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Supplementary Information

HYDROXYLAMINE-O-SULFONAMIDE IS A VERSATILE LEAD COMPOUND FOR THE DEVELOPMENT OF CARBONIC ANHYDRASE INHIBITORS

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Experimental methods

Chemistry

General. All reagents and solvents were of commercial quality and used without further purification, unless otherwise specified. All reactions were carried out under an inert atmosphere of nitrogen. TLC analyses were performed on silica gel 60 F_{254} plates (Merck Art.1.05554). Spots were visualized under 254 nm UV illumination, or by ninhydrin solution spraying. Melting points were determined on a Büchi Melting Point 510 and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DRX-400 spectrometer using DMSO-d_6 as solvent and tetramethylsilane as internal standard. For ^{1}H NMR spectra, chemical shifts were expressed in δ (ppm) downfield from tetramethylsilane, and coupling constants (*J*) were expressed in Hertz. Electron Ionization mass spectra were recorded in positive or negative mode on a Water MicroMass ZQ.

N-Boc-O-sulfamoylhydroxylamine (B)

Sulfamoyl chloride (3 equiv) was added at 0 °C to a solution of N-Boc-hydroxylamine (1 g, 7.51 mmol) in 30 mL of dimethylacetamide. The reaction mixture was stirred at room temperature for 2 h, then poured into ethyl acetate, and washed several times with water. The organic phase was dried over anhydrous sodium sulfate filtered and then concentrated *in vacuo*. The residue was purified by silica gel chromatography (Petroleum ether/AcOEt 5/5) to afford the expected compound in quantitative yield (1.6 g).

^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.92 (s, 1H, NH), 7.91 (s, 2H, NH_2), 1.43 (s, 9H, $C(CH_3)_3$). ^{13}C (DMSO-d_6, 101 MHz) δ 155.61 (C=O), 81.46 ($\underline{C}(CH_3)_3$), 28.12 ($C(\underline{C}H_3)_3$).

MS ESI^+ m/z 235.8 $[M+Na]^+$.

Hydroxylamine-O-sulfonamide (1)

Compound **B** (1 g, 4.7 mmol) was dissolved in a solution of 10% TFA-CH_2Cl_2 at 0°C. The reaction mixture was stirred at room temperature and monitored by TLC until the complete disappearance of starting material. The solvent was then removed under reduced pressure to give the expected compound **1** in 80% yield (0.42 g) as trifluoroacetate salt.

^{1}H NMR (DMSO-*d6,* 400 MHz) δ 2.95 (s, 2H, NH_2SO_2-), 3.03 (s, 2H, NH_2O-), 11.5 (s, 1H, CF_3COOH).

^{13}C NMR (101 MHz, DMSO) δ 116.3 (CF_3COOH), 161.5 (C=O).

MS ESI^- m/z 111.0 $[M-H]^-$; m/z 113.0 $[CF_3COOH-H]^-$.

CA inhibition assay

An SX.18MV-R Applied Photophysics stopped-flow instrument was used for assaying the CA-catalyzed CO_2 hydration activity by using the method of Khalifah.[1] Inhibitor and enzyme were preincubated for 15 min. IC_{50} values were obtained from dose response curves working at seven different concentrations of test compound (from 0.1 nM to 50 μM), by fitting the curves using PRISM (www.graphpad.com) and non-linear least squares methods; values represent the mean of at least three different determinations.[2,3] The inhibition constants (K_{Is}) were then derived by using the Cheng-Prusoff equation, as follows: $K_I = IC_{50}/(1 + [S]/K_m)$ where [S] represents the CO_2 concentration at which the measurement was carried out, and K_m the

concentration of substrate at which the enzyme activity is at half maximal. All enzymes used were recombinant, produced as reported earlier.[4-7] The enzyme concentrations used in the assay were ranging between 8.5 and 12.8 nM.

Raman microspectroscopy

A confocal Raman microscope (Jasco, NRS-3100) was used to obtain Raman spectra of single crystals and of carbonic anhydrase inhibitor (CAI) powders. The 514 nm line of an Ar^+ laser was injected into an integrated Olympus microscope and focused to a spot size of approximately 2 μm by a 20 x objective. A holographic notch filter was used to reject the excitation laser line. The Raman backscattering was collected at 180°, using a 0.1 mm slit and a 1200 grooves/mm grating, corresponding to an average spectral resolution of 7 cm^{-1}. It took 60 s to collect a complete data set by a Peltier-cooled 1024 x 128 pixel CCD photon detector (Andor DU401BVI). Wavelength calibration was performed by using cyclohexane as a standard. Raman microspectroscopy measurements on hCA II crystals have been conducted keeping crystals into a drop of mother liquor, using a typical hanging drop crystallization plate.

Crystallization, X-ray data collection and refinement

Crystals of hCA II in complex with **1** have been obtained by the crystal soaking technique.[8, 9] In particular, enzyme crystals were grown at room temperature by the vapor diffusion hanging drop method. Equal volumes of protein solution (10 mg/mL in 0.1 M TRIS-HCl pH 8.0) and of a solution containing 1.3 M sodium citrate and 0.1 M Tris-HCl, pH 8.5 were mixed and equilibrated against a 500 μL reservoir containing the same precipitant solution. A few crystals were then transferred in a 2 μL drop of freshly prepared precipitant solution containing the inhibitor at the concentration of 40 mM, and the cryoprotectant (15% v/v glycerol). These crystals were kept in the soaking solution for about one hour, and then quickly flash-frozen in a nitrogen stream at 100 K. A complete X-ray data set was collected up to 1.45 Å resolution by a copper rotating anode generator developed by Rigaku, equipped with a Rigaku Saturn CCD detector. Crystallographic data were processed with HKL2000.[10] The crystal belonged to the $P2_1$ space group with one molecule in the asymmetric unit. Crystal parameters and data collection statistics are given in Table S1.

The structure of hCA II/**1** adduct was analyzed by difference Fourier techniques, using the hCA II native structure refined at 2.0 Å resolution (PDB entry 1CA2) as the starting model.[11] The structure refinement and map calculation were performed by using the program CNS 1.3.[12,13] In particular, an initial round of rigid body refinement was followed by simulated annealing and isotropic thermal factor (B-factor) refinement. At this stage of refinement, the analysis of |Fo|-|Fc| maps in the active site region showed the presence of one inhibitor molecule, which was gradually built into the model. hCA II/**1** complex structure was then refined by alternating positional and temperature refinement with manual building by using O.[14] The final crystallographic R-factor and R-free values calculated for the 39182 observed reflection (in the 50.00-1.45 Å resolution range) were 0.162 and 0.192, respectively. The stereochemical quality of the model was assessed by PROCHECK.[15] The statistics for

refinement are summarized in Table S1. Coordinates and structure factors have been deposited with the Protein Data Bank (accession code 4YVY).

Results and discussion

Raman assignment

Since the electron density maps around Zn^{2+} did not allow to discriminate unambiguously the binding mode of inhibitor **1** to hCA II, Raman microspectroscopy on the hCA II single crystals in the presence and absence of the inhibitor was performed (Figure 3 in the text). The additional reproducible Raman band at 322 cm^{-1} appearing upon addition of compound **1** can be attributed to a vibrational mode associated to the binding between hCA II and the inhibitor. Particularly, the low frequency value can be putatively assigned to some stretching modes between Zn^{2+} and O and/or N atoms. Unfortunately, Zn^{2+}-O and Zn^{2+}-N frequencies sometimes are reported not too far.[16, 17] Indeed, in literature the Zn^{2+}-N frequency spans from 250 cm^{-1} up to 450 cm^{-1}: at 252 cm^{-1} in ftalocyanin,[18] at 260 cm^{-1} for Zn^{2+}-histidines complexes,[19] at 334 cm^{-1} in $Zn^{2+}(Cys)_2$ complex,[17] and about 450 cm^{-1} with Zn^{2+}-amines.[20] Concerning the Raman data for Zn^{2+}-O stretching, in crystals of 1,2-diacetamidocyclohexane with Zn^{2+} the Raman signal for the Zn^{2+} stretching with the carbonyl oxygen atom was reported at 313 cm^{-1},[21] while in the $Zn^{2+}(Cys)_2$ complex it was reported to 296 cm^{-1}.[17]

In the case under investigation, the Zn^{2+}-His stretching mode, due to the coordination of the zinc ion by the three histidines His94, His96 and His119 and expected around 260 cm^{-1},[19] was absent both in hCA II and hCA II/**1** crystals. This absence was probably due to a signal too weak or covered by a significant contribution from mother liquor (see Figure 3 in the text).

As it concerns the binding of **1** to hCA II, since literature was not definitive on the Raman assignment of this frequency in terms of Zn^{2+}-N or Zn^{2+}-O binding, we performed additional Raman experiments on the same hCA II crystals soaked with two other inhibitors, whose coordination was already defined in previous crystallographic investigations.[22, 23] Particularly, we investigated Raman microspectroscopy of hCA II crystals in the presence of compound **2** of Figure 1 (binding to Zn^{2+} *via* O atoms[22]), and compound **3** (**AZM**, binding to Zn^{2+} exclusively *via* the sulfonamide nitrogen atom[23]). Raman experiments on hCA II with compound **2** did not show any additional band around 322 cm^{-1} (Figure S1), whereas Raman experiments on hCA II with compound **3** showed an additional Raman signal around 324 cm^{-1} comparable to that observed in hCA II/**1** adduct (Figure S2). The proximity of these two frequency values of 324 and 322 cm^{-1} (for compounds **3** and **1**, respectively) suggested in the case of the hCA II/**1** adduct at least a partial binding of Zn^{2+} via the sulfonamide nitrogen atom, though other binding coordinations cannot be completely ruled out.

Our electron density maps suggested also the presence of a second binding coordination where the hydroxylamine moiety coordinated to the catalytic zinc ion in a side-on (η^2) fashion.[24] Unfortunately, no Raman data were available for side-on Zn^{2+}-ligand coordination. However, structural and Raman data corresponding to other metal with nitrosyl or peroxo species[25-27] indicated a significant switch in the low frequency mode between end-on and side-on metal-ligand coordination.[25] Analyzing our Raman spectra we did not observed any additional signal beyond that at 322 cm^{-1} correspondent to the **AZM**-like coordination. Thus, we hypothesized that the alternative side-on coordination of the hydroxylamine moiety is not detectable

in our spectra due to low Raman cross-section. Finally, reminding that **3** binds Zn^{2+} exclusively via N-binding,[23] our Raman analysis allowed also a quantitative evaluation of the N-binding. Indeed, since the binding of compounds **1** and **3** to hCA II through the sulfonamide nitrogen atom is expected to be very similar, we can tentatively assume that the Raman cross-section of the Zn^{2+}-N band around 322 cm^{-1} is the same. As a consequence, considering binding of compound **3** as 100 % and using the proteic signal of Phe band at 1007 cm^{-1} for spectral normalization, we can evaluate a fraction 0.4 ± 0.1 of N-binding for **1** (Figure S3). Thus, Raman data suggest that in hCA II/**1** adduct 40% of the active sites are in a **AZM-**like coordination, whereas the remaining 60 % is in a binding coordination not identifiable from Raman experiments.

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Table S1. Data collection and refinement statistics for hCA II/**1** complex. Values in parentheses refer to the highest resolution shell (1.50-1.45 Å).

Crystal parameters	
Space group	$P2_1$
a (Å)	42.24
b (Å)	41.32
c (Å)	72.15
β (°)	104.39
Data collection statistics	
Resolution range (Å)	50.0-1.45
Temperature (K)	100
Total reflections	166302
Unique reflections	39182
Completeness (%)	91.1 (74.1)
R-merge*	0.049 (0.412)
Mean I/sigma(I)	22.64 (2.81)
Refinement statistics	
Resolution range (Å)	50.0-1.45
R-factor ** (%)	16.2
R-free** (%)	19.2
r.m.s.d. from ideal geometry:	
Bond lengths (Å)	0.014
Bond angles (°)	1.7
Number of protein atoms	2091
Number of inhibitor atoms	12
Number of water molecules	332
Average B factor (Å^2)	
All atoms	14.2
Protein atoms	12.3
Inhibitor atoms	7.7
Water molecules	26.5

* R-merge = $\Sigma_{hkl}\Sigma_i|I_i(hkl)-\langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an observation and $\langle I(hkl)\rangle$ is the mean value for its unique reflection; summations are over all reflections.

** R-factor = $\Sigma_h||Fo(h)|-|Fc(h)||/\Sigma_h|Fo(h)|$, where Fo and Fc are the observed and calculated structure-factor amplitudes, respectively. R-free was calculated with 5% of the data excluded from the refinement.

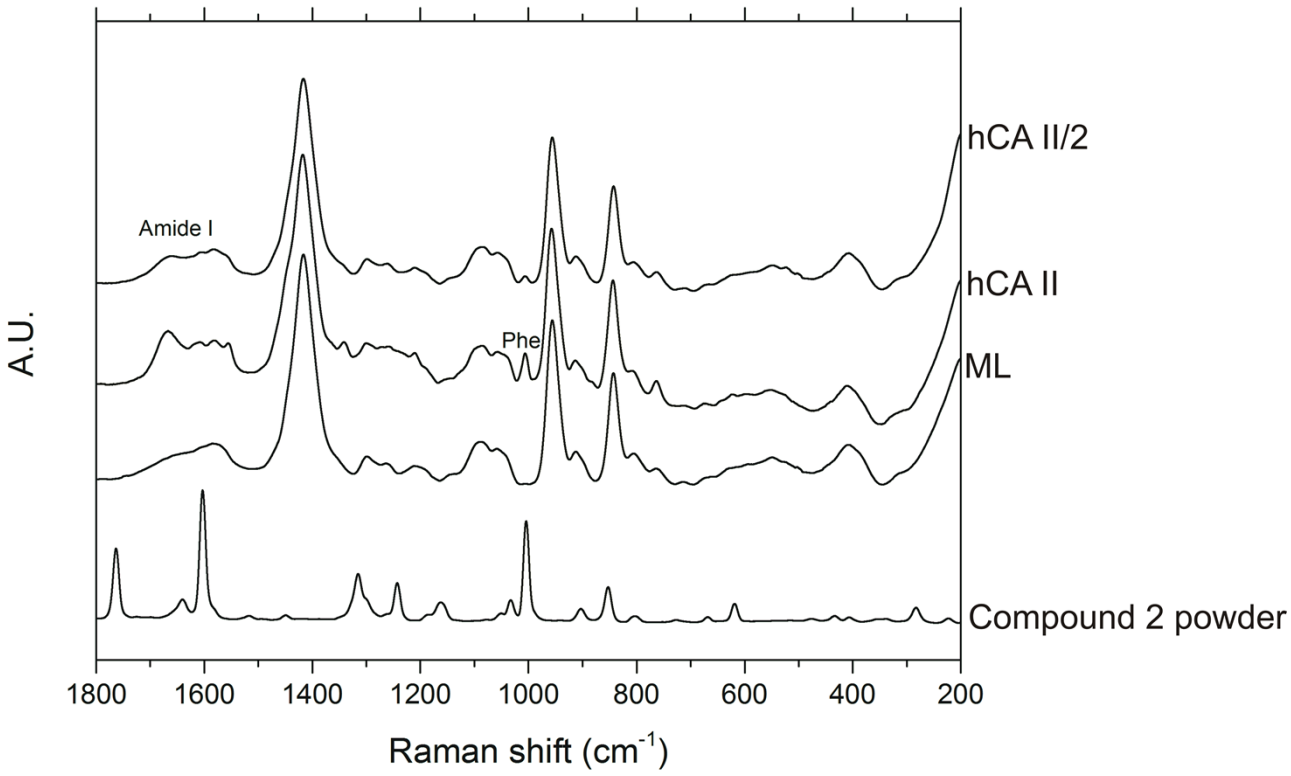

Figure S1. Raman spectra of the hCA II native crystals, inhibitor **2**, hCA II/**2** complex crystals and the mother liquor (ML).

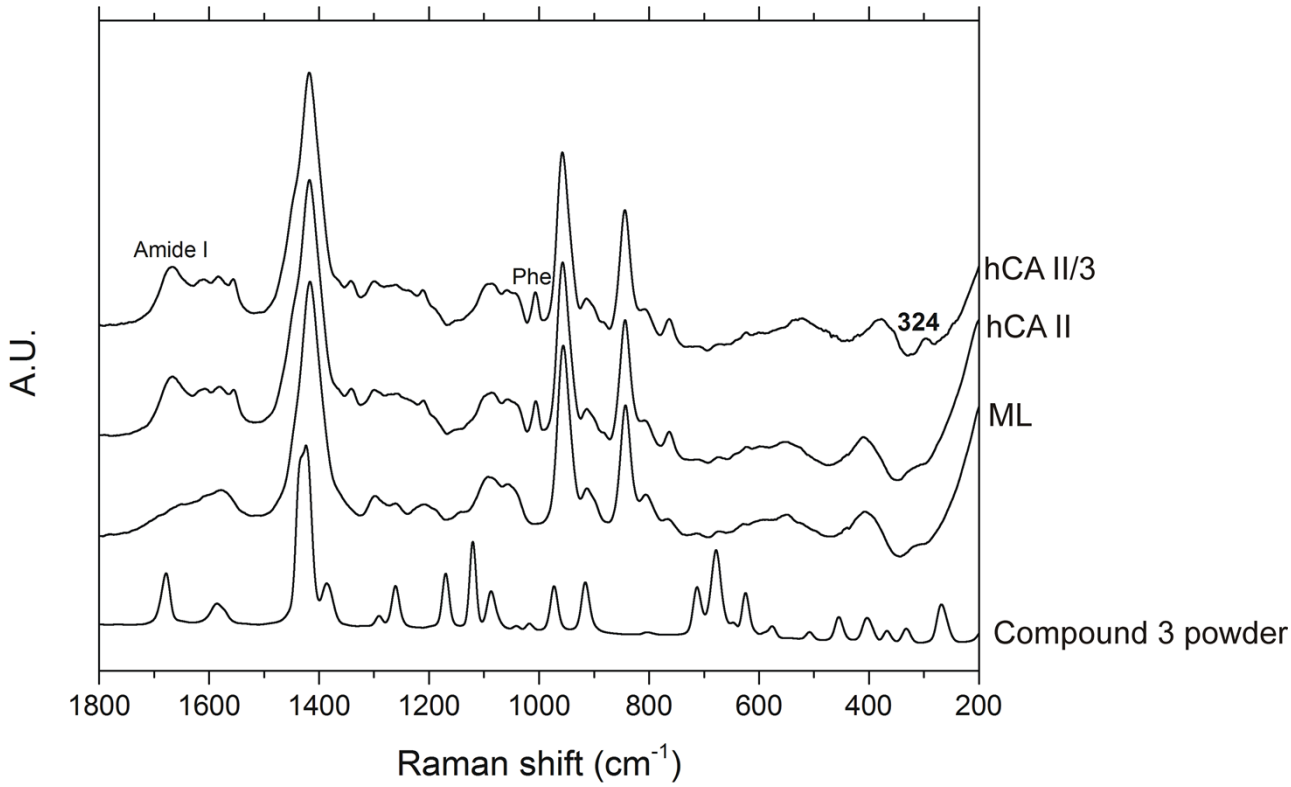

Figure S2. Raman spectra of the hCA II native crystals, inhibitor **3**, hCA II/**3** complex crystals and the mother liquor (ML).

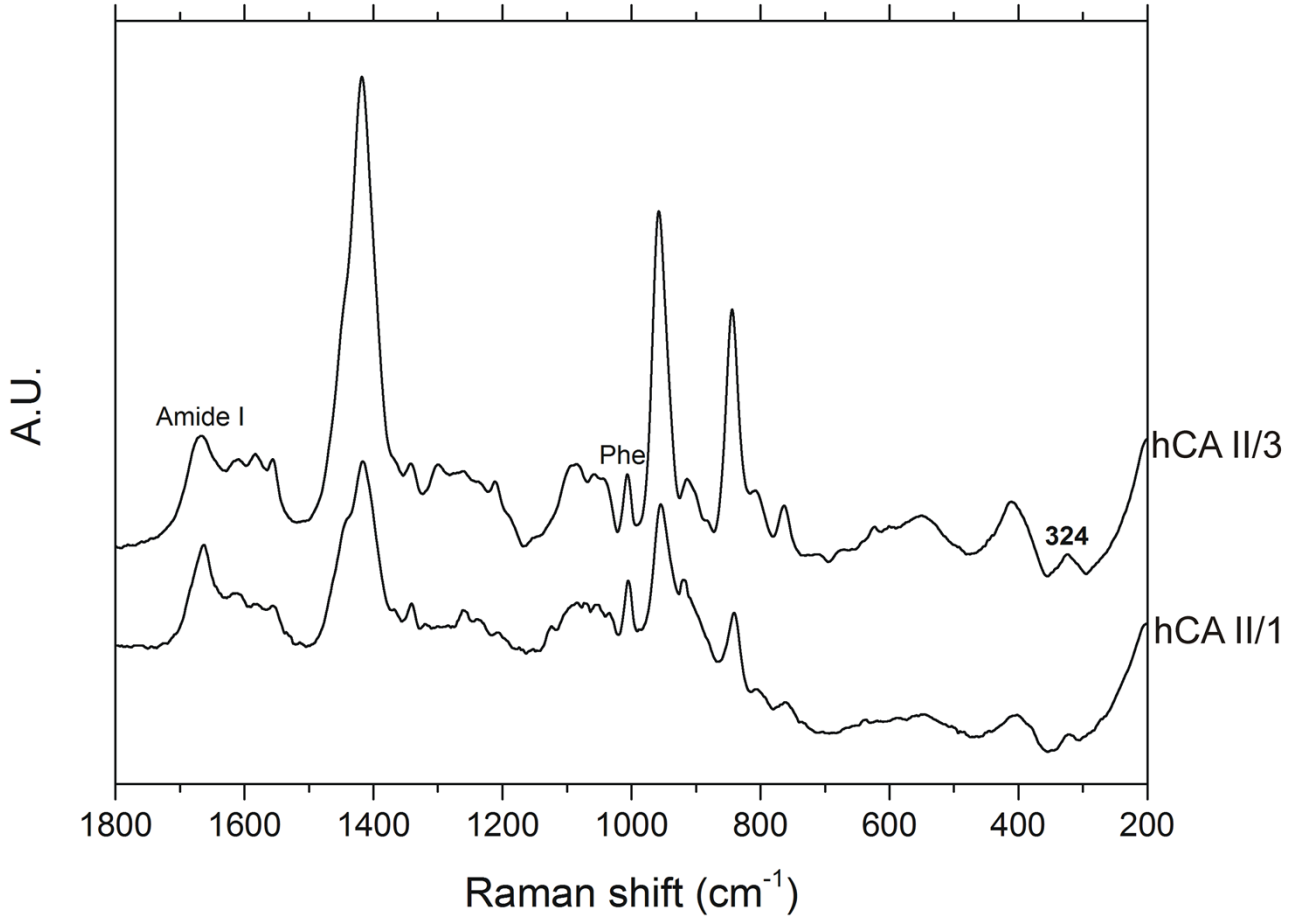

Figure S3. Comparison of putative Zn-N stretching for hCA II crystals in the presence of compounds **1** and **3**. Raman spectra were normalized using Phe signal.