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Supplementary Information

Catechols: a new class of carbonic anhydrase inhibitors

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Experimental methods

Crystallization, data collection, and refinement

Recombinant hCA II has been expressed and purified as previously described.[1] To obtain crystals of the hCA II/**1** adduct a soaking experiment was carried out. In detail, first crystallization experiments on hCA II native crystals were performed at 20 °C by the hanging-drop vapor-diffusion method. Drops were prepared by mixing equal volumes of protein (10 mg/mL in 0.1 M TRIS-HCl pH 8.5) and precipitant solution (1.3 M sodium citrate and 0.1 M Tris-HCl, pH 8.5), and were then equilibrated against 1 mL reservoir containing the same precipitant solution. Crystals grew in a couple of days and few of them were then transferred in a 2 µL drop, containing the precipitant solution, chlorogenic acid (**CGA**) at saturated concentration and 10% glycerol (*w/v*). The crystals were kept in the soaking solution for about 24 hours and then flash-frozen in gas nitrogen stream. Complete X-ray data were collected at a temperature of 100 K, using a copper rotating-anode generator developed by Rigaku equipped with a Rigaku Saturn CCD detector. Diffraction data were processed and scaled using program HKL2000 (HKL Research).[2] Unit cell parameters and data reduction statistics are summarized in Table S2.

The protein/inhibitor complex structure was analyzed by difference Fourier techniques, using the atomic coordinates of the native hCA II (PDB entry 1CA2)[3] as starting model. The refinement was performed with the program *CNS*,[4] while the model building and map inspection were obtained using the *O* program.[5] 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 an inhibitor molecule in the enzyme active site. However, the shape of this density was not compatible with compound **CGA**, but instead well matched with caffeic acid (**CFA)**. After initial refinement limited to the enzyme, a **CFA** molecule was built into the model for further refinement. Restraints on inhibitor bond angles and distances were taken from similar structures in the Cambridge Structural Database[6] and standard restraints were utilized on protein bond angles and distances during 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, assuring 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_{work}/R_{free} values of 0.159 and 0.192. The correctness of stereochemistry was finally checked using PROCHECK.[7] Coordinates and structure factors have been deposited in the Protein Data Bank (accession code 6YRI).

hCA II crystals were also soaked in the precipitant solution containing **CFA** at saturated concentration. The structure of the obtained adduct was analyzed with the same procedure described above. Data collection and refinement statistics are reported in Table S2.

HPLC-DAD analysis

HPLC-DAD analysis of **CGA** and **CFA** was carried out by a Perkin-Elmer apparatus equipped with a series LC 200 pump, a series 200 diode array detector and a series 200 autosampler. Data acquisition and processing were carried out with a Perkin-Elmer Totalchrom software. The chromatographic separation was performed using a Phenomenex Luna C18 column (250 x 4.6 mm, i.d. 5 μm). The mobile phase consisted of acetonitrile and water acidified by formic acid (5%) in ratio 20:80 in isocratic conditions at a flow rate of 1 mL/min. The used detection wavelength was 280 nm and the injection volume was 20 μL. Peaks were identified respect to commercial and high-purity grade standards of **CGA** and **CFA** (Sigma-Aldrich, Milan, Italy). Calibration curves were available for quantitative analyses (**CGA** (R^2 = 0.9987), **CFA** (R^2 = 0.9997)). Standard chromatograms are reported in **Figure S3**.

Analysis of CGA into CFA conversion in the crystallization buffer

4.5 mL of a mixture containing 1.3 M sodium citrate and 0.1 M Tris HCl (pH 8.5) were added with 0.5 mL glycerol and put under stirring. 70.0 mg of **CGA** (FW = 354.31) were weighed, solubilized with 0.1 mL DMSO and added with 4.9 mL of the freshly prepared mixture (final concentration of chlorogenic acid in the crystallization buffer: 40 mM; final concentration of DMSO: 2%). The

obtained yellow solution was maintained under stirring at T = 20 ± 1 °C for 24 h in the dark. Withdrawals were made at the following discrete time-points (t) = 3, 8, 16 and 24 h and the samples directly analyzed according to HPLC-DAD described method. Each experiment was performed in triplicate (**Figure S4**).

Analysis of CGA into CFA conversion in the inhibition assays buffer

A solution containing hCA II 10^{-7} M, **CGA** 10^{-2} M, 20 mM Hepes (pH 7.5) and 20 mM Na_2SO_4 (inhibition assays conditions) was directly analyzed after 1:10 dilution according to the HPLC-DAD described method. Each experiment was performed in triplicate (**Figure S5**).

Inhibition assays at different enzyme/inhibitor incubation times

An Applied Photophysics stopped-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity.[8] Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.5) as buffer, and 20 mM Na2SO4 (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 the determination of the kinetic parameters and inhibition constants. For each inhibitor 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 inhibitors (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min/3h/6h/24h 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 PRISM 3 and the Cheng–Prusoff equation, as reported earlier,[9] and represent the mean from at least three different determinations.

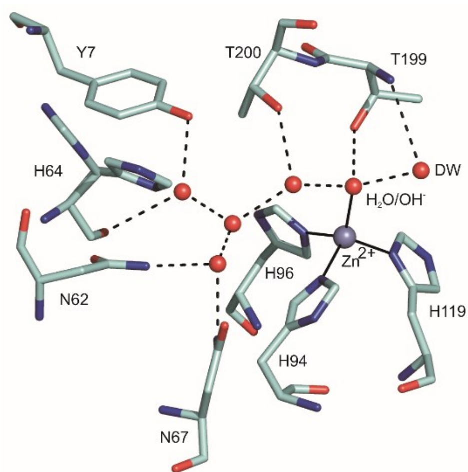

Figure S1. Active site of hCA II (PDB accession code 1TE3),[10] which has been chosen as representative CA. The Zn^{2+} ion is tetrahedrally coordinated by three histidines and a water molecule/hydroxide ion, which is in turn involved in a network of hydrogen bonds. Water molecules are indicated as red circles. DW indicates the “Deep Water”.

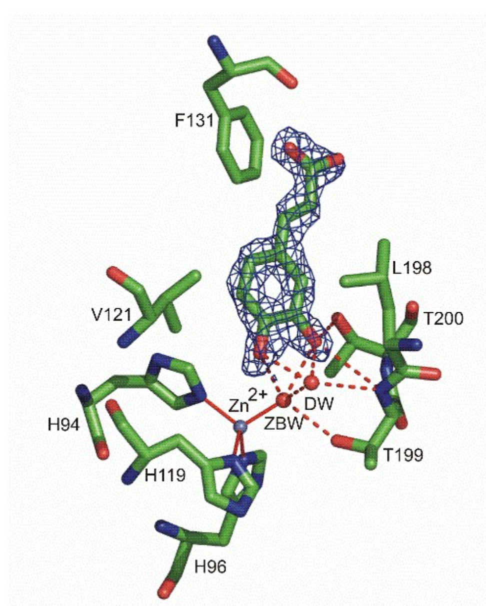

Figure S2. Active site region of the complex obtained by soaking hCA II crystals in a **CFA** solution, showing the σA-weighted │2Fo-Fc│ map (contoured at 1.0 σ) relative to the inhibitor molecule. The zinc ion and residues involved in inhibitor recognition are shown. Continuous lines indicate zinc ion coordination, whereas dashed lines indicated hydrogen bond distances. Water molecules are indicated as red circles. DW and ZBW indicate the "Deep Water" and the zinc-bound water molecule.

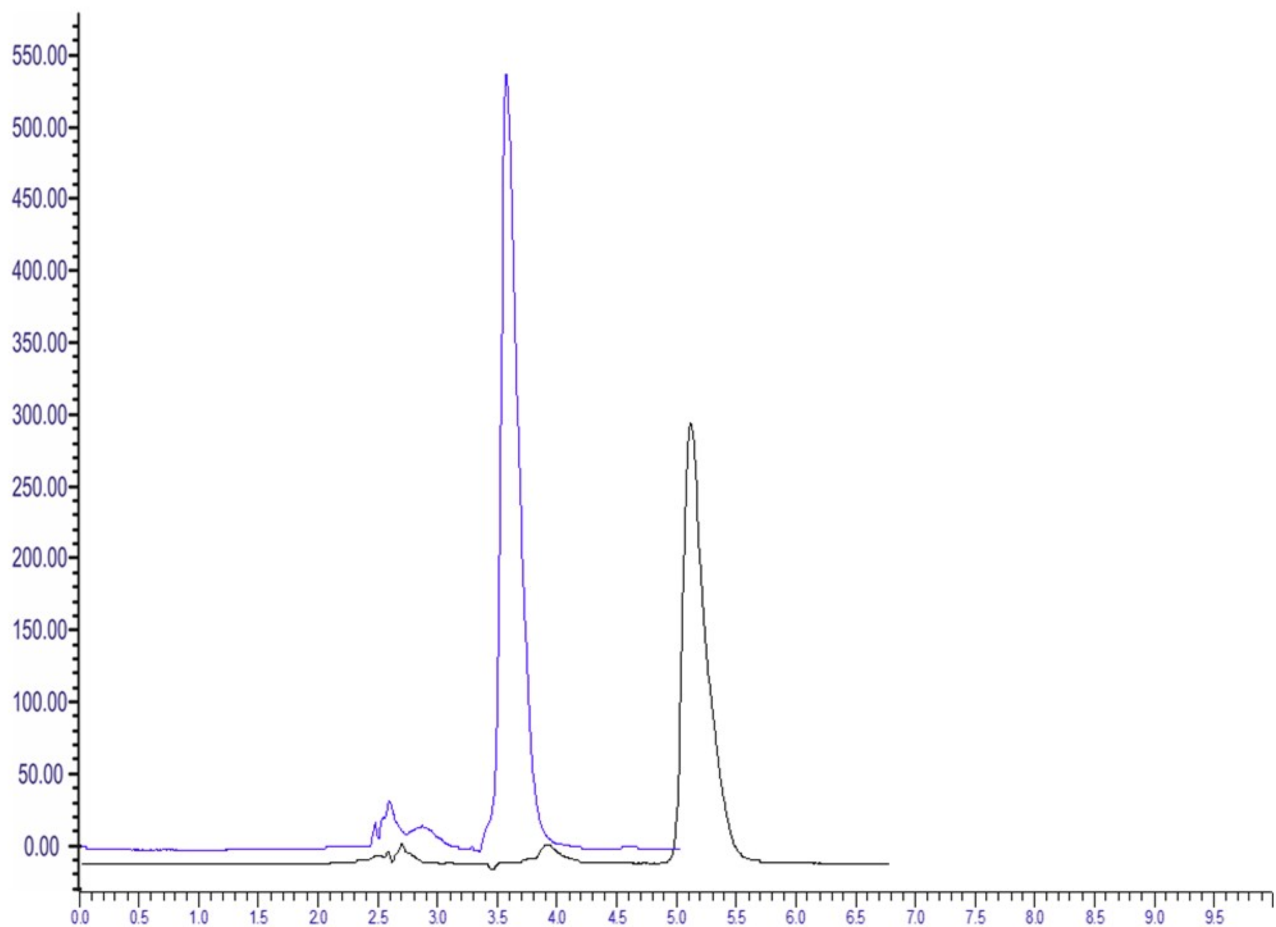

Figure S3. Chromatogram of the standard compounds: **CGA** (t_R = 3.6 min) and **CFA** (t_R 5.1 min).

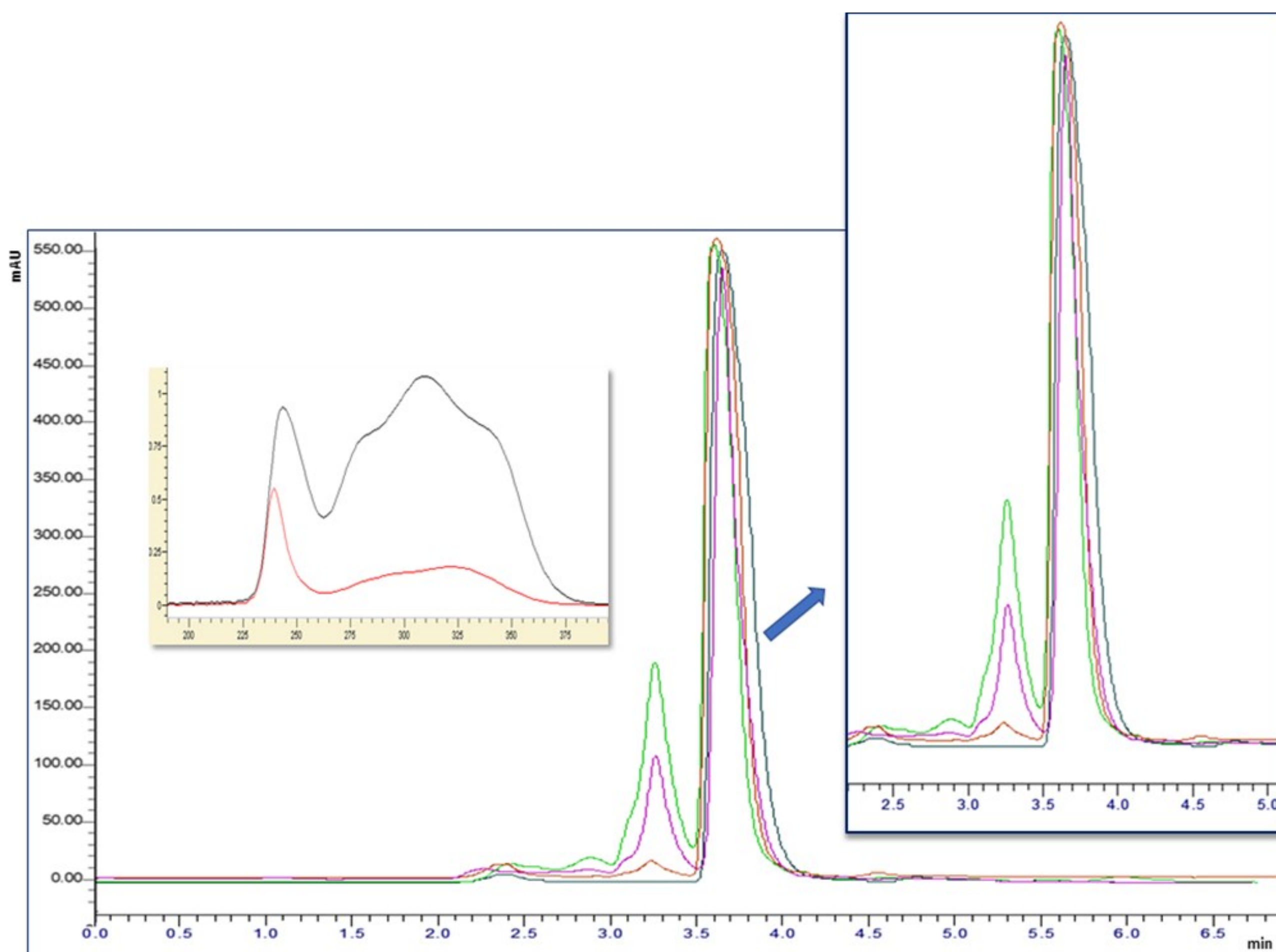

Figure S4. Analysis by HPLC at different times of **CGA** in the crystallization buffer. Chromatograms of withdrawals at 3 (dark green), 8 (orange), 16 (magenta), and 24 (green) hours. Peak with t_R of 3.2 min: tentatively identified as 1-caffeoylquinic acid according to literature.[11] Peak with t_R of 3.6 min: **CGA**. The inset on the left shows spectral data of the revealed peaks (1-caffeoylquinic acid reported in red and chlorogenic acid reported in blue).

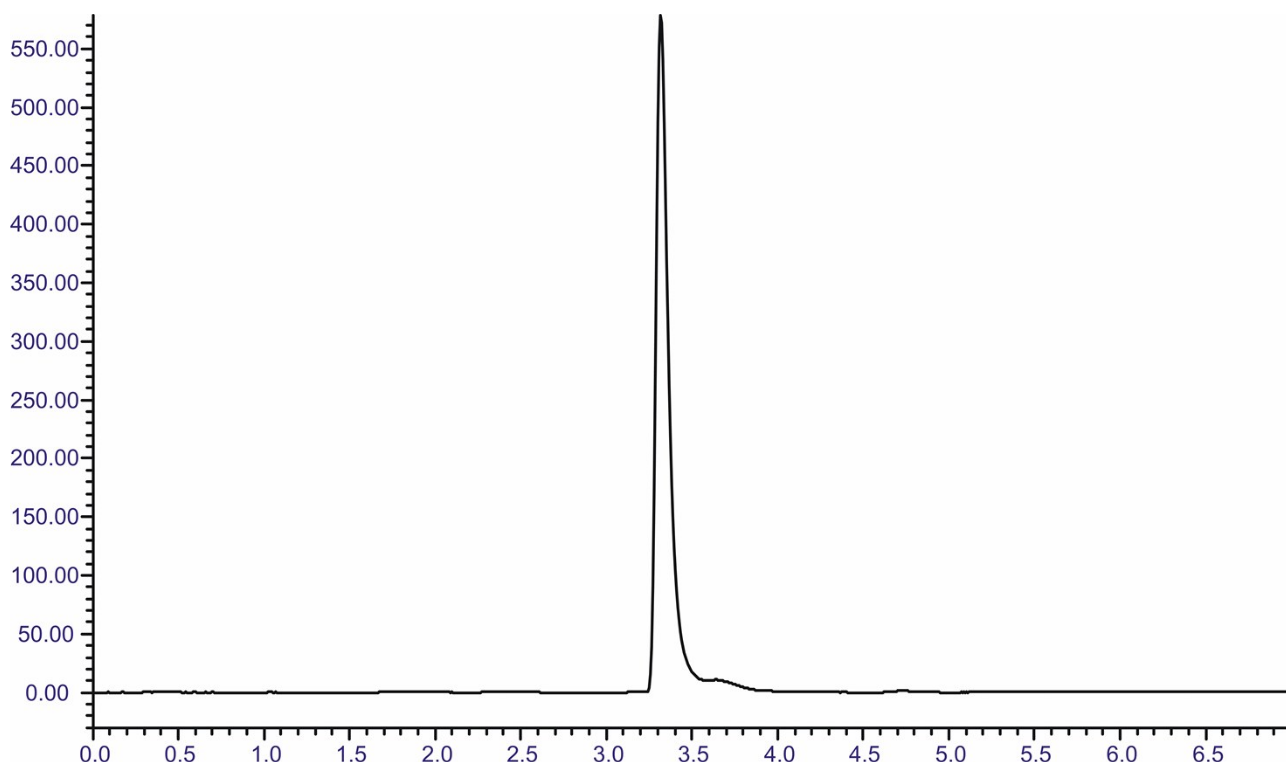

Figure S5. Chromatogram of the solution containing hCA II/**CGA** in the inhibition assay conditions after 36 hour of incubation.

Table S1. Inhibition of hCA I, hCA II, hCA VA and hCA VII with **CGA** and **CFA** and the standard CAI acetazolamide (**AAZ**).

		K_I (μM)*		
Compound	**hCA I**	**hCA II**	**hCA VA**	**hCA VII**
CGA[a]	25.0	30.1	0.05	>100
CFA[b]	2.38	1.61	6.49	6.42
AAZ[b]	0.25	0.012	0.063	0.0025

[a]Data taken from Mollica et al.[12] [b]Data taken from Innocenti et al.[13]
*Values obtained by using an enzyme/inhibitor incubation time of 15 minutes.

Table S2 Data collection and refinement statistics for hCA II/**1** and hCA II/**2** complexes.

Crystal parameters	**hCA II/1**	**hCA II/2**
Space group	$P2_1$	$P2_1$
a (Å)	42.2	42.2
b (Å)	41.3	41.4
c (Å)	72.1	72.0
β (°)	104.3	104.3
Data collection statistics		
Resolution (Å)	25.0-1.60	32.0-1.68
Temperature (K)	100	100
Total reflections	153395	112036
Unique reflections	31182	27669
Completeness (%)	97.1 (85.1)	99.5 (95.7)
R-merge*	0.051 (0.153)	0.060 (0.233)
Rmeas§	0.056 (0.183)	0.069 (0.305)
Rpim¶	0.022 (0.097)	0.031 (0.193)
Mean I/sigma(I)	26.5 (7.8)	19.05 (3.65)
Refinement statistics		
Resolution limits (Å)	25.0-1.60	32.0-1.68
Rwork** (%)	15.9	16.4
Rfree** (%)	19.2	20.1
r.m.s.d. from ideal geometry:		
Bond lengths (Å)	0.014	0.009
Bond angles (°)	1.6	1.6
Number of protein atoms	2107	2129
Number of inhibitor atoms	13	13
Number of water molecules	278	273
Average B factor ($Å^2$)		
All atoms	12.2	10.6
Protein atoms	10.6	9.1
Inhibitor atoms	18.0	26.3
Water molecules	23.1	21.1

R-merge = ΣhklΣi|Ii(hkl)-<I(hkl)>|/ΣhklΣiIi(hkl), where Ii(hkl) is the intensity of an observation and <I(hkl)> is the mean value for its unique reflection; summations are over all reflections;

§Rmeas=Σhkl{N(hkl)/[N(hkl)-1]}1/2xΣi|Ii(hkl)-<I(hkl)>|/ΣhklΣiIi(hkl);

¶Rpim=Σhkl{1/[N(hkl)-1]}1/2xΣi|Ii(hkl)-<I(hkl)>|/ΣhklΣiIi(hkl);

Rwork = Σhkl||Fo(hkl)|−|Fc(hkl)||/Σhkl|Fo(hkl)| calculated for the working set of reflections. Rfree is calculated as for R-work, but from data of the test set that was not used for refinement (Test Set Size =3.1% for hCA II/1**, 3.8% for hCA II/**2**). Values in parentheses are referred to the highest resolution shell (1.63–1.60 Å for hCA II/**1** and 1.71–1.68 Å for hCA II/**2**).

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