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Carbonic anhydrase inhibitors: Synthesis and inhibition studies against mammalian isoforms I–XV with a series of 2-(hydrazinocarbonyl)-3-substituted-phenyl-1*H*-indole-5-sulfonamides

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ABSTRACT

A series of 2-(hydrazinocarbonyl)-3-substitutedphenyl-1*H*-indole-5-sulfonamides possessing various 2-, 3- or 4- substituted phenyl groups with methyl-, halogeno- and methoxy- functionalities, as well as the perfluorophenyl moiety have been synthesized and evaluated as inhibitors of 13 catalytically active, mammalian carbonic anhydrase (CA, EC 4.2.1.1) isoforms, that is, CA I–CA XV (of human (h) or murine (m) origin). The new compounds were ineffective inhibitors of isozymes hCA III, hCA IV, hCA VA, hCA VB, hCA VI and mCA XIII, moderate inhibitors of hCA I, hCA VII, hCA IX and mCA XV, and excellent, low-nanomolar inhibitors of hCA II and hCA XIV. The substitution pattern of the aromatic group in the 3-position of the indole ring influenced biological activity and isozyme inhibition profiles in this series of sulfonamides. Some of the best and most selective hCA XIV and mCA XV inhibitors ever reported have been identified in this study.

1. Introduction

In a recent preliminary work from this group,[1] we investigated the interaction between 2-(hydrazinocarbonyl)-3-phenyl-1*H*-indole-5-sulfonamide (compound **L**), and 12 carbonic anhydrase (CA, EC 4.2.1.1) isoforms of mammalian (human or murine) origin. This sulfonamide behaved as a very potent inhibitor of CA I and II (K_Is of 7.2–7.5 nM), a medium potency inhibitor of CA VII, IX, XII and XIV and a weak inhibitor against the other isoforms, making it thus an interesting clinical candidate for situations in which a strong inhibition of CA I and II is needed. Furthermore, the inhibition profile of **L** was quite distinct from that of the clinically used compounds such as acetazolamide **AZA**, methazolamide, **MZA**, ethoxzolamide **EZA** or dichlorophenamide **DCP** which promiscuously inhibit most of these mammalian isoforms in the low-nanomolar range.[2] Indeed, many of these CAs are medicinal chemistry targets for the development of diuretics, antiglaucoma, antiobesity, anticonvulsant or anticancer drugs/diagnostic tools.[2–4]

The crystal structure of the hCA II adduct with sulfonamide **L** reported by us,[1] also revealed many favorable interactions between the inhibitor and the enzyme which explain its strong low-nanomolar affinity for isoform II (K_I of 7.2 nM) but may also be exploited for the design of effective inhibitors incorporating such bicyclic moieties.

As shown in Figure 1, where a schematic representation for the binding of sulfonamide **L** to the human CA II (hCA II) active site is presented, the inhibitor coordinates to the Zn(II) ion within the enzyme active site in the deprotonated state, the same SO_2NH^- functionality of **L** also making a hydrogen bond with the OH of Thr199. The $CONHNH_2$ fragment of the inhibitor then participates in a network of three hydrogen bonds with a water molecule (Wat101) and two amino acid residues known to interact with various inhibitors bound to the hCA II active site, that is, Asn62 and Asn67.[5–7] A host of van der Waals interactions between the inhibitor and amino acid residues lining the active site also favorably influence the strong binding of **L** to the enzyme cavity (data not shown).[1] But what is more interesting from the drug design point of view, the 3-phenyl moiety of **L** is accommodated in a hydrophobic region of the active site[1] where enough additional space is available for various moieties to be introduced as substituents to the phenyl ring, allowing thus for interesting structure–activity correlations. Using this X-ray crystal structure as a starting point and the observation that probably the 3-phenyl moiety of **L** can be further substituted without losing the potent CA inhibitory properties, we report here the synthesis and inhibitory properties against all the mammalian catalytically active CA isozymes (CA I–XV) of a series of 2-(hydrazinocarbonyl)-3-substituted-phenyl-1*H*-indole-5-

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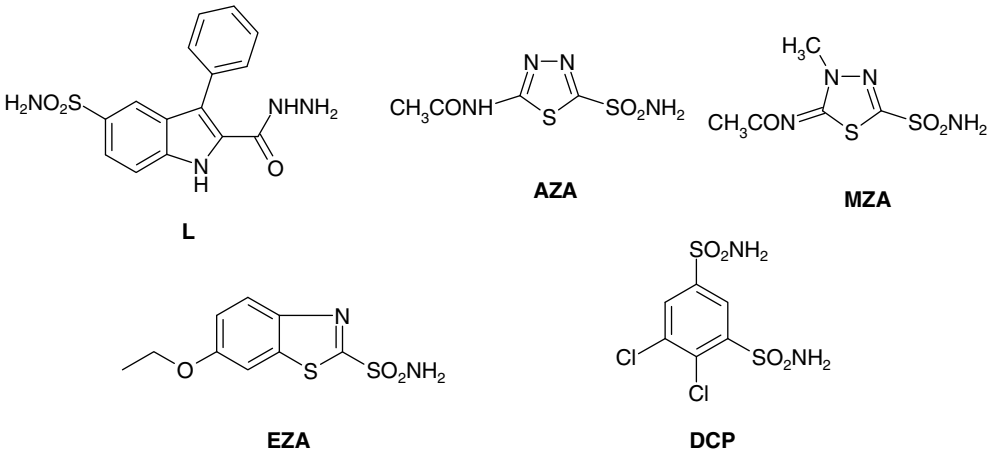


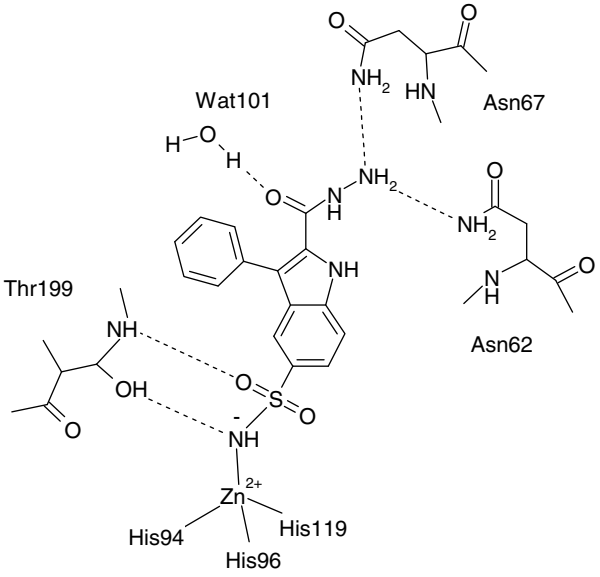


Figure 1. Schematic interactions to which **L** participates when bound within the hCA II active site, as determined by X-ray crystallography (PDB code 3B4F).[1] Hydrogen bonds to which several moieties of the inhibitor participate with amino acid residues 62, 67 and 199 from the enzyme active site and a water molecule (Wat101) are shown as dotted lines.

sulfonamides. In contrast to the lead molecule **L**, these compounds incorporate 3-phenyl moieties variously substituted in different positions with methyl-, halogeno (fluoro-, chloro- and bromo-), and methoxy groups or the 3-perflorophenyl group.

2. Results and discussion

2.1. Chemistry

Sulfonamide **L** was previously reported by Salman's group,[8] being easily prepared from sulfanilamide as starting material. Diazotization of sulfanilamide followed by condensation of the diazonium salt with ethyl 2-benzylacetoacetate led to an intermediate which was cyclized in acidic medium with formation of the ethyl ester derivative of **L**, which was then converted to the lead compound by treatment with hydrazine.[8] We used a similar approach for the preparation of the series of congeners of **L** bearing different moieties in position 3 of the indole ring (Scheme 1).

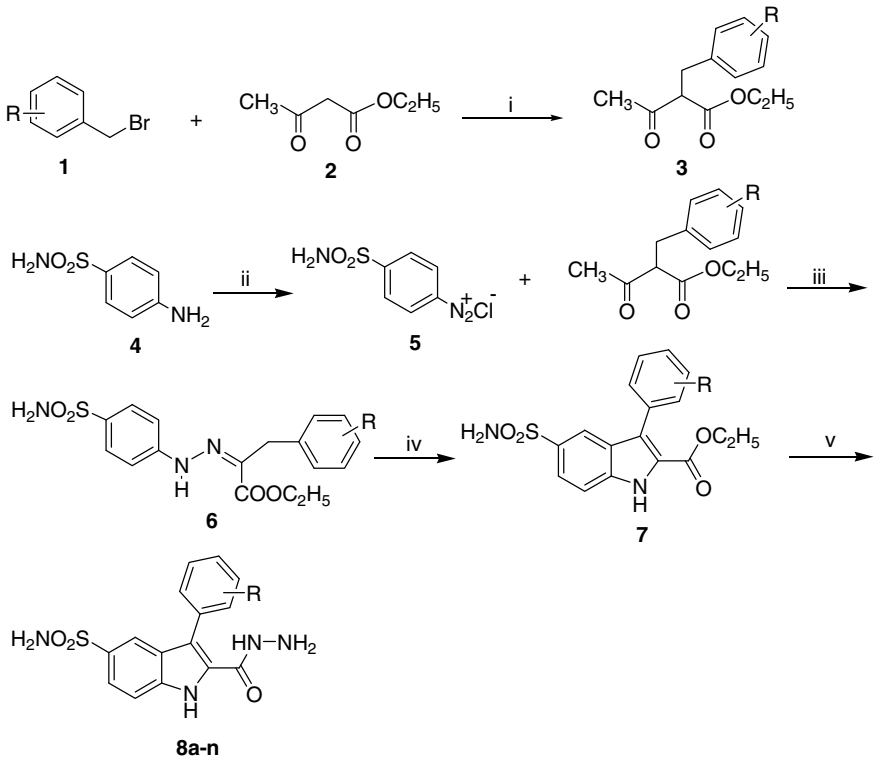
Condensation of ring-substituted benzyl bromides **1** with ethyl acetoacetate **2** gave the key intermediates, ethyl 2-acetyl-3-(substitutedphenyl)propionates **3**, by literature procedures.[9] Diazotization of sulfanilamide **4** led to the diazonium salt **5** which has been coupled with the key intermediates **3** allowing the preparation of the hydrazones **6**, which were cyclized in the presence of concentrated acid (HCl) to the indoles **7**. The last step consisted in conversion of the ester moieties of **7** to the corresponding hydrazides by treatment with hydrazine hydrate at reflux, leading thus to the desired series of compounds **8a–8n** (Scheme 1).

We have chosen the various substituents of the 3-phenyl group of indoles **8a–8n** by considering both the limited available space within the hydrophobic pocket of the enzyme active site, as discussed above (see Fig. 1), as well as general medicinal chemistry considerations, for example, moieties that may increase lipo- or hydrosolubility of the new compounds, and eventually also interacting in a positive manner with amino acid residues present in the active site region where this moiety of the inhibitor lies (such as among others Trp5, Asn62, His64 and Pro201).[1,5–7] Thus, we have incorporated 2-, 3- or 4-substituted phenyl groups possessing methyl-, halogeno- and methoxy- functionalities in the 3-position of the indolesulfonamides **8**, as well as the perfluorophenyl moiety, since such substitution patterns would not lead to excessively bulky groups, and presumably, these derivatives will bind to the enzyme similarly to the lead molecule **L**. On the other hand, their different chemical nature, ensued by the presence of the additional functionality in the 3-phenyl ring may lead to diverse interactions of compounds **8** with amino acid residues within the various isoforms active sites cavity, and possibly to an inhibition profile which will be different for this new series of compounds as compared to the lead **L** or the classical sulfonamide inhibitors **AZA**–**DCP** (see discussion later in the text). In fact, the main interest in this class of compounds is that of detecting derivatives with a more isoform-selective profile as compared to the clinically used sulfonamides.[2–4]

2.2. Carbonic anhydrase inhibition

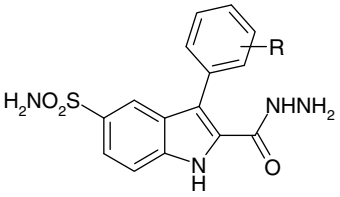
Inhibition data against the 13 catalytically active mammalian (h = human, m = murine) CA isoforms, that is, CA I–CA XV, with the new sulfonamides **8a–8n** reported here as well as the lead **L** are shown in Table 1 and Figure 2.

The following structure–activity relationship (SAR) can be drawn by considering data of Table 1: (i) Unlike the lead **L**, which behaves as a very potent hCA I inhibitor (K_I of 7.5 nM), the congeners **8a–8n** reported here showed moderate–weak inhibitory activity against this isozyme, with inhibition constants in the range of 104–659 nM. The most effective hCA I inhibitors in this series of derivatives incorporated 2-Me-, 4-Me-, 3-F-, 4-F-, 4-Cl, 2-Br-phenyl and pentaflurophenyl groups (all of them with K_Is around



Scheme 1. Preparation of 2-(hydrazinocarbonyl)-3-substitutedphenyl-1*H*-indole-5-sulfonamide derivatives **8a**–**n**. Reagents and conditions: (i) EtOH, Na, (ii) $NaNO_2$, 37% HCl, 0 °C; (iii) KOH, 0 °C; (iv) 37% HCl, reflux, 4 h; (v) $H_2NNH_2 \cdot H_2O$, reflux, 6 h.

Table 1
Inhibition of CA isozymes I–XV (of human = h, and murine = m origin) with sulfonamides **8a**–**n** and the lead molecule **L** as standard[13]



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Compound **8**	R	K_I (nM)[#,*]								K_I (nM)[#]				
		hCA I	hCA II	hCA III	hCA IV	hCA VA	hCA VB	hCA VI	hCA VII	hCA IX[**]	hCA XII[**]	mCA XIII	hCA XIV	mCA XV
L	H	7.5	7.2	1.4×10^6	9000	1100	1100	2650	89	102	110	2633	48	66
8a	2-Me	107	11.6	7.8×10^6	9096	775	906	7384	80	79	76	894	9.3	64
8b	3-Me	730	48.4	6.9×10^6	7760	748	814	6325	69	77	68	820	8.5	47
8c	4-Me	104	60.5	6.7×10^6	3035	727	690	4230	62	61	55	738	7.6	28
8d	2-F	621	36.0	7.3×10^6	9500	804	941	7610	85	75	83	893	9.2	59
8e	3-F	116	8.6	7.1×10^6	6260	784	910	6070	69	71	69	801	8.4	54
8f	4-F	108	15.5	7.5×10^6	3650	705	576	3980	54	65	58	740	7.8	49
8g	2-Cl	640	38.8	7.4×10^6	8680	855	937	7440	82	77	76	935	9.5	58
8h	3-Cl	311	9.2	6.2×10^6	6140	743	844	5570	72	73	70	816	8.5	53
8i	4-Cl	112	11.6	6.9×10^6	4030	658	506	3525	61	59	56	734	7.4	47
8j	2-Br	110	48.5	3.9×10^6	7325	842	947	6950	83	74	77	992	9.3	70
8k	3-Br	510	54.1	5.5×10^6	5525	790	858	5760	72	70	682	867	8.5	57
8l	4-Br	659	40.8	5.2×10^6	2912	619	672	4450	58	63	60	759	7.0	47
8m	3-OMe	342	7.4	5.5×10^6	5740	791	924	7490	69	71	78	810	8.8	49
8n	F_5	110	7.0	5.4×10^6	5215	750	862	5620	68	70	79	734	8.7	48

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

110 nM), whereas the remaining compounds were typically three to six times less effective, with K_Is in the range of 310–659 nM (Table 1). It is thus clear that any substitution in the 3-phenyl moiety of the lead **L** has as a consequence a drastic loss of hCA I inhibitory

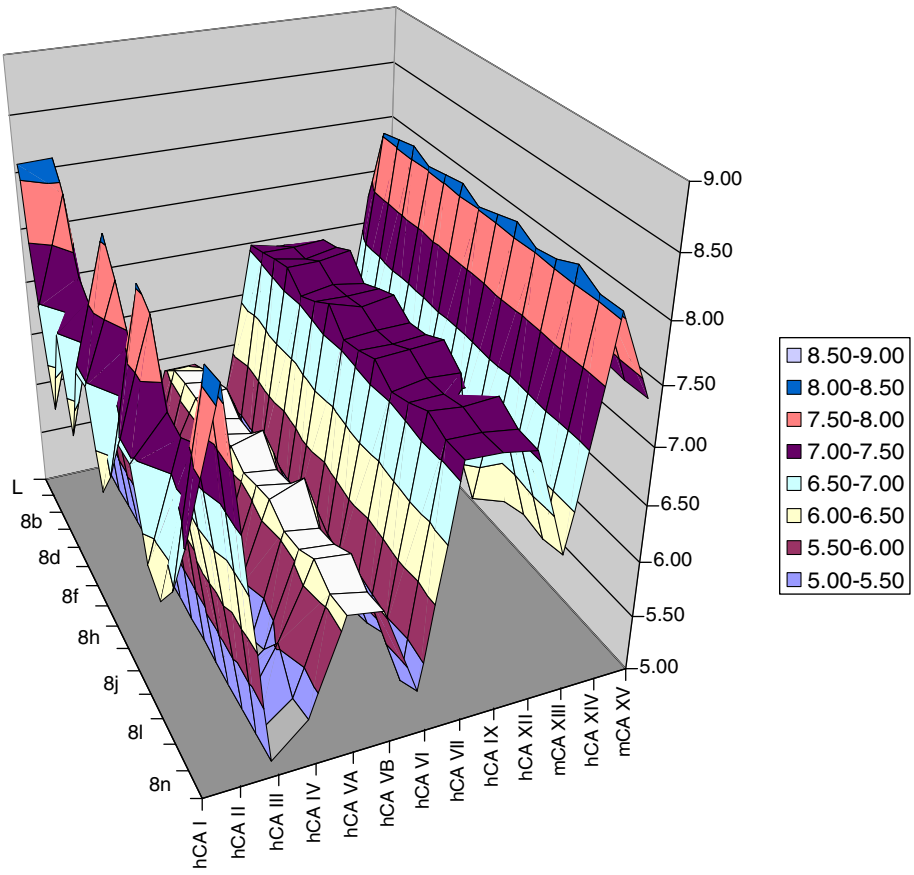

Figure 2. 3D-graph showing isozyme type on the x-axis versus compound **L** and **8a**–**8n** along the abscissa versus $-\log K_I$ on the vertical axis.

power, the most effective inhibitors among derivatives **8** reported here (i.e, sulfonamides **8a** and **8c**) being 14.2 times weaker inhibitors against this isozyme as compared to **L**. This may be due to the fact that the hCA I active site is rather restricted (as compared to that of hCA II for example) due to the presence of the bulky residue His200 (which in CA II is Thr) near the Zn(II) ion of the enzyme, at the bottom of the active site.[10,11]

(ii) Against the physiologically dominant isoform hCA II, the new compounds **8** investigated here showed interesting inhibitory activity, with inhibition constants in the range of 7.0–60.5 nM. Thus, similarly to the lead **L** (K_I of 7.2 nM), several of the new derivatives showed excellent inhibitory activity. These compounds incorporate 2-Me-, 3-F-, 3-Cl-, 4-Cl-, 3-methoxy-phenyl and perfluorophenyl moieties in position 3 of the indole ring. Thus, for hCA II which possesses a wider active site cavity as compared to hCA I,[5–7,10,11] many substitution patterns at the 3-phenyl ring are tolerated without loss of inhibitory activity, but also without a net gain. Indeed, all these derivatives mentioned above (**8a, 8e, 8h, 88i, 8m** and **8n**) showed comparable activity with the lead **L**, making thus SAR rather complicated. It is for example obvious that the phenyl ring can bear a small and compact group in either *ortho*-, *meta*- or *para*-positions, while maintaining very potent CA II inhibitory activity, but many of the various isomers (compare **8a**, **8b** and **8c**; **8d–8f; 8g–i**, and **8j**–**8l**, respectively) show very different CA II inhibitory activity. Less effective CA II inhibition was observed for derivatives **8b, 8c, 8d, 8g** and **8j–8l**, which showed K_Is in the range of 36–60.5 nM, appreciably inhibiting the enzyme. The only compound with an intermediate activity between the very efficient and less efficient CA II inhibitors was the 4-fluorophenyl substituted analogue **8f**, which with a K_I of 15.5 nM is a potent CA II inhibitor, comparable with the clinically used compounds **AZA**–**DCP** (data not shown).[2–4]

(iii) As most sulfonamides investigated up to now,[12] derivatives **8** reported here as well as the lead molecule **L**, show very weak hCA III inhibitory activity, with inhibition constants in the range of 1.4–7.8 mM. The clinically used sulfonamides also inhibit this isoform in the millimolar range.[12]

(iv) The membrane-bound isoform hCA IV is also not very sensitive to all sulfonamide inhibitors, as shown earlier by us.[13] Thus, the lead **L** behaves as a weak hCA IV inhibitor,[1] with a K_I of 9.0 μM. The same trend has been observed here for its congeners **8**, which possess inhibition constants in the range of 2.9–9.5 μM (Table 1). It may be observed that some of the substitution patterns present in the new derivatives reported here, such as the 4-Me- or 4-Br-phenyl ones of **8c** and **8l**, led to a threefold improvement of the hCA IV inhibitory activity of these derivatives over the lead **L**, with K_Is of 2.9–3.0 μM.

(v) Against the two mitochondrial isoforms hCA VA and hCA VB, the lead molecule **L** showed weak inhibitory activity (K_Is of 1.1 μM).[1] While this activity was improved against both isoforms and with all the new congeners of **L** reported here, they still remain weak–medium potency inhibitors of hCA VA (K_Is in the range of 0.619–0.855 μM) and hCA VB (K_Is in the range of 0.506–0.941 μM). A second salient feature is that SAR is very flat for these two isoforms, with all the compounds possessing a rather similar inhibitory activity. This is probably due to the particular active site environments of these isozymes which are not so well studied at this moment.[14]

(vi) The secretory isoform hCA VI[15] was also weakly inhibited by **L** (K_I of 2.65 μM)[1] and by its congeners **8a**–**8n** (K_Is of 3.525–7.490 μM, Table 1). However, in contrast to the results or for hCA VA and VB inhibition discussed above, in this case all substitution patterns present in the new sulfonamides **8** were detrimental to the hCA VI inhibitory activity as compared to the lead **L**, with the

new derivatives being generally 1.34–2.13 times less inhibitory as compared to **L**.

(vii) The brain-associated cytosolic isoform hCA VII[16]was moderately inhibited by the lead **L** (K_I of 89 nM) and by its congeners **8a–8n**, with inhibition constants in the range of 54–85 nM. It may be observed again a rather flat SAR with all these compounds behaving as effective, but not very potent hCA VII inhibitors, irrespective of the substitution pattern of the 3-phenyl moiety of the indole ring, a similar trend to that observed for the inhibition of hCA VA and hCA VB (but compounds **8a–8n** are much better inhibitors of this cytosolic isoform as compared to the mitochondrial ones discussed above).

(viii) The tumor-associated isozyme hCA IX[17,18] was modestly inhibited by the lead L and its congeners **8**, with inhibition constants in the range of 59–102 nM. SAR was rather flat, with all the new derivatives 8 being more effective inhibitors than the lead, but the increase in the inhibitory properties were rather modest, the best new CA IX inhibitor being the 4-Cl-phenyl derivative **8i**, with a K_I of 59 nM.

(ix) The second transmembrane, tumor-associated isoform, hCA XII[19] was weakly inhibited by the lead **L** (K_I of 110 nM), but all the new compounds **8** (except the *meta*-bromophenyl-substituted one, **8k**) showed better inhibitory activities against this medicinally relevant isoform, with inhibition constants in the range of 55–83 nM. It is difficult to explain the weak inhibitory activity of **8k** compared to its isomers **8j** and **8l**, which are 8.85 and 11.36 times better inhibitors, respectively. Some of these compounds were roughly two times better CA XII inhibitors as compared to the lead **L**, with K_Is around 55–58 nM (Table 1).

(x) The lead **L** was an ineffective inhibitor of the last cytosolic isoform, mCA XIII[20] (K_I of 2.633 μM) whereas its substituted congeners **8** behaved as more effective inhibitors with K_Is in the range of 734–992 nM. Again there are no significant differences of activity for the various substitution patterns of the 3-phenyl group in compounds **8**, except for this enhancement of the inhibitory power as compared to the lead **L**.

(xi) Although the lead **L** behaved as a medium potency inhibitor of the transmembrane isoform hCA XIV[21] (K_I of 48 nM), all the new derivatives **8** investigated here showed excellent, low-nanomolar inhibitory properties against this physiologically relevant isoform, with K_Is in the range of 7.0–9.5 nM. This is a very interesting result, as very few low-nanomolar hCA XIV inhibitors were reported up to now[2,21] and this isoform is quite abundant in several organs (such as the brain, kidneys, liver, etc.)[21] where its functions are not very well understood. In addition, some of these derivatives (e.g., **8l**) can be indeed considered as isoform XIV selective CA inhibitors, since the selectivity ratios for the inhibition of CA XIV over CA VII (the next most inhibited isozyme by this compound among the 13 CAs investigated here) was of 8.28. Indeed, CA XIV shows a strong expression in the cortex region of kidneys, where it is restricted to the proximal tubules of S1 and S2 segments.[22] The expression is high in the apical plasma membrane, while the basolateral membrane shows lower signal. CA XIV is also expressed in the initial portion of the thin descending limbs of Henle. The high expression of CA XIV in the proximal tubulus suggested that this isozyme may have an important role in the bicarbonate reabsorption in the kidneys.[22] CA XIV has also been found to be expressed on neuronal membranes and axons in both mouse and human brain. The most intense expression has been observed on large neuronal bodies and axons in the anterolateral part of the pons and medulla oblongata. In addition, CA XIV was found to be expressed in the hippocampus, corpus callosum, cerebellar white matter and peduncles, pyramidal tract and choroid plexus.[22] CA XIV has been also identified in the murine liver, where it was found to be expressed at the plasma membrane of hepatocytes.[22] It may be observed that the physiological role of this isoform is not well understood for the moment. Identifying highly potent and isoform-selective inhibitors as the ones reported here for the first time, may thus help understanding the involvement and physiological function of this isoform.

(xii) The last mammalian isoform discovered so far, mCA XV,[23] was never investigated up to now for its interaction with sulfonamide inhibitors, except acetazolamide (which is a moderate inhibitor, with a K_I of 72 nM).[22b] Data of Table 1 show that many of the new compounds investigated here as well as the lead **L**, show better mCA XV inhibitory activity as compared to the clinically used drug acetazolamide, with K_Is in the range of 28–70 nM. The substitution pattern leading to the most effective CA XV inhibitor is that present in **8c**, with a 4-tolyl group in the 3-position of the indole ring, whereas the one leading to the CA XV 'worst' inhibitor (which has the same potency as acetazolamide) is the 2-bromophenyl substitution present in **8j** (K_I of 70 nM).

The 3D plot of Figure 2 shows the variation of the inhibitory power of these derivatives (as $-\log K_I$ on the *z*-axis) as a function of the substitution pattern of compounds **L** and **8a–8n** (*y*-axis) for the 13 CA isozymes investigated here, CA I–CA XV, shown on the *x*-axis. As far as we know, this is the first study reporting the full inhibition profile against all 13 mammalian CA isozymes for a series of congeneric sulfonamides bearing various substitution patterns. The complicated landscape observed in Figure 2 is a clear proof that achieving some isoform-selective sulfonamide CA inhibitors is possible, since the blue, CA XIV peaks of most of these new derivatives are quite prominent. This type of study, although time consuming and difficult to be done (and presented) might shed new light on specificity issues when designing inhibitors of enzymes which possess a large number of isoforms, as the mammalian CA family

3. Conclusions

Using 2-(hydrazinocarbonyl)-3-phenyl-1*H*-indole-5-sulfonamide as the lead molecule, a compound for which the X-ray crystal structure in adduct with hCA II has been reported, a series of congeners possessing various 3-aryl groups has been synthesized and evaluated as inhibitors of 13 catalytically active, mammalian CA isoforms, that is, CA I–CA XV (of human or murine origin). The new compounds reported here incorporated in the 3-position of the indole ring 2-, 3- or 4- substituted phenyl groups with methyl-, halogeno- and methoxy-functionalities, as well as the perfluorophenyl moiety. The new compounds were ineffective inhibitors of isozymes hCA III, hCA IV, hCA VA, hCA VB, hCA VI and mCA XIII, moderate inhibitors of hCA I, hCA VII, hCA IX and mCA XV, and excellent inhibitors of hCA II and hCA XIV. The substitution pattern of the aromatic group in the 3-position of the indole ring generally influenced biological activity and isozyme inhibition profiles in this series of sulfonamides. Some of the best and most selective hCA XIV and mCA XV inhibitors ever reported have been identified in this study.

4. Experimental

4.1. Chemistry

Buffers, and chemicals 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]

4.1.1. Ethyl 2-acetyl-3-(substitutedphenyl)propionate 3[9]

An ethanolic solution of sodium ethoxide was prepared by the addition of sodium (0.5 g, 22 mmol) to dry ethanol (20 mL). Ethyl acetoacetate (10.4 g, 80.0 mmol) was added to the reaction

mixture, and the solution was stirred for 10 min. at room temperature. Substitutedbenzyl bromide (5 g, 20 mmol) was added, and the reaction mixture was heated under reflux for 15 h. The mixture was concentrated under reduced pressure and the residue was taken up in ether (100 mL). The ether solution was washed with water (50 mL) and was dried. The residue after removal of solvent under reduced pressure was purified by fractional distillation.

4.1.2. 2-Substitutedbenzyl-2-[*N*-(4-sulfonamidophenyl)hydrazono]ethanoates 6

To a solution of 0.01 mol sulfanilamide in 4 ml of 37% HCl, 10 ml of 7% $NaNO_2$ aqueous solution was added dropwise at 0 °C. This solution, containing diazonium salt, was poured into an ice-cold mixture of 2.3 g (a little excess of 0.01 mol) ethyl 2-substitutedbenzylacetoacetate, 10 ml of EtOH, 20 ml of H_2O and 2.7 g of KOH. The mixture was kept cold overnight. The hydrazone produced as an oil was separated, dissolved in Et_2O, washed with H_2O and dried over anhydrous Na_2SO_4. Et_2O was distilled, the oily residue was treated with 5 ml of 37% HCl and set aside for 5 h at room temperature. The resulting solid substance was recrystallized from EtOH.

4.1.3. Ethyl 5-(aminosulfonyl)-3-substitutedphenyl-1*H*-indole-2-carboxylates 7[8]

A mixture of 0.01 mol ethyl 2-substitutedbenzyl-2-[*N*-(4-sulfonamidophenyl)hydrazono]ethanoate **6** and about 10 ml of 37% HCl was heated on a water bath for 4 h, cooled and poured into 100 ml of H_2O, the crude product was filtered, washed with H_2O, and recrystallized from EtOH.

4.1.4. 2-(Hydrazinocarbonyl)-3-substitutedphenyl-1*H*-indole-5-sulfonamides 8a–8n

Ethyl 5-(aminosulfonyl)-3-substitutedphenyl-1*H*-indole-2-carboxylate **7** (0.01 mol, 3.45 g) was dissolved in 20 ml of EtOH, 4 ml of $H_2NNH_2.H_2O$ was added and refluxed for 6 h, cooled and kept cold overnight. The resulting crystals were filtered off, washed with Et_2O and recrystallized from EtOH/DMF.

4.1.4.1. 3-(2-Methylphenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8a. Yield 29%; mp 249–50 °C; IR (KBr) (υ, cm^{-1}), 1646 (C=O); ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.04 (3H, s, phenyl 2-CH_3), 4.46 (2H, s, NHNH_2), 7.11 (2H, s, SO_2NH_2), 7.26 (1H, d, J = 7.81 Hz, Ar-H), 7.32 (1H, d, J = 7.32 Hz, Ar-H), 7.35–7.41 (2H, m, Ar-H), 7.60 (2H, d, J = 9.28 Hz, indole $C_{4,7}$–H), 7.67 (1H, dt, J = 8.30, 1.71 Hz, indole C_6–H), 8.03 (1H, s, CONH), 12.16 (1H, br s, indole NH). Anal. Calcd for $C_{16}H_{16}N_4O_3S$ (344.388): C, 55.80, H, 4.68; N, 16.27; S, 9.31. Found: C, 55.66; H, 4.89; N, 16.59; S, 9.61.

4.1.4.2. 3-(3-Methylphenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8b. Yield 58%; mp 313–4 °C; IR (KBr) (υ, cm^{-1}), 1643 (C=O); ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.38 (3H, s, phenyl 3-CH_3), 4.48 (2H, s, NHNH_2), 7.13 (2H, s, SO_2NH_2), 7.21 (1H, d, J = 7.32 Hz, Ar-H), 7.25 (1H, d, J = 7.81 Hz, Ar-H), 7.29 (1H, s, Ar-H), 7.38 (1H, t, J = 7.32 Hz, Ar-H), 7.56 (1H, d, J = 8.79 Hz, indole C_7–H), 7.67 (1H, dd, J = 8.78, 1.46 Hz, indole C_6–H), 7.96 (1H, d, J = 1.46 Hz, indole C_4–H), 8.74 (1H, s, CONH), 12.06 (1H, brs., indole NH). Anal. Calcd for $C_{16}H_{16}N_4O_3S$ (344.388): C, 55.80, H, 4.68; N, 16.27; S, 9.31. Found: C, 55.31; H, 4.63; N, 16.65; S, 9.47.

4.1.4.3. 3-(4-Methylphenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8c. Yield 19%; mp 262–4 °C; IR(KBr) (υ, cm^{-1}), 1643 (C=O); ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 2.38 (3H, s, phenyl 4-CH_3), 4.47 (2H, s, NHNH_2), 7.11 (2H, s, SO_2NH_2), 7.30 (2H, d, J = 7.81 Hz, Ar-H), 7.36 (2H, d, J = 7.81 Hz, Ar-H), 7.56 (1H, d, J = 8.79 Hz, indole C_7–H), 7.67 (1H, dd, J_1 = 8.79, J_2 = 1.46 Hz, indole C_6–H), 7.97 (1H, d, J = 1.47 Hz, indole C_4–H), 8.73 (1H, s, CONH). Anal. Calcd for $C_{16}H_{16}N_4O_3S$ (344.388): C, 55.80, H, 4.68; N, 16.27; S, 9.31. Found: C, 55.54; H, 4.33; N, 16.24; S, 9.49.

4.1.4.4. 3-(2-Fluorophenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8d. Yield 60%; mp 246–8 °C; IR(KBr) (υ, cm^{-1}), 1644 (C=O); ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 4.53 (2H, s, NHNH_2), 7.21 (2H, s, SO_2NH_2), 7.38 (2H, t, J = 7.81 Hz, Ar-H), 7.50–7.56 (2H, m, Ar-H), 7.67 (1H, d, J = 8.78 Hz, indole C_7–H), 7.76 (1H, dd, J = 8.78, 1.46 Hz, indole C_6–H), 7.92 (1H, s, indole C_4–H), 9.14 (1H, s, CONH), 12.24 (1H, br s, indole NH). Anal. Calcd for $C_{15}H_{13}FN_4O_3S$ (348.352): C, 51.72, H, 3.76; N, 16.08; S, 9.20. Found: C, 51.69; H, 3.54; N, 15.75; S, 8.84.

4.1.4.5. 3-(3-Fluorophenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8e. Yield 47%; mp 274–7 °C; IR(KBr) (υ, cm^{-1}), 1645 (C=O); ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 4.52 (2H, s, NHNH_2), 7.15 (2H, s, SO_2NH_2), 7.20 (1H, td, J = 8.78, 2.44 Hz, Ar-H), 7.29 (2H, t, J = 6.84 Hz, Ar-H), 7.52 (1H, q, J = 6.88 Hz, Ar-H), 7.58 (1H, d, J = 8.78 Hz, indole C_7–H), 7.69 (1H, dd, J = 8.79, 1.46 Hz, indole C_6–H), 8.03 (1H, d, J = 1.46 Hz, indole C_4–H), 9.19 (1H, s, CONH), 12.13 (1H, br s, indole NH). Anal. Calcd for $C_{15}H_{13}FN_4O_3S$ (348.352): C, 51.72, H, 3.76; N, 16.08; S, 9.20. Found: C, 52.17; H, 4.07; N, 15.88; S, 8.84.

4.1.4.6. 3-(4-Fluorophenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8f. Yield 48%; mp 269–71 °C; IR(KBr) (υ, cm^{-1}), 1645 (C=O); ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 4.49 (2H, s, NHNH_2), 7.13 (2H, s, SO_2NH_2), 7.31 (2H, t, J = 8.78 Hz, Ar-H), 7.50 (2H, t, J = 6.84 Hz, Ar-H), 7.57 (1H, d, J = 8.30 Hz, indole C_7–H), 7.68 (1H, dd, J = 8.78, 1.47 Hz, indole C_6–H), 7.98 (1H, d, J = 0.98 Hz, indole C_4–H), 9.02 (1H, s, CONH), 12.10 (1H, br s, indole NH). Anal. Calcd for $C_{15}H_{13}FN_4O_3S$ (348.352): C, 51.72, H, 3.76; N, 16.08; S, 9.20. Found: C, 51.79; H, 3.53; N, 15.79; S, 9.13.

4.1.4.7. 3-(2-Chlorophenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8g. Yield 35%; mp 259–61 °C; IR(KBr) (υ, cm^{-1}), 1640 (C=O); ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 4.45 (2H, s, NHNH_2), 7.13 (2H, s, SO_2NH_2), 7.45 (3H, d, J = 2.93 Hz, Ar-H), 7.58–7.62 (2H, m, indole C_7-H and Ar-H), 7.69 (2H, d, J = 8.78 Hz indole C_4-H and indole C_6–H), 8.72 (1H, s, CONH), 12.10 (1H, br s, indole NH). Anal. Calcd for $C_{15}H_{13}ClN_4O_3S$ (364.806): C, 49.39, H, 3.59; N, 15.36; S, 8.79. Found: C, 48.98; H, 3.76; N, 15.50; S, 8.40.

4.1.4.8. 3-(3-Chlorophenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8h. Yield 59%; mp 301–4 °C; IR(KBr) (υ, cm^{-1}), 1643 (C=O); ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 4.52 (2H, s, NHNH_2), 7.16 (2H, s, SO_2NH_2), 7.42 (2H, t, J = 9.76 Hz, Ar-H), 7.49–7.52 (2H, m, Ar-H), 7.59 (1H, d, J = 8.78 Hz, indole C_7–H), 7.70 (1H, dd, J = 8.79, 1.46 Hz, indole C_6–H), 8.00 (1H, s, indole C_4–H), 9.23 (1H, s, CONH), 12.60 (1H, br s, indole NH). Anal. Calcd for $C_{15}H_{13}ClN_4O_3S$ (364.806): C, 49.39, H, 3.59; N, 15.36; S, 8.79. Found: C, 49.26; H, 3.70; N, 14.98; S, 8.42.

4.1.4.9. 3-(4-Chlorophenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8i. Yield 70%; mp 273–5 °C; IR(KBr) (υ, cm^{-1}), 1640 (C=O); ^1H NMR (DMSO-d_6, 500 MHz) δ (ppm): 4.50 (2H, s, NHNH_2), 7.13 (2H, s, SO_2NH_2), 7.48 (2H, d, J = 8.79 Hz, Ar-H), 7.53 (2H, d, J = 8.30 Hz, Ar-H), 7.58 (1H, d, J = 8.30 Hz, indole C_7–H), 7.68 (1H, dd, J = 8.78,1.46 Hz, indole C_6–H), 8.01 (1H, d, J = 0.98 Hz, indole C_4–H), 9.14 (1H, s, CONH), 12.15 (1H, br s, indole NH). Anal. Calcd for $C_{15}H_{13}ClN_4O_3S$ (364.806): C, 49.39, H, 3.59; N, 15.36; S, 8.79. Found: C, 49.05; H, 3.77; N, 14.98; S, 8.43.

4.1.4.10. 3-(2-Bromophenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8j. Yield 85%; mp 260–2 °C; IR(KBr) (υ, cm^{-1}),

1639 (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 4.46 (2H, s, NHNH_2), 7.13 (2H, s, SO_2NH_2), 7.38 (1H, td, J = 7.32, 1.95 Hz, Ar-H), 7.43 (1H, dd, J = 7.32, 1.47 Hz, Ar-H), 7.50 (1H, t, J = 7.32 Hz, Ar-H), 7.60 (1H, d, J = 8.79 Hz, Ar-H), 7.64 (1H, s, indole C_4–H), 7.69 (1H, dd, J = 8.78, 0.98 Hz, indole C_6–H), 7.77 (1H, d, J = 7.81 Hz, indole C_7–H), 8.55 (1H, s, CONH), 12.10 (1H, br s, indole NH). Anal. Calcd for $C_{15}H_{13}BrN_4O_3S$ (409.257): C, 44.02, H, 3.20; N, 13.69; S, 7.83. Found: C, 43.65; H, 2.92; N, 13.34; S, 7.45.

4.1.4.11. 3-(3-Bromophenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8k. Yield 64%; mp 302–3 °C; IR(KBr) (υ, cm^{-1}), 1641 (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 4.51 (2H, s, NHNH_2), 7.16 (2H, s, SO_2NH_2), 7.42–7.46 (2H, m, Ar-H), 7.56–7.58 (1H, m, Ar-H), 7.59 (1H, d, J = 8.30 Hz, indole C_7–H), 7.65 (1H, s, Ar-H), 7.69 (1H, dd, J = 8.30, 1.47 Hz, indole C_6–H), 7.99 (1H, d, J = 1.46 Hz, indole C_4–H), 9.23 (1H, s, CONH), 12.60 (1H, br s, indole NH). Anal. Calcd for $C_{15}H_{13}BrN_4O_3S$ (409.257): C, 44.02, H, 3.20; N, 13.69; S, 7.83. Found: C, 43.75; H, 3.03; N, 13.47; S, 7.50.

4.1.4.12. 3-(4-Bromophenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8l. Yield 59%; mp 261–3 °C; IR(KBr) (υ, cm^{-1}), 1641 (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 4.50 (2H, s, NHNH_2), 7.14 (2H, s, SO_2NH_2), 7.42 (2H, d, J = 8.78 Hz, Ar-H), 7.58 (1H, d, J = 8.78 Hz, indole C_7–H), 7.66–7.70 (3H, m, indole C_6-H and Ar-H), 8.01 (1H, d, J = 1.46 Hz, indole C_4–H), 9.15 (1H, s, CONH), 12.16 (1H, brs., indole NH). Anal. Calcd for $C_{15}H_{13}BrN_4O_3S$ (409.257): C, 44.02, H, 3.20; N, 13.69; S, 7.83. Found: C, 43.64; H, 3.09; N, 13.32; S, 7.56.

4.1.4.13. 3-(3-Methoxyphenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8m. Yield 70%; mp 286–8 °C; IR(KBr) (υ, cm^{-1}), 1643 (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 3.80 (3H, s, phenyl 3-OCH_3), 4.50 (2H, s, NHNH_2), 6.96–6.98 (1H, m, Ar-H), 7.03–7.04 (2H, m, Ar-H), 7.14 (2H, br s, SO_2NH_2), 7.41 (1H, t, J = 7.30 Hz, Ar-H), 7.57 (1H, d, J = 8.78 Hz, indole C_7–H), 7.67 (1H, dd, J = 8.30, 1.47 Hz, indole C_6–H), 8.01 (1H, d, J = 1.47 Hz, indole C_4–H), 8.86 (1H, s, CONH), 11.43 (1H, br s, indole NH). Anal. Calcd for $C_{16}H_{16}N_4O_4S$ (360.387): C, 53.32, H, 4.47; N, 15.55; S, 8.90. Found: C, 53.33; H, 4.26; N, 15.41; S, 8.83.

4.1.4.14. 3-(2,3,4,5,6-Pentafluorophenyl)-2-(hydrazinocarbonyl)-1*H*-indole-5-sulfonamide 8n. Yield 52%; mp 86–7 °C; IR(KBr) (υ, cm^{-1}), 1658 (C=O); ^{1}H NMR (DMSO-d_6, 500 MHz) δ (ppm): 4.43 (2H, s, NHNH_2), 7.10 (2H, s, SO_2NH_2), 7.52 (1H, d, J = 8.79 Hz, indole C_7–H), 7.59 (1H, dd, J = 8.78, 3.42 Hz, indole C_6–H), 7.66–7.69 (1H, m, indole C_4–H), 9.28 (1H, s, CONH), 12.20 (1H, br s, indole NH). Anal. Calcd for $C_{15}H_9F_5N_4O_3S$ (420.313): C, 42.86, H, 2.16; N, 13.33; S, 7.63. Found: C, 42.45; H, 2.55; N, 13.25; S, 7.32.

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 0.01 nM done with the assay buffer mentioned above. At least seven different inhibitor concentrations have been used for measuring the inhibition constant. 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] All CAs used here were recombinant proteins obtained as reported earlier by our groups.[12–22]

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