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Supporting Information

OUT OF THE ACTIVE SITE BINDING POCKET FOR CARBONIC ANHYDRASE INHIBITORS

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EXPERIMENTAL SECTION

Synthesis

All the chemicals for the synthesis and physico-chemical characterization were purchased by Sigma-Aldrich (Italy) and used without further purification. Column chromatography was carried out using Sigma-Aldrich® silica gel (high purity grade, pore size 60 Å, 230-400 mesh particle size). Analytical thin-layer chromatography was carried out on Sigma-Aldrich® silica gel on TLA aluminum foils with fluorescent indicator. Visualization was carried out under ultra-violet irradiation (254 nm). The synthesized product has been fully characterized by melting point (FP62 apparatus, Mettler-Toledo, Italy), IR (neat, without diluting or mixing it in anhydrous KBr) (FT-IR Spectrometer Spectrum 1000, Perkin-Elmer, United Kingdom), ^{1}H (400 MHz) and ^{13}C NMR (101 MHz) spectroscopy (Bruker Avance 400, USA), and ESI-MS analysis in the positive and negative ion modes (Thermo Finnigan LXQ ion trap, Germany). Coupling constants *J* are valued in Hertz (Hz). Chemical shifts are expressed as δ units (parts per million), based on appearance rather than interpretation, and are referenced to the residual non deuterated solvent peak. The assignment of exchangeable protons (-COOH) was confirmed by the addition of D_2O. Temperatures are reported in °C. Where given, systematic compound names are those generated by ChemDraw ultra following IUPAC conventions.

2-(Benzylsulfinyl)benzoic acid (3): The corresponding sulfoxide was obtained by adding dropwise hydrogen peroxide (33%, 4.0 equiv.) to a stirring solution of the proper thioether compound **4** (2-(benzylthio)benzoic acid, 1.0 equiv.) in glacial acetic acid (15 mL) at room temperature. After 2 h the reaction was quenched with NaOH 2 N (15 mL) and extracted with chloroform (3 × 20 mL). The organic phases were reunited, dried over anhydrous sodium sulfate and concentrated *in vacuo*. Purification by column chromatography on silica gel gave the title compound in good yield (88%) as a white crystalline solid (as a racemic mixture). Mp 160-162 °C; IR ν_{max} 3098 (ν C_{sp2}-H + a broad band due to ν O-H), 2883 (ν C_{sp3}-H), 1689 (ν C=O), 1589 (ν C=C), 999 (ν S=O) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.78-3.81 (d, *J*= 12.8 Hz, 1H, CH_2), 4.43-4.46 (d, *J*= 12.8 Hz, 1H, CH_2), 7.14-7.16 (m, 2H, ArH), 7.30-7.31 (m, 3H, ArH), 7.63-7.67 (m, 1H, ArH), 7.78-7.79 (m, 2H, ArH), 8.09-8.11 (d, J_o= 7.6 Hz, 1H, ArH); ^{13}C NMR (101 MHz, DMSO-d_6) δ 62.3 (*C*H_2), 125.03 (*C*H=), 128.18 (*C*H=), 128.25 (*C*H=), 128.55 (*C*H=), 130.72 (*C*H=), 130.93 (*C*H=), 131.35 (*C*H=), 132.25 (*C*H=), 133.68 (*C*H=), 148.01 (*C*H=), 167.25 (*C*OOH); MS (ESI) positive mode *m*/*z*: 261.08 [(M + H)$^+$], 283.08 [(M + Na)$^+$], 520.75 [$(M)_2$ + H)$^+$], 542.83 [$(M)_2$ + Na)$^+$]; MS (ESI) negative mode *m*/*z*: 259.08 [(M - H)$^-$], 518.75 [$(M)_2$ - H)$^-$].

CA catalytic activity and inhibition assay. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[1] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 – 20 mM Hepes (pH 7.5) and 20 mM Na_2SO_4 (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10-100 s. The CO_2 concentrations ranged from 1.7 to 17 mM. For each inhibitor concentration 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 the inhibitor (10 mM) were prepared in distilled-deionized water and dilutions up to 0.001 μM were done thereafter with distilled-deionized water. Inhibitor 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-I complex. The inhibition constants were obtained by non-linear least-squares methods using the Cheng-Prusoff equation, as reported earlier,[2] and represent the mean from at least three different determinations. All CA isoforms were recombinant ones obtained in house as reported earlier.[3-5]

Crystallization and X-ray data collection

Crystals of hCA II/inhibitor adduct have been obtained by the soaking technique, as previously reported.[6-9] In particular, crystallization experiments on hCA II native crystals were carried out using the hanging-drop vapor-diffusion method at 20 °C. Drops were prepared by mixing equal volumes of protein (10 mg/ml in 0.1 M TRIS-HCl pH 8.5) and precipitant solution, which contained 2.5 M $(NH_4)_2SO_4$, 0.3 M NaCl, 100 mM Tris-HCl (pH 8.2) and 5 mM 4-(hydroxymercurybenzoic) acid, and were then equilibrated against 1 mL reservoir. Crystals grew in a couple of days and a few of them were then transferred in a 2 μL drop, containing the precipitant solution, the inhibitor at saturated concentration and 10% glycerol (*w/v*). These crystals were kept in the soaking solution for 48 hours and then flash-frozen in gas nitrogen stream.

A complete dataset was collected at 1.50 Å resolution at a temperature of 100 K, using a copper rotating-anode generator developed by Rigaku equipped with a Rigaku Saturn CCD detector. Diffracted data were processed using the HKL crystallographic data reduction package,[10] and were indexed in the monoclinic space group $P2_1$ with one molecule in the asymmetric unit. Unit cell parameters and data reduction statistics are summarized in Table S1.

Structure resolution and refinement

The enzyme-inhibitor complex structure was analyzed by difference Fourier techniques, using the atomic coordinates of the native hCA II (PDB entry 1CA2)[11] as starting model. The refinement was carried out with the program *CNS*,[12] whereas the model building and map inspection were performed using the *O* program.[13] In particular, an initial round of rigid body refinement was followed by simulated annealing and isotropic thermal factor (B-factor) refinement. The inspection of electron density maps, at various stages of crystallographic refinement, clearly showed the binding of one inhibitor molecule in a cavity on the protein surface (Figures 2-3). These maps were well defined for the phenylsulfinyl moiety of the inhibitor**,** while a poorer definition was observed for the benzyl group. Interestingly, although the racemic mixture of compound **3** was present in the crystallization solutions, only the *S* enantiomer was observed bound to the protein.

After initial refinement limited to the enzyme, an inhibitor molecule was gradually built into the model for further refinement. Restraints on inhibitor bond angles and distances were taken from similar structures in the Cambridge Structural Database[14] and standard restraints were used on protein bond angles and distances throughout refinement. The ordered water molecules were added automatically and checked individually. Each peak contoured at 3σ in the |Fo| - |Fc| maps was identified as a water molecule, provided that hydrogen bonds would be allowed between this site and the model. Several alternating cycles of energy minimization, individual temperature factor refinement and manual model building gave the final model with R-factor/R-free values of 0.163 and 0.184. The correctness of stereochemistry was finally checked using PROCHECK.[15] 88.4% of the non-glycine residues were located in the most favored regions of the Ramachandran plot. The final model statistics are reported in Table S1. All crystallographic figures were generated by *PyMOL* (http://www.pymol.org). Coordinates and structure factors have been deposited with the Protein Data Bank (accession code 4QY3).

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Table S1 Data collection and refinement statistics for hCA II/**3** complex. Values in parentheses refer to the highest resolution shell (1.55-1.50 Å).

Crystal parameters	
Space group	$P2_1$
a (Å)	42.1
b (Å)	41.5
c (Å)	72.2
β (°)	104.5
Data collection statistics	
Resolution (Å)	50.0-1.50
Temperature (K)	100
Total reflections	171447
Unique reflections	36283
Completeness (%)	92.7 (77.8)
R_{merge}*	0.061 (0.320)
Mean I/sigma(I)	19.53 (3.13)
Refinement statistics	
Resolution (Å)	50.0-1.50
R_{factor}** (%)	18.4
R_{free}** (%)	16.3
r.m.s.d. from ideal geometry:	
Bond lengths (Å)	0.012
Bond angles (°)	1.6
Number of protein atoms	2042
Number of inhibitor atoms	18
Number of water molecules	284
Average B factor (Å^2)	
All atoms	13.2
Protein atoms	11.6
Inhibitor atoms	24.4
Water molecules	23.9

*$R_{merge} = \Sigma_{hkl}\Sigma_i|I_i(hkl)-\langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$, 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.

**Rfactor $= \Sigma_h||Fo(h)|-|Fc(h)||/\Sigma_h|Fo(h)|$, 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.