Carbonic anhydrase activators: Activation of human isozymes I, II and IX with phenylsulfonylhydrazido L-histidine derivatives

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ABSTRACT

Activation of the human carbonic anhydrase (CA, EC 4.2.1.1) isozymes I, II (cytosolic) and IX (transmembrane, tumor-associated isoform) with a series of arylsulfonylhydrazido-L-histidines incorporating 4-substituted-phenyl, pentafluorophenyl- and β-naphthyl moieties was investigated. The compounds showed a weak hCA I activation profile, but were more efficient as hCA II and IX activators. The 4-iodo-phenyl-substituted derivative behaved as a strong and isozyme selective hCA II activator, with an activation constant of 0.21 μM. This is the first isoform-selective, potent CA activator reported to date.

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Carbonic anhydrases (CAs, EC 4.2.1.1)[1] are activated by amines and amino acids,[2] and most of the 16 mammalian isozymes known to date were investigated for their interaction with this type of compounds.[3–6,11c] CA activators (CAAs), unlike the CA inhibitors (CAIs)[1] are not used clinically at this time, but some pharmacological studies[7] emphasized their potential use as agents to enhance memory or for the management of Alzheimer's disease. Indeed, many CA isozymes (of the 15 known to date in humans—since CA XV, the last isoform is not present in primates) are present in reduced amounts in the brain of such patients.[8] The CA activation mechanism started to be understood only in recent years after the report of the first X-ray crystal structure of isozyme CA II with histamine.[9] This, and subsequent kinetic and X-ray crystallographic studies,[10–12] showed the activators to bind at the entrance of the active site cavity, in a region different of the inhibitor or substrate binding sites, and to facilitate the rate-determining step of the catalytic turnover, that is, the transfer of a proton from the zinc-bound water molecule to the environment, with generation of the catalytically active species of the enzyme, with hydroxide coordinated to the zinc ion.[1–4,11c] In many mammalian isozymes a histidine residue (His64, CA I numbering system) or a His cluster (His3, 4, 10, 15 and 64 in the case of CA II,[9] the best catalyst together with CA IX among the many investigated such enzymes)[13] assists this proton transfer process. Thus, in the presence of a CAA, due to the supplementary proton release pathway in the enzyme–activator complex, a dramatic enhancement of k_{cat} is observed (with no modification of K_M) as reported by us and by Silverman's and Lindskog's groups.[3–6,10–12] The overall effect is an enhancement of the catalytic turnover by a factor of 2–7 for the various CA isozymes, generally in the presence of micromolar concentrations of activators, some of which are derivatives with physiological relevance (e.g., histamine, catecholamines, amino acids such as L-His, L-Phe, L-Tyr, L-DOPA, L-Trp, etc.) present in high amounts in various tissues.[3–6,10–12] In this way, the investigation of CAAs may lead not only to new types of therapeutic agents but also to a better understanding of these enzymes, present in a multitude of isoforms and in various tissues/organs,[3–6,10–12] in some of which their role is not clearly established.

A well known CAA is L-His, the interactions of which with various isoforms has been characterized in detail both kinetically and by means of X-ray crystallography by our group.[10–12] As seen from Figure 1, L-His binds at the entrance of the human (h) hCA II active site, in the activator binding site previously discovered by us for the histamine (Hsm)—hCA II adduct,[9] participating in three strong hydrogen bonds with His64, Gln92 and Asn67.[12c] In addition, the activator molecule participates to an extended

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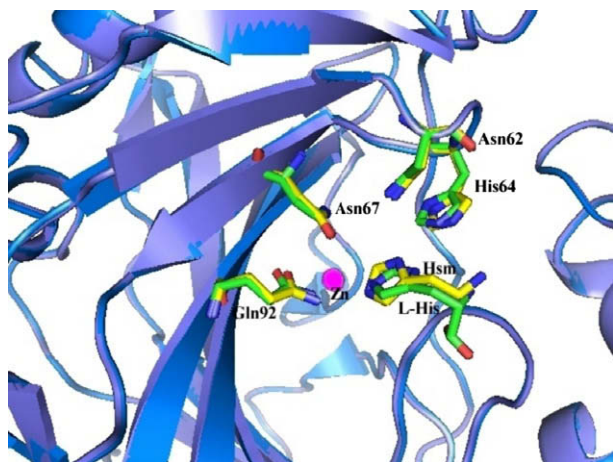
Figure 1. Superposition of hCA II—histamine, Hsm (pdb file 1AVN)[9] and hCA II—L-His (pdb file 2ABE)[12c] adducts as obtained by X-ray crystallographic experiments. The catalytic Zn(II) ion (violet sphere) and amino acid residues involved in the binding of activators (Asn67, Gln92, Asn62, and His64) are shown. Hsm is in yellow (together with the corresponding amino acids involved in its binding) whereas L-His (and the corresponding amino acids) are in green. The protein backbones/Zn(II) ions are totally superposable for the two adducts and are in blue and violet, respectively.

network of hydrogen bonds which involve its imidzolic Nε moiety, and three water molecules from the active site, which connect the activator and the zinc-bound water molecule, presumably leading to the supplementary proton transfer pathway mentioned above. All these hydrogen bonds are strong ones, the distances between two adjacent oxygen atoms being in the range of 2.83–2.91 Å.[12c] It may be observed that the amino group of L-His, similarly with the amino group of histamine (from its aminoethyl moiety) are freely fluctuating, being orientated towards the exit of the active site and making few or no contacts with amino acid residues or water molecules (Fig. 1).[12c] From the medicinal chemistry point of view, this offers an interesting opportunity to derivatize these moieties, in order to obtain different classes of CAAs. Indeed, many types of such compounds have been reported by using histamine as lead molecule,[11] but no CAAs were yet obtained by considering L-His as lead. Here we report a first study of this type, and investigate some L-His derivatives which incorporate sulfonyl-hydrazine moieties. The hydrazide function is a structural motif found in a large variety of compounds of biological interest such as the anti-tuberculosis drug isoniazide. The use of this moiety in the design of CAIs has been applied successfully by this group, and new classes of interesting compounds have been identified, among which derivatives of types **A** and **B**.[14,15]
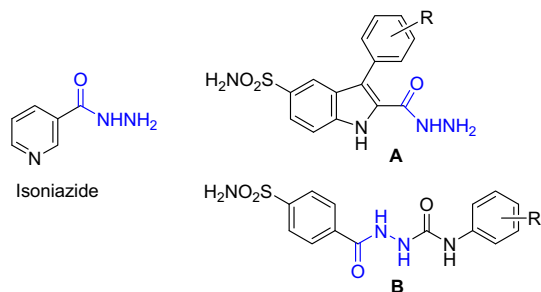
In our ongoing research on the preparation of new modulators of CA activity, we report here an investigation of the phenylsulfonylhydrazide derivatives of L-His as potential CAAs. These compounds were designed using L-His as lead, since its binding mode to CA II shown in Figure 1 is well understood. They were reported earlier to behave as weak inhibitors of the prokaryotic en-

zyme histidinol dehydrogenase.[16] The compounds 1–13 investigated here as activators of several CA isozymes were prepared by reaction of L-histidine methyl ester with arylsulfonyl hydrazides of the type $ArSO_2NHNH_2$.[16]

	R
1	-CH$_3$
2	-F
3	-Br
4	-I
5	-Cl
6	-tBu
7	-Ph
8	-OCH$_3$
9	-CN
10	-NO$_2$
11	-CF$_3$
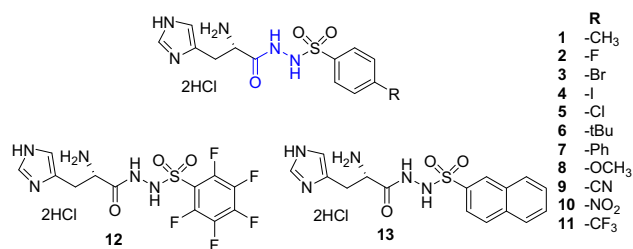
Data of Table 1 show that the L-His derivatives 1–13 show interesting activating properties against the three CA isozymes hCA I, II (cytosolic isoforms) as well as hCA IX (transmembrane, tumor-associated isozyme) investigated in this study. The following structure activity relationship (SAR) can be observed for this small series of compounds:

(i) against hCA I, all new derivatives 1–13 showed moderate activating properties, with activation constants (K_A-s) in the range of 33.1–78.9 μM, being thus much less efficient activators as compared to the lead molecule L-His, which is a very effective activator (K_A of 0.03 μM, Table 1). Thus, the introduction of the bulky arylsulfonylhydrazido moiety in the lead molecule L-His is detrimental for the hCA I activating properties of compounds 1–13, this class of derivatives behaving as weak activators of this isoform. In fact, the X-ray crystal structure of isoform hCA I in adduct with L-His showed the activator to be bound quite deep within the active site.[17a] On the contrary, the binding site of L-His and other amino acid derivatives to hCA II is orientated towards the entrance of the active site cavity,[10,12] where much more space is available for the binding of activators (or inhibitors).[17b,18]

(ii) Indeed, against the ubiquitous and physiologically highly relevant isoform hCA II the new compounds showed a more interesting activation profile. Again, most of these compounds (1, 2, and 6–12) were rather weak activators, pos-

Table 1
Activation constants of hCA I and hCA II (cytosolic isozymes), as well as hCA IX (transmembrane, tumor-associated isoform) with compounds 1–13 and L-His as lead molecule

No.	Compound	K_A (μM)[*]		
		hCA I[a]	hCA II[a]	hCA IX[b]
—	L-His	0.03 ± 0.002	10.9 ± 0.7	9.71 ± 0.5
1		46.8 ± 3.1	25.0 ± 1.1	18.1 ± 0.9
2		63.4 ± 4.5	44.8 ± 2.8	19.5 ± 1.4
3		55.7 ± 2.8	19.6 ± 0.9	13.8 ± 1.2
4		76.3 ± 5.9	0.21 ± 0.01	14.1 ± 0.8
5		70.1 ± 6.0	6.53 ± 0.40	35.4 ± 1.8
6		78.9 ± 4.7	45.2 ± 1.7	29.8 ± 2.0
7		33.1 ± 1.9	51.7 ± 4.2	28.5 ± 1.7
8		44.8 ± 2.5	38.0 ± 1.9	27.4 ± 1.3
9		40.3 ± 3.6	28.7 ± 2.0	17.8 ± 0.5
10		54.7 ± 4.1	45.6 ± 1.9	30.5 ± 1.1
11		38.9 ± 3.2	49.5 ± 4.7	22.4 ± 1.5
12		42.5 ± 2.0	50.2 ± 2.1	30.2 ± 2.6
13		63.8 ± 3.3	18.0 ± 0.9	24.1 ± 1.4

[*] Mean ± standard error (from three determinations) by a stopped-flow, CO$_2$ hydrase method.[18]
[a] Human recombinant isozymes.[12]
[b] Catalytic domain, human recombinant enzyme.[13]

sessing K_As in the range of 25.0–51.7 μM. Three compounds (**3, 5** and **13**) were better CAAs, with activation constants in the range of 6.53–19.6 μM (in the same range as the lead molecule, L-His which shows a K_A of 10.9 μM), whereas compound **4** showed excellent activating properties, with a K_A of 0.21 μM (Table 1). Thus, the nature of the arylsulfonylhydrazido moiety present in derivatives **1–13** is highly important for the CA II activating properties of these derivatives. The only effective such substitution patterns were those incorporating 4-halogeno-phenylsulfonyl (where the halogen is bromine, iodine and chlorine) and 2-naphthyl-sulfonyl moieties. The best activator was the iodo-substituted derivative **4** which was 51.9 times a better hCA II activator as compared to the lead molecule L-His. The next most efficient CAA was the 4-chloro-derivative **5**, which was 1.67 times more effective than L-His, but 31 times less effective compared to the structurally-related 4-iodo derivative **4**. Thus, very small structural changes in the molecules of these activators lead to dramatic changes in the CA activating properties, but the interactions responsible for them are unknown for the moment, since no X-ray crystal structure of such activators with hCA II are available at the moment.

(iii) The tumor-associated isoform hCA IX was also activated by all these compounds, with several of them behaving as moderately effective hCA IX activators (i.e., compounds **1–4** and **9** showed K_As in the range of 14.1–19.5 μM, of the same order of magnitude as L-His itself). Most of them were however weaker hCA IX activators, with activation constants in the range of 22.4–35.4 μM.

(iv) An important observation which emerged during this research is related to the specificity of compound **4** for the activation of hCA II and not of the other two isoforms investigated here, that is, hCA I and IX. Indeed, the problem of designing isozyme-selective CAIs and CAAs for this enzyme family with 16 different members, is not a trivial one.[1–4,11c] Most of the sulfonamide CAIs and many CAAs belonging to the amino acid/amine type of compounds indiscriminately inhibit or activate, respectively, all CA isoforms investigated so far.[1–4,11c] This may constitute a problem when such an enzyme modulator is used as a pharmacological agent that should target only one isoform, and also explains the various side effects seen when CAIs are clinically used as diuretics, antiglaucoma or antiobesity agents.[1–4,11c] Some progress has been registered ultimately for the design of isozyme-selective CAIs but no reports regarding isozyme-selective CAAs are available so far. Here we detect the first such compound (i.e., derivative **4**) which shows such an activating profile of specifically activating only one isoform. Indeed, **4** is a submicromolar activator of isoform II and shows much higher affinities for hCA I and IX (K_As in the range of 14.1–76.3 μM).

In conclusion, we report an activation study with a series of arylsulfonylhydrazido-L-histidine derivatives, incorporating 4-substituted-phenyl, pentafluorophenyl- and β-naphthyl moieties. These compounds showed a weak hCA I activation profile, but were more efficient as hCA II and IX activators. The 4-iodophenyl-sulfonyl substituted derivative behaved as a strong and isozyme selective hCA II activator, with an activation constant of 0.21 μM.

Acknowledgments

References and notes

1. Supuran, C. T. *Nat. Rev. Drug Disc.* **2008**, *7*, 161.
2. (a) Temperini, C.; Scozzafava, A.; Supuran, C. T. *Curr. Pharm. Des.* **2008**, *14*, 708; (b) Supuran, C. T.; Scozzafava, A. Activation of Carbonic Anhydrase Isozymes. In *The Carbonic Anhydrases—New Horizons*; Chegwidden, W. R., Carter, N., Edwards, Y., Eds.; Birkhauser: Basel, Switzerland, 2000; pp 197–219.
3. (a) Ilies, M.; Scozzafava, A.; Supuran, C. T. Carbonic Anhydrase Activators. In *Carbonic Anhydrase—Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton (FL), USA, 2004; pp 317–352; (b) Clare, B. W.; Supuran, C. T. *J. Pharm. Sci.* **1994**, *83*, 768.
4. (a) Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2002**, *45*, 284; (b) Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1177; (c) Ilies, M.; Banciu, M. D.; Ilies, M. A.; Scozzafava, A.; Caproiu, M. T.; Supuran, C. T. *J. Med. Chem.* **2002**, *45*, 504.
5. (a) Parkkila, S.; Vullo, D.; Puccetti, L.; Parkkila, A. K.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3955; (b) Vullo, D.; Nishimori, I.; Innocenti, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1336.
6. (a) Vullo, D.; Innocenti, A.; Nishimori, I.; Scozzafava, A.; Kaila, K.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4107; (b) Nishimori, I.; Onishi, S.; Vullo, D.; Innocenti, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem.* **2007**, *15*, 5351.
7. (a) Sun, M.-K.; Alkon, D. L. *Trends Pharmacol. Sci.* **2002**, *23*, 83; (b) Sun, M. K.; Alkon, D. L. *J. Pharmacol. Exp. Ther.* **2001**, *297*, 961.
8. Lakkis, M. M.; O'Shea, K. S.; Tashian, R. E. *J. Histochem. Cytochem.* **1997**, *45*, 657.
9. (a) Briganti, F.; Mangani, S.; Orioli, P.; Scozzafava, A.; Vernaglione, G.; Supuran, C. T. *Biochemistry* **1997**, *36*, 10384; (b) Temperini, C.; Innocenti, A.; Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 628; (c) Vullo, D.; Nishimori, I.; Innocenti, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1336; (d) Vullo, D.; Innocenti, A.; Nishimori, I.; Scozzafava, A.; Kaila, K.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4107; (e) Pastorekova, S.; Vullo, D.; Nishimori, I.; Scozzafava, A.; Pastorek, J.; Supuran, C. T. *Bioorg. Med. Chem.* **2008**, *16*, 3530.
10. (a) Bhatt, D.; Fisher, S. Z.; Tu, C.; McKenna, R.; Silverman, D. N. *Biophys. J.* **2007**, *92*, 562; (b) Elder, I.; Han, S.; Tu, C.; Steele, H.; Laipis, P. J.; Viola, R. E.; Silverman, D. N. *Arch. Biochem. Biophys.* **2004**, *421*, 283; (c) Duda, D.; Govindasamy, L.; Agbandje-McKenna, M.; Tu, C.; Silverman, D. N.; McKenna, R. *Acta Crystallogr., Sect. D* **2003**, *59*, 93; (d) An, H.; Tu, C.; Duda, D.; Montanez-Clemente, I.; Math, K.; Laipis, P. J.; McKenna, R.; Silverman, D. N. *Biochemistry* **2002**, *41*, 3235; (e) Elleby, B.; Sjöblom, B.; Tu, C.; Silverman, D. N.; Lindskog, S. *Eur. J. Biochem.* **2000**, *267*, 5908.
11. (a) Briganti, F.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2043; (b) Scozzafava, A.; Supuran, C. T. *Eur. J. Med. Chem.* **2000**, *35*, 31; (c) Scozzafava, A.; Iorga, B.; Supuran, C. T. *J. Enz. Inhib.* **2000**, *15*, 139; (d) Supuran, C. T.; Scozzafava, A. *Bioorg. Med. Chem.* **1999**, *7*, 2915.
12. (a) Temperini, C.; Scozzafava, A.; Puccetti, L.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5136; (b) Temperini, C.; Scozzafava, A.; Vullo, D.; Supuran, C. T. *J. Med. Chem.* **2006**, *49*, 3019; (c) Temperini, C.; Scozzafava, A.; Vullo, D.; Supuran, C. T. *Chemistry* **2006**, *12*, 7057; (d) Temperini, C.; Innocenti, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem.* **2008**, *16*, 8373.
13. Hilvo, M.; Baranauskiene, L.; Salzano, A. M.; Scaloni, A.; Matulis, D.; Innocenti, A.; Scozzafava, A.; Monti, S. M.; Di Fiore, A.; De Simone, G.; Lindfors, M.; Janis, J.; Valjakka, J.; Pastorekova, S.; Pastorek, J.; Kulomaa, M. S.; Nordlund, H. R.; Supuran, C. T.; Parkkila, S. *J. Biol. Chem.* **2008**, *283*, 27799.
14. Winum, J. Y.; Dogné, J. M.; Casini, A.; de Leval, X.; Montero, J. L.; Scozzafava, A.; Vullo, D.; Innocenti, A.; Supuran, C. T. *J. Med. Chem.* **2005**, *48*, 2121.
15. (a) Güzel, Ö.; Temperini, C.; Innocenti, A.; Scozzafava, A.; Salman, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 152; (b) Güzel, Ö.; Innocenti, A.; Scozzafava, A.; Salman, A.; Parkkila, S.; Hilvo, M.; Supuran, C. T. *Bioorg. Med. Chem.* **2008**, *16*, 9113.
16. Abdo, M. R.; Joseph, P.; Boigegrain, R. A.; Montero, J. L.; Köhler, S.; Winum, J. Y. *J. Enzyme Inhib. Med. Chem.* **2008**, *23*, 357.
17. (a) Temperini, C.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5152; (b) Maresca, A.; Temperini, C.; Vu, H.; Pham, N. B.; Poulsen, S. A.; Scozzafava, A.; Quinn, R. J.; Supuran, C. T. *J. Am. Chem. Soc.* **2009**, *131*, 3057.
18. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An Applied Photophysics stopped-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s at 25 °C. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation constants. For each activator at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activators **1–13** (10 mM) were prepared in distilled-deionized water and dilutions up to 0.001 μM were done thereafter with distilled-deionized water. Activator and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–A complex. The activation constant (K_A), defined similarly with the inhibition constant K_I,[1–4,11c] can be obtained by considering the classical Michaelis–Menten equation (Eq. 1), which has been fitted by non-linear least squares by using PRISM 3:

$$\nu = \nu_{max} / \{1 + K_M/[S](1 + [A]_f/K_A)\} \tag{1}$$

where $[A]_f$ is the free concentration of activator.

Working at substrate concentrations considerably lower than K_M ([S] << K_M), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by Eq. 2:[9,11,12]

$$v = v_0 \cdot K_A / \{K_A + ([A]_t - 0.5\{([A]_t + [E]_t + K_A) - ([A]_t + [E]_t + K_A)^2 - 4[A]_t \cdot [E]_t)^{1/2}\}\} \tag{2}$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[9,11,12]