Supplementary Information for

# Polypharmacology of Epacadostat: a Potent and Selective Inhibitor of the Tumor Associated Carbonic Anhydrases IX and XII

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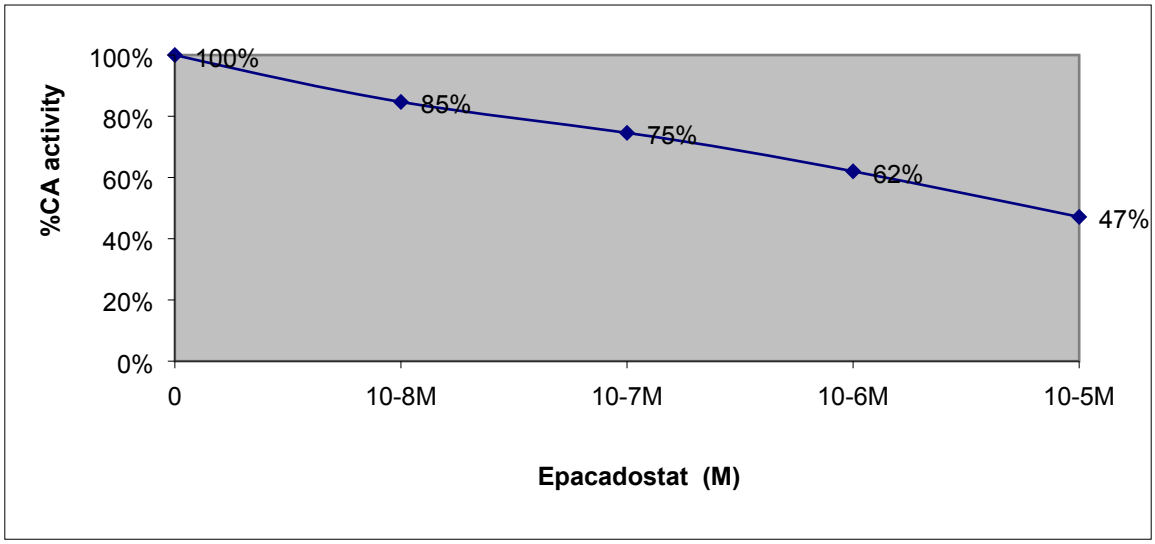
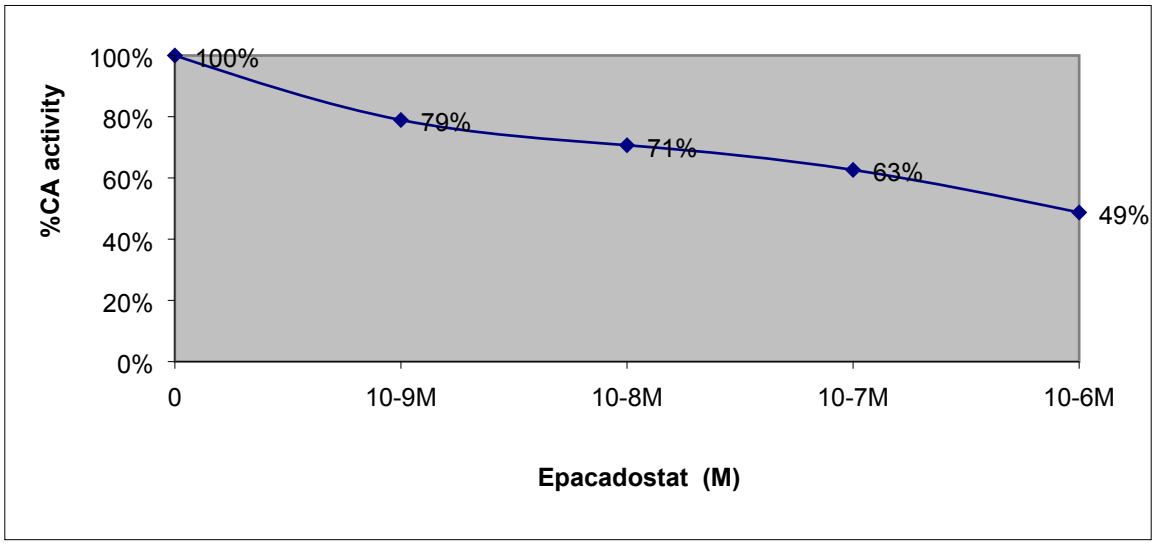
**Carbonic anhydrase inhibition**

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 20 mM Hepes (pH 7.5) as buffer, 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 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 inhibitor (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 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 ($[IC_{50}=K_I(1+[S]/Km)]$), as reported earlier [2], and represent the mean from at least three different determinations. Human CA I and II isoforms were obtained from Sigma-Aldrich (Milan, Italy), whereas all the remaining ones considered were recombinant and obtained in-house as reported earlier [3-5].

**Enzymatic Activity Curves**




hCA I

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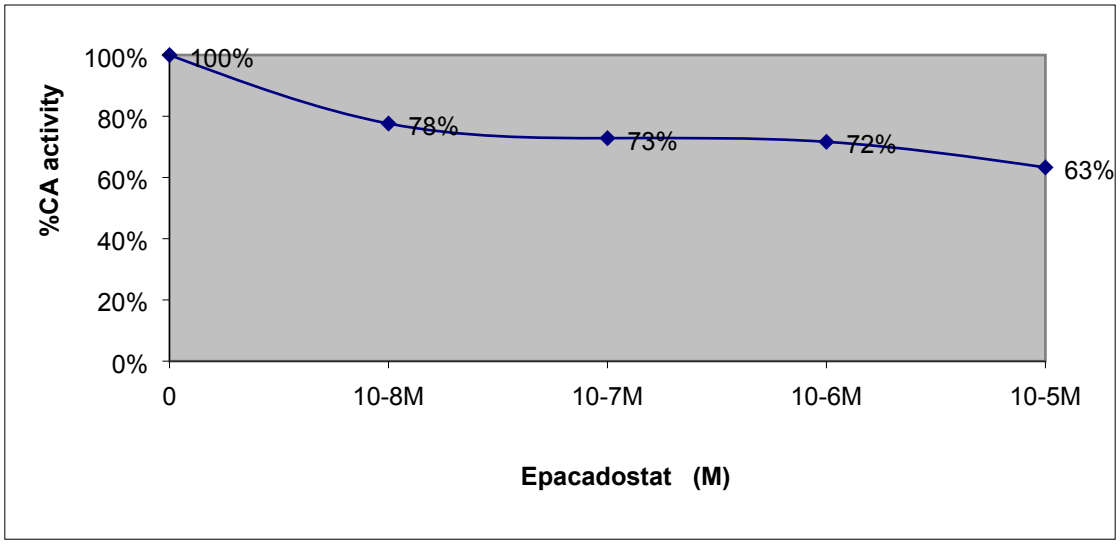


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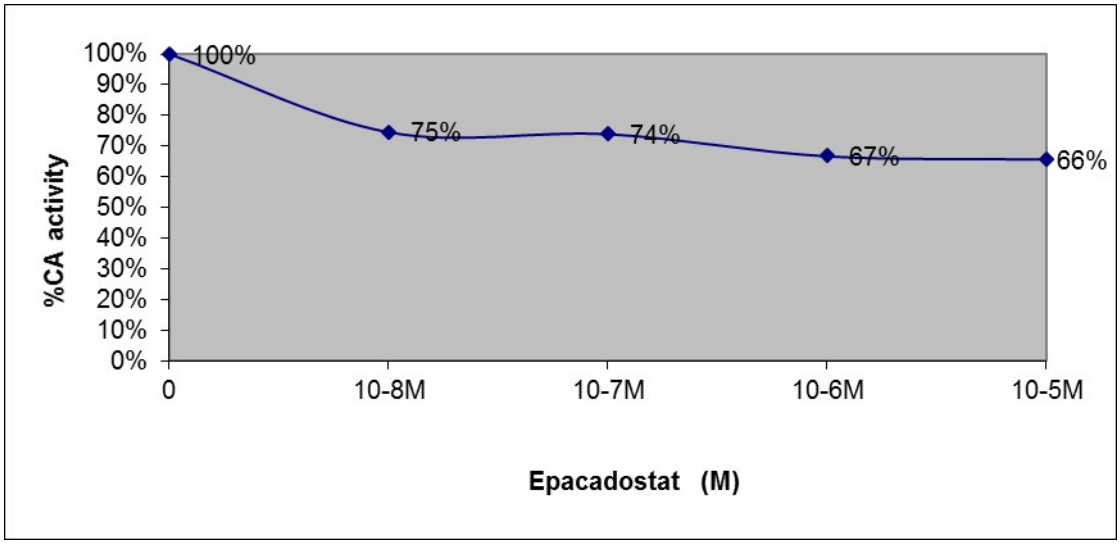
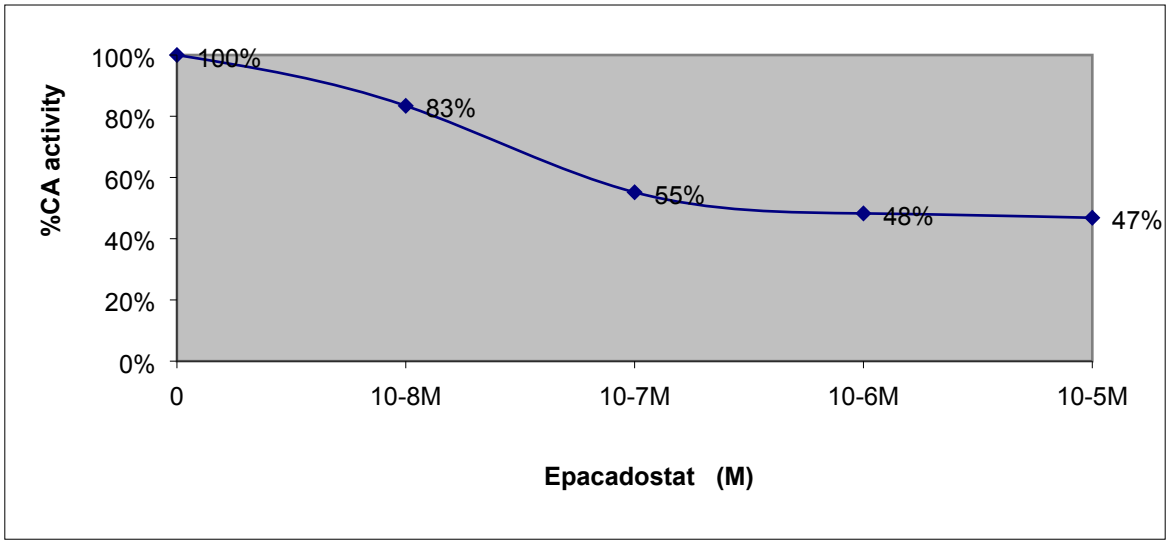



hCA IV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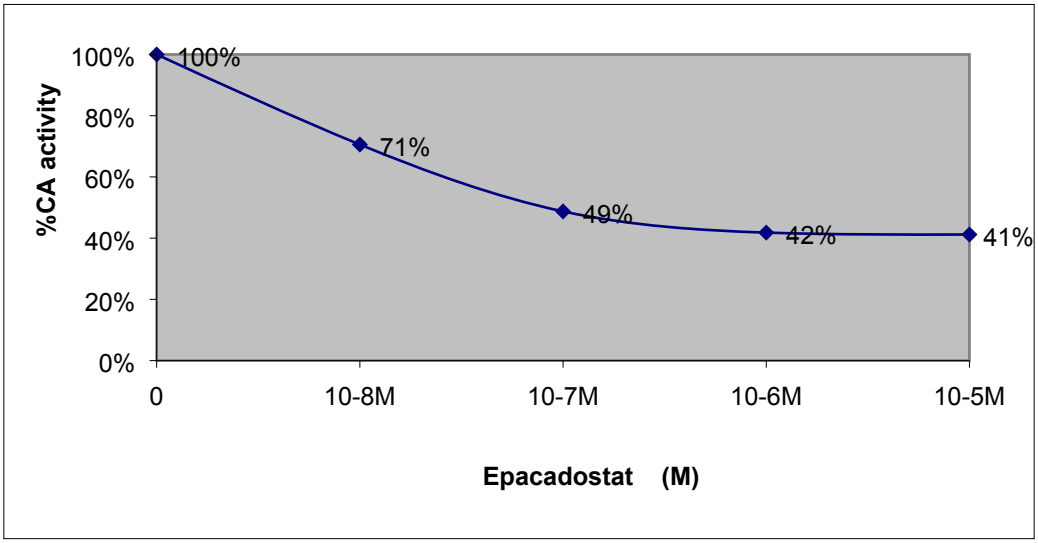
80%
60%
40%
20%
0%
100%
83%
55%
48%
47%
0
10-8M
10-7M
10-6M
10-5M
Epacadostat (M)

hCA VA


%CA activity
100%
80%
60%
40%
20%
0%
100%
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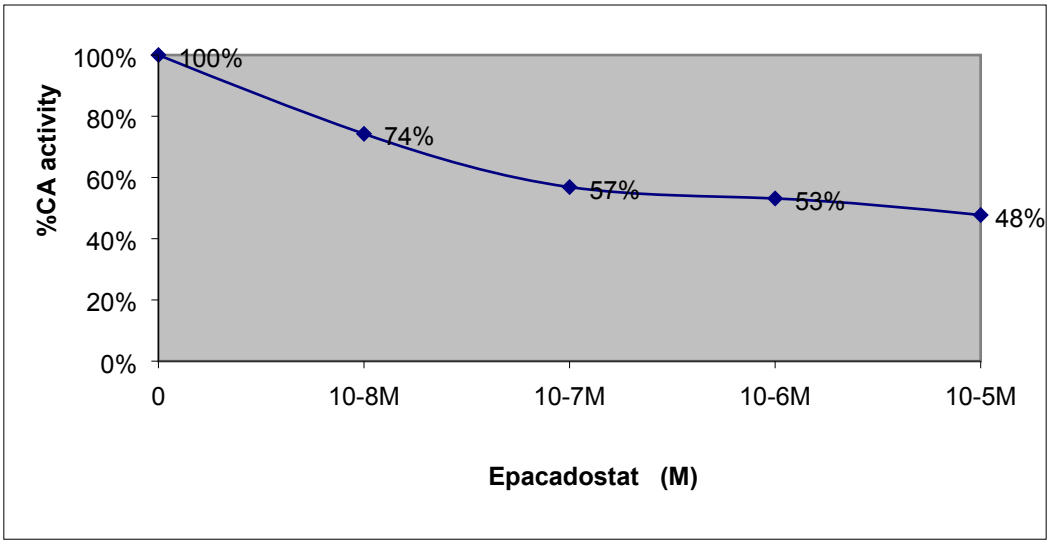
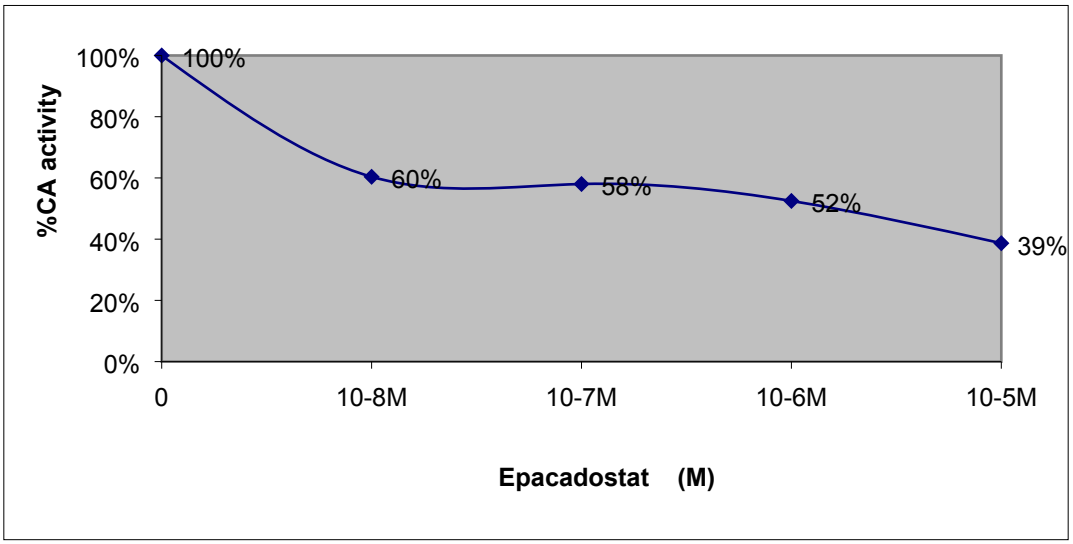
49%
42%
41%
0
10-8M
10-7M
10-6M
10-5M
Epacadostat (M)

hCA VB




hCA VI

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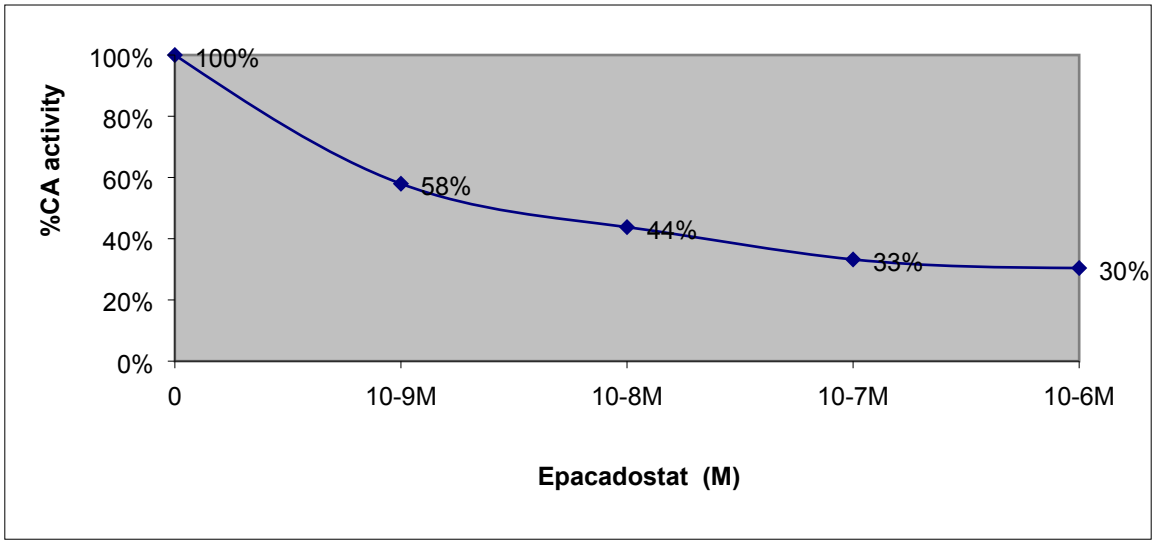
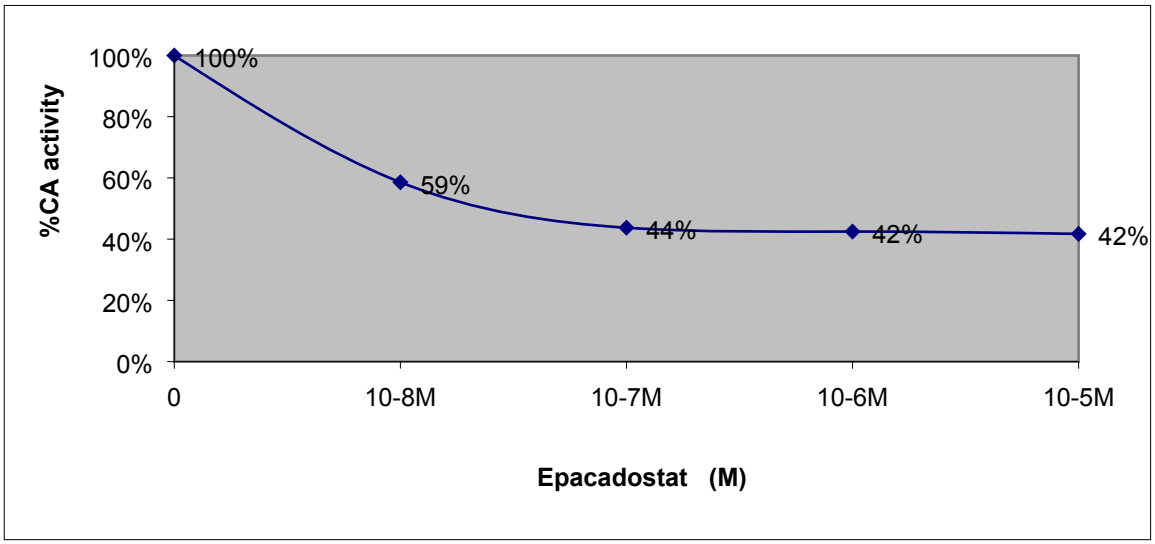
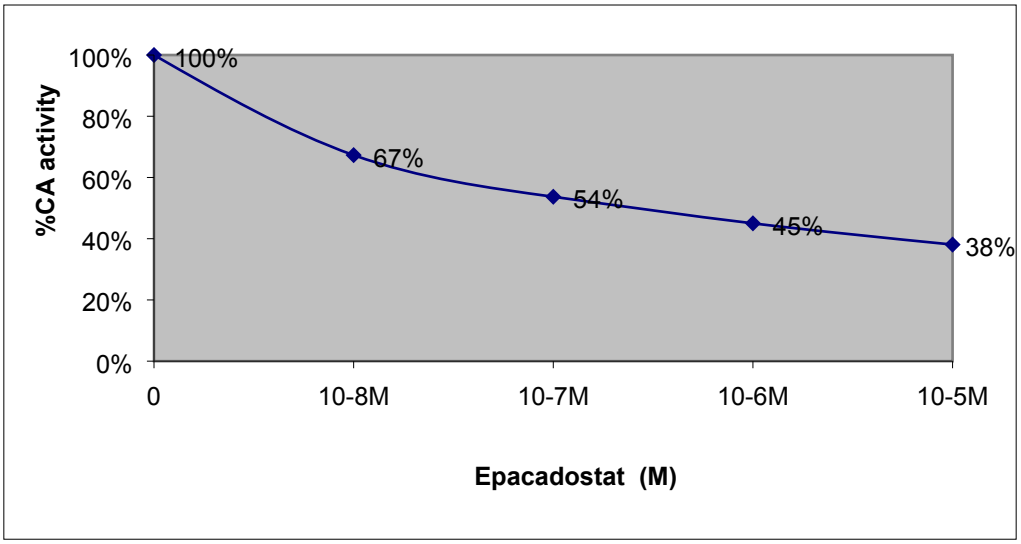


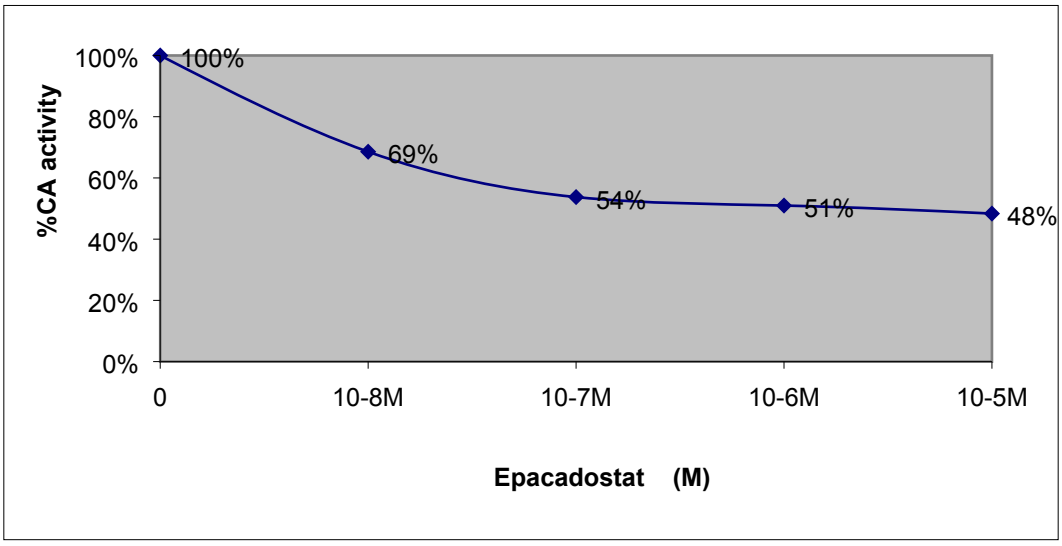

hCA IX




hCA XII




hCA XIII




hCA XIV

**Expression, Cloning and Purification of hCAs IV, IX and XII were carried out by modification of the procedures reported in the literature and below described [3, 4].**

The GeneArt Company, specializing in gene synthesis, designed the synthetic Carbonic Anhydrase 4,9,12 containing a NdeI and XhoI site at the 5′ and 3′ ends of the CA4/9/12 gene, respectively. The resulting plasmid was amplified into E. coli DH5 α cells. The Carbonic Anhydrase 4/9/12 DNA fragments were separated on 1% agarose gel. The recovered Carbonic Anhydrase gene and the linearized expression vector (pET 29b+) were ligated by T4 DNA ligase to form the expression vector pET29b+/Ca IV/IX/XII. In order to confirm the integrity of the CA IV/IX/XII gene and the fact that no errors occurred at the ligation sites, the vector containing the fragment was sequenced. Competent E. coli Artic Cells (BL21 DE3 for CA IV) were transformed with pET29b+/CA IV/IX/XII, grown at 20 °C, induced with 1 mM IPTG, and grown over night.

*Protein Preparation and purification*

After additional growth for all night, cells were harvested and disrupted by sonication at 4 °C. Following centrifugation, the sample was centrifuged at 1200g at 4 °C for 30 min. The precipitate was resuspended in 20 mM buffer phosphate, pH 8.0, and loaded onto a His-select HF nickel affinity gel. The protein was eluted with 250 mM imidazole. At this stage of purification the enzyme was at least 95 % pure and the obtained recovery was 3 mg of the recombinant human CA.

**Expression, Cloning and Purification of hCAs III, VA, VB, VI, VII and XIV were carried out by modification of the procedure reported in the literature and below described for the CA XIV isoform [5].**

CA XIV-GST construct: A putative full-length cDNA of hCA XIV (Accession No. AB025904) was obtained by RT-PCR with poly(A) RNA from the human spinal cord (Clontech, Palo Alto, CA) and the 50 and 30 rapid amplification of cDNA ends (RACE) has been performed by the previously described method. [6] The cDNA fragment encoding the open reading frame of hCA XIV was amplified by PCR using adopter primers including EcoRI and SalI recognition sequences (underlined in the following sequences, respectively): 50 -CCGAATTCGGACTGC ATGTTGTTCTCCGCCCTCCT-30 and 50 -TTTGTCG ACTTATGCCTCAGTCGTGGCTT-30 . The PCR was hot-started with incubation for 5 min at 94 °C and consisted of 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. The PCR products were cleaved with the corresponding restriction enzymes, purified, and cloned inframe into a modified pGEX-4T2 vector using T4-ligase (Promega). The proper cDNA sequence of the hCA XIV insert included in the vector was reconfirmed by DNA sequencing. The constructs were then transfected into E. coli strain BL21 for production of the hCA XIV protein [7] The protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside, cells were harvested when the OD600 arrived at 1.00 and lysed by sonication in PBS. The cell homogenate was incubated at room temperature for 15 min and homogenized twice with a Polytron (Brinkmann) for 30 s each at 4 °C. Centrifugation at 30,000g for 30 min afforded the supernatant containing the soluble proteins. The obtained supernatant was then applied to a prepacked glutathione–Sepharose 4B column (Amersham). The column was extensively washed with buffer and then the fusion (GST–hCA XIV) protein was eluted with a buffer consisting of 5 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0. Finally, the GST part of the fusion protein was cleaved with

thrombin. The advantage of this method is that hCA XIV is purified quite easily and the procedure is quite simple. The obtained hCA XIV was further purified by sulfonamide affinity chromatography, the amount of enzyme being determined by spectrophotometric measurements and its activity by stopped-flow experiments, with $CO_2$ as substrate [1].

**Crystallization and X-ray data collection**

Crystals were obtained using the hanging drop vapor diffusion method using 24 well Linbro plate. 2 µl of 0.8 mM solution of hCA II in Tris-HCl pH=8.0 were mixed with of a solution of 1.5, 1.6 and 1.7 M sodium citrate, 50 mM Tris pH 8.0 and were equilibrated against 500 µl of the same solution at 296 K. Crystals of the protein grew in a few days. hCAII crystals were soaked in 5mM inhibitor solution for 2 days. The crystals were flash-frozen at 100K using a solution obtained by adding 25% (*v/v*) glycerol to the mother liquor solution as cryoprotectant. Data on crystals of the complexes were collected using synchrotron radiation at the ID-30-B beamline at ESRF (Grenoble, France) with a wavelength of 0.826 Å and a Pilatus3_6M Dectris CCD detector. Data were integrated and scaled using the program XDS. [8]

**Structure determination**

The crystal structure of hCA II (PDB accession code: 4FIK) without solvent molecules and other heteroatoms was used to obtain initial phases of the structures using Refmac5. [9] 5% of the unique reflections were selected randomly and excluded from the refinement data set for the purpose of Rfree calculations. The initial |Fo - Fc| difference electron density maps unambiguously showed the inhibitor molecules. Atomic models for inhibitors were calculated and energy minimized using the program JLigand 1.0.40. [10] Refinements proceeded using normal protocols of positional, anisotropic atomic displacement parameters alternating with manual building of the models using COOT. [11] Solvent molecules were introduced automatically using the program ARP. [12] The quality of the final models were assessed with COOT and RAMPAGE. [13] Atomic coordinates were deposited in the Protein Data Bank (PDB accession code: 6IC2). Graphical representations were generated with Chimera. [14]

**Summary of Data Collection and Atomic Model Refinement Statistics.**

**Table S1** Summary of Data Collection and Atomic Model Refinement Statistics

| | HCAII + epacadostat |
|---|---|
| PDB ID | 6IC2 |
| Wavelength (Å) | 0.826 |
| Space Group | P21 |
| Unit cell (a, b, c, α, β, γ) (Å,°) | 42.41, 41.38, 72.32, 90.0, 104.54, 90.0 |
| Limiting resolution (Å) | 41.06 -1.15 (1.19 – 1.15) |
| Unique reflections | 81956 (14234) |
| Rsym (%) | 9.5 (52.5) |
| Rmeas (%) | 10.9 (65.0) |
| Redundancy | 3.26 (3.18) |
| Completeness overall (%) | 94.9 (88.3) |
| <I/σ(I)> | 11.39 (2.85) |
| CC (1/2) | 99.7 (78.9) |
| Refinement statistics | |
| Resolution range (Å) | 41.06 – 1.15 |
| Unique reflections, working/free | 77585/3937 |
| Rfactor (%) | 24.25 |
| Rfree(%) | 26.84 |
| r.m.s.d. bonds(Å) | 0.0044 |
| r.m.s.d. angles (°) | 1.1657 |
| Ramachandran statistics (%) | |
| Most favored | 96.9 |
| additionally allowed | 3.1 |
| outlier regions | 0.0 |
| Average B factor (Å$^2$) | |
| All atoms | 19.798 |
| inhibitors | 12.038 |
| solvent | 31.340 |

***In silico* studies**

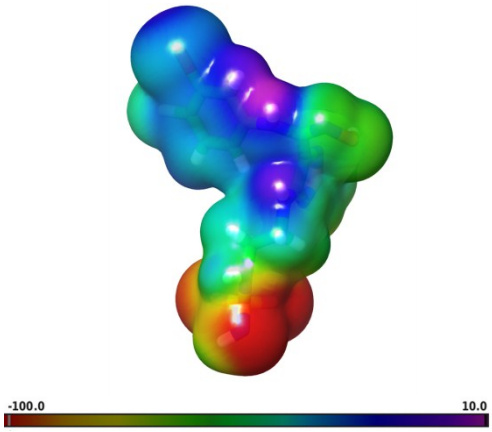



**Figure S1.** Electrostatic potential surface of Epacadostat computed at the B3LYP/LACVP**$^{++}$ level of theory. Scale -100 (red) to + 10 (purple) kcal/mol

**References**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