INSIGHTS TOWARDS SULFONAMIDE DRUG SPECIFICITY IN α-CARBONIC ANHYDRASES

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Abstract

Carbonic anhydrases (CAs, EC 4.2.1.1) are a group of metalloenzymes that play important roles in carbon metabolism, pH regulation, CO_2 fixation in plants, ion transport etc., and are found in all eukaryotic and many microbial organisms. This family of enzymes catalyzes the interconversion of CO_2 and HCO_3^-. There are at least 16 different CA isoforms in the alpha structural class (α-CAs) that have been isolated in higher vertebrates, with CA isoform II (CA II) being ubiquitously abundant in all human cell types. CA inhibition has been exploited clinically for decades for various classes of diuretics and anti-glaucoma treatment. The characterization of the overexpression of CA isoform IX (CA IX) in certain tumors has raised interest in CA IX as a diagnostic marker and drug target for aggressive cancers and therefore the development of CA IX specific inhibitors. An important goal in the field of CA is to identify, rationalize, and design potential compounds that will preferentially inhibit CA IX over all other isoforms of CA. The variations in the active sites between isoforms of CA are subtle and this causes non-specific CA inhibition which leads to various side effects. In the case of CA IX inhibition, CA II along with other isoforms of CA provide off-target binding sites which is undesirable for cancer treatment. The focus of this article is on CA IX inhibition and two different structural approaches to CA isoform specific drug designing: tail approach and fragment addition approach.

Introduction

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes that catalyze the reversible hydration/dehydration of CO_2/HCO_3^-[1,2]. All CAs identified to date use a metalhydroxide mechanism to mediate the two-step ping-pong catalytic mechanism.

Classification

CA has been divided into five classes: α (found in mammals, prokaryotes, algae and fungi), β (found mainly in plants and some prokaryotes), γ (present only in some forms of bacteria), and two other sub-classes: δ and ζ (similar to class β, found in diatoms)[3–8]. Human class α, the most studied of all classes, consists of at least 15 isoforms[6,9]. Of these 15 isoforms, three are non-catalytic because they lack the Zn-metal in the active site which is essential for the enzyme function. These non-catalytic isoforms (CA VIII, X and XI) are referred to as CA related proteins (CARPs). The remaining 12 catalytic isoforms differ from

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each other in catalytic activity, tissue localization and cellular distribution (cytosolic (CA I-III, VII, XIII), membrane bound (CA IV, IX, XII, XIV), secretory (CA VI) or mitochondrial (CA VA, VB))[4,10–25]. Table 1 provides a list of one PDB ID for each solved crystal structures of catalytic isoforms of α-CA (mostly human); Table 2 and 3 summarize primary sequence identity and structural similarity among these isoforms, respectively.

Structure and function

CA isoform II (CA II) is a monomeric 29 kDa protein composed of 260 amino acids with a mixed α/β globular protein fold. It is the best studied and most abundant isoform of CA present in humans, comprising a 10 β-strand sheet flanked by 7 α-helices. The active site zinc present at the base of the 15 Å deep conical cleft is tetrahedrally coordinated by three histidine residues (H94, H96 and H119) and a hydroxyl ion. Clustering of various amino acid residues in and around the rim of the active site of CA, forms various amino acid selective pockets. The active site cavity is partitioned into two very different but conserved environments comprising a cluster of hydrophobic amino acids (V121, V143, L198, V207 and W209) on one side, and hydrophilic amino acids (Y7, N62, H64, N67, T199 and T200) lining the surface on the other side. While the rim of the active site exhibits surface pockets that are characteristic to the various isoforms (Fig. 1A).

The contrasting environments within the active site aid in enhancing the catalytic efficiency of the enzyme. The first step, hydration of CO_2 that occurs in the hydrophobic pocket (Fig. 1B), is the nucleophilic attack on incoming CO_2 by a Zn-bound OH^- to produce HCO_3^-. The binding of HCO_3^- at the metal is weak and accordingly is displaced by a water molecule. In the second step, the $Zn-H_2O$ loses a proton (H^+) leaving the Zn in its original $Zn-OH^-$ conformation through a network of well-ordered H-bonded waters to several hydrophilic residues (Y7, N62 and N67 in CA II) that line the active site (Fig. 1B). These residues are found to be highly conserved among various isoforms of α-CA. This network stretches from the Zn-bound solvent to the proton shuttling residue H64.

$$EZnOH^- + CO_2 \leftrightarrow EZnHCO3^- \leftrightarrow EZnH_2O + HCO_3^- \quad \text{step1:}$$

$$EZnH_2O + B \leftrightarrow EZnOH^- + BH^+ \quad \text{step2:}$$
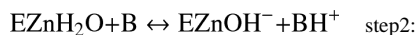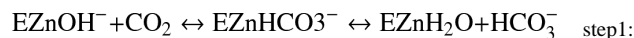
Extensive research has shown that this proton transfer step (step 2) is rate limiting in CA catalysis and His64 to be the proton donor/acceptor in the process [26–31]. The enzyme displays very strong pH dependence for both k_{cat} and k_{cat}/K_M that are defined by a single ionization corresponding to a pK_a of ~7 [30,32–35].

Physiological role

CA is involved in several physiological roles including rapid access to bicarbonate in red blood corpuscles (RBCs), fluid secretion, acid/base balance and thus pH regulation, gluconeogenesis, ureagenesis, gastric acid production, tumorigenesis, lipogenesis, electrolyte secretion, and transport of CO_2 from tissues[4,10,11,13–18,36]. CO_2 released as a part of respiration by tissues is weakly soluble in blood and thus, in order to be transported, is converted to HCO_3^- by CA in the RBCs. Furthermore, the role of CA in diseases such as glaucoma has long been known. Over secretion of aqueous humor in the eye causes increased intra-ocular pressure. Reduction in CA activity decreases the secretion of HCO_3^- and aqueous humor, thereby reducing the pressure. Recent research on the role of CA in epilepsy, obesity, pain, and cancers of breast and intestine has drawn attention[3–6,8,37,38].

CA Inhibition

As CAs are involved in many physiological reactions as mentioned above, CA isoforms are therapeutic targets with the potential to be inhibited to treat diseases like glaucoma, edema, epilepsy and obesity. Broadly, CA inhibitors (CAIs) that bind to the metal center can be divided into two main classes, by forming: (1) trigonal-bipyramidal species (e.g. formates, cyanates) by adding an extra functional group to the already hydroxyl bound zinc or, (2) tetrahedral adducts (e.g. sulfonamides, bisulfites) with zinc, by displacing the zinc-bound hydroxyl. However, inhibitors like coumarins (that bind away from Zn), nitrates (that bind near the Zn but do not coordinate with the metal), and trithiocarbonates (that bind to the Zn in a distorted tetrahedron coordination) have also been reported[39–44].

Extensive research has been performed on various classes of CAIs[45–49]. The sulfonamide derived compounds are buried deep in the active site, displacing solvent molecules, with the sulfonamide amine nitrogen binding directly to the active site zinc atom (distance ~ 2.0 Å) and accepting a hydrogen bond from OG1 of T199 and with the O2 of the sulfonamide accepting a hydrogen bond from the main-chain nitrogen atom of T199 at a distance ~ 3.0 Å. The overall $Zn(N)_4$ coordination can be described as a distorted tetrahedron. The compound moieties extend out of the active site and are stabilized by both hydrophilic and hydrophobic residues (Fig. 1B).

CA IX and Cancer

Cancer continues to be the second most leading cause of death in the United States, after cardiovascular diseases. Accounting for 1.6 million cases in 2011, cancer was estimated to kill 571,000 people in the U.S.[50].

It has now been well documented that tumor hypoxia is associated with poor prognosis and increased tumor aggressiveness, and CA IX (an endogenous marker for tumor hypoxia) upregulation that regulates the pH of tumor cells. In contrast to the other CA isoforms, CA IX has been implicated to play a role in regulation of cell proliferation, adhesion, and malignant cell invasion. Interestingly, CA IX is over expressed in human epithelial tumors derived from tissues that normally do not express these isoforms, including carcinomas of the cervix, lungs, kidneys, prostate, and breast. There is also evidence that CA IX allows tumors to acclimate to a hypoxic microenvironment, promoting tumor cell proliferation, and that CA IX expression is related to poor survival in patients[51].

Most tumors experience a structurally and functionally disturbed microcirculation of oxygen which pathophysiologically causes an inadequate supply of oxygen (or hypoxia)[4,37,50,52–55]. In tumors such as gliomas/ependymomas[4], mesotheliomas[4], tumors of kidney[56], carcinomas of bladder[57], uterus/cervix[58,59], pharynx[60], head/neck[61], breast[62–64], esophagus, follicles, brain, vulva, squamous/basal cells[4] and lungs[65], hypoxia or a mutation in von Hippel-Lindau (VHL) factor leads to an overexpression of CA IX (up to 150 fold)[4,37,62] through hypoxia inducible factor (HIF) cascade[4,37,50,52–55,64].

CA IX is a transmembrane protein[37]. Based on sequence similarity, CA IX has been recognized as a multidomain protein consisting of an N-terminal proteoglycan like (PG) domain, a CA catalytic domain, a transmembrane segment (TM), and an intracytoplasmic (IC) portion[66]. The CA catalytic domain presents a significant sequence identity (30-40%) to other catalytic CA isozymes. Both CA and PG domains are glycosylated[67]. A cDNA coding for the CA IX protein was first cloned by Pastorek *et al.*[68], and the *CA9* gene was further characterized[66]. According to recent biochemical and structure reports, the CA IX is a dimer[67] and shows similarity with CA II not only in terms of catalytic efficiency, but also affinity for inhibitors[3,4,15,37] like sulfonamides[69–75] and sulfamates[74–76].

So far, only one structure of CA IX[77] crystallized in complex with acetazolamide (a classic inhibitor of CA) is available in the PDB. The structures of CA II (PDB ID: 3KS3)[78] and CA IX (PDB ID: 3IAI)[77] are very similar and have a Cα RMSD of 1.5 Å (Fig. 2). However, there are several amino acid residues differences in and around the rim of the active site of the two isoforms. Ala65, Phe131, Asn67, Gln69, Ile91 and Leu204 in CA II are structurally equivalent to Ser65, Val131, Gln67, Thr69, Leu91 and Ala204 in CA IX, respectively. It is interesting to note that 3 of these differences lie in a region that forms a hydrophobic pocket on the rim of the active site where some of the already known inhibitors bind CA II[49,73,79–89]. It is these differences which could be exploited to design inhibitors which prefer binding to one CA compared to another. Differences in the active site residues of CA II and CA IX also account for a variation in the size of their active site cavities. With a volume of 290.4 Å^3 and surface area of 245.3 Å^2, the active site of CA II is larger than that of CA IX (volume = 270.7 Å^3, surface area = 215.3 Å^2). These calculations were performed using the online web server of CASTp[90].

Isoform Specific Drug Design

One of the major issues in targeting CA IX for the treatment of tumors is its structural similarity with CA II. In fact, most isoforms of CA bear only few differences in the active site which makes it difficult for designing inhibitors that would specifically bind to one isoform over the others. This non-specific binding (or off-target inhibition) to other isoforms of CA by clinically used (U.S. Food and Drug Administration approved) CA II inhibitory drugs is what causes various side effects during the course of treatment of glaucoma, such as eye irritation, watering, blurred vision, taste changes, constipation and diarrhea[4]. This has given rise to the need to get greater insights into the fine structural differences within the isoforms and exploit these to design novel and more specific inhibitors in order to circumvent the problem of off-target binding. Although, not many isoform specific inhibitors are known yet, but new sulfonamides and other non-sulfur based compounds are continuously being reported to find derivatives with better inhibition profiles as compared to the promiscuous, first generation inhibitors [14,91,92]. Various docking and kinetic studies to test and characterize novel and already existing inhibitors for isoform specificity are going on[51,93].

Rational drug design or ligand design is the process of finding new compounds based on the knowledge of a biological target. However, once a ligand has been designed and proven to be effective in inhibiting or enhancing its appropriate target, there are certain issues that need to be resolved before the ligand can be used as a drug. These issues or rules were first defined as the *Lipinski's rule of five*[94]. The rule describes pharmacokinetic properties like absorption, distribution, metabolism, excretion (ADME) of an ideal orally administered drug. According to this rule, for a drug (ligand) to be orally administered, it should not violate more than one of the following criteria: the ligand should have no more than 5 H-bond donors, 10 H-bond acceptors, 500 Dalton of molecular weight, and an octanol-water partition coefficient (log P) of 5. However, over the years there have been various changes to this rule, like the ones suggested by Ghose *et al.*[95] and thus it is no longer followed verbatim. Some of the most successful results yielded by rational, structure based computer aided drug design have produced drugs like Saquinavir (HIV protease inhibitor)[96], Dorzolamide (CA inhibitor)[97,98] and Iminatib (tyrosine kinase inhibitor)[99].

One of the most common approaches that have been used for designing CAIs is referred to as the *ring approach* where an aromatic ring is attached directly to the sulfonamide group to enhance binding affinities. This approach has been calibrated and exploited over the last few decades[8]. There is already enough information available about high affinity inhibitors against CA and more compounds are added to an ever growing database. However, it is not

the binding affinity but isoform-specificity that is lacking and hence, the ring approach has become less important. *Combinatorial chemistry* is another widely used, although not a very successful approach to rapidly synthesize or computationally simulate a large number of structurally related molecules. The failure to take molecular flexibility of drugs into account could well be considered as the biggest shortcoming of this approach[100].

As mentioned earlier, CA IX has been observed to be a key player in tumorigenesis and tumor progression[101]. One of the major issues with cancer treatment is the inability of most drugs to distinguish cancerous cells from normal cells. Overexpression of CA IX specifically in cancerous cells makes it a good drug target.

Protein Data Bank (PDB) Mining Approach

As shown in Fig. 1A earlier, clustering of various amino acid residues in and around the active site of CA, forms various pockets. Different sulfonamide inhibitors' "tails" prefer different orientations and active site rim pockets as they extend out of from the Zn. Their orientation within a pocket depends largely on the interactions between the terminal tail atoms of the inhibitor and the residues that form the pocket. A list of all the inhibitors deposited in the PDB in complex with CA II, has been compiled. The inhibitors were then categorized based on their structural similarity and only one coordinate file for each inhibitor was included, shortening the original list of 400 structures to 145 non-redundant structures. Each of these structures were visualized in *COOT*[102] and analyzed based on which pocket the inhibitors bind in. As shown in the flowchart (Fig. 3), 115 inhibitors were found to bind in one general pocket, while 14 inhibitors (Fig. 4) occupied a "selective" pocket (Fig. 5). Based on the observed active site binding interactions of these inhibitors in the crystal structures and their respective inhibition constants, the goal is to structurally characterize aspects of these interactions that would lead to more potent, selective CA IX sulfonamide inhibitors.

The PDB deposited structure of CA IX, solved to a resolution of 2.2 Å, crystallized in $P6_1$ space group as a dimer. Coordinates of the aforementioned 129 inhibitors from the non-redundant list were used to model the respective inhibitors into the crystal structure of CA IX (PDB ID: 3IAI)[77]. The structures of CA II (PDB ID: 3KS3)[78] and CA IX (PDB ID: 3IAI)[77] are very similar although, there is a difference in three residues (N67Q, E69T and I91L) that form part of this "selective" pocket in the active site. It is this pocket where 14 inhibitors from the non-redundant list of PDBs were found to be bound. This raises opportunities for designing and altering the tails of these 14 inhibitors in a manner that might preferentially enhance their K_Is for CA IX over CA II.

With the objective of enhancing the CA II inhibitor database to enable studies using the tail approach, more and more crystal structures of CA II in complex with various novel inhibitors belonging to different structural classes are being solved[45–49].

To attain selective inhibition of CA IX and to relate its catalytic activity to extracellular acidification, the hypothesis is that the inhibition characteristics for CA IX will vary from CA II (based on crystal structures). The specific differences in amino acids projecting into the active site (namely, residues at positions 65, 67, 69. 91 and 131 (CA II numbering)) will have a direct effect on the specificity of inhibitor binding.

Among various other approaches that are currently under study, *tail approach* (a word coined by Supuran and co-workers)[8] can be used to enhance the aqueous solubility of inhibitors in order to decrease the side effects caused by drugs which are only soluble in highly acidic solvents. The approach (Fig. 6) could also involve altering the terminal regions

of an already known inhibitor such that the tail interacts specifically with residues on the surface of one isoform (CA IX) and not others.

Fragment Addition Approach

This is an entirely different approach to drug design as it uses *ab initio* methodology to find random fragments that may bind to a protein. Once such fragments are identified, the forces and interactions that stabilize this non Zn-bound molecule within the active site can be mapped using structure activity relationships (SARs) among different isoforms of CA and exploited to design an isoform specific inhibitor. Thereafter, several of these binding regions could be used in fragment addition based drug design, as explained by SAR by NMR by Shuker *et al.*[103].

There are various fragment libraries available that can be used for binding studies and drug design. The choice of which fragment library to choose depends largely on the experiment that is going to be performed. One of such libraries is the "*Zenobia* fragment library" that comprises of 352 compounds (soluble up to 200 mM in DMSO). Moreover, all these "fragment" compounds have molecular weights, polar surface area, hydrogen bond donors/acceptors in the range defined by the Lipinski's rule of five.

Fragment addition approach could also be combined with tail approach by exploiting the zinc binding property of sulfonamide groups. Compounds (fragments) which bind at unique regions in CA IX could be covalently linked to the zinc binding sulfonamide group in classical CAIs.

Conclusion and Discussion

Carbonic anhydrase IX (CA IX) has been shown to play a critical role in cancer proliferation. CA IX overexpression in certain types of cancers has led to the use of CA IX as a biomarker for cancer diagnosis and prognosis. In addition, much knowledge has been acquired on CA II inhibition for various classes of diuretics and systemically acting antiglaucoma agents. The differences in the active sites of different isoforms of CA are subtle and this causes non-specific CA inhibition which leads to side effects. The PDB was mined for previously solved α-CA inhibitor complex structures (mainly CA II), and a sub-set of selected structures were superposed with the structure of CA IX. For example, the inhibitor from a CA II complex (PDB ID: 3N2P)[49] when modeled in CA IX (PDB ID 3IAI)[77] appears to form one extra hydrogen bond with the protein (Fig. 7). This is because Q97 in CA IX is one C-C bond longer than the N67 in CA II and thus extends further towards the inhibitor to be close enough to form a H-bond. Surface area (SA) buried by an inhibitor in a crystal structure with CA II, has been compared with that of the same inhibitor modeled into CA IX. A reduction of buried SA by 10 Å^2 corresponds to a decrease in Gibbs free energy by 0.3 kcal/mol[104]. Although the buried SA in almost all the 14 inhibitors with CA IX is smaller (larger ΔG) than that with CA II, the presence of extra hydrogen bonds in the former compensates for the gain in ΔG by a larger magnitude (H-bond energy values obtained from Wendler *et al.*[105]). These findings can be hereafter correlated to the inhibition of CA II and CA IX to provide SARs that may help in understanding the varied binding affinities of the inhibitors toward different α-CA isoforms (Table 4). This data can further be used to guide *in silico* design of new CA inhibitors (CAIs) that would preferentially bind to CA IX (on-target) over others CAs (off-target).

Acknowledgments

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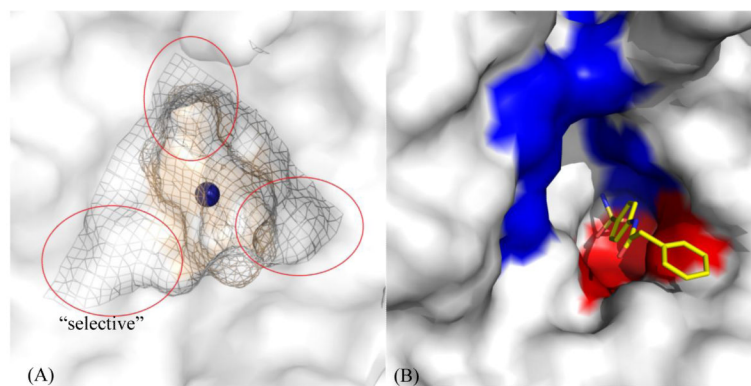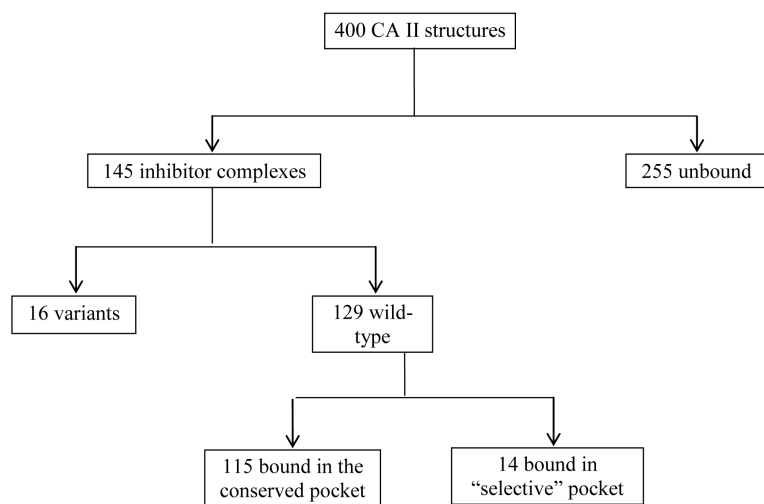
Figure 1.
(A) Various pockets (encircled mesh) within and around the active site of CA II[78]. (B): An inhibitor, PDB ID: 3OYS[48], extending out of the active site of CA II (white surface), stabilized by hydrophobic (red) and hydrophilic (blue) regions. Active site Zn is shown as a blue sphere.
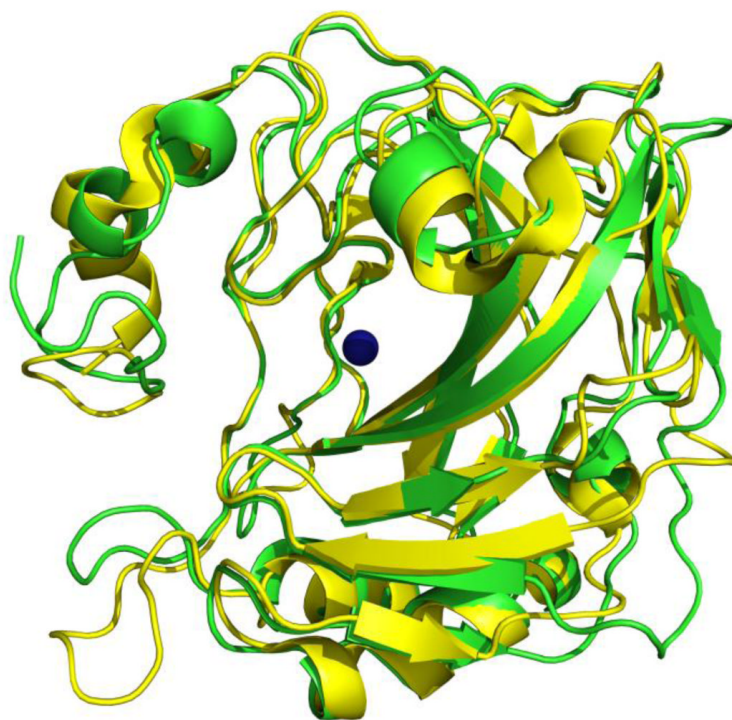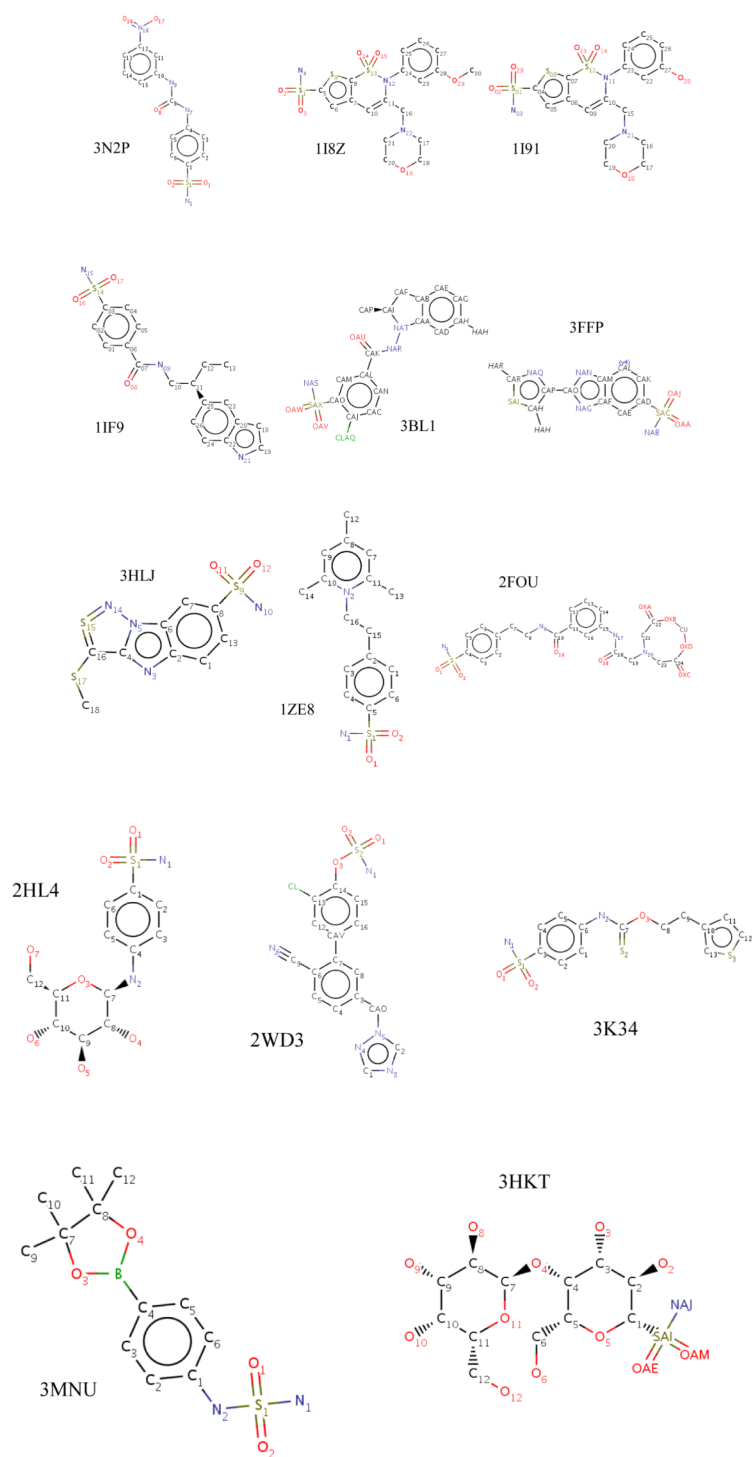
Figure 2.
Superposition of CA II[78] (yellow) and CA IX[77] (green), showing high conservation in secondary structures. Active site Zn is shown as a blue sphere.
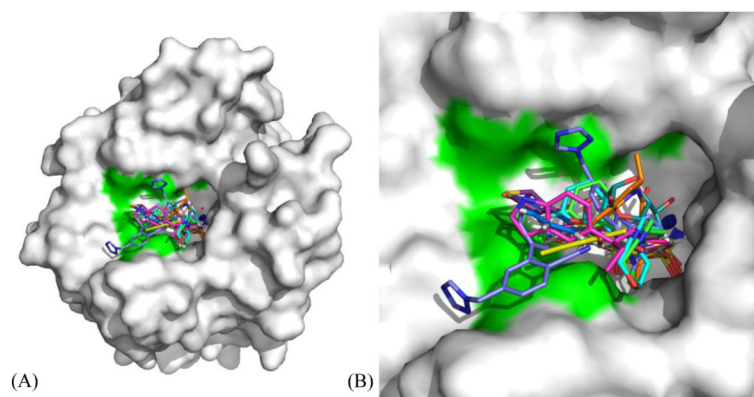
Figure 3.
Stepwise findings from Protein Data Bank mining. 14 inhibitors deposited in the PDB were found to be bound in the "selective" pocket which has different hydrophobicity in CA II and CA IX.

Figure 4.
Structures of the 14 inhibitors that occupy the "selective" pocket in CA II.

Figure 5.
(A) The 14 inhibitors[49,73,79–89] occupying the "selective" pocket in CA II (white surface), where 3 residues differ from CA IX (shown in green). Active site Zn is shown as a blue sphere. (B) Active site zoomed.
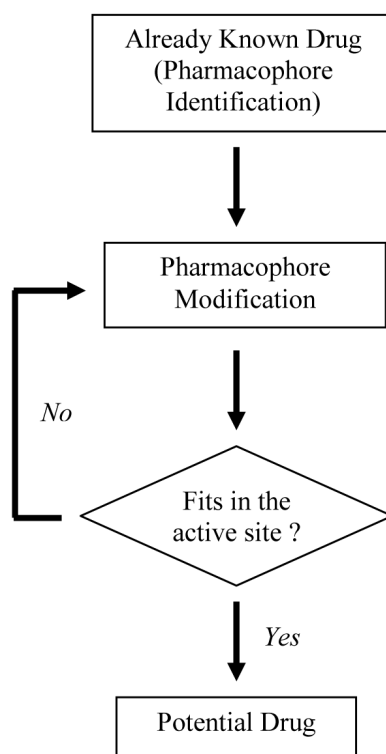
Figure 6.
Approach to isoform specific drug design and enhancement of inhibition profiles for currently known drugs.
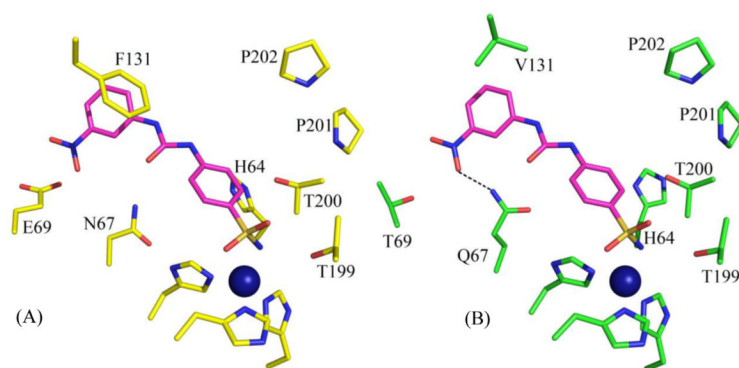
Figure 7.
Stick representation of (A) PDB ID 3N2P[49], and (B) the same inhibitor modeled into the active site of CA IX, PDB ID 3IAI[77]. When modeled into CA IX, this inhibitor forms an extra H-bond (shown with dotted black line). Active site Zn is sown as a blue sphere. Residues are as labeled.

Table 1

List of isoforms of α-CA crystal structures along with their respective PDB IDs, and source organism.

α - Carbonic Anhydrase (catalytic)	PDB IDs	Source Organism
I	3LXE[106]	Human
II	3KS3[78]	Human
III	1Z93[107]	Human
IV	1ZNC[38]	Human
V	1DMX[108]	Murine
VI	3FE4[109]	Human
VII	3ML5[110]	Human
IX	3IAI[77]	Human
XII	1JCZ[111]	Human
XIII	3D0N[112]	Human
XIV	1RJ5[113]	Human

Table 2

Primary sequence identity % (upper right) and number of conserved residues (lower left) among various α-CA isoforms.

	I	II	III	IV	V	VI	VII	IX	XII	XIV
I	-	60.5	54.4	28.8	43.7	30.9	50.6	31.3	29.8	32.6
II	158	-	58.9	32.4	49.4	32.5	55.9	31.4	28.4	36.0
III	142	153	-	30.2	43.3	32.8	52.9	29.5	27.3	33.0
IV	78	88	82	-	27.9	31.3	33.1	29.4	26.0	34.2
V	114	129	113	76	-	28.6	47.9	29.0	24.3	32.6
VI	83	87	88	85	77	-	35.3	41.0	35.4	41.4
VII	132	146	138	90	125	95	-	34.6	31.6	36.3
IX	85	85	80	80	79	110	94	-	31.2	41.9
XII	79	75	72	70	64	93	84	83	-	37.8
XIV	86	82	88	93	87	110	97	113	99	-

Table 3

Structural main chain RMSDs (Å) among various isoforms of human CAs.

	I	II	III	IV	V*	VI	VII	IX	XII	XIII	XIV
I	-	0.98	0.87	2.48	1.02	1.55	0.99	2.02	2.07	0.85	1.52
II		-	0.96	2.29	0.98	1.48	0.77	2.05	2.17	0.76	1.50
III			-	2.37	1.01	1.69	0.86	1.79	1.99	0.85	1.41
IV				-	1.65	1.52	2.36	2.81	2.78	1.39	2.48
V*					-	1.47	0.83	1.82	1.89	1.30	1.42
VI						-	1.52	1.35	1.58	1.58	1.49
VII							-	1.79	1.97	0.77	1.42
IX								-	1.87	2.06	1.88
XII									-	1.17	1.86
XIII										-	1.18
XIV											-

*
Murine CA

Table 4

Differences in buried surface area, hydrogen bonds, and difference in Gibbs free energy (ΔG) associated with H-bonds among published CA II-inhibitor crystal complexes and modeled CA IX-inhibitor complexes.

PDB IDs	Buried Surface Area (Å²)		Gain in ΔG in CA IX (kcal/mol)	# of Hydrogen Bonds	
	CA II	CA IX		CA II	CA IX
3N2P[49]	369.10	362.83	0.19	4	5
1I8Z[79]	443.37	423.51	0.60	5	5
1I9I[79]	434.65	426.42	0.25	5	5
1IF9[80]	373.02	354.52	0.55	4	4
3BL1[81]	406.18	375.43	0.92	4	4
3FFP[82]	335.25	313.03	0.67	4	4
3HLJ[83]	339.61	316.01	0.71	3	3
1ZE8[73]	362.12	336.70	0.76	3	4
2FOU[84]	358.57	345.67	0.39	4	5
2HL4[85]	358.58	342.58	0.48	5	6
2WD3[86]	400.05	414.40	−0.43	5	5
3K34[87]	398.95	367.60	0.94	4	4
3MNU[88]	341.60	322.31	0.58	5	5
3HKT[89]	376.31	356.40	0.60	5	2