inhibitor as compared to *p*-aminophenol **9**. Without an X-ray crystal structure of this isozyme[2] it is difficult to explain these very interesting results. It is also interesting to note that hCA VII has a very high affinity for sulfonamide inhibitors, with many compounds (among which acetazolamide **12**) showing subnanomolar activity for its inhibition.[15a]

(ix) The tumour-associated isoform hCA IX was better inhibited by most of these phenols as compared to the cytosolic hCA VII (Table 1). Thus, pyrocatechol **2**, *p*-hydroxybenzoic acid **8** and *p*-aminophenol **9** showed the weakest inhibitory activity, with K_Is in the range of 115–517 μM. Medium potency inhibition was observed with derivatives **3**–**5**, **7**, **10** and **11** (K_Is in the range of 32.5–78.7 μM), whereas phenol 1 and its 3,5-difluoroderivative 6 were the best hCA IX inhibitors in this series of compounds, with inhibition constants of 8.8–9.4 μM. It should be noted that most substitution patterns in the molecule of **1** (except for the 3,5-difluoro one mentioned above) led to a loss of potency as hCA IX inhibitors to the corresponding phenols as compared to the lead **1**, although most of these compounds maintain a profile of medium potency inhibitors (Table 1).

(x) Phenols **5** and **9** showed weak inhibitory activity against the second tumour-associated isozyme, hCA XII with inhibition constants in the range of 479–598 μM. The remaining difluoro derivative **6** was a medium potency inhibitor (K_I of 70.2 μM), whereas all other derivatives were much better hCA XII inhibitors, with inhibition constants less than 10 μM. The best inhibitor was paracetamol **11** (K_i of 4.1 μM), which was again (as for the inhibition of hCA VII) 116.8 times a better inhibitor as compared to the deacetylated compound **9**. Other favourable substitution patterns for hCA XII inhibition were those present in the diphenols **2**–**4** and the hydroxybenzoic acids **7** and **8**; these compounds showing inhibition constants of 4.8–8.9 μM.

(xi) Phenol **1** and *p*-aminophenol **9** were quite weak inhibitors of mCA XIII, another cytosolic isoform[2] (K_Is in the range of 657–697 μM) whereas the remaining derivatives behaved as medium potency inhibitors, with compact inhibition constants in the range of 30.3–86 μM. The most potent inhibitor for mCA XIII was pyrocatechol **2**, which with an inhibition constant of 12.2 μM was 57.1 times a better mCA XIII inhibitor as compared to phenol **1**.

(xii) The last human isoform, hCA XIV (transmembrane one, similarly to hCA IX and XII, but not tumour-associated)[15b] was generally well inhibited by all the investigated phenols **1**–**11**, which showed K_Is in the range of 10.1–49.8 μM (Table 1). The most effective inhibitors were phenol **1**, resorcinol **3**, 3,5-difluorophenol **6**, *p*-aminophenol **9** and paracetamol **11**, with K_Is in the range of 10.1–11.5 μM, whereas the remaining phenols were slightly less effective as hCA XIV inhibitors.

(xiii) Even for this small series of investigated phenols, SAR is quite different for the 12 CA isozymes assayed here, proving that

this class of less investigated CAIs may lead to selective inhibition of some isoforms. For example, resorcinol **3** is an effective inhibitor of isozymes hCA II, hCA VA, hCA VB, hCA XII and hCA XIV (K_Is in the range of 7.1–10.7 μM) but is a much weaker as inhibitor of the remaining isozymes, with inhibition constants in the range of 62.6–795 μM. Thus, exploring phenol scaffolds incorporating groups that allow a distinct inhibition profile of various mammalian CAs, may lead to more selective CAIs. Indeed, some of the scaffolds investigated here such as among others **2**–**4**, **7**, **8** and **9**, may easily be derivatized at one of their OH, COOH or NH_2 moieties, leading probably to compounds with very different CA inhibitory activity. Work is in progress in our laboratory for designing phenols with improved activity and selectivity for the various mammalian CA isoforms with medicinal chemistry applications.[2]

3. Conclusions

A detailed inhibition study of 12 mammalian isoform of the metalloenzyme CA, that is, CA I–XIV, with a series of phenols was performed. The inhibition profile by phenols of these CAs was distinct of those of the sulfonamides and their isosteres, the main class of clinically used inhibitors. Phenol and some of its 2-, 3- and 4-substituted derivatives incorporating hydroxy-, fluoro-, carboxy-, amino-, cyano- and acetamido-moieties were generally effective low micromolar CA inhibitors, with inhibition constants in the range of 9.8–4003 μM against CA I, of 0.090–870 μM against CA II, of 0.71–885 μM against CA III, of 9.5–809 μM against CA IV, of 8.7–867 μM against CA VA, of 4.2–649 μM against CA VB, of 11.4–658 μM against CA VI, of 9.1–1359 μM against CA VII, of 8.8–517 μM against CA IX, of 4.1–598 μM against CA XII, of 12.2–697 μM against CA XIII and of 10.1–49.8 μM against CA XIV, respectively. The different mechanism of inhibition by phenols as compared to sulfonamides, and their diverse inhibition profile for these mammalian isozymes, makes this class of derivatives of great interest for the design of compounds with selectivity and/or specificity for some of the medicinal chemistry targets belonging to this enzyme family.

4. Experimental

4.1. Chemistry

Buffers, phenols **1**–**11** and acetazolamide **12** were from Sigma–Aldrich (Milan, Italy) of highest purity available, and were used without further purification. All CA isozymes were recombinant ones produced and purified in our laboratory as described earlier.[14–16]

4.2. CA catalytic/inhibition assay

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic/inhibition of various CA isozymes as reported by Khalifah.[13] 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 7.4) as buffer, 0.1 M Na_2SO_4 or $NaClO_4$ (for maintaining constant the ionic strength; these anions are not inhibitory in the used concentration),[12–15] following the CA-catalyzed CO_2 hydration reaction for a period of 5–10 s. Saturated CO_2 solutions in water at 25 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO/water 1:1, v/v) and dilutions up to 0.001 μM done with the assay buffer mentioned above. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. K_Is were obtained from Lineweaver–Burk plots, as reported earlier.[12–16]

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