Electronic Supplementary Material (ESI) for Chemical Communications

Nucleophile recognition as an alternative inhibition mode for benzoic acid based carbonic anhydrase inhibitors

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Supporting Information

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Experimental

General Methods. All chemicals were purchased from commercial suppliers (Aldrich, Alfa Aesar, TCI, or Fisher) and used as received. ^{1}H NMR spectra were recorded on a Varian FT-NMR instrument running at 400 MHz at the Department of Chemistry and Biochemistry, University of California, San Diego. Mass spectrometry was performed at the Small Molecule Mass Spectrometry Facility in the Department of Chemistry and Biochemistry at the University of California, San Diego.

4-Mercaptomethyl benzoate (9). 4-Mercaptobenzoic acid (100 mg, 0.605 mmol), MeOH (3 mL), and a catalytic amount of H_2SO_4 were heated in the microwave at 70 °C for 30 min. The solvent was removed and the product purified by flash chromatography to yield a white powder (104 mg, 96%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.89 (d, 2H, J = 8.0 Hz), 7.28 (d, 2H, J = 8.0 Hz), 3.89 (s, 3H), 3.60 (s, 1H). ESI-MS(-) *m/z* 167.20 $(M-H)^-$.

hCAII Activity Assay. The plasmid encoding human carbonic anhydrase isozyme II (hCA II) and containing a T7 RNA polymerase promoter and an ampicillin resistance gene (pACA) was a generous gift from Thomas Ward, University of Basel (Switzerland) and Carol Fierke, University of Michigan (U.S.A.). The protein was expressed and purified as previously described (Monnard et al., *Chem. Commun.* **2011**, *47*, 8238-8240). hCAII was incubated with varying concentrations of inhibitor (500 nM to 45 mM, depending on solubility) in 50 mM Tris-SO_4 pH 8.0 containing 5% DMSO for 10 min before adding 4-nitrophenylacetate (500 μM final concentration). The assay was run at 30 °C with a final well volume of 100 μL. Cleavage of the substrate to 4-nitrophenol was monitored by the increase in absorbance at 405 nm measured on a BioTek ELx808 absorbance plate reader. Initial rates were then compared to those for both inhibitor-free and fully inhibited hCAII (full inhibition was achieved with 50 μM benzenesulfonamide) in order to determine percent inhibition. Every plate also had control wells containing benzenesulfonamide at its reported IC_{50} value as a control (Iyer *et al.*, *J. Biomol. Screen.* **2006**, *11*, 782-791). IC_{50} values were determined by fitting plots of inhibitor concentration vs. % inhibition in GraphPad Prism. Experiments indicate that at the high ligand concentrations required to observe inhibition by hydroxybenzoic acids that there is a significant change in pH of the assay solution (~7 to 7.5). Examination of the catalytic activity of hCAII at

these pH values (adjusted with HCl) shows that the activity of the enzyme is diminished under conditions of low pH. Therefore, at least part of the inhibition observed with these compounds can be attributed to the resulting change in pH of the solution. Our reading of the literature does not discuss this issue with ligands that would be expected to elicit a similar change in pH. In light of the wide range of values that have been obtained for ligands of this class (see main text), further studies may be required to isolate the effects of pH and ligand binding to obtain a better estimate of the inhibitory ability of these compounds.

Protein Crystallization. Crystals of hCAII were obtained by the sitting drop vapor diffusion method. The protein solution consisted of 20 mg/mL hCAII and 1 mM 4-chloromercuribenzoic acid in 50 mM Tris-SO_4 pH 8. The precipitant solution contained 2.7–2.9 M $(NH_4)_2SO_4$ in 50 mM Tris-SO_4 pH 8.15. Drops consisted of 3 μL of protein solution plus 3.5-4 μL of precipitant solution and were equilibrated at 18 °C against 1 mL of precipitant solution. Crystals of roughly 0.3×0.3×0.3 mm size appeared after 2 d to 3 weeks. Once formed, crystals were transferred to soak solutions (10 μL) containing inhibitor and 3 M $(NH_4)_2SO_4$ in 50 mM Tris-SO_4 pH 8. The soak solutions also contained 10% glycerol as a cryoprotectant. Inhibitor soak concentrations and times: **1**, 20 mM for 2 d; **2**, saturated solution for 6 d; **3**, saturated solution for 8 d; **4**, 50 mM for 4 d; **5**, 50 mM for 8 d; **6**, 10 mM for 4 d. Crystals were taken directly from the soak solution for data collection. All structures include a 4-mercuribenzoic acid ligand bound to Cys206.

Crystal Structure Determination. X-ray diffraction studies were carried out on a Bruker D8 Smart 6000 CCD diffractometer equipped with Cu K_α radiation (λ = 1.5478 Å) at 100 K. The data were integrated using the Bruker SAINT software program and scaled using the SADABS software program. All crystals belong to the monoclinic space group $P2_1$. The data were phased by molecular replacement using a previously reported hCAII structure (PDB ID 3KS3; B. S. Avvaru et al. *Biochemistry* **2010**, *49*, 249-251) with water molecules removed. Models were built by alternating refinement using REFMAC5 (G. Murshudov et al. *Acta Cryst.* **1997***, D53*, 240-255) and manual model building and visualization using Coot (P. Emsley et al. *Acta Cryst.* **2004**, *D60*, 2126-2132). Inhibitor topologies were generated using the PRODRG server (A.W. Schüttelkopf et al. *Acta Cryst.* **2004**, *D60*, 1355-1363).

Table S1. Refinement data for hCAII crystals.

Ligand	**1**	**2**	**3**	**4**	**5**	**6**
PDB ID	**4E3D**	**4E3F**	**4E3G**	**4E3H**	**4E49**	**4E4A**
a (Å)	42.204(2)	42.2732(7)	42.1438(5)	42.115(2)	42.179(1)	42.86(1)
b (Å)	41.479(2)	41.4072(6)	41.5407(6)	41.624(2)	41.502(1)	42.12(2)
c (Å)	72.119(4)	72.227(1)	72.1667(9)	71.920(3)	71.824(2)	71.80(2)
β (deg)	104.323(3)	104.2330(8)	104.4292(7)	104.138(2)	104.106(2)	104.33(2)
Resolution Range [a]	69.88-1.60 (1.642-1.60)	35.64-1.50 (1.539-1.50)	40.81-1.55 (1.59-1.55)	23.80-1.50 (1.539-1.50)	40.92-1.45 (1.488-1.45)	40.77-1.45 (1.488-1.45)
Completeness (%)	99.66 (98.84)	99.92 (99.90)	99.65 (98.41)	99.48 (97.66)	99.44 (97.76)	99.53 (98.77)
R_{work}	0.169 (0.181)	0.167 (0.156)	0.191 (0.196)	0.172 (0.223)	0.158 (0.179)	0.185 (0.230)
R_{free} [b]	0.211 (0.269)	0.204 (0.220)	0.228 (0.276)	0.212 (0.265)	0.185 (0.228)	0.221 (0.256)
Bond Length RMS (Å)	0.025	0.025	0.026	0.027	0.030	0.027
Bond Angle RMS (deg)	2.103	2.221	2.128	2.235	2.321	2.307

[a] Parentheses indicate values for the highest resolution shell. [b] Calculated using 5% of the data excluded from refinement.

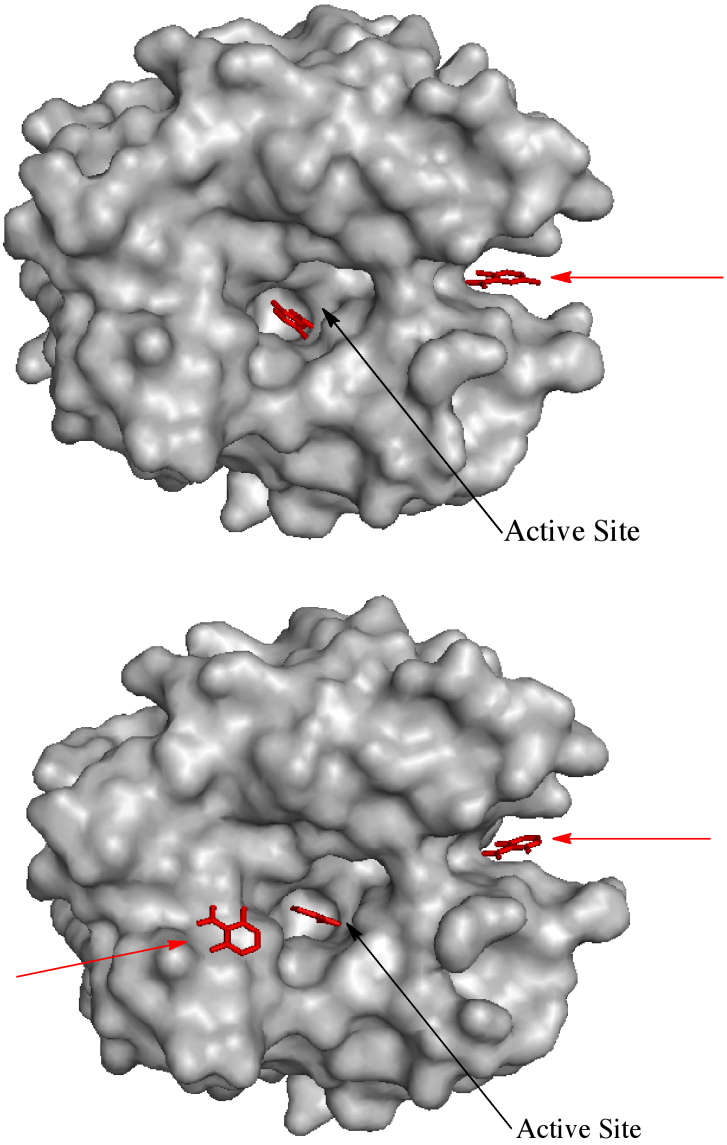

Figure S1. Additional binding sites (red arrows) of compounds **1** (top) and **2** (bottom).

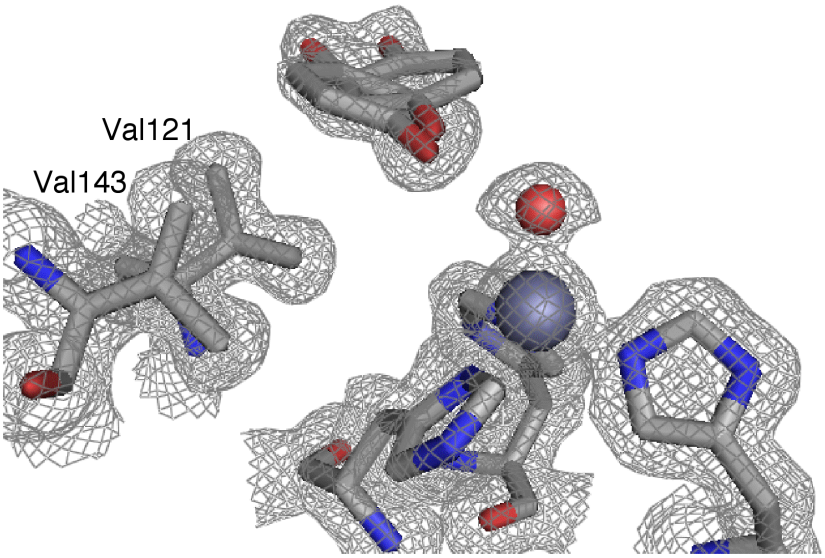

Figure S2. Structure of compound **4** bound in the active site of hCAII with the $2|F_o| - |F_c|$ electron density map contoured to 0.8σ. Both disordered positions maintain interactions with the hydrophobic wall.

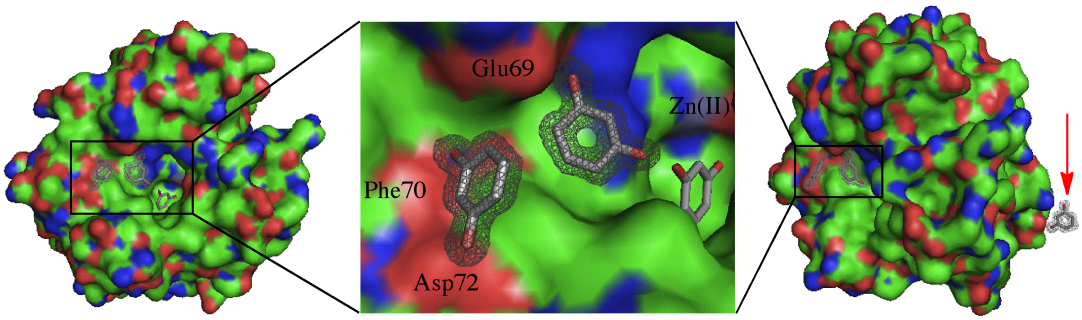

Figure S3. Additional binding sites of compound **5**. Two molecules are located on the hydrophilic side of the active site tunnel (middle) while the other is located on the opposite side of the protein (right, red arrow).

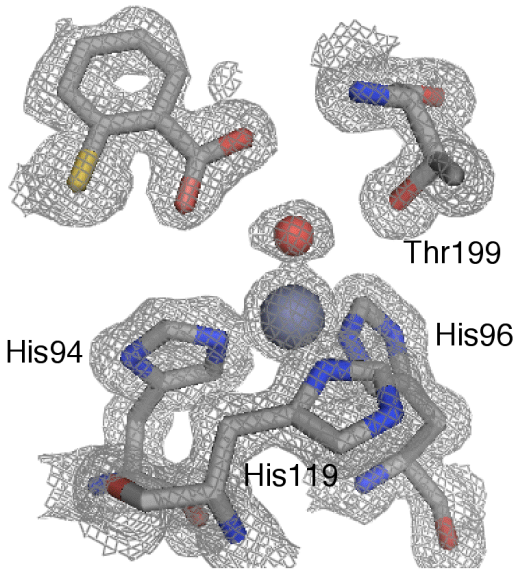

Figure S4. Crystal structure of compound **6** bound in the hCAII active site. The catalytic Zn(II) ion and bound water are shown as gray and red spheres, respectively. The $2|F_o| - |F_c|$ electron density map is contoured to 1.2σ.