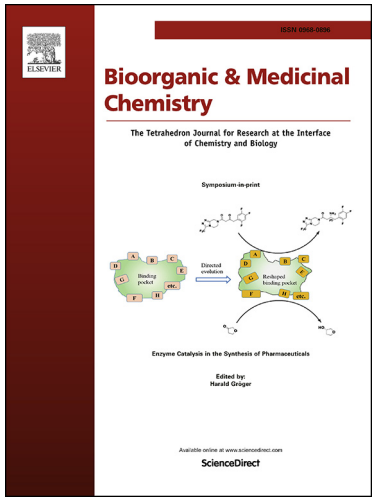


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# α-Carbonic anhydrases are strongly activated by spinaceamine derivatives

Suleyman Akocak [a*], Nabih Lolak [a], Silvia Bua [b], Alessio Nocentini [b], Gulcin Karakoc [a], Claudiu T. Supuran [b*]

[a] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Adiyaman University, 02040 Adiyaman, Turkey
[b]Università degli Studi di Firenze, Dipartimento Neurofarba, Sezione di Scienze Farmaceutiche e Nutraceutiche, Via U. Schiff 6, 50019 Sesto Fiorentino (Florence), Italy.

**Abstract**. A series of 4-substituted-spinaceamine (4,5,6,7-tetrahydro-imidazolo[4,5-*c*]pyridine) were prepared from histamine and aromatic aldehydes Schiff bases, and investigated as activators of four human (h) carbonic anhydrase (CA, EC 4.2.1.1) isoforms, the cytosolic hCA I, II and VII, and the membrane-associated hCA IV. All isoforms were effectively activated by the new derivatives, and the nature of the moiety in position 4 of the bicyclic system was the factor influencing activation properties against all isoforms. For hCA I, these compounds showed $K_A$s in the range of 2.52 – 21.5 μM, the most effective activator being 4-(2-hydroxyphenyl)-spinaceamine. For hCA II the activation constants ranged between 0.60 – 17.2 μM, with 4-(2,3,5,6-tetrafluorophenyl)- spinaceamine the best activator. Affinity for hCA IV was in the range of 0.52 – 63.8 μM, and the same compound as for hCA II was the most effective activator. The most sensitive isoform for activation was the brain-associated hCA VII, for which $K_A$s in the range of 82 nM – 4.26 μM were observed. Effective hCA VII activators were the (2-bromophenyl)-, 2,3,5,6-tetrafluorophenyl- and furyl-substituted spineaceamines ($K_A$s of 82-95 nM). As CA activators may have pharmacologic applications in various fields, this work provides interesting derivatives for further studies.

**Keywords**: carbonic anhydrase; activator; spinaceamine; histamine; proton shuttle.

*Corresponding authors: Tel: +90 (416) 223 3800-2836, Fax: +90 (416) 223 3842, E-mail: akocaksuleyman@gmail.com, sakocak@adiyaman.edu.tr, (Suleyman Akocak);
Tel: +39-055-4573729, Fax: +39-055-4573385, E-mail: claudiu.supuran@unifi.it (Claudiu T. Supuran)

## 1. Introduction

Activation of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) was already reported in the early '40s by several independent groups[1-5], with amines (e.g., histamine), amino acids and some short peptides. However, this field became rather controversial soon thereafter, since many researchers working on CAs considered that such effective catalysts do not "need to be activated"[6]. It has even been suggested that this phenomenon does not exist at all, being an artefact due to the complexation of adventitious heavy metals (which inhibit the enzyme activity) present in the enzyme preparations by the activators[6-8]. This rather long period of controversy dated till the early '90s, when work with highly purified enzymes and very precise techniques, such as the stopped-flow assay and the X-ray crystallography, undoubtedly demonstrated that the CA activators (CAAs) exist and that they take part to the catalytic cycle[9-11]. In 1990, a general mechanism of action for the CAAs has been proposed based on equation *1* [9]:

$$EZn^{2+}\text{—}OH_2 + A \Leftrightarrow [EZn^{2+}\text{—}OH_2 - A] \Leftrightarrow [EZn^{2+}\text{—}HO^- - AH^+] \Leftrightarrow EZn^{2+}\text{—}HO^- + AH^+ \quad (1)$$

enzyme - activator complexes

According to this model, the activator binds within the enzyme active site with the formation of enzyme – activator complexes[9], in which the activator molecule participates to the rate-determining step of the catalytic cycle, i.e., proton shuttling between the zinc-coordinated water molecule and the environment, with the formation of the zinc hydroxide species of the enzyme. In fact, in many CA isoforms, it has been shown that residue His64 placed in the middle of the active site cavity is involved in this phenomenon, acting as a proton shuttle during the catalytic cycle[12]. Tu et al.[12] demonstrated this by means of site-directed mutagenesis and extensive kinetic measurements, showing that His64 through its imidazole moiety (with a pKa of around 7), is crucial for proton shuttling and the generation of the nucleophilically active species of the enzyme, according to equations *2* and *3* which describes the CA catalytic cycle:

$$EZn^{2+}\text{—}OH^- + CO_2 \Leftrightarrow EZn^{2+}\text{—}HCO_3^- \Leftrightarrow EZn^{2+}\text{—}OH_2 + HCO_3^- \quad (2)$$

$$EZn^{2+}\text{—}OH_2 \Leftrightarrow EZn^{2+}\text{—}HO^- + H^+ \quad (3)$$

Work from several groups showed that the rate limiting step of the entire catalytic cycle for the $CO_2$ hydration reaction catalyzed by CAs is the proton transfer step (equation *3*) [11-13]. Confirmation that activators have a similar role to His64, i.e., shuttling of the protons from the active site to the environment and facilitation of the formation of the nucleophilic enzyme species, came from the first X-ray crystallographic structure of isoform CA II complexed with histamine as activator[11]. Histamine was observed bound at the entrance of the active site cavity, far away from the metal ion, and participating in a network of hydrogen bonds involving several water molecules, which, as in the case of His64, favor the release of the proton from the water molecule coordinated to the zinc, to the reaction medium[11]. X-ray crystal structures with many other amine and amino acid CAAs were thereafter reported[14-21], which reinforced the above findings: all activators bind in the same active site region, at the entrance of the cavity, from where they can enhance the formation of the zinc hydroxide species of the enzyme, by favoring the proton shuttling between the cavity and the reaction medium[13]. Furthermore, recently it has also been shown the CAAs may have pharmacological applications for enhancing cognition, in the management of CA deficiencies, for therapy memory and for obtaining artificial tissues[22,23]. Thus, there is a strong interest in designing CAAs belonging to various chemical classes, with a more effective and isoform-selective profile compared to amines and amino acids from the first generation of such derivatives[13], such as histamine, dopamine, serotonin and natural amino acids, which may have other pharmacological activities connected to the histaminergic, dopaminergic, serotoninergic activities, in addition to their activating effects in many CA isoforms. Here, we explored the synthesis and CA activating effects on four human (h) CA isoforms with a series of spinaceamine derivatives.

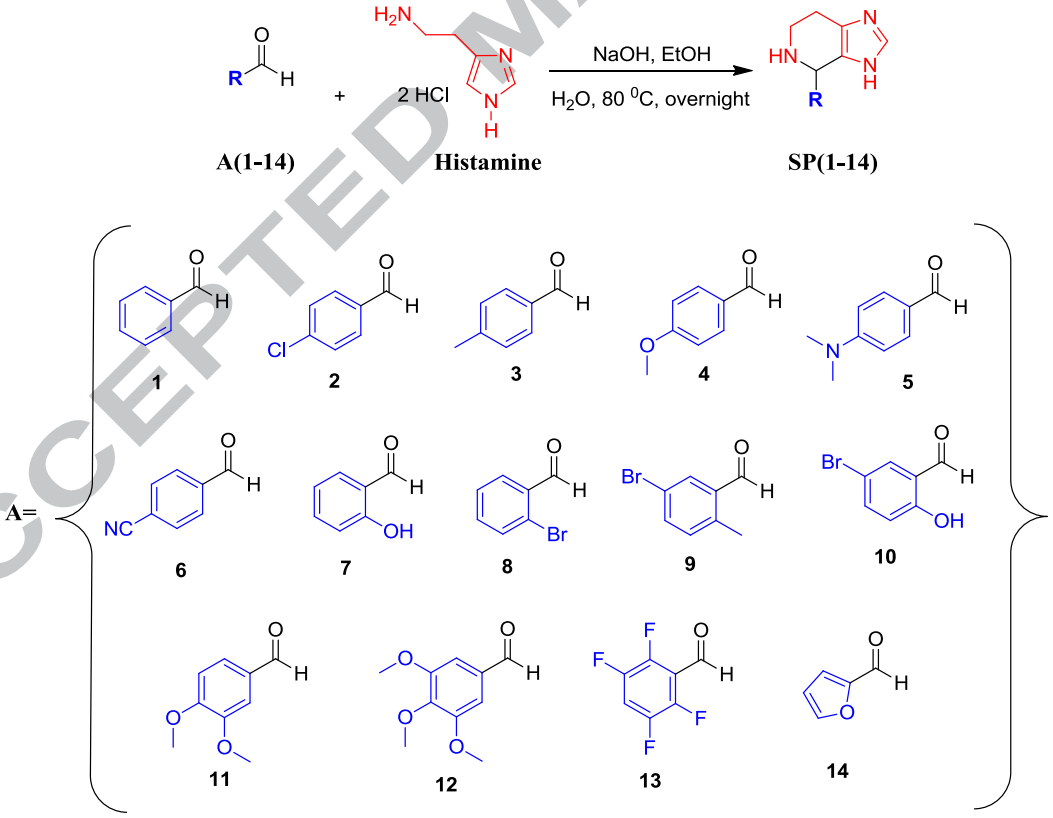
## 2. Results and Discussion

### 2.1. Chemistry

Spinaceamine (**SP**), an alkaloid isolated from the skin of amphibians from Australia and Papua New Guinea, may be considered a cyclization product of histamine[24]. This bicyclic derivative incorporates two basic nitrogen atoms (in the imidazolic and six-membered cycle) which in principle may be protonated and thus participate to proton shuttling when bound to the CA active site. The rationale for designing CAAs presented in this work is based on our previous

data which showed efficient CA activating effects for derivatized histamine Schiff base compounds[25]. Therefore, in this work, a number of structurally diverse spinaceamine substituted compounds (ring-closure product of histamine Schiff bases)[26,27] were synthesized according to general synthetic route illustrated in Scheme 1. In order to generate chemical diversity, different substituted aldehydes were chosen, possessing aromatic and heterocyclic moieties, and they were reacted with histamine leading to the new spinaceamine substituted compounds **SP(1-14)** (Scheme 1). All the synthesized compounds **SP(1-14)** were fully characterized by using several analytical and spectral data (see experimental part for details).

In the current work, the synthesis of the series of substituted spinaceamines **SP(1-14)** was carried out with some modifications of the literature procedures[26,27]. Briefly, histamine dihydrochloride was coupled with substituted aromatic/heterocyclic aldehydes, leading after oxidative ring-closure under heating to the desired bicylic derivatives. The structures of substituted spinaceamines **SP(1-14)** were confirmed by using several analytical and spectral data (FT-IR, $^{1}$H-NMR, $^{13}$C-NMR, etc.) as described in the experimental part.




**Scheme 1.** General synthetic route for the synthesis of 4-substituted spinaceamines **SP(1-14)**.

### 2.2. Carbonic anhydrase activation

Considering the fact that the new heterocyclic derivatives **SP(1-14)** reported here incorporate in their molecules functionalities with an appropriate pKa for acting as proton shuttles in the CA catalytic cycle, we have investigated them as CAAs against the following four CA isoforms with important physiological functions: the three cytosolic enzymes hCA I, II and VII[29], and the membrane-associated hCA IV[30]. They are involved in various pathologies, both in the CNS, kidneys, eyes and other organs in which they are highly abundant[29-31].

**Table 1**. CA activation data with histamine spinaceamines **SP1-SP4** and histamine (HST) as a standard activator by a stopped-flow $CO_2$ hydrase assay[28].

| Compound | $K_A$ (μM)* | | | |
|---|---|---|---|---|
| | hCA I | hCA II | hCA IV | hCA VII |
| **SP1** | 8.12 | 16.3 | 63.8 | 2.69 |
| **SP2** | 17.6 | 8.49 | 50.1 | 0.84 |
| **SP3** | 6.27 | 17.2 | 7.06 | 1.03 |
| **SP4** | 6.01 | 7.31 | 6.37 | 0.73 |
| **SP5** | 3.45 | 7.44 | 3.14 | 0.59 |
| **SP6** | 4.89 | 7.25 | 5.80 | 4.26 |
| **SP7** | 2.52 | 1.62 | 3.59 | 0.11 |
| **SP8** | 4.30 | 2.80 | 4.05 | 0.082 |
| **SP9** | 12.7 | 15.5 | 4.42 | 0.38 |
| **SP10** | 4.90 | 1.03 | 10.9 | 0.12 |
| **SP11** | 3.76 | 0.71 | 6.17 | 0.26 |
| **SP12** | 9.28 | 3.26 | 2.84 | 0.093 |
| **SP13** | 9.92 | 0.60 | 0.52 | 0.11 |
| **SP14** | 21.5 | 5.12 | 2.40 | 0.095 |
| **HST** | 2.10 | 125 | 4.03 | 37.6 |

* Mean from 3 different determinations (errors in the range of 5-10 % of the reported values, data not shown).

The following structure-activity relationship (SAR) can be evidenced from data of Table 1:

(i) The cytosolic isoform hCA I was moderately activated by all compounds reported here with $K_A$ values in the range of 2.52-21.5 µM. The substituted spinaceamines were slightly weaker activators compared to the starting, standard compound histamine (**HST**, $K_A$ = 2.10 µM), which has a moderate activity on hCA I. In particular, compound **SP7** bearing the 2-OH-phenyl substitution showed the best activation against this isoform with a $K_A$ of 2.52 µM. The activation constants of the other derivatives were close to each other and were ranging between 3.45 and 9.92 µM, except for compounds **SP2** (4-Cl), **SP9** (2-Me, 5-Br) and **SP14** (furyl) which were less effective activators compared to the other derivatives ($K_A$ values of 17.6, 12.7 and 21.5 µM, respectively).

(ii) All compounds reported here **SP(1-14)** were more active CAAs against one of most abundant cytosolic isoform, hCA II, compared to histamine, which is a quite weak activator of this isoform with a $K_A$ of 125 µM. In this series of compounds, the best hCA II activators were **SP7** (2-OH), **SP10** (2-OH, 5-Br), **SP11** (3,4-diMeO) and **SP13** (2,3,5,6-F) with $K_A$s in the range of 0.60-1.62 µM, being thus several orders of magnitude better CAAs compared to histamine. Compounds **SP1** (4-H), **SP3** (4-Me) and **SP9** (2-Me, 5-Br) were the least active CAAs in the series, with $K_A$ values of 16.3, 17.2, and 15.5 µM. The structure-activity data demonstrate that the spinaceamine derivatives **SP(1-14)** show much better hCA II activation potency than their counterparts, non-cyclic histamine Schiff base derivatives, which were reported recently by us and showed ineffective activity against this isoform[25].

(iii) The membrane-bound isoform hCA IV was moderately activated by most of the compounds reported here. **SP13** (2,3,5,6-F) showed a good potency, with an activation constant of 0.52 µM. The activation constants of the remaining compounds ranged from 2.40 µM to 10.9 µM, except the **SP1** (4-H) and **SP2** (4-Cl), which were the weakest activators in the series, with $K_A$s of 63.8 and 50.1 µM, respectively.

(iv) The other cytosolic isoform investigated here, hCA VII, was efficiently activated by most of the compounds reported in this work. All derivatives showed much better activation profile than the lead histamine ($K_A$ of 37.6 μM) against hCA VII. Only three compounds showed $K_A$s > 1 μM, i.e., compounds **SP1** (4-H), **SP3** (4-Me) and **SP6** (4-CN) with $K_A$ values of 2.69, 1.03 and 4.26 μM, respectively. The remaining derivatives were much more effective CAAs: **SP8** (2-Br), **SP12** (2,3,4-triMeO) and **SP14** (furyl) showed nanomolar activation potency, with $K_A$s in the range of 82-95 nM. As this isoform is one of the most widely spread in the brain, and probably involved in crucial metabolic/pH regulation processes, our results seem to be very promising in the search of more effective CA VII activators than the currently available such derivatives. The rest of the investigated compounds were also good activators, with $K_A$ values ranging between 0.11 μM and 0.84 μM. Another important finding in the current work is the good selectivity observed for hCA VII activation over the other physiologically related isoforms, such as hCA I, II and IV (e.g., **SP8** was a nanomolar hCA VII activators whereas it has micromolar activity against the remaining three isoforms – Table 1).

## 3. Conclusions

In the present study, a series of 4-substituted-spinaceamine derivatives **SP(1-14)** were synthesized from histamine by using aromatic and heterocyclic aldehydes. The obtained compounds were investigated and assessed as activators of four human (h) carbonic anhydrase isoforms, the cytosolic hCA I, II and VII, and the membrane-associated hCA IV. All the compounds from this study showed great activity against the tested isoforms that most of the compounds were having better affinity than starting compound histamine. Specifically, hCA VII, was greatly activated by most of the tested compounds which some of them having nanomolar potency (82-95 nM). Since CAAs are getting more attention in the memory therapy and cognitive neurodegenerative disorders, and hCA VII is a key isoform involved in brain metabolism, these potent hCA VII activators reported here may be considered of interest for *in vivo* investigations for possible therapeutic applications.

## 4. Experimental

### 4.1. Chemistry

All chemicals and anhydrous solvents were purchased from Sigma-Aldrich, Merck, Alfa Aesar and TCI and used without further purification. Melting points (mp) were determined with SMP30 melting point apparatus in open capillaries and are uncorrected. FT-IR spectra were recorded by using Perkin Elmer Spectrum 100 FT-IR spectrometer. Nuclear Magnetic Resonance ($^1$H-NMR and $^{13}$C-NMR) spectra of compounds were recorded using a Bruker Advance III 300 MHz spectrometer in DMSO-$d_6$ and TMS as an internal standard operating at 300 MHz for $^1$H-NMR and 75 MHz for $^{13}$C-NMR. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 $F_{254}$ plates

#### 4.1.1. General procedure for the synthesis of Spinaceamine substituted compounds SP(1-14)

To solution of 5 mmol of histamine dihydrochloride in 10 ml of water were added solutions of 15 mmol of sodium hydroxide (NaOH) and 5 mmol of appropriate aldehyde derivatives in 15 ml of ethanol. The reaction mixture was heated overnight at around 80 $^0$C and allowed to slow evaporation of the solvent. The completion of the reaction was monitored by TLC and FT-IR. After that, the mixture was allowed to cool to room temperature and separated precipitate was filtered off. The obtained final compounds **SP(1-14)** recrystallized from hot water and dried under vacuum at 40 $^0$C and fully characterized by FT-IR, $^1$H-NMR, $^{13}$C-NMR, and melting points.

**4-phenyl-4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridine (SP1):** Yield: 66%; Color: white powder, mp: 195-198 $^0$C; FT-IR (cm$^{-1}$): 3312, 3024, 2956, 2917, 2804, 1619, 1438, 1364, 972, 813; $^1$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 7.80 (s, 1H, H-2 Im), 7.62 (m, 3H, Ar-H), 7.35 (m, 2H, Ar-H), 5.12 (s, 1H, -CH-), 3.32 (m, 2H,-$CH_2CH_2$-Im), 2.95 (t, 2H, $J$ = 6.2, -$CH_2CH_2$-Im): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 140.5, 135.3, 134.4, 129.7, 128.2, 127.4, 113.8, 64.1, 39.7, 30.1.

**4-(4-chlorophenyl)-4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridine (SP2):** Yield: 52%; Color: white powder, mp: 170-173 $^{0}$C; FT-IR (cm$^{-1}$): 3334, 3036, 2955, 2906, 2806, 1622, 1441, 1015, 805; $^{1}$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 7.72 (s, 1H, H-2 Im), 7.50 (d, 2H, *J* = 7.5, Ar-H), 7.28 (d, 2H, *J* = 7.5, Ar-H), 5.15 (s, 1H, -CH-), 3.25 (m, 2H,-$CH_2CH_2$-Im), 2.96 (t, 2H, *J* = 6.0, -$CH_2CH_2$-Im), 2.41 (s, 3H, -$CH_3$): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 139.8, 136.7, 134.8, 134.1, 129.1, 127.8, 114.5, 64.5, 40.6, 30.8, 21.5.

**4-(p-tolyl)-4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridine (SP3):** Yield: 45%; Color: white powder; mp: 180-183 $^{0}$C; FT-IR (cm$^{-1}$): 3345, 3012, 2921, 2841, 1607, 1435, 973, 809; $^{1}$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 7.68 (s, 1H, H-2 Im), 7.48 (d, 2H, *J* = 7.2, Ar-H), 7.22 (d, 2H, *J* = 7.2, Ar-H), 5.12 (s, 1H, -CH-), 3.18 (m, 2H,-$CH_2CH_2$-Im), 2.90 (t, 2H, *J* = 6.2, -$CH_2CH_2$-Im), 2.38 (s, 3H, -$CH_3$): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 139.5, 136.1, 134.6, 133.9, 128.2, 127.5, 114.7, 64.2, 40.3, 30.3, 21.7.

**4-(4-methoxyphenyl)-4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridine (SP4):** Yield: 50%; Color: light yellow; mp: 180-183 $^{0}$C; FT-IR (cm$^{-1}$): 3329, 3028, 2955, 2809, 1609, 1442, 1365, 1009, 813; $^{1}$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 7.81 (s, 1H, H-2 Im), 7.58 (d, 2H, *J* = 7.0, Ar-H), 7.33 (d, 2H, *J* = 7.0, Ar-H), 5.16 (s, 1H, -CH-), 3.88 (s, 3H, -$OCH_3$), 3.26 (m, 2H,-$CH_2CH_2$-Im), 2.97 (t, 2H, *J* = 6.0, -$CH_2CH_2$-Im): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 158.6, 139.5, 136.2, 134.3, 133.6, 129.4, 127.5, 113.7, 64.0, 55.8, 39.4, 29.3.

**4-N,N-dimethyl-phenyl-(4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridine (SP5):** Yield: 62%; Color: orange; mp: 90-93 $^{0}$C; FT-IR (cm$^{-1}$): 3330, 3024, 2914, 2845, 1605, 1443, 1354, 946, 816; $^{1}$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 7.85 (s, 1H, H-2 Im), 7.63 (d, 2H, *J* = 7.7, Ar-H), 7.42 (d, 2H, *J* = 7.5, Ar-H), 5.22 (s, 1H, -CH-), 3.32 (m, 2H,-$CH_2CH_2$-Im), 3.18 (s, 6H, -$N(CH_3)_2$), 2.99 (t, 2H, *J* = 5.5, -$CH_2CH_2$-Im): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 150.4, 139.1, 136.7, 134.3, 133.2, 129.1, 126.9, 114.5, 64.2, 40.8, 39.8, 29.7.

**4-(4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridin-4-yl)benzonitrile (SP6):** Yield: 55%; Color: light yellow; mp: 200-203 $^{0}$C; FT-IR (cm$^{-1}$): 3322, 3158, 3020, 2963, 2848, 2231, 1615, 1452, 1388, 971, 841; $^{1}$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 7.88 (s, 1H, H-2 Im), 7.53 (d, 2H, *J* = 6.9,

Ar-H), 7.33 (d, 2H, *J* = 7.2, Ar-H), 5.20 (s, 1H, -CH-), 3.28 (m, 2H,-$CH_2CH_2$-Im), 2.98 (t, 2H, *J* = 6.2, -$CH_2CH_2$-Im): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 141.2, 137.3, 134.9, 134.0, 128.7, 127.6, 118.8, 114.3, 64.5, 40.4, 30.5.

**2-(4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridin-4-yl)phenol (SP7):** Yield: 48%; Color: light brown; mp: 195-198 $^0$C; FT-IR ($cm^{-1}$): 3342, 3320, 3150, 3035, 2962, 28021, 1610, 1458, 1345, 978, 815; $^1$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 13.45 (br.s, 1H, -OH), 7.80 (s, 1H, H-2 Im), 7.50 (m, 3H, Ar-H), 7.38 (m, 2H, Ar-H), 5.15 (s, 1H, -CH-), 3.29 (m, 2H,-$CH_2CH_2$-Im), 2.95 (t, 2H, *J* = 6.2, -$CH_2CH_2$-Im): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 159.3, 136.2, 134.5, 133.6, 128.2, 126.9, 118.1, 113.3, 64.2, 39.4, 29.5.

**4-(2-bromophenyl)-4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridine (SP8):** Yield: 55%; Color: white powder; mp: 120-122 $^0$C; FT-IR ($cm^{-1}$): 3125, 3028, 2975, 2816, 1633, 1463, 967, 832; $^1$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 7.92 (s, 1H, H-2 Im), 7.63 (m, 3H, Ar-H), 7.45 (m, 2H, Ar-H), 5.21 (s, 1H, -CH-), 3.38 (m, 2H,-$CH_2CH_2$-Im), 2.99 (t, 2H, *J* = 6.5, -$CH_2CH_2$-Im): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 141.2, 137.3, 134.9, 133.8, 128.1, 126.5, 118.5, 113.9, 64.7, 39.9, 30.1.

**4-(5-bromo-2-methylphenyl)-4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridine (SP9):** Yield: 70%; Color: white powder; mp: 198-200 $^0$C; FT-IR ($cm^{-1}$): 3345, 3121, 3020, 2942, 2829, 1613, 1479, 971, 832; $^1$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 7.88 (s, 1H, H-2 Im), 7.63 (d, 1H, Ar-H), 7.48 (s, 1H, Ar-H), 7.12 (s, 1H, Ar-H), 5.11 (s, 1H, -CH-), 3.32 (m, 2H,-$CH_2CH_2$-Im), 2.94 (t, 2H, *J* = 5.5, -$CH_2CH_2$-Im), 2.12 (s, 3H, -$CH_3$): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 141.8, 137.9, 134.5, 133.5, 128.7, 126.9, 118.3, 115.6, 113.2, 64.2, 39.5, 30.5, 20.3.

**4-bromo-2-(4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridin-4-yl)phenol (SP10):** Yield: 73%; Color: light yellow; mp: 136-139 $^0$C; FT-IR ($cm^{-1}$): 3284, 3032, 2959, 2816, 1609, 1479, 1251, 963, 816; $^1$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 13.65 (br.s, 1H, -OH), 7.82 (s, 1H, H-2 Im), 7.58 (d, 1H, Ar-H), 7.42 (s, 1H, Ar-H), 7.10 (s, 1H, Ar-H), 5.05 (s, 1H, -CH-), 3.30 (m, 2H,-$CH_2CH_2$-Im), 2.92 (t, 2H, *J* = 5.8, -$CH_2CH_2$-Im): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 157.2, 136.3, 133.9, 133.1, 128.5, 126.3, 118.6, 115.3, 113.1, 64.0, 39.2, 29.4.

**4-(3,4-dimethoxyphenyl)-4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridine (SP11):** Yield: 43%; Color: brown; mp: 162-165 $^{0}$C; FT-IR (cm$^{-1}$): 3321, 3018, 2939, 2833, 1627, 1450, 1261, 952, 808; $^{1}$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 7.70 (s, 1H, H-2 Im), 7.48 (s, 1H, Ar-H), 7.31 (d, 1H, *J* = 6.9, Ar-H), 6.95 (d, 1H, *J* = 6.9, Ar-H), 5.01 (s, 1H, -CH-), 3.88 (s, 3H, -$OCH_3$), 3.79 (s, 3H, -$OCH_3$), (3.25 (m, 2H,-$CH_2CH_2$-Im), 2.89 (t, 2H, *J* = 5.8, -$CH_2CH_2$-Im): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 150.3, 149.6, 134.5, 133.7, 128.2, 126.8, 118.2, 115.1, 113.5, 64.1, 55.7, 55.1, 39.7, 29.8.

**4-(3,4,5-trimethoxyphenyl)-4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridine (SP12):** Yield: 65%; Color: white powder; mp: 148-150 $^{0}$C; FT-IR (cm$^{-1}$): 3298, 3109, 2947, 2831, 1588, 1419, 1238, 970, 818; $^{1}$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 7.72 (s, 1H, H-2 Im), 7.38 (s, 1H, Ar-H), 7.25 (s, 1H, Ar-H), 5.03 (s, 1H, -CH-), 3.90 (s, 6H, -$OCH_3$), 3.82 (s, 3H, -$OCH_3$), (3.22 (m, 2H,-$CH_2CH_2$-Im), 2.85 (t, 2H, *J* = 5.5, -$CH_2CH_2$-Im): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 151.6, 138.6, 134.2, 133.5, 128.1, 126.3, 118.7, 115.6, 114.0, 64.3, 55.9, 55.2, 40.3, 30.4.

**4-(2,3,5,6-tetrafluorophenyl)-4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridine (SP13):** Yield: 55%; Color: white powder; mp: 220-222 $^{0}$C; FT-IR (cm$^{-1}$): 3320, 3105, 2967, 2848, 1620, 1435, 1243, 971, 820; $^{1}$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 7.75 (s, 1H, H-2 Im), 6.95 (s, 1H, Ar-H), 5.01 (s, 1H, -CH-), (3.20 (m, 2H,-$CH_2CH_2$-Im), 2.87 (t, 2H, *J* = 5.5, -$CH_2CH_2$-Im): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 149.6, 137.5, 134.1, 133.7, 128.5, 126.2, 118.4, 115.1, 114.3, 64.5, 40.5, 29.7.

**4-(furan-2-yl)-4,5,6,7-tetrahydro-3H-imidazo[4,5-*c*]pyridine (SP14):** Yield: 55%; Color: light brown mp: 95-97 $^{0}$C; FT-IR (cm$^{-1}$): 3284, 3085, 2961, 2825, 1613, 1446, 1365, 1009, 813; $^{1}$H-NMR ($CD_3OD$, 300 MHz, δ ppm): 7.89 (s, 1H, H-2 Im), 7.78 (d, 1H, *J* = 5.5, furyl ), 6.45 (d, 1H, *J* = 5.5, furyl), 6.28 (m, 1H, furyl), 5.33 (s, 1H, -CH-), 3.35 (m, 2H,-$CH_2CH_2$-Im), 2.99 (t, 2H, *J* = 6.5, -$CH_2CH_2$-Im): $^{13}$C-NMR ($CD_3OD$, 75 MHz, δ ppm): 151.7, 143.8, 139.2, 134.1, 118.3, 114.5, 106.3, 63.5, 39.1, 28.7.

### 4.2. CA activation

An Sx.18Mv-R Applied Photophysics (Oxford, UK) stopped-flow instrument has been used to assay the catalytic activity of various CA isozymes for $CO_2$ hydration reaction[28]. 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 $NaClO_4$ (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 inhibition 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 (at 0.1 mM) were prepared in distilled-deionized water and dilutions up to 1 nM were made thereafter with the assay buffer. Enzyme and activator solutions were pre-incubated together for 15 min prior to assay, in order to allow for the formation of the enzyme–activator complexes. The activation constant ($K_A$), defined similarly with the inhibition constant $K_I$, can be obtained by considering the classical Michaelis–Menten equation (equation *4*), which has been fitted by non-linear least squares by using PRISM 3:

$$v = v_{max}/\{1+(K_M/[S])(1+[A]_f/K_A)\} \qquad (4)$$

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 equation *5*:

$$v=v_0.K_A/\{K_A+([A]_t-0.5\{([A]_t+[E]_t+K_A)-([A]_t+[E]_t+K_A)^2-4[A]_t.[E]_t)^{1/2}\}\} \qquad (5)$$

where $v_0$ represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator[32-35].

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**References and notes**

1. Leiner, M. Das Ferment Kohlensäureanhydrase im Tierkörper. *Naturwiss.* 1940; 28: 316-317

2. Leiner M, Leiner G. Die Aktivatoren der Kohlensäureanhydrase. *Naturwiss.* 1941; 29: 195-197.

3. Leiner M, Leiner G. Die Messmethoden zur Untersuchung der katalytischen Wirksamkeit der Kohlensäureanhydrase. *Biochem. Z.* 1941; 311: 119-145.

4. Main RE, Locke A. Activation of carbonic anhydrase by histamine. *J. Biol. Chem.* 1941; 140: LXXXI.

5. Kiese M. Die Aktivierung der Kohlensäureanhydrase. *Naturwiss.* 1941; 29: 116-117.

6. Clark AM, Perrin DD. A re-investigation of the question of activators of carbonic anhydrase. *Biochem. J.* 1951; 48: 495-503.

7. Ho C, Sturtevant JM. Activation of bovine carbonic anhydrase by ethylenediamine tetraacetic acid. *Biochem. Biophys. Res. Commun.* 1960; 3: 20-23.

8. Silverman DN, Backman L, Tu C. Role of hemoglobin in proton transfer to the active site of carbonic anhydrase. *J Biol Chem.* 1979; 254: 2588-2591.

9. Supuran CT. Carbonic anhydrase activators. Part 4. A general mechanism of action for activators of isozymes I, II and III. *Rev. Roum. Chim.* 1992; 37: 411-421.

10. Supuran CT, Balaban, AT. Carbonic anhydrase activators. Part 8. $pK_a$ - Activation relationship in a series of amino acid derivatives activators of isozyme II. *Rev. Roum. Chim.* 1994; 39: 107-113.

11. Briganti F, Mangani S, Orioli P, Scozzafava A, Vernaglione G, Supuran CT. Carbonic anhydrase activators: X-ray crystallographic and spectroscopic investigations for the interaction of isozymes I and II with histamine. *Biochemistry.* 1997; 36: 10384-92.

12. Tu CK, Silverman DN, Forsman C, Jonsson BH, Lindskog S. Role of histidine 64 in the catalytic mechanism of human carbonic anhydrase II studied with a site-specific mutant. *Biochemistry.* 1989; 28: 7913-7918.

13. Supuran CT. Carbonic anhydrase activators. *Future Med Chem.* 2018; 10: 561-573.

14. Temperini C, Scozzafava A, Supuran CT. Carbonic anhydrase activation and the drug design. *Curr Pharm Des.* 2008; 14: 708-715.

15. Temperini C, Scozzafava A, Vullo D, Supuran CT. Carbonic anhydrase activators. Activation of isozymes I, II, IV, VA, VII, and XIV with l- and d-histidine and crystallographic analysis of their adducts with isoform II: engineering proton-transfer processes within the active site of an enzyme. *Chemistry.* 2006; 12: 7057-7066.

16. Temperini C, Scozzafava A, Vullo D, Supuran CT. Carbonic anhydrase activators. Activation of isoforms I, II, IV, VA, VII, and XIV with L- and D-phenylalanine and crystallographic analysis of their adducts with isozyme II: stereospecific recognition within the active site of an enzyme and its consequences for the drug design. *J Med Chem.* 2006; 49: 3019-3027.

17. Temperini C, Innocenti A, Scozzafava A, Supuran CT. Carbonic anhydrase activators: kinetic and X-ray crystallographic study for the interaction of D- and L-tryptophan with the mammalian isoforms I-XIV. *Bioorg Med Chem.* 2008; 16: 8373-8378.

18. Temperini C, Innocenti A, Scozzafava A, Mastrolorenzo A, Supuran CT. Carbonic anhydrase activators: L-Adrenaline plugs the active site entrance of isozyme II, activating better isoforms I, IV, VA, VII, and XIV. *Bioorg Med Chem Lett.* 2007; 17: 628-635.

19. Temperini C, Scozzafava A, Puccetti L, Supuran CT. Carbonic anhydrase activators: X-ray crystal structure of the adduct of human isozyme II with L-histidine as a platform for the design of stronger activators. *Bioorg Med Chem Lett.* 2005; 15: 5136-5141.

20. Temperini C, Scozzafava A, Supuran CT. Carbonic anhydrase activators: the first X-ray crystallographic study of an adduct of isoform I. *Bioorg Med Chem Lett.* 2006; 16: 5152-5156.

21. Bhatt A, Mondal UK, Supuran CT, Ilies MA, McKenna R. Crystal Structure of Carbonic Anhydrase II in Complex with an Activating Ligand: Implications in Neuronal Function. *Mol Neurobiol.* 2018; 55: 7431-7437.

22. Canto de Souza L, Provensi G, Vullo D, Carta F, Scozzafava A, Costa A, Schmidt SD, Passani MB, Supuran CT, Blandina P. Carbonic anhydrase activation enhances object recognition memory in mice through phosphorylation of the extracellular signal-regulated kinase in the cortex and the hippocampus. *Neuropharmacology.* 2017; 118: 148-156.

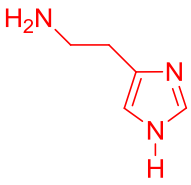
23. a) Wang X, Schröder HC, Schlossmacher U, Neufurth M, Feng Q, Diehl-Seifert B, Müller WE. Modulation of the initial mineralization process of SaOS-2 cells by carbonic anhydrase activators and polyphosphate. *Calcif Tissue Int.* 2014; 94: 495-509; b) Sanku RKK, John JS, Ilies MA, Walker EA. Potential learning and memory disruptors and enhancers in a simple, 1-day operant task in mice. *Behavioural Pharmacol.* 2018; 29: 482-492.

24. Roseghini M, Endean R, Temperilli A. New and uncommon indole- and imidazole-alkylamines in skins of amphibians from Australia and Papua New Guinea. *Z Naturforsch C.* 1976; 31: 118-120.

25. Akocak S, Lolak N, Vullo D, Durgun M, Supuran CT. Synthesis and biological evaluation of histamine Schiff bases as carbonic anhydrase I, II, IV, VII, and IX activators. *J Enzyme Inhib Med Chem.* 2017; 32: 1305-1312.

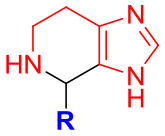
26. Smolyar NN, Abramyants MG, Yutilov YM. Dehydrogenation of 4-Phenyl-Substituted Spinaceamine and Spinacine. *Russ. J. Org. Chem.* 2006; 42: 541-544.
27. Smolyar NN, Abramyants MG, Zavyazkina TI, Matveeva DI, Borodkin YS, Voloskii IA. Synthesis and dehydrogenation of Spinaceamine and Spinacine 4-hetaryl derivatives. *Russ. J. Org. Chem.* 2009; 45: 1219-1223.
28. Khalifah RG. The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow kinetic studies on the native human isoenzymes B and C. *J Biol Chem.* 1971; 246: 2561-2573.
29. Supuran CT. Carbonic anhydrase inhibitors and their potential in a range of therapeutic areas. *Expert Opin Ther Pat.* 2018; 28: 709-712.
30. a) Supuran CT. Applications of carbonic anhydrases inhibitors in renal and central nervous system diseases. *Expert Opin Ther Pat.* 2018; 28: 713-721; b) Lolak N, Akocak S, Bua S, Koca M, Supuran CT. Design and synthesis of novel 1,3-diaryltriazene-substituted sulfonamides as potent and selective carbonic anhydrase II inhibitors. *Bioorg. Chem.* 2018; 77: 542-547; c) Lolak N, Akocak S, Bua S, Supuran CT. Design, synthesis and biological evaluation of novel ureido benzenesulfonamides incorporating 1,3,5-triazine moieties as potent carbonic anhydrase IX inhibitors. *Bioorg. Chem.* 2019; 82: 117-122.
31. a) Supuran CT. Advances in structure-based drug discovery of carbonic anhydrase inhibitors. *Expert Opin Drug Discov.* 2017; 12: 61-88; b) Akocak S, Ilies MA. Next-generation primary sulfonamide carbonic anhydrase inhibitors. In: Supuran CT, Capasso C (Eds) Targeting Carbonic Anhydrases, *Future Science*, London, 2014; 35-51; c) Akocak S, Alam MR, Shabana AM, Sanku RKK, Vullo D, Thompson H, Swenson ER, Supuran CT, Ilies MA. PEGylated bis-sulfonamide carbonic anhydrase inhibitors can efficiently control the growth of several carbonic anhydrase IX-expressing carcinomas. *J. Med. Chem.* 2016; 59: 5077-5088.
32. Angeli A, Kuuslahti M, Parkkila S, Supuran CT. Activation studies with amines and amino acids of the α-carbonic anhydrase from the pathogenic protozoan Trypanosoma cruzi. *Bioorg Med Chem.* 2018; 26: 4187-4190.
33. a) Angeli A, Del Prete S, Alasmary FAS, Alqahtani LS, AlOthman Z, Donald WA, Capasso C, Supuran CT. The first activation studies of the η-carbonic anhydrase from the malaria parasite Plasmodium falciparum with amines and amino acids. *Bioorg Chem.* 2018; 80: 94-98; b) Draghici B, Vullo D, Akocak S, Walker EA, Supuran CT, Ilies MA. Ethylene bis-imidazoles are highly potent and selective activators for isozymes VA and VII of carbonic anhydrase, with a potential nootropic effect. *Chem. Commun.* 2014; 50: 5980-5983; c) Akocak S, Lolak L, Nocentini A, Karakoc G, Tufan A, Supuran CT. Synthesis and biological evaluation of novel aromatic and heterocyclic bis-sulfonamide Schiff bases as carbonic anhydrase I, II, VII and IX inhibitors. *Bioorg. Med. Chem.* 2017; 25(12): 3093-3097.
34. Stefanucci A, Angeli A, Dimmito MP, Luisi G, Del Prete S, Capasso C, Donald WA, Mollica A, Supuran CT. Activation of β- and γ-carbonic anhydrases from pathogenic bacteria with tripeptides. *J Enzyme Inhib Med Chem.* 2018; 33: 945-950.
35. a) Angeli A, Alasmary FAS, Del Prete S, Osman SM, AlOthman Z, Donald WA, Capasso C, Supuran CT. The first activation study of a δ-carbonic anhydrase: TweCAδ from the diatom Thalassiosira weissflogii is effectively activated by amines and amino acids. *J Enzyme Inhib Med Chem.* 2018; 33: 680-685; b) Akocak S, Lolak N, Bua S, Supuran CT. Discovery of novel 1,3-diaryltriazene sulfonamides as carbonic anhydrase I, II, VII and IX inhibitors. *J Enzyme Inhib Med Chem.* 2018; 33: 1575-1580; c) Akocak S, Lolak L, Bua S, Turel I, Supuran CT. Synthesis and biological evaluation of novel N,N'-diaryl cyanoguanidines acting as potent and selective carbonic anhydrase II inhibitors. *Bioorg. Chem.* 2018; 77: 245-251.

**Graphical Abstract**




$K_A$s
CA I: 2.10 μM
CA II: 125 μM
CA IV: 4.03 μM
CA VII: 37.6 μM

**Histamine**




**SP(1-14)**

$K_A$s
CA I: 2.52 - 21.5 μM
CA II: 0.60 - 17.2 μM
CA IV: 0.52 - 63.8 μM
CA VII: 82 nM - 4.26 μM

**Highlights**

- In the current study, a series of 4-substituted-spinaceamine derivatives **SP(1-14)** were synthesized.
- The spinaceamine derivatives were investigated as hCA I, II, IV and VII activators.
- Some of the compounds showed nanomolar potency against hCA VII, which is a brain involved isoform.
- Very good selectivity observed for hCA VII activation over the other physiologically related isoforms, such as hCA I, II and IV.