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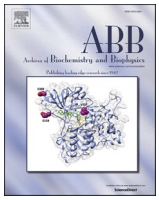

Phosphorylation strongly affects the inhibition of human carbonic anhydrase I CO_2 hydration activity

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A B S T R A C T

Human carbonic anhydrases (hCAs) have essential roles in respiration, acid-base balance, and fluid secretion, with implications in diseases such as glaucoma, epilepsy, obesity, and cancer. Of the fifteen known hCAs, human CA I (hCA I) is particularly abundant in erythrocytes, playing a critical role in CO_2 transport. Despite extensive research on hCA I, the impact of post-translational modifications (PTMs), particularly phosphorylation, on its catalytic activity and inhibitor binding remains poorly understood. Although multiple phosphorylation sites have been identified in hCA I *in vivo* through high-throughput proteomics studies including at the highly conserved Ser51 residue, the functional consequences of these modifications are not well characterized. We investigated the effects of a phosphomimetic mutation at Ser51 on hCA I, examining its catalytic efficiency and susceptibility to inhibition by sulfonamides and anions. Using a recombinant expression system and a stopped-flow kinetic assay, we characterized the CO_2 hydration activity and inhibition profiles of S51E hCA I compared to the wild type enzyme. Our results demonstrate that the S51E mutation increases the catalytic turnover rate (k_{cat}) from 2.0 × 10^5 s^{-1} to 2.6 × 10^5 s^{-1} but significantly decreases substrate affinity, raising the Michaelis constant (K_M) from 4.0 mM to 13.9 mM, reducing overall catalytic efficiency by over 50 %. Inhibition studies with a panel of 41 sulfonamides revealed that the S51E mutation dramatically alters inhibitor sensitivity, particularly for the most effective inhibitors. For example, 15 of the 16 most effective sulfonamide inhibitors for hCA I (with K_Is <350 nM) were an average of over 35-fold less effective in inhibiting S51E hCA I than the wild type. The K_I of the anticonvulsant zonisamide increased from 31 nM for the wild type hCA I to 4.0 μM. The inhibition profile with a panel of 37 small anions further indicated that the S51E mutant exhibited significantly reduced susceptibility to inhibition by 24 out of 37 tested anions, with some K_I values increasing by up to 11,000-fold for inhibitors like hydrogen sulfide. This study underscores the significant impact that phosphorylation may have on hCA I function and inhibition. By characterizing the effects of phosphorylation on the CO_2 hydration activity and inhibitor sensitivity of hCA I, these findings represent early steps in developing more selective proteoform-specific inhibitors, which could lead to more effective treatments for diseases involving carbonic anhydrases.

1. Introduction

Carbonic anhydrases (CAs) are a ubiquitous metalloenzyme superfamily found across all kingdoms of life, including animals, plants, fungi, archaea, and bacteria [1]. These enzymes catalyze the reversible hydration of carbon dioxide (CO_2) to bicarbonate (HCO_3^-) and protons (H^+), a reaction that is crucial for maintaining pH balance, ion transport, and CO_2 transport and fixation [1,2]. In mammals, 15 different CA isozymes have been identified, categorized into α, β, γ, δ, ζ, η, θ, and ι families based on their structural and functional characteristics [1]. Notably, human carbonic anhydrases (hCAs) belong exclusively to the α class and play vital roles in various physiological processes [2,3].

hCAs are involved in critical physiological functions such as respiration, acid-base balance, bone resorption, and the formation of bodily fluids like aqueous humor, cerebrospinal fluid, saliva, and gastric acid [3]. The enzymatic activity of hCAs has been implicated in multiple diseases, including glaucoma, epilepsy, obesity, cancer, and infectious diseases [2–4]. There is tremendous interest in developing CA inhibitors for therapeutic purposes [4–7]. However, *in vivo*, hCAs are heavily modified, most prevalently by phosphorylation, and the impact of these modifications on their activity and inhibition is not well understood [8–10].

Among the fifteen known hCAs, including the twelve that are catalytically active, human CA I (hCA I) is one of the most extensively studied due to its abundance in erythrocytes and its role in facilitating CO_2 transport from tissues to the lungs [3]. Despite its

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well-characterized function, recent emerging evidence suggests that post-translational modifications (PTMs) such as phosphorylation can significantly alter its activity and interaction with inhibitors [10], thereby impacting its physiological and pathological roles.

Phosphorylation is a common PTM that regulates enzyme activity, stability, and interaction with other molecules [11]. While over ten phosphorylation sites have been identified in hCA I and hCA II through high-throughput proteomics experiments [8,10], the specific functional consequences of these modifications remain unclear. Among the serine residues, Ser51 in hCA I is particularly promising for investigation due to its surface accessibility [12], allowing for kinase-mediated phosphorylation. It is part of a Ser-Tyr motif that may be recognised by PAS domain-containing serine/threonine-protein kinase (PASK) according to a recent proteomic survey [13] designed to uncover kinase target motifs and an associated kinase prediction algorithm [14]. PASK is a stress-response kinase that regulates energy homeostasis and protein translation and its substrates include glycogen synthase GYS1 and ribosomal proteins eEF1A1 and RPS6, highlighting its role in key metabolic pathways. Ser51 in hCA I is located on a beta-sheet (residues 48–51 and 79–82, Fig. 1) within 14 residues of His65, which is crucial for proton shuttling in the rate-limiting step of the enzyme's catalytic cycle [15]. His65 is located on the loop connecting two beta-strands that form another small beta-sheet (residues 57–62 and residues 67–71, Fig. 1). Beta-sheets can propagate allosteric effects throughout a protein's structure due to the correlation of motions between residues that participate in the beta-sheet structural motif [16]. Since His65 has a key role in maintaining catalytic efficiency, phosphorylation of Ser51 (located ~20 Å from the zinc active site) could induce allosteric effects through the two beta-sheets, potentially influencing overall enzyme activity and inhibition. *In vitro* studies using serine-to-glutamate (S > E) phosphomimetic mutations at specific serine residues have shown that these modifications can alter the enzyme's catalytic efficiency and inhibitor binding affinity [10], suggesting a potential regulatory mechanism. Phosphomimetic mutations, such as S > E substitutions in which glutamate mimics the size and charge of phosphoserine [17], are often used to simulate the effects of phosphorylation on protein structure and function. Recently, phosphomimetic mutations at Ser51 in hCA I and Ser50 in hCA II have been reported to affect the esterase activity and interactions of hCA II with a few sulfonamide inhibitors [10].

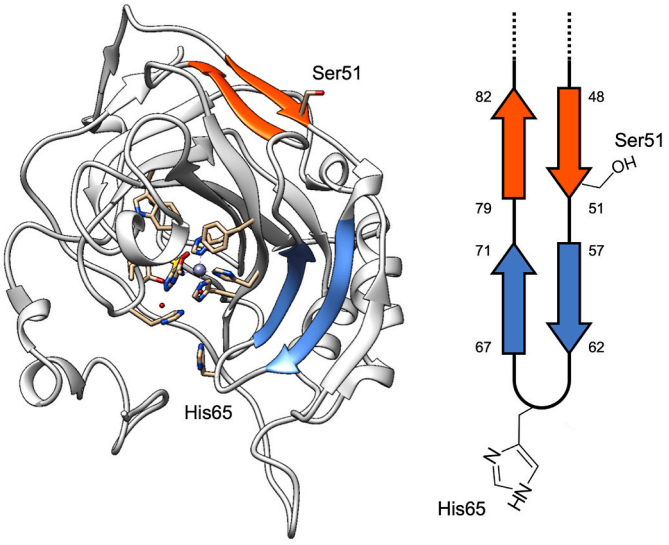

Fig. 1. Crystal structure of human carbonic anhydrase I (hCA I) highlighting key residues involved in catalysis and regulation. His65, located in the loop connecting two beta-strands, is crucial for proton shuttling in the rate-limiting step of CO_2 hydrolysis. Ser51, positioned on a beta-sheet (residues 48–51 and 79–82), is a surface-exposed residue that can be phosphorylated. Phosphorylation of Ser51 may propagate allosteric effects through adjacent beta-sheets and influence the enzyme's catalytic efficiency and inhibitor binding. PDB ID: 1AZM.

The inhibition of CAs has significant therapeutic implications, particularly for diseases such as glaucoma, epilepsy, and certain cancers [2–4,6]. Sulfonamides are the most well-known CA inhibitors, acting by binding to the catalytically active zinc ion at the active site, thereby inhibiting the hydration of CO_2 [6]. This binding disrupts the normal coordination environment of the zinc ion, which is necessary for the hydration reaction, thus preventing the conversion of CO_2 to bicarbonate and protons [5]. Similar to sulfonamides, small anions can also bind to the metal ion center, thereby inhibiting the enzyme's catalytic activity [18]. Profiling the inhibition of CA activity by sulfonamides and small anions provides insights into the fundamental activity of the enzyme. Additionally, these inhibitors serve as a basis for developing more effective inhibitors by allowing researchers to elaborate on their structures and design more potent compounds.

Previous studies have shown that phosphomimetic mutations can either increase or decrease the esterase catalytic efficiencies of CAs, depending on both the position of the modification and the CA isoform [10]. These effects suggest that phosphorylation might serve as a mechanism for modulating enzymatic activity and drug-binding affinities, thereby affecting the specificity and efficacy of therapeutic agents targeting CAs. Previous work investigating the effects of phosphorylation has primarily focused on the esterase activity of CAs [10] or used broad-specificity kinases [19], but these studies did not explore the detailed impact of phosphorylation on CO_2 hydration activity kinetics or evaluate the effects of inhibitors in a functional assay. Moreover, as analytical technology increasingly enables proteoforms – distinct forms of proteins arising from genetic variations, alternative splicing, and post-translational modifications – to be more comprehensively characterized [20,21], there may be an increasing interest in developing inhibitors specific to distinct proteoforms. For example, if modifications like phosphorylation can significantly reduce the susceptibility of the enzyme to inhibition, designing inhibitors that specifically target these modified forms is imperative. Understanding how these modifications influence enzyme function and inhibitor binding will aid the development of more selective and potent therapeutic agents.

Here, we address these gaps by investigating the phosphorylation effects on human CA I in the context of CO_2 hydration activity using phosphomimetic mutation. We examine the influence of sulfonamide and anion inhibitors on the activity of the serine to glutamic acid mutation at Ser51 (S51E) of human CA I, providing insights into enzyme regulation and potential therapeutic strategies. This research seeks to deepen our understanding of how PTMs influence hCA function and facilitate the development of more effective inhibitors for clinical applications.

2. Methods

2.1. Recombinant protein expression and purification

Artic Express (DE3)RP competent cells (Agilent) were transformed with a previously reported plasmid [10] containing the hCA I S51E nucleotide sequence. The plasmid was engineered with an N-terminal 6xHis tag for affinity purification. The cells were cultured at 30 °C with shaking at 180 rpm. When the culture reached an optical density at 600 nm of 0.7, it was chilled on ice and then transferred to a 15 °C environment. Protein expression was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 30 min, 0.5 mM $ZnSO_4$ was added to the culture. The induced culture was allowed to grow overnight for 18 h.

Cells were harvested in 20 mM Tris HCl lysis buffer (pH 8.3) by centrifugation at 5000 rpm for 20 min. The harvested cells were sonicated with 10 cycles of 10 s 'on' and 120 s 'off' at 50 % amplitude (Bandelin Sonopuls TT213). After sonication, the lysate was centrifuged at 12,000×*g* for 45 min. The supernatant was then incubated for 30 min

with nickel affinity gel resin (His Select HF, Sigma) that had been equilibrated in lysis buffer. Following a centrifugation step at 1500×g, the resin was washed with 50 mM Tris/HCl buffer (pH 8.3), 500 mM NaCl, and 20 mM imidazole. The protein was eluted with the same buffer containing 200 mM imidazole. The collected fractions were dialyzed against 20 mM Tris/HCl (pH 8.3). At this stage of purification, the recombinant proteins were 90 % pure with a yield of approximately 4.5 mg/L of bacterial culture. The purity of the mutant enzyme was determined by SDS-PAGE analysis, performed on the affinity-purified enzyme based on the relative intensity of the band compared to minor impurities (data not shown). The SDS-PAGE gel showed a single predominant band corresponding to the expected molecular weight of the mutant carbonic anhydrase. The concentration of the active carbonic anhydrase enzyme was adjusted based on the SDS-PAGE purity estimate to ensure that the k_{cat} and catalytic efficiency values reflect only the active enzyme in the preparation.

2.2. Stopped flow kinetic CO_2 hydration assay and carbonic anhydrase inhibition

A stopped-flow instrument (SX.18 MV-R, Applied Photophysics, Surrey, UK) was used to measure the activity of the S51E mutant for catalyzing the CO_2 hydration reaction [22]. Phenol red (0.2 mM) served as the indicator with its absorbance measured at the maximum wavelength of 557 nm. The buffer solution consisted of 20 mM HEPES (pH 7.4) and 20 mM Na_2SO_4 to maintain a constant ionic strength. Initial rates of the CA-catalyzed CO_2 hydration reaction were observed over 10–100 s, with CO_2 concentrations ranging from 1.7 to 17 mM for determining kinetic parameters and inhibition constants [2]. Enzyme concentrations were between 5 and 12 nM. For each inhibitor, at least six traces of the initial 5–10 % of the total reaction time were analyzed to determine the initial velocity. Uncatalyzed rates were measured similarly and subtracted from the total observed rates. Stock solutions of the inhibitor (0.1 mM) were prepared in distilled-deionized water and diluted up to 0.01 nM with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature to allow for the formation of the enzyme-inhibitor (E–I) complex. Inhibition constants were calculated using non-linear least-squares methods with PRISM 3 (GraphPad Software, USA) and the Cheng-Prusoff equation [23], representing the mean of at least three determinations. The catalytic efficiency (k_{cat}/K_M) for each variant was calculated based on the Michaelis–Menten Equation (1):

$$\frac{1}{v} = \frac{K_M}{V_{max}[S]} + \frac{1}{V_{max}} \quad (1)$$

where v and V_{max} represent the initial rate and maximum rate of the enzymatic reaction, respectively, and [S] is the substrate concentration. The Lineweaver-Burk equation can be obtained by using GraphPad Prism 3 to fit the double reciprocal curves of $1/v$ versus $1/[S]$.

3. Results and discussion

3.1. Effects of S51E mutation on enzyme kinetics for human carbonic anhydrase I

The kinetic parameters and acetazolamide inhibition constants for the S51E mutant and wild type hCA I are shown in Table 1. The S51E mutation of hCA I significantly increases the catalytic turnover rate (k_{cat}) with a value of 2.6×10^5 s^{-1} compared to 2.0×10^5 s^{-1} for the wild type enzyme. This suggests that the S51E mutation enhances the rate at which the enzyme catalyzes the conversion of CO_2. However, this mutation also significantly reduces substrate affinity. Specifically, the Michaelis constant (K_M) for the S51E mutant is 13.9 mM, considerably higher than the 4.0 mM observed for the wild type enzyme. A higher K_M indicates a lower affinity for the substrate, suggesting that the S51E mutation decreases the enzyme's substrate binding affinity. Consequently, the catalytic efficiency (k_{cat}/K_M) of the S51E mutant is reduced to 1.9×10^7 M^{-1} s^{-1} from 5.0×10^7 M^{-1} s^{-1} for the wild type enzyme. Despite the increase in k_{cat}, the substantial rise in K_M leads to an overall decrease in catalytic efficiency, highlighting the tradeoff between increase turnover rate and decreased substrate binding efficiency due to the S51E mutation. The S51E mutation also decreases the catalytic efficiency for the esterase activity of hCA I as previously reported [10].

Overall, the data reported here confirm that the catalytic efficiency for CO_2 hydration activity of hCA I substantially decreases upon the introduction of a phosphomimic site at Ser51 due to a decrease in substrate affinity, despite an increase in the catalytic turnover rate. Ser51 is located on a surface-accessible beta sheet, approximately 20 Å from the Zn center, near the base of a loop containing His65 [15]. His65 is pivotal in the rate-limiting step of the catalytic cycle, facilitating proton shuttling from the active site to regenerate the enzyme's active form [15]. Phosphorylation at Ser51 could allosterically impact the positioning and basicity of His65, potentially altering the proton-shuttling dynamics and thereby affecting the enzyme's overall catalytic efficiency.

3.2. Effects of S51E mutation on the inhibition of hCA I CO_2 hydration activity by sulfonamides

The inhibition constant (K_I) for acetazolamide (Table 1) is higher for the S51E mutant (392 nM) than the wild type enzyme (250 nM) indicating that the S51E mutation reduces the enzyme's affinity for this inhibitor by ~57 %. This result suggests that the phosphomimetic mutation at Ser51 makes hCA I less susceptible to inhibition by acetazolamide. Thus, to further explore the impact of the S51E mutation on sulfonamide inhibition, we conducted a detailed inhibition study of hCA I with a panel of 38 other sulfonamides and one sulfamate (compounds **1**–**24** and **AAZ** to **EPA**; Fig. 2). The latter 17 compounds in the inhibition panel are clinically used as diuretics, antiglaucoma, antiepileptic and antiobesity agents, targeting many of the human CA isoforms involved in these pathologies, namely acetazolamide (**AAZ**), methazolamide (**MZA**), ethoxzolamide (**EZA**), dorzolamide (**DZA**), brinzolamide (**BRZ**), saccharin (**SAC**), benzolamide (**BZA**), topiramate (**TPM**), zonisamide (**ZNS**), sulpiride (**SLP**), indisulam (**IND**), valdecoxib (**VLX**), celecoxib (**CLX**), sultiame (**SLT**), hydrochlorothiazide (**HCT**), famotidine (**FAM**), and epacadostat (**EPA**).

The sulfonamide inhibition data (Fig. 3A and Table 2) reveal that the S51E mutation significantly affects the inhibition profile of hCA I by various sulfonamides by either increasing or decreasing the susceptibility of the enzyme to inhibition. For 18 of the 40 inhibitors that were tested, the phosphomimic substantially reduces the susceptibility of the enzyme to inhibition. For example, of the sixteen most effective wild type hCA I inhibitors with K_I values < 350 nM, fifteen were substantially less effective on the S51E mutant with K_I values that were an average of over 35-fold higher for the mutant compared to the wild type. For example, inhibitor **20** is the most effective of the panel at inhibiting wild

Table 1
Kinetic parameters for the CO_2 hydration reaction catalyzed by hCA I and S51E hCA I phosphomimic enzyme (20 °C, pH 7.5 in 10 mM HEPES buffer). The inhibition constant for the clinically relevant sulfonamide acetazolamide is also reported.

Enzyme	Class	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	K_I (acetazolamide) (nM)
hCA I	α	2.0×10^5	4.0	5.0×10^7	250
S51E hCA I	α	2.6×10^5	13.9	1.9×10^7	392

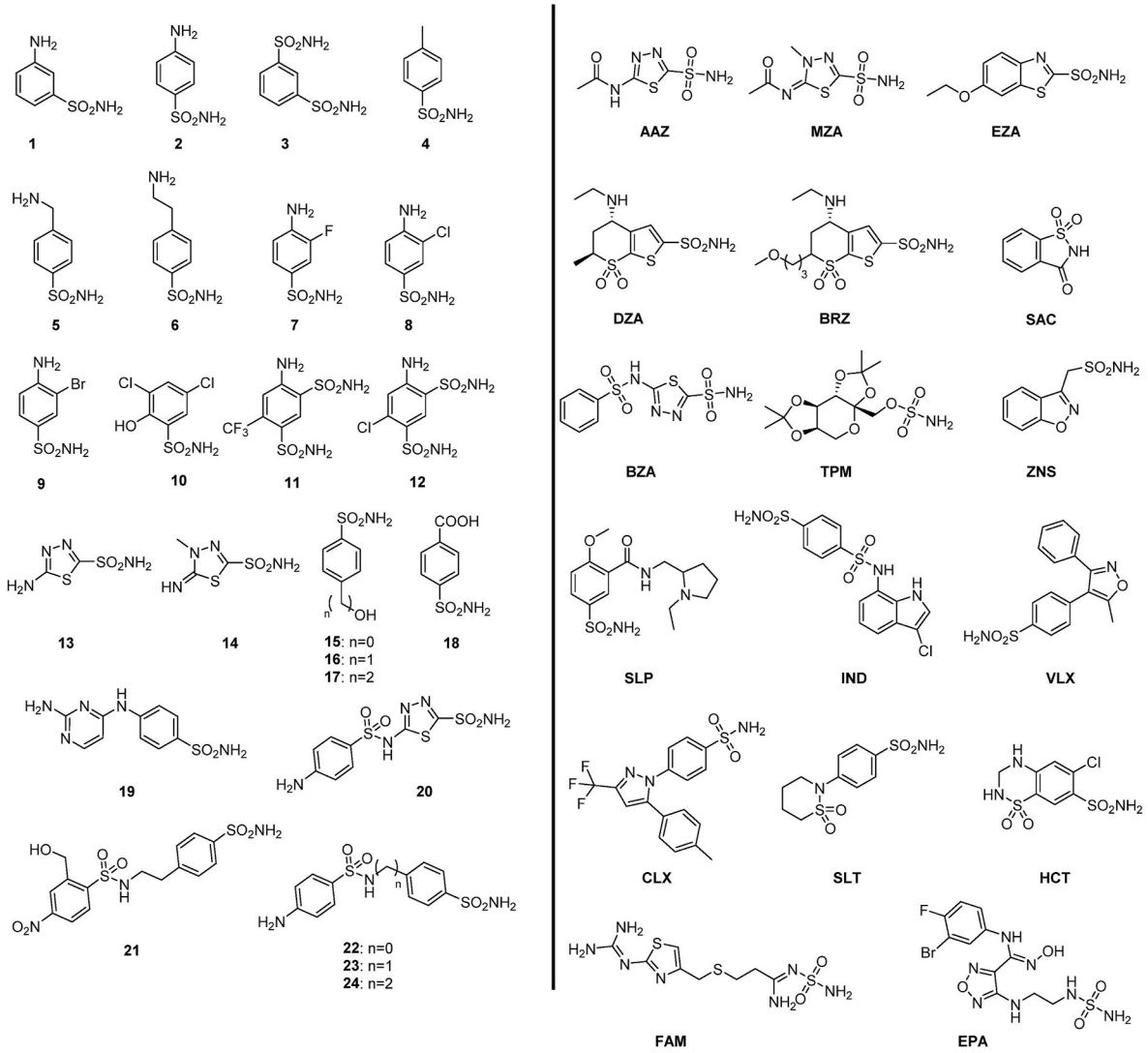

Fig. 2. Sulfonamide and sulfamate inhibitors **1**–**24** and **AAZ** to **EPA**.

type hCA I. However, the phosphomimic results in the inhibition constant for the sulphonamide **20** increasing by over two orders of magnitude (from a K_I of 6 nM to 700 ± 57 nM; >120-fold increase in K_I). Likewise, brinzolamide inhibits wild type hCA I with a K_I of 15 nM, which increases by over 6.0-fold to 94.2 ± 6.1 nM for S51E hCA I and the K_I for sulpiride increases by over 140-fold from 56 nM to 7700 ± 400 nM with the S51E mutation. These data indicate that the phosphomimic can dramatically reduce the effectiveness of the strongest inhibitors in the panel.

While the 16 most effective inhibitors of the wild type hCA I (K_Is < 350 nM) resulted in their effectiveness decreasing by an average of over 35-fold when inhibiting the S51E phosphomimetic mutant (with the exception of inhibitor **24**) compared to the wild type enzyme, the other 24 compounds (with K_Is > 350 nM for the wild type) had K_I values for the mutant that were either comparable to or lower than those for the wild type enzyme, except for **FAM** and sulfonamide **5**. Specifically, the K_I values for these weaker inhibitors were on average 1.3-fold more effective inhibitors for the mutant than for the wild type. These data indicate that the mutation can more strongly impact the effectiveness of the strongest inhibitors of the wild type enzyme disproportionately compared to the weaker inhibitors.

The S51E mutation in human carbonic anhydrase I (hCA I) affects inhibitors differently, depending on their interactions with the zinc ion and surrounding binding pocket (Table 2). For example, the small monocyclic sulphonamide **AAZ** shows a moderate 1.6-fold reduction in affinity potentially owing to its interaction mechanism being dominated by zinc-sulphonamide ionic interaction with minimal engagement of the broader binding pocket [12]. In contrast, the affinity for **BZA** for the mutant decreases 6.3-fold, while the affinity of **DZA** increases substantially compared to the mutant (Table 2). This suggests that the mutation alters the binding pocket significantly, impacting the larger multi-ring structures of BZA and DZA, which interact more extensively beyond the zinc center. These findings suggest that the S51E mutation can strongly affect inhibitors with greater reliance on binding pocket interactions.

3.3. Anion inhibition profiles for S51E mutant and wild type hCA I enzymes

Next, we sought to profile the anion inhibition profile of S51E hCA I. Anions are a particularly important class of inhibitors for metalloenzymes because they can bind the metal ion in the active site cavity [7,18]. While small anion inhibitors are often not highly effective, they are relevant for understanding detailed inhibition mechanisms of metalloenzymes and for drug design purposes. Simple chemical entities are easily modifiable to develop more elaborate scaffolds, leading to effective inhibitors. Many small anions, such as bicarbonate and chloride, are physiologically relevant and present in high concentrations.

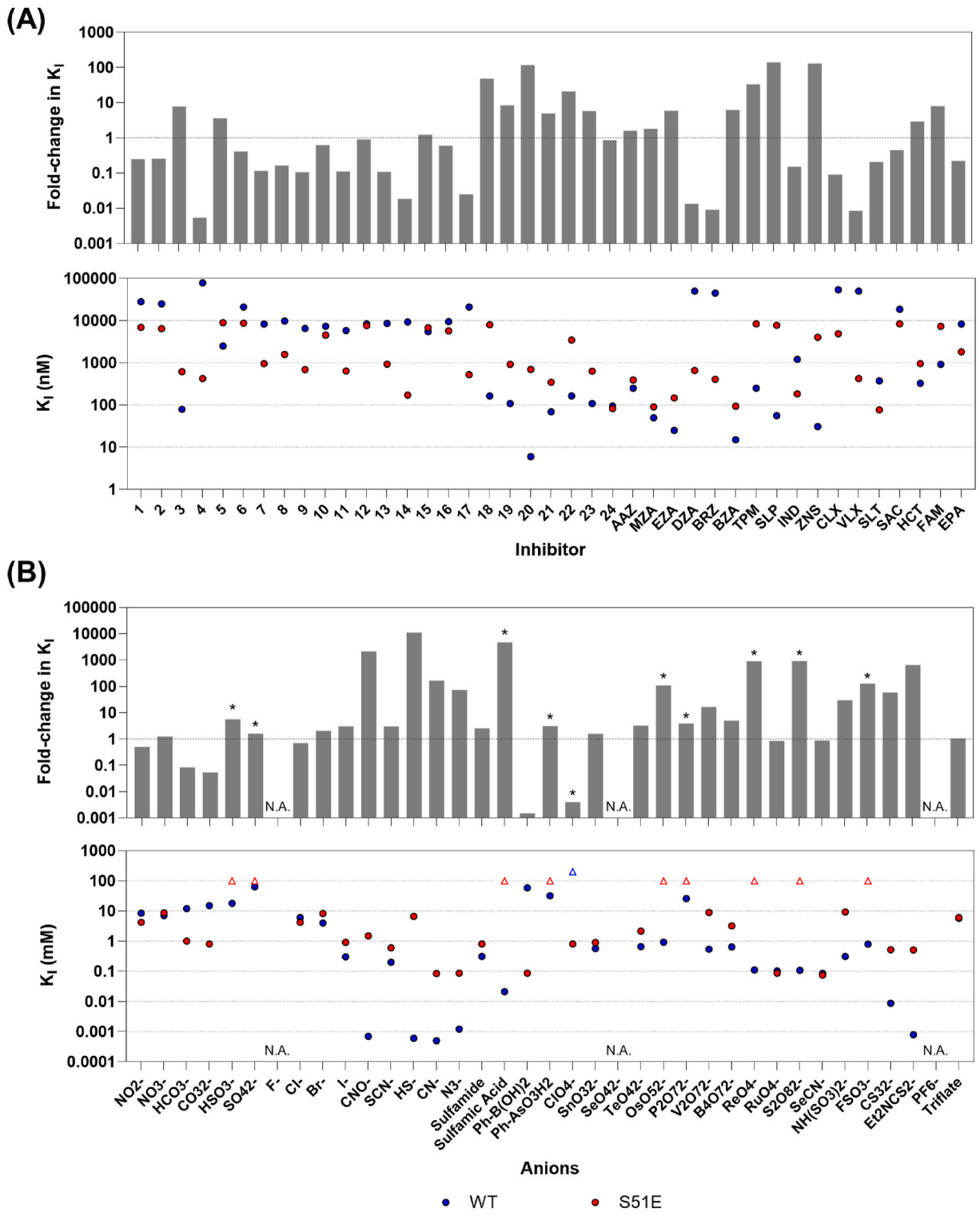

Fig. 3. Effect of the S51E phosphomimetic mutation on hCA I catalytic activity and inhibition profiles for the CO_2 hydration reaction. Fold-change in lnK_I and K_I values for S51E hCA I and wild type hCA I for (A) sulfonamide and (B) anion inhibitors. Open triangles correspond to lower limits of the CO_2 hydration inhibition assay with asterisks denoting the corresponding fold change bars. Three anions had a negligible effect on the activity of both hCA I and the mutant, denoted as not applicable (N.A.). Refer to Tables 2 and 3

In Table 3 and Fig. 3B, the measured inhibition constants of 37 anions are shown for the S51E mutation of hCA I compared to the wild type enzyme. The effects of the phosphomimic on different anions inhibition can be classified broadly into three types: (i) strongly decreasing susceptibility to inhibition (>1.5-fold increase in K_I value), (ii) moderate impact on inhibition (0.5- to 1.5-fold change in K_I value), and (iii) strongly increasing susceptibility to inhibition (>1.5-fold decrease in K_I value). The results indicate that 24 of 37 anions are substantially less effective inhibitors of the S51E mutant with K_I values 1.5-fold higher than the wild type, while 5 anions are more effective for the mutant, displaying >1.5-fold lower K_I values. An additional five anions had K_I values for the mutant that were within 50 % of the wild type, while three

Table 2
Inhibition constants of S51E hCA I phosphomimic and hCA I with sulfonamides **1**–**24** and clinically relevant drugs **AAZ-EPA** obtained using a kinetic stopped-flow CO_2 hydration assay.

Compound	K_I (nM)[a]		Fold Change[c]	Compound	K_I (nM)[a]		Fold Change[c]
	S51E hCA I	hCA I[b]			S51E hCA I	hCA I[b]	
1	6890 ± 410	28000	0.25	**22**	3450 ± 180	164	21
2	6440 ± 370	25000	0.26	**23**	634 ± 35	109	5.8
3	616 ± 32	79	7.8	**24**	81.9 ± 6.7	95	0.86
4	423 ± 31	78500	0.0054	**AAZ**	392 ± 26	250	1.6
5	8920 ± 590	2500	3.6	**MZA**	90.5 ± 8.0	50	1.8
6	8680 ± 660	21000	0.41	**EZA**	147.3 ± 9.8	25	5.9
7	953 ± 59	8300	0.11	**DZA**	661 ± 41	50000	0.013
8	1580 ± 130	9800	0.16	**BRZ**	408 ± 21	45000	0.0091
9	690 ± 55	6500	0.10	**BZA**	94.2 ± 6.1	15	6.3
10	4540 ± 410	7300	0.62	**TPM**	8340 ± 820	250	33
11	640 ± 33	5800	0.11	**SLP**	7700 ± 400	56	140
12	7610 ± 460	8400	0.91	**IND**	184 ± 18	1200	0.15
13	930 ± 53	8600	0.11	**ZNS**	4050 ± 280	31	130
14	172 ± 15	9300	0.019	**CLX**	4880 ± 420	54000	0.090
15	6760 ± 370	5500	1.2	**VLX**	422 ± 22	50000	0.0084
16	5690 ± 420	9500	0.60	**SLT**	77.0 ± 5.5	374	0.21
17	524 ± 46	21000	0.025	**SAC**	8360 ± 770	18540	0.45
18	8010 ± 440	164	49	**HCT**	962 ± 50	328	2.9
19	919 ± 48	109	8.4	**FAM**	7330 ± 500	922.4[d]	7.9
20	700 ± 57	6	120	**EPA**	1820 ± 100	8262[e]	0.22
21	345 ± 32	69	5.0				

[a] Mean from three replicates by a stopped flow technique.
[b] C.T. Supuran *Nat. Rev. Drug Disc.*, 7 (2008), p. 168.
[c] $K_{I,mutant}/K_{I,wild\ type}$.
[d] Angeli A, Ferraroni M, Supuran CT. Famotidine, an Antiulcer Agent, Strongly Inhibits *Helicobacter pylori* and Human Carbonic Anhydrases. ACS Med Chem Lett. 2018 Sep 4; 9(10):1035–1038.
[e] Angeli A, Ferraroni M, Nocentini A, Selleri S, Gratteri P, Supuran CT, Carta F. Polypharmacology of epacadostat: a potent and selective inhibitor of the tumor associated carbonic anhydrases IX and XII. Chem Commun. 2019 May 14; 55(40):5720–5723.

Table 3
Inhibition constants of anions for S51E hCA I and hCA I activity obtained using a kinetic stopped flow CO_2 hydration assay.

Anion	K_I (mM)[a]		Fold Change[c]	Anion	K_I (mM)[a]		Fold Change[c]
	hCA I mutant	hCA I[b]			hCA I mutant	hCA I[b]	
NO_2^-	4.17 ± 0.35	8.4	0.50	**ClO_4^-**	0.809 ± 0.041	>200	0.0040
NO_3^-	8.68 ± 0.77	7.0	1.2	**SnO_3^{2-}**	0.891 ± 0.068	0.57	1.6
HCO_3^-	0.960 ± 0.060	12	0.080	**SeO_4^{2-}**	>100	>100	N.A.
CO_3^{2-}	0.792 ± 0.046	15	0.053	**TeO_4^{2-}**	2.13 ± 0.14	0.66	3.2
HSO_3^-	>100	18	>5.6	**OsO_5^{2-}**	>100	0.92	>110
SO_4^{2-}	>100	63	>1.6	**$P_2O_7^{2-}$**	>100	25.8	>3.9
F^-	>100	>300	N.A.	**$V_2O_7^{2-}$**	8.94 ± 0.74	0.54	17
Cl^-	4.21 ± 0.24	6	0.70	**$B_4O_7^{2-}$**	3.22 ± 0.29	0.64	5.0
Br^-	8.22 ± 0.79	4	2.1	**ReO_4^-**	>100	0.11	>910
I^-	0.910 ± 0.053	0.3	3.0	**RuO_4^-**	0.0863 ± 0.0062	0.101	0.85
CNO^-	1.543 ± 0.091	0.0007	2200	**$S_2O_8^{2-}$**	>100	0.107	>930
SCN^-	0.617 ± 0.055	0.2	3.1	**$SeCN^-$**	0.0747 ± 0.0046	0.085	0.88
HS^-	6.60 ± 0.59	0.0006	11000	**$NH(SO_3)^{2-}$**	9.31 ± 0.63	0.31	30
CN^-	0.0848 ± 0.0049	0.0005	170	**FSO_3^-**	>100	0.79	>130
N_3^-	0.0877 ± 0.0050	0.0012	73	**CS_3^{2-}**	0.513 ± 0.049	0.0087	59
Sulfamide	0.806 ± 0.045	0.31	2.6	**Et_2NCS^{2-}**	0.509 ± 0.028	0.00079	640
Sulfamic Acid	>100	0.021	>4800	**PF_6^-**	>100	>100	N.A.
Ph-B(OH)$_2$	0.0857 ± 0.0068	58.6	0.0015	**Triflate**	6.04 ± 0.41	5.7	1.1
Ph-AsO$_3$H$_2$	>100	31.7	>3.2				

[a] Mean from three replicates by a stopped flow technique.
[b] Del Prete S, De Luca V, Nocentini A, Scaloni A, Mastrolorenzo MD, Supuran CT, Capasso C. Anion Inhibition Studies of the Beta-Carbonic Anhydrase from *Escherichia coli*. Molecules. 2020 May 31; 25(11):2564.
[c] $K_{I,mutant}/K_{I,wild\ type}$.

had a minimal effect on the activity of both the wild type and mutant (PF_6^-, SeO_4^{2-} and F^-). However, as with the sulfonamides, the mutation generally resulted in a much larger relative effect on the K_I values of the most effective anionic inhibitors. For example, for the seven anions that most effectively inhibit the wild type hCA I enzyme, the K_I values for the mutant increased by an average of over 2500-fold vs an average decrease in K_I values of only 1.3-fold for the 15 least effective anion inhibitors.

Some of the most notable changes were observed for the three most effective anions against the wild type. Cyanide (CN^-) is the most effective anion inhibitor of the wild type hCA I with a K_I of 0.5 μM, which increased to 84.8 ± 4.9 μM for the S51E mutant, representing a >170-fold increase. Hydrogen sulfide (HS^-) showed an even more pronounced change, with a K_I of 0.6 μM in the wild type increasing to 6.60 ± 0.59 mM in the mutant, an 11,000-fold increase. Fulminate (CNO^-) had a K_I of 0.7 μM in the wild type, which increased to 1.543 ±

0.091 mM in the mutant, showing over a 2000-fold increase. In contrast, the five anions that were the most effective against the mutant relative to the wild type showed a decrease in K_I values for the mutant by only between 1.5- to 2-fold. These anions include nitrite (NO_2^-), bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), and phenylboronic acid (Ph-B(OH)$_2$) with K_I values for the wild type ranging from low to high mM (8.4–59 mM). In addition, perchlorate ClO_4^- has no measurable inhibition activity (>200 mM) for the wild type, but has a K_I of 0.809 ± 0.041 mM for the mutant. These trends demonstrate that the S51E phosphomimetic mutation can dramatically alter the enzyme's sensitivity to various anion inhibitors.

4. Conclusions

A phosphomimetic mutation at serine 51 (S51E) of human carbonic anhydrase I significantly decreases the catalytic efficiency for the CO_2 hydration reaction. The kinetic parameters demonstrate that the catalytic turnover rate (k_{cat}) increases from 2.0 × 10^5 s^{-1} for the wild type enzyme to 2.6 × 10^5 s^{-1} for the S51E mutant. However, this mutation also results in a considerable increase in the Michaelis constant (K_M), from 4.0 mM in the wild type to 13.9 mM in the mutant. This higher K_M indicates a lower substrate affinity, leading to an overall decrease in catalytic efficiency by over 50 %. Based on a stopped flow kinetic assay for the CO_2 hydration reaction, the results for the panel of 41 sulfonamide and sulfamate inhibitors revealed that the S51E mutation strongly affects the inhibition profile of hCA I. For the sixteen most effective wild type hCA I inhibitors (with K_I values < 350 nM), fifteen were substantially less effective on the S51E mutant with K_I values increasing by an average of over 35-fold. For example, the K_I of anticonvulsant zonisamide and polypharmaceutical agent sulpiride increased from 31 to 56 nM for the wild type enzyme to 4050 ± 280 and 7700 ± 400 nM respectively for the S51E phosphomimic. Conversely, for the 24 inhibitors with K_I values > 350 nM for the wild type enzyme, the S51E mutation had either comparable or slightly stronger inhibition in the mutant. This suggests that the most effective inhibitors of the wild type enzyme are more strongly affected by the phosphomimetic mutation. In the hCA I crystal structure, Ser51 is ~20 Å from the active site metal ion and located on the loop containing His65, a residue crucial for proton shuttling in the enzyme's catalytic cycle [12]. Thus, phosphomimic-induced conformational changes may allosterically affect inhibitor binding, potentially by altering the positioning or function of His65. This influence on His65 could modulate the enzyme's activity by impacting proton transfer dynamics. The anion inhibition profiles also indicate that the S51E mutation can significantly alter the activity of hCA I by anionic inhibitors. For 24 of the 37 anions tested, the S51E mutant was significantly less susceptible to inhibition. Some of the most notable changes were observed for cyanide (CN^-), hydrogen sulfide (HS^-), and fulminate (CNO^-), where K_I values increased by over 150-fold, 10,000-fold, and 2000-fold, respectively.

This study highlights the significant impact phosphorylation may have on the catalytic efficiency and inhibitor sensitivity of hCA I. The results indicate that a phosphomimetic mutation can either enhance or reduce inhibitor binding, depending on the specific inhibitor in question. These findings underscore the importance of considering post-translational modifications in the development of therapeutic agents targeting carbonic anhydrases and represents early steps in the development of proteoform-specific inhibitors for carbonic anhydrases. By characterizing the effects of phosphorylation on CO_2 hydration activity and inhibitor sensitivity of hCA I, insights into enzyme regulation can be obtained and pave the way for designing more selective and potent inhibitors. Future studies should explore the structural and functional consequences of other post-translational modifications to develop a comprehensive understanding of CA regulation.

CRediT authorship contribution statement

Andrea Angeli: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Vivian De Luca:** Writing – review & editing, Methodology, Formal analysis. **Xiaojing Huang:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Daniel L. Winter:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Clemente Capasso:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Claudiu T. Supuran:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Conceptualization. **William A. Donald:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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