# Drug Repurposing: Identification and X-Ray Crystallographic Analyses of US-FDA Approved Drugs against Carbonic Anhydrase-II

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## Abstract

Of all isoforms, human carbonic anhydrase II (PF00194; EC 4.2.1.1), which is mostly found in red cells, kidneys, and the eyes, plays a pivotal role in numerous physiological processes, and its dysregulation has been linked to the wide range of illnesses, such as glaucoma. Finding new inhibitors that target Carbonic anhydrase II, therefore has great potential in drug discovery. Using drug repurposing approach, this study focused on the investigation of different drugs as Carbonic anhydrase II inhibitors and their structural studies using X-ray crystallography. For this purpose, 100 different drugs were evaluated for bovine and human carbonic anhydrase II inhibitory activity. Among all, two drugs, *i.e.* acetohexamide (**1**) and levosulpiride (**54**) were found to be active, with $IC_{50}$=437.0±0.2 and 1128±0.75 $\mu$M, respectively. Mechanistic studies suggested that both drugs are competitive inhibitors of the human carbonic anhydrase II enzyme. The X-ray crystal structure analysis revealed that acetohexamide (**1**) interacts *via* terminal acetyl group with the active site residues of the carbonic anhydrase II enzyme, and showed strong hydrogen bonding with Zn, His94, His119, and Asn67. The sulfonamide group of levosulpiride was involved in strong hydrogen bonding with Zn, His94, His119, and Thr199. From *in vivo* studies, we found that carbonic anhydrase activity was significantly inhibited by the intraperitoneal administration of levosulpiride for up to 5 h. Our findings provide comprehensive insights for the optimization of the pharmacological profile of these drugs, and provide avenues for the exploration of different derivatives of these drugs with enhanced efficacy and fewer adverse effects.

## Keywords

Acetohexamide, carbonic anhydrase inhibitors (CAIs), human carbonic anhydrase II (hCA II), levosulpiride, X-ray crystallography.

**PDB IDs:** 8J2O and 8JEE

## 1. Introduction

Carbonic anhydrases (CAs) are zinc-binding metalloenzymes, catalyzing the hydration of $CO_2$ to bicarbonate ions and protons. Sixteen distinct isoforms of CA are distributed in the living system [1]. Among many isoforms, carbonic anhydrase II (CA II) is found to be the most catalytically active and physiologically relevant enzyme. CA II is engaged in a wide range of cellular functions, and disruption in CA II activity can lead to pathological conditions like glaucoma, edema, epilepsy, altitude sickness, obesity, and renal diseases [2]. The exponential increase in CA II-associated disorders is becoming a major health care issue in the world. To date, several carbonic anhydrase II inhibitors have been reported, with some also being in clinical trials. X-ray crystallography has been used to comprehensively analyze the synthetic derivatives of different classes of compounds, such as sulfonamides, hydroxamates, sulfocoumarins, thioxocoumarins, mono- and dithiocarbamates as CA II inhibitors [3]. About 25 CA inhibitors (CAIs) are commercially available, and most of them belong to the sulfonamide family. Among them, acetazolamide, methazolamide, ethoxzolamide, dichlorphenamide, dorzolamide, and brinzolamide are primarily used to treat CA II-associated diseases [4]. However, some drugs are found to be effective inhibitors of CA II enzymes, such as celecoxib and valdecoxib (COX-2 inhibitors), indisulam (anticancer agent), topiramate (antiepileptic drug), sulpiride (an antipsychotic drug), EMATE (breast cancer therapy), and ibuprofen (NSAID) [5,6]. The idea to search for more than one therapeutic application of the already approved FDA drugs further strengthens the concept and applications of drug repurposing. Drug repurposing is the process of identifying new therapeutic applications for previously approved medications. It allows to reuse existing clinical data and safety profiles. Drug repurposing is a smart drug development technique since it can minimize the time and expense associated with bringing a new medicine to the market [7].

Drug repurposing has received a great deal of interest in recent years, and a variety of computational and experimental methodologies, such as network-based approaches, high-throughput screening, and drug-disease association databases, have been established [8]. It is expected to see more repurposed pharmaceuticals on the market in the coming years as researchers continue to investigate the potential of existing drugs for new purposes. This study

focused on the CA II inhibitory activity of 100 clinically approved and widely used drugs (Table S1). Among all, acetohexamide (**1**) and levosulpiride (**54**) showed CA II inhibitory activity.

Acetohexamide is the oral drug used to manage type 2 diabetes mellitus [9]. It is a sulfonylurea derivative and has been extensively employed for hyperglycemic conditions [10]. Acetohexamide interacts with a membrane receptor which belongs to ATP-dependent potassium channel (also known as the sulfonylurea receptor) and stimulates insulin secretion from pancreatic β-cells. The drug-receptor interaction inhibits the potassium channels, resulting in disturbed resting potential and a strong calcium influx. These events stimulate insulin secretion that travels to the hepatic portal veins to reduce glucose production [11-13]. Acetohexamide is absorbed through the gastrointestinal tract, metabolized in the liver, and excreted in urine. In the bloodstream, it strongly binds to serum transport proteins especially human serum albumin [14].

Levosulpiride, an enantiomer of sulpiride, is an atypical antipsychotic drug primarily used for the treatment of psychosis and depression. Besides its role in psychiatric illnesses, it is also effective in gastrointestinal indications [15]. Levosulpiride is effective in the treatment of schizophrenia, depression, burning mouth syndrome, emesis, dyspepsia, and unilateral labyrinthine dysfunction [16]. Levosulpiride has an antagonistic effect on the dopaminergic receptors ($D_2$ and $D_3$), where drug-receptor binding slows down the release of dopamine at both pre-synaptic and post-synaptic receptors. This increases gastrointestinal motility while decreasing nausea and vomiting. By regulating the serotonergic and noradrenergic systems, it also possesses anxiolytic and antidepressant properties [17].

In the current study, acetohexamide (**1**) and levosulpiride (**54**) were identified for a new indication, *i.e.* CA II inhibitory activity, with $IC_{50}$ values of 437.0 ± 0.2 and 1128 ± 0.75 $\mu$M, respectively. Mechanistic studies suggested that both drugs were found to be competitive inhibitors of hCA II enzyme. The X-ray crystal structure analysis was employed to study the molecular interaction of these drugs with hCA II enzyme. Finally, levosulpiride (**54**) was found to significantly inhibit the *in vivo* carbonic anhydrase inhibitory activity in mice.

## 2. Materials and methods

Bovine carbonic anhydrase II (C3934), 4-nitrophenyl acetate (N8130), Tris-HCl, and acetazolamide (A6011) were purchased from Sigma-Aldrich, USA. Reagent-grade ethanol and DMSO were used in experiments. Commercially available drugs were obtained from the *in-house* Molecular Bank of Dr. Panjwani Center for Molecular Medicine and Drug Research (PCMD), ICCBS, University of Karachi, Karachi, Pakistan.

### 2.1. Protein expression and purification:

Recombinant hCA II gene was cloned into the pET23-b vector followed by expression in *E. coli* B21 (DE3) competent cells. *E. coli* BL21 (DE3) competent cells were cultured at 37 °C in sterile LB media supplemented with ampicillin (100 mg/mL) until the $O.D._{595}$ = 0.6. Expression of hCA II was induced by adding isopropyl β-thiogalactopyranoside (IPTG, 1 mM) at 37 °C for three hours. Cells were harvested by centrifugation and later lysed through sonication. Finally, the CA II enzyme was purified using affinity chromatography (*p*-aminomethylbenzenesulfonamide affinity column). Affinity column was equilibrated with 100 mM Tris–HCl/200 mM sodium sulfate (pH 9), and washed with 100 mM Tris–HCl/200 mM sodium sulfate (pH 7) to remove unbound proteins. Finally, hCA II was eluted with 50 mM Tris-HCl containing 400 mM sodium azide and 100 mM NaCl (pH 7.8). In order to remove sodium azide, and for buffer-exchange (into 50 mM Tris-HCl (pH 8.5)), purified hCA II was concentrated, and subjected to size-exclusion chromatography (using HiLoadTM 16/600 Superdex 75 pg column) [18,19].

After enzyme purification, SDS-PAGE was applied to monitor the purity of the protein (with 10% running and 3% stacking gels under reducing conditions). Protein sample was prepared by mixing enzyme (10 $\mu$L) with sample diluting buffer (10 $\mu$L) and heating at 95 °C for 5 min. Samples (10 $\mu$L) and protein marker were loaded into wells of prepared gel. Electrophoresis was carried out at 75 V for 45min and 150 V for 60min at room temperature. The running buffer contained glycine (192 mM), Tris (25 mM), and SDS (0.1%). Gels were stained in staining solution (containing 1% Comassie brilliant blue, methanol, and glacial acetic acid) for 45min, and then de-stained using 25% methanol and 70% acetic acid.

The enzyme activity of expressed hCA-II was measured utilizing *p*-nitrophenyl acetate as substrate (0.7 mM) [20]. Briefly, purified hCA II enzyme (0.15 mg/mL, 20 $\mu$L) was incubated with Tris-HCl buffer (160 $\mu$L) for 15min at 25 °C, absorbance was recorded at 400 nm as a pre-read. The enzyme reaction was initiated by addition of substrate, *p*-nitrophenyl acetate (20 $\mu$L). Rate of product formation was monitored for 30min using a microplate reader (Multiskan GO Spectrophotometer, Thermo Scientific, USA) and compared with blank (containing no enzyme).

Highly purified hCA II was concentrated with centrifugal filters (Amicon® Ultra-15 centrifugal filters; 10 kDa MWCO) for subsequent inhibition studies and crystallization experiments.

### 2.2. Carbonic anhydrase II inhibition studies:

The carbonic anhydrase inhibition studies were carried out by following the method reported by Shank and colleagues [20]. Reactions were performed in 96-well plates at 25 °C. Total reaction volume was 200 $\mu$L per well, which comprised of 20 $\mu$L of test compounds (dissolved in DMSO; final concentration 10%), 140 $\mu$L of Tris-HCl buffer (pH 7.4), 20 $\mu$L of bovine or human CA II (0.15 mg/mL), and 20 $\mu$L of substrate, *i.e*. *p*-nitrophenyl acetate.

In each well, test compound (n =3) was incubated with CA II enzyme and Tris-HCl buffer for 15min. After incubation, absorbance at 400 nm was recorded as a pre-read. The reaction was initiated by addition of substrate, and final rate of product formation was monitored for 30min (with regular intervals of 1min) using a microplate reader (Multiskan GO Spectrophotometer, Thermo Scientific, USA). $IC_{50}$ values for active drugs were calculated using the EZ-fit enzyme kinetics software (Perella Scientific, Amherst, NH, USA).

### 2.3. Mechanistic studies:

The kinetic parameters of test compounds were determined utilizing different concentrations of test compounds (drugs) and substrate. Selected drugs were incubated for 15min with enzyme (hCA II) and buffer. Reaction was started by adding different concentrations of substrate (*i.e.* 0.175, 0.35, 0.7, and 1.4 mM), and allowed to run for 30min at 25 °C. The rate of product formation was determined by recording the change in absorbance at 400 nm (with one minute time interval).

### 2.4.Crystallization of hCA II in complex with drugs:

Sitting drop vapor diffusion method was used for the crystallization of hCA II complexed with either of two active drugs, acetohexamide (**1**) and levosulpiride (**54**). The co-crystals of hCA II were prepared using MRC Maxi 48 well crystallization plates. The drugs were dissolved in deionized water. hCA II (10 mg/mL) was mixed with equal volume of ligand solution (5 mM) and reservoir buffer. Reservoir buffer comprised of ammonium sulfate (2.9 M) and Tris-HCl (50 mM; pH 7.4) [21]. Crystals were obtained in 5-6 days. Glycerol (20-25% v/v) was used as cryoprotectant.

#### 2.4.1. X-Ray data collection, processing, and refinement:

X-Ray diffraction data sets were collected on Bruker D8 Venture (Bruker, USA) equipped with a PHOTON 100 detector. Data was collected at 100 K. Data collection and initial processing was performed utilizing Proteum3 software. The indexed data set was further scaled in the Aimless program of CCP4i suite [22]. Molecular replacement was used to provide initial phases for data sets, employing MolRep program [23]. The newly generated model was built up through multiple iterative refinement cycles in Refmac5 and COOT [24]. After constructing the main chain, water molecules were added. The electron density map (2Fo-Fc) with occupancy 1.0 was used to add ligands, ions, and buffer molecules. The data collection and refinement parameters of hCA II in complex with acetohexamide (**1**) and levosulpiride (**54**) are presented in Table 3. The final refined models of hCA II-acetohexamide complex and hCA II-levosulpiride complex were validated and deposited into the Protein Data Bank (PDB) with accession IDs 8J2O and 8JEE, respectively. Figures were generated using Pymol [25].

### 2.5. *In vivo* carbonic anhydrase inhibition studies:

Levosulpiride (**54**) was selected for *in vivo* studies. The Institutional Animal Care and Use Committee (IACUC) of the International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi, Pakistan, approved the experimental protocols described in this manuscript (Approved ASP # ICCBS-ASP-03-2024-07).

NMRI mice (20 ± 2g) were obtained from the in-house Animal Research Facility (ARF), ICCBS, University of Karachi. All animals were housed in clean cages with free access to standard

laboratory chow and filtered water and acclimatized for 4 days. Mice were housed in separate cages during the study, and divided into following groups:

**Group 1 (control group, n=8):** This group of mice received saline *via* intraperitoneal administration.

**Test group (n=8):** levosulpiride (100 mg/kg of body weight) was intraperitoneal administered to the mice of this group.

Blood samples were taken intracardially from each mouse at the first, third, and fifth hour after injection of the test compound and saline, under anesthesia (ketamine + Xylazine). The blood was collected into EDTA-containing vacutainer and centrifuged at 2500xg for 15min at 4°C. Plasma and buffy coat (leucocytes) were removed carefully, and erythrocyte pellet was washed three times with cold 0.9% NaCl. Erythrocyte pellet was suspended in ice-cold water (1 mL: 5 mL) to give an erythrocyte hemolysate and then centrifuged at 10,000xg for 10 minutes to remove the ghost and intact cells [26].

### 2.5.1. Protein determination:

Quantity of the protein in erythrocyte samples was determination spectrophotometrically according to Bradford's method [27]. Protein samples (4 $\mu$L) were mixed with 196 $\mu$L of Bradford solution (1X), and incubated for 5min at room temperature, after incubation absorbance was recorded at 595 nm by using a microplate reader (Multiskan GO Spectrophotometer, Thermo Scientific, USA). Bovine serum albumin (BSA) in the range of 1-0.2 mg/ mL was utilized to generate a standard curve.

### 2.5.2. Measurement of CA activity:

Esterase activity was analyzed spectrophotometrically using *p*-nitrophenyl acetate as a substrate. Esterase activity was measured in 96 well plates. The total reaction volume was 300 $\mu$L per well, containing 140 $\mu$L of 0.05M Tris-HCl buffer (pH= 7.4), 100 $\mu$L of 3 mM 4-NPA, 50 $\mu$L of deionized $H_2O$, and 10 $\mu$L of erythrocyte hemolysate. Blank was obtained by replacing the erythrocyte hemolysate with deionized water. After the addition of substrate, the change in absorbance was recorded over 20min (with 1-minute intervals) at 348 nm [26, 28].

### 2.6. Statistical analysis:

The $IC_{50}$ values and coefficient of determination ($R^2$) were calculated using the GraphPad Prism version 7.0.0 (Boston, Massachusetts, USA), and results were presented as mean ± standard error of the mean (SEM). The kinetic parameters $K_m$, $K_i$, $V_{max}$, and K*i* coefficient of determination ($R^2$) were determined and graphs were plotted using the Grafit 7.0 program (Erithacus Software Ltd., UK). The Lineweaver Burk Plot between 1/S and $1/V_{max}$ was used to calculate the $K_m$ values. To determine the K*i* values, a double reciprocal plot was generated between different concentrations of the inhibitor [I] and slope, which equals $K_m/V_{max}$. Finally, the Dixon plot (drawn between inhibitor concentrations [I] and $1/V_{max}$) was used to validate the type of inhibition.

## 2. Results and discussion:

## 3.1. Protein expression and purification:

Human carbonic anhydrase II enzyme was successfully expressed in *E. coli* BL21 (DE3) cells, and purified through *p*-aminomethylbenzenesulfonamide affinity chromatography [29]. The purity of the hCA II enzyme was examined by SDS-PAGE analysis, which showed a distinct band at approximately 29 kDa.

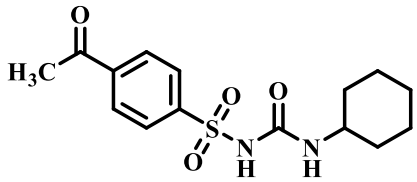
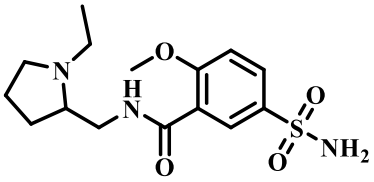
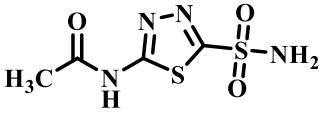
## 3.2. Carbonic anhydrase II inhibition studies:

Expression and purification of human carbonic anhydrase II (hCA II) enzyme is costly process. Consequently, we first conducted a screening of 100 drugs against bovine carbonic anhydrase II (bCA II), which is affordable and commercially available. Drugs identified as active against bCA II were then assessed for their effectiveness against hCA II enzyme. Out of 100 screened drugs, two drugs *i.e.*, acetohexamide (**1**) and levosulpiride (**54**) were found to be active against both bCA II and hCA II (Table 1), while the remaining 98 drugs showed less than 50% inhibition, and considered as inactive (Table S1).

Results showed that acetohexamide (**1**) was found to be a moderate inhibitor of bovine and human CA II with $IC_{50}$ values of 435 ± 0.07 and 437 ± 0.2 $\mu$M, respectively (Table 1). Similarly, levosulpiride (**54**) exhibits a weak CA II inhibitory profile against both human and bovine

isoforms with $IC_{50}$ values of 1259 ± 0.50 and 1128 ± 0.75 $\mu$M, respectively. Both drugs inhibited the activity of CA II in a dose-dependent manner (Figure S1). Previously, the dextro enantiomer of levosulpiride, *i.e.* the antipsychotic drug sulpiride was reported as an inhibitor of hCA I, II, and IV isozymes [27], but there is no report describing the CA II inhibitory activity of levosulpiride.

**Table 1:** *In vitro* inhibitory activity of acetohexamide (**1**) and levosulpiride (**54**) against carbonic anhydrase II enzyme.

| Drug | Bovine Carbonic Anhydrase-II inhibitory activity | | Human Carbonic Anhydrase-II inhibitory activity | |
|---|---|---|---|---|
| | **$IC_{50}$ ± S.E.M ($\mu$M) *** | **$R^2$** | **$IC_{50}$ ± S.E.M ($\mu$M) *** | **$R^2$** |
|  Acetohexamide (**1**) | 435.0 ± 0.07 | 0.982 | 437.0 ± 0.2 | 0.980 |
|  Levosulpiride (**54**) | 1259 ± 0.50 | 0.920 | 1128 ± 0.75 | 0.923 |
|  Acetazolamide (Standard Inhibitor) | 0.13 ± 0.01 | 0.971 | 0.10 ± 0.002 | 0.988 |

*$IC_{50}$ values are presented as mean ± standard error of the mean (n=3).

## 3.3. Kinetic studies of active drugs against CA II enzyme:

Kinetic studies were performed to assess the mode of inhibition of two active drugs (*i.e.*, acetohexamide (**1**) and levosulpiride (**54**)) against hCA II enzyme. The kinetic studies revealed that both acetohexamide (**1**) and levosulpiride (**54**) showed a competitive mode of inhibition with

K*i* values of 447 ± 0.015, and 1075 ± 0.02 M, respectively. It was observed that in the presence of these drugs the $K_m$ was increased, while $V_{max}$ of the enzyme in the absence and presence of inhibitors remained the same. The line-weaver burk, secondary, and dixon plots further support the graphical basis of competitive inhibition for these drugs (Figure 1 and 2; Table 2).

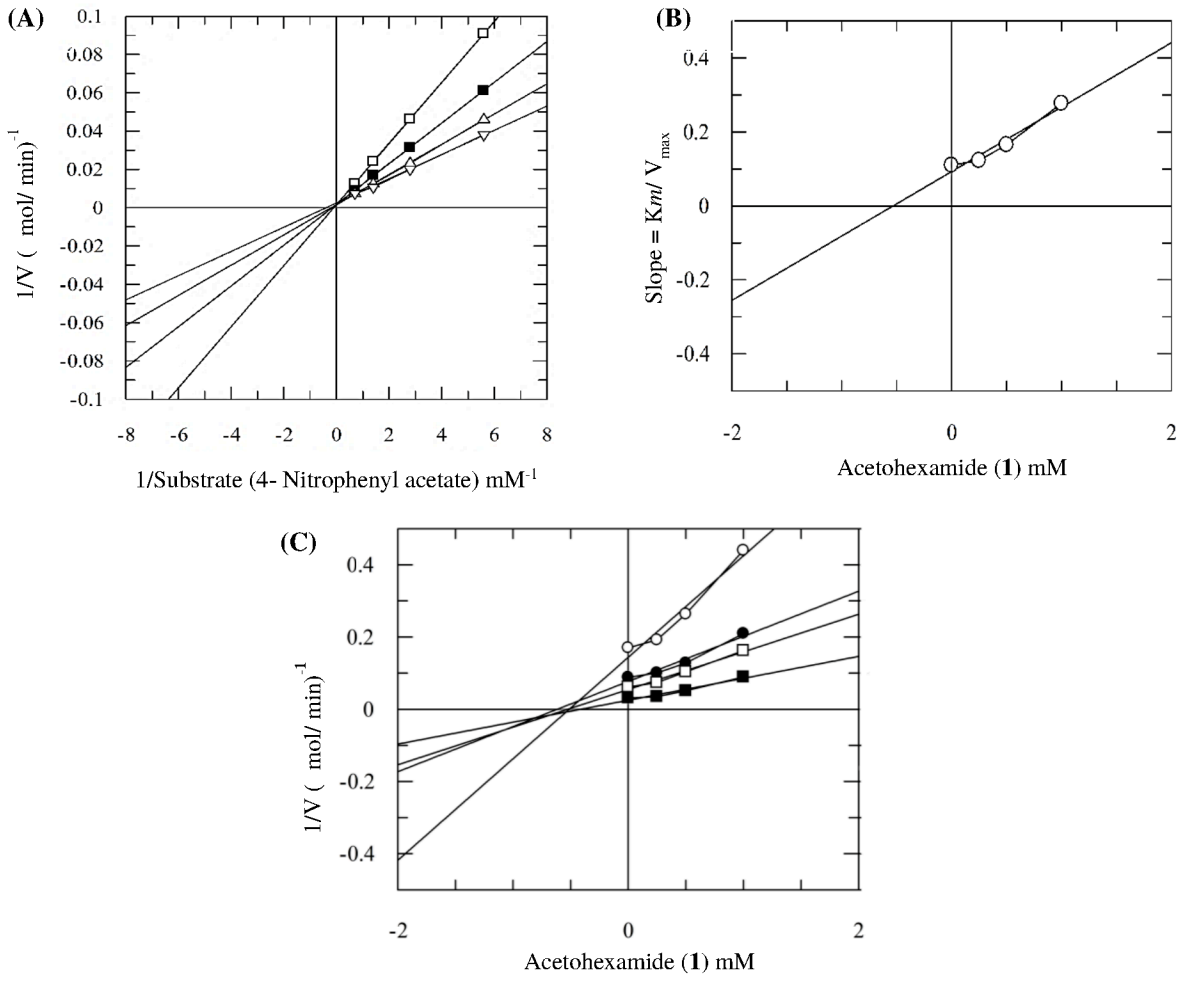



**Figure 1**: Mode of inhibition by acetohexamide (**1**). (**A**) Lineweaver-Burk plot in the absence (▽), and in the presence of 1 mM (□), 0.5 mM (■), and 0.25 mM (Δ) concentration of acetohexamide (**1**). (**B**) Secondary replot of Lineweaver-Burk plot between the slopes of each line on Lineweaver-Burk plot *versus* different concentrations of acetohexamide (**1**) (**C**) Dixon plot of reciprocal of rate of reaction (velocities) versus different concentrations of acetohexamide (**1**).

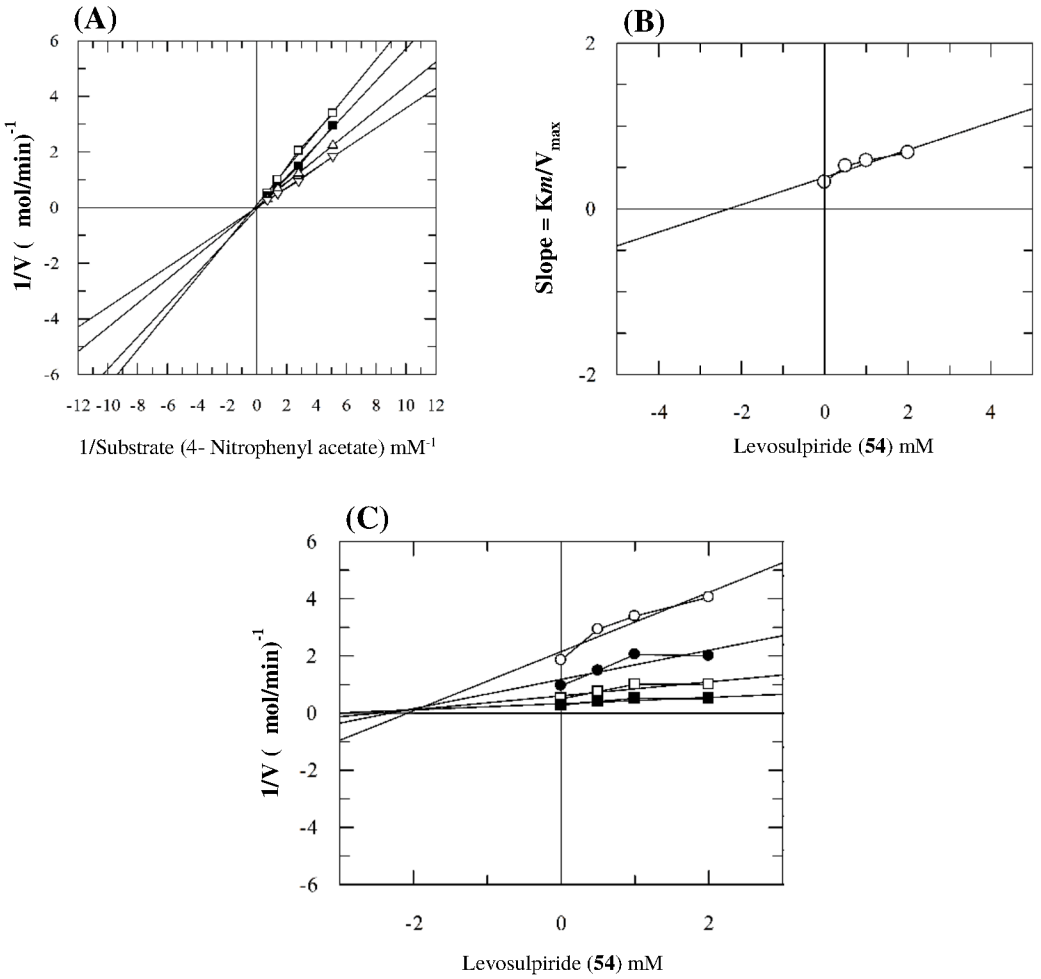



**Figure 2**: Mode of inhibition by levosulpiride (**54**) (**A**) Lineweaver-Burk plot in the absence (∇), and in the presence of 2 mM (□), 1 mM (■), and 0.5 mM (Δ) concentration of levosulpiride. (**B**) Secondary replot of the Lineweaver-Burk plot between the slopes of each line on Lineweaver-Burk plot *versus* different concentrations of acetohexamide (**1**) (**C**) Dixon plot of reciprocal of rate of reaction (velocities) versus different concentrations of levosulpiride.

**Table 2**: Kinetic parameters of acetohexamide (**1**), and levosulpiride (**54**) against hCA II

| **Drugs** | **$V_{max}$ [a] ( M/L/min)$^{-1}$** | **$V_{max\ app}$ [a] ( M/L/min)$^{-1}$** | **$K_m$ [b] (mM)** | **$K_{m\ app}$ [b] (mM)** | **$K_i$ [c] ( M) ± S.E.M.** | **$R^2$** | **Type of Inhibition** |
|---|---|---|---|---|---|---|---|
| Acetohexamide (**1**) | 64.1 | 64.5 | 5.8 | 7.0 | 447.2 ± 0.01 | 0.9769 | Competitive |
| Levosulpiride (**54**) | 66.6 | 66.2 | 9.4 | 23.8 | 1075 ± 0.02 | 0.9337 | Competitive |

***Km*** = Michaelis-Menten constant in the absence of inhibitor, ***Km app*** = Michaelis-Menten constant in the presence of inhibitor, **V*max*** = maximum velocity of reaction in the absence of inhibitor, **V*max app*** = maximal velocity of reaction in the presence of inhibitor, ***Ki*** = inhibition constant

## 3.4. X-Ray crystallographic analysis:

Based on the findings from the *in vitro* CA II inhibition experiments, X-ray crystallographic studies were performed to analyze the three-dimensional structures of hCA II-drug complexes, including the drug-protein interactions at the atomic level.

### 3.4.1. X-Ray crystallographic analysis of hCA II-acetohexamide complex:

Fully grown flattened plate-shaped co-crystals of purified hCA II, in complex with acetohexamide (**1**) were used for single-crystal X-ray data collection. Crystal parameters, data collection and refinement statistics for hCA II- acetohexamide complex are presented in Table 3. The co-crystals of acetohexamide with hCA II diffracted at 2.60 Å resolution in the $P2_1$ space group. The refined structure contains 1 molecule of acetohexamide (**1**), a zinc atom, sulfate ion, glycerol and 85 water molecules. Analysis of the electron density revealed that acetohexamide was found in the catalytic cleft of hCA II enzyme (Figure 3).

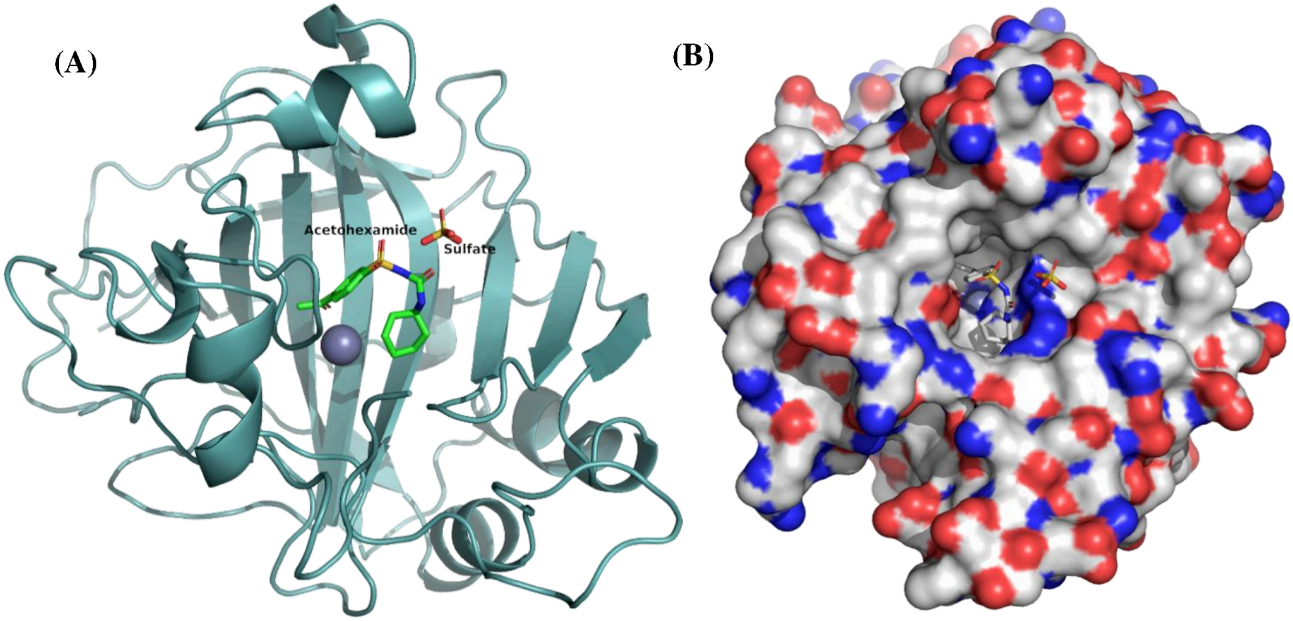



**Figure 3**: Crystal structure of hCA II in complex with acetohexamide (**1**). (**A**) Cartoon view and (**B**) surface view (acetohexamide is colored green, the grey sphere is a zinc ion, and the red/yellow stick model is a sulfate ion).

### 3.4.2. Binding interactions of acetohexamide (1) with hCA II enzyme:

Acetohexamide (**1**) makes strong hydrogen bond interactions with Zn and key amino acid residues of the active site (Figure 4). The oxygen atom (*i.e.* O1) of the acetyl group displayed strong hydrogen bonding with the Zn at a distance of 2.7 Å. Additionally, the O1 atom also showed interactions with the key histidine residues (*i.e.* His119 and 94). The O1 atom forms hydrogen bonding with nitrogen (*i.e.* ND1) of His119, and also with nitrogen (*i.e.* NE2) of His94 at a distance of 3.1 and 3.4 Å, respectively. Moreover, the nitrogen (N2) of acetohexamide showed hydrogen bonding with ND2 of Asn67 at a bond distance of 3.5 Å. One sulfate molecule was also present near the active site, and displayed two hydrogen bond interactions with acetohexamide. The nitrogen (*i.e.* N1) of acetohexamide was bonded to O3 of sulfate at a distance of 2.9 Å. In addition, the O4 atom of acetohexamide also showed a hydrogen bonding interaction with O1 of the sulfate molecule at a distance of 3.0 Å. Additionally, hydrophobic interaction with Leu198 and His94/Ala65/Thr200 were also observed which further stabilize the two six-membered rings.

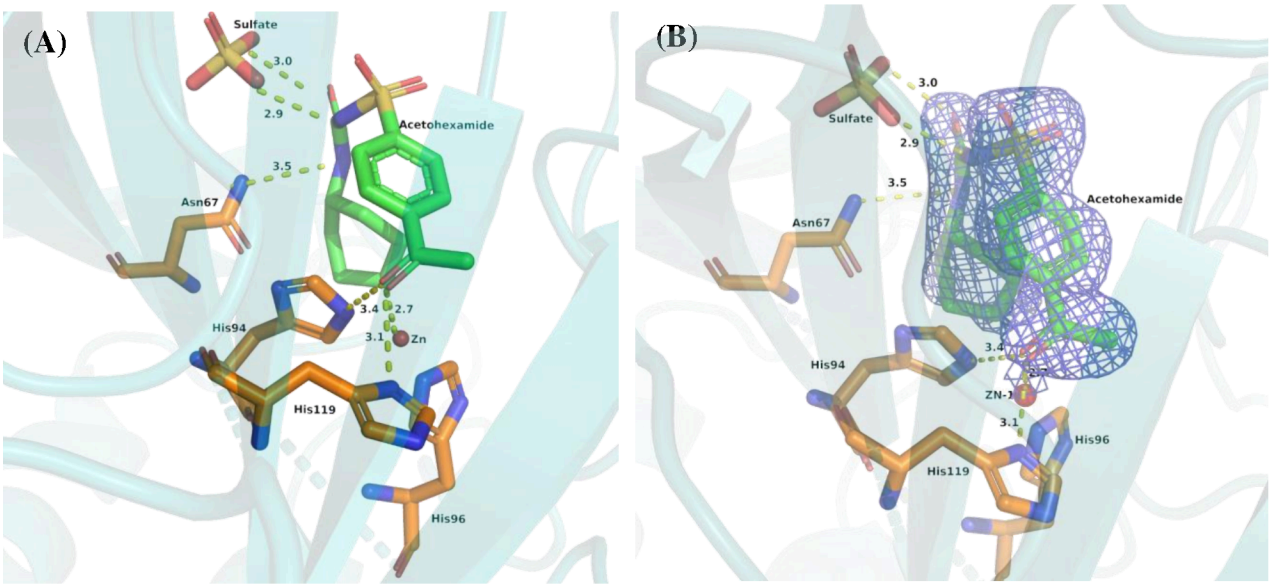



**Figure 4:** (A) Key amino acids of hCA II enzyme showing interactions with acetohexamide (**1**) (B) Electron density map 2Fo-Fc at a sigma level of 1 for acetohexamide (**1**) (blue mesh).

The final refined model was superposed with a previously reported native structure (PDB 3KS3) in order to study any possible conformational changes in the overall structure, and amino acids of the active site before and after binding of acetohexamide (**1**). The root mean square deviation (RMSD) of the superposed structures was found to be 0.198 $Å^2$, showing no significant changes in the backbone of hCA II. Similarly, the amino acids present in the vicinity of acetohexamide (**1**) also retained their conformations (Figure 5).

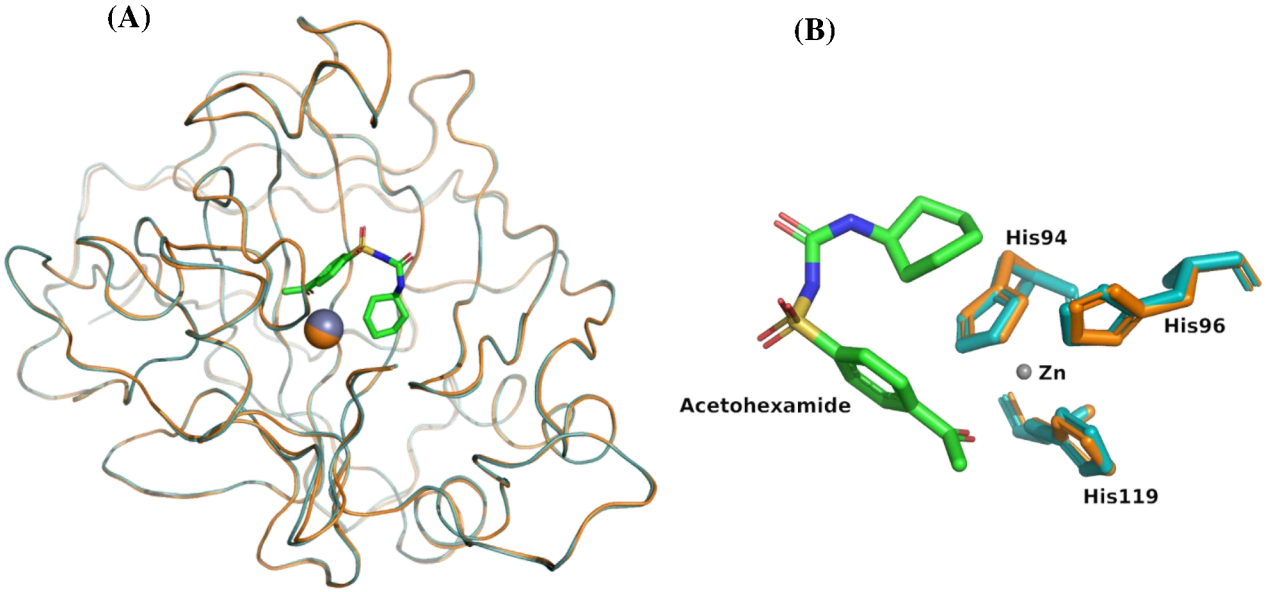



**Figure 5:** **(A)** Superposition of the main chain of hCA II (blue color) with previously reported native structure (PDB 3KS3) (orange color). **(B)** Conformation of amino acids in the active site of hCA II (cyan color showing the residues of final refined model, while orange color for the reference structure (PDB 3KS3)).

Carbonic anhydrase II inhibition has been strongly associated with sulfonamide containing compounds. Drugs that are efficient CA II inhibitors such as topiramate, indisulam, EMATE, celecoxib, valdecoxib, *etc.* contain a free sulfonamide moiety. These drugs have potent CA II inhibitory activities ranging from 5-40 nM [30-31]. However, acetohexamide is an *N*-sulfonylurea bearing a *p*-acetylphenylsulfonyl group and a cyclohexyl group. In other words, the sulfonamide group attains a central position in the molecule, perhaps this might be the reason that acetohexamide exhibits a weak CA II inhibitory activity in contrast with sulfonamide tail-end-containing drugs.

### 3.4.3. X-Ray crystallographic analysis of hCA II- levosulpiride complex:

Crystal parameters, data collection, and refinement statistics for the hCA II- levosulpiride complex are presented in Table 3. The co-crystals of hCA II- levosulpiride complex diffracted at 2.96 Å resolution in $P2_1$ space group with unit cell parameters of a= 42.32 Å, b= 41.77 Å, c= 71.73 Å and $\alpha=\gamma= 90°$, $\beta=104.17°$. The structure contains 45 water molecules, a zinc atom, and 1 molecule of levosulpiride (**54**), 2 sulfate ions, and 1 glycerol molecule. Analysis of the electron density revealed levosulpiride in the catalytic cleft of hCA II and oriented in a way that its sulfonamide moiety makes strong hydrogen bond interactions with $Zn^{+2}$ and with key amino acid residues of the active site (Figure 6).

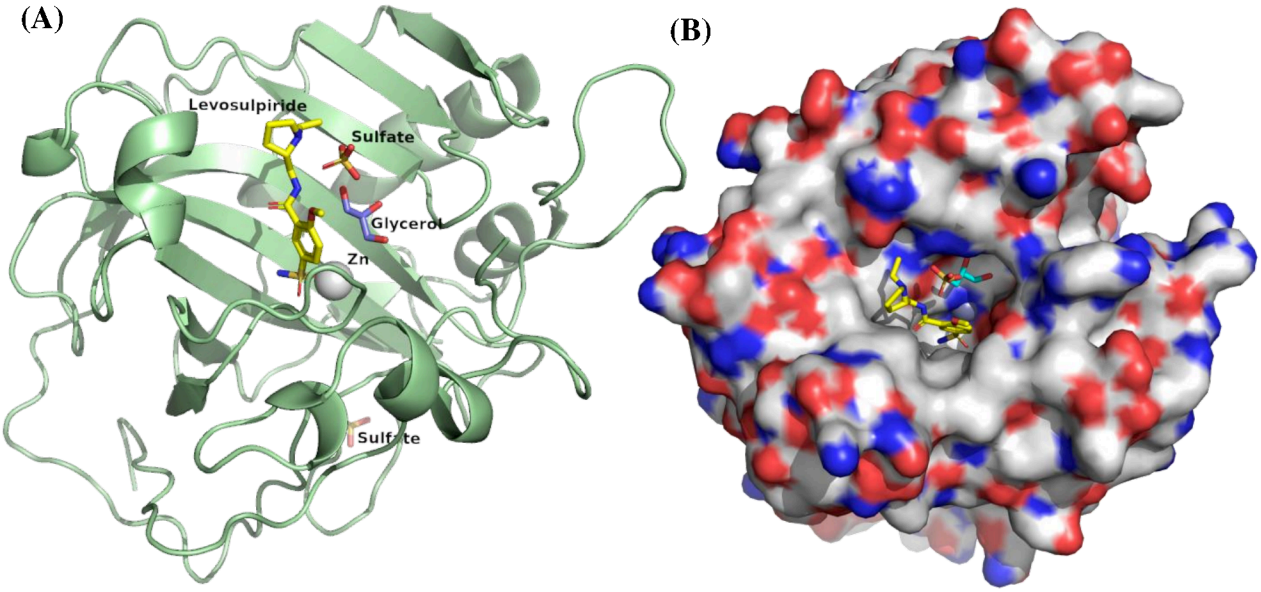



**Figure 6**: Crystal structure of hCA II in complex with levosulpiride (**54**). (**A**) Cartoon view is displayed next to the (**B**) surface view (levosulpiride is colored yellow, the grey sphere is a zinc ion).

### 3.4.4. Binding interactions of levosulpiride (54) with hCA II enzyme:

The sulfonamide group showed hydrogen bonding interactions with the amino acids of active site of hCA II enzyme (Figure 7). The oxygen atom (O3) of levosulpiride displayed a Zn-O bond at a distance of 1.89 Å. However, the Zn atom maintained its characteristic tetrahedral geometry with the surrounding histidine amino acids in the active site. The O2 atom of levosulpiride showed strong hydrogen bonding with OG1 and N of Thr199 at a distance of 3.0 and 2.6 Å,

respectively. Moreover, the O3 of levosulpiride also displayed strong hydrogen bonding with ND1 of His119 at a bond distance of 3.0 Å, and with NE2 of His94 at 2.6 Å.

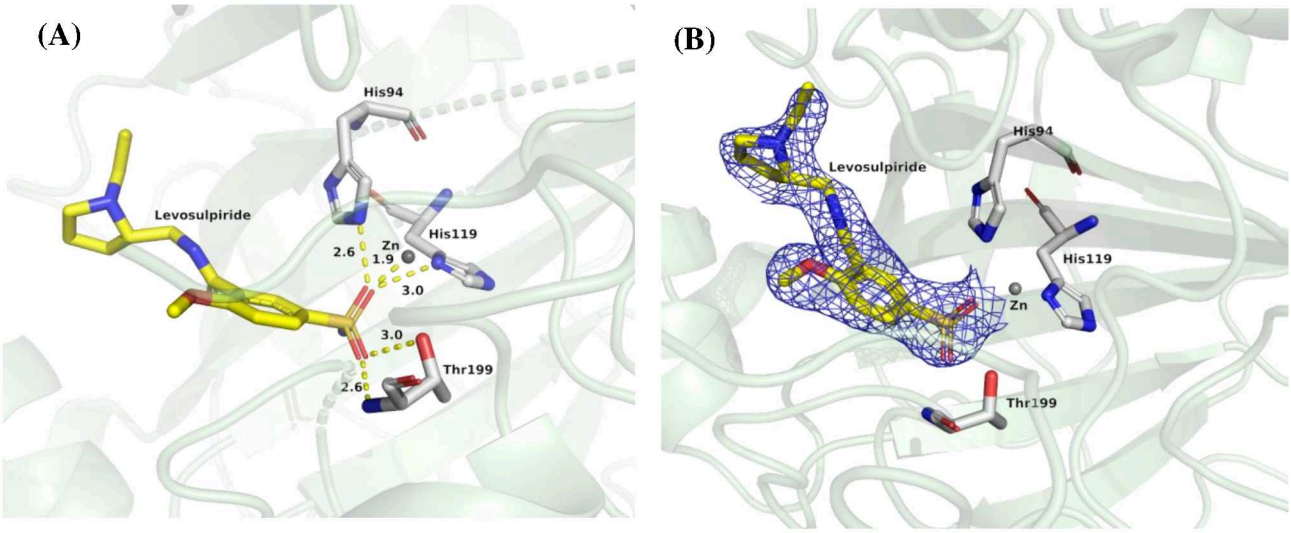



**Figure 7**: (**A**) Key amino acids of hCA II enzyme showing interactions with levosulpiride (**54**) (B) Electron density map 2Fo-Fc (sigma=1) for levosulpiride (**54**) (blue mesh).

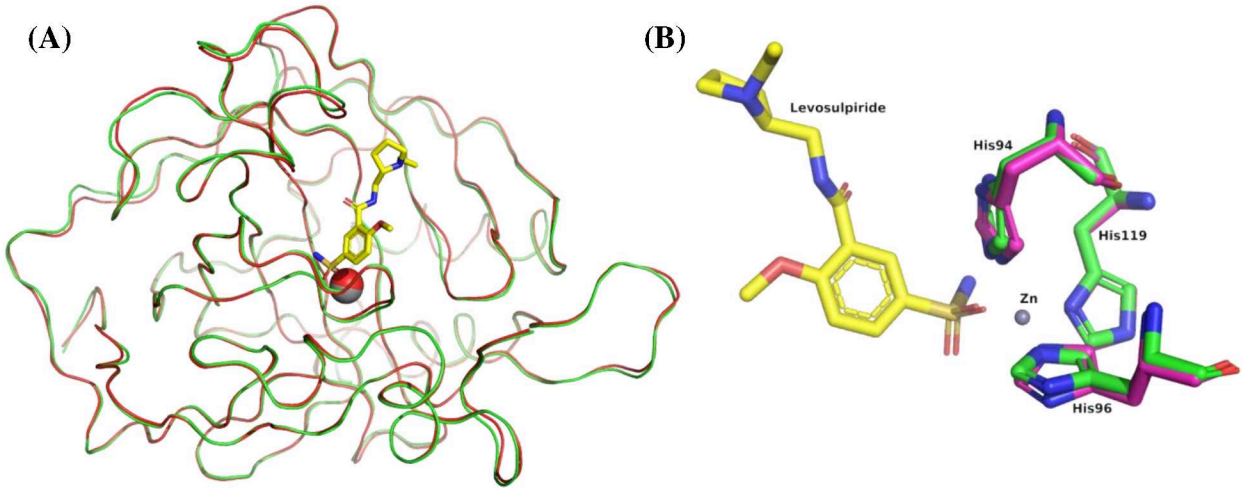



**Figure 8: (A)** Superposition of the main chain of hCA II (green color) with a previously reported native structure (PDB 3KS3) (red color). **(B)** Conformation of amino acids in the active site of hCA II (cyan color showing the residues of final refined model, while orange color for the reference structure (PDB 3KS3)).

The refined model of the hCA II-Levosulpiride complex was finally superposed with a previously reported native structure (PDB 3KS3). The RMSD of the superposed structures was 0.255 Å, showing no significant changes in the backbone of hCA II upon binding of the levosulpiride molecule. Similarly, the amino acids present in the active site of hCA II retained their conformations upon binding of levosulpiride (Figure 8).

Previously, the X-ray structure of CA II- sulpride complex was reported by Abbate and co-workers in 2004 [30]. The molecular interactions exhibited by sulpride with hCA II has few key differences when compared with our reported hCA II-levosulpiride complex. In sulpiride, the nitrogen of the sulfonamide group not only makes hydrogen bonding with Thr199 but also with Glu 106. while in our reported structure, the sulfonamide nitrogen atom did not show these interactions. In a classical CA II enzymatic reaction, Thr199 and Glu106 are considered gate-keeping residues. These ensure the proper orientation of the substrate for a favorable nucleophilic attack and thus initiate the reaction. In an inhibition reaction, this strong binding of sulpride reflects one of the possible reasons for the better efficacy against hCA II than levosulpiride. Additionally, in the reported structure of hCA II- sulpride complex, the Zn metal in the active site has shown interactions with both nitrogen and oxygen atoms of sulpride, while in our reported structure of hCA II- levosulpiride complex, only the oxygen of sulfonamide group showed interaction with Zinc metal. Finally, in the reported hCA II- sulpiride complex, the sulfonamide group did not show any hydrogen bonding with any key histidine residue of the active site, while in our refined structure of hCA II- levosulpiride complex, oxygen of the sulfonamide group is forming hydrogen bonds with His119 and His94 residues of the active site of hCA II enzyme. These observations indicate that both enantiomers although showed binding interactions within the active site, but in different manners.

**Table 3:** Data collection, processing, and refinement parameters of hCA II in complex with acetohexamide (**1**) and levosulpiride (**54**).

| **Data Collection and Processing Parameters** | **Acetohexamide (1)** | **Levosulpiride (54)** |
|---|---|---|
| Instrument | Bruker D8 Venture | |
| X-Ray source | Cu | |
| Temperature (K) | 100 | |
| Wavelength (Å) | 1.54 | |
| Detector | PHOTON 100 | |
| Resolution range (Å) | 2.60 | 2.96 |
| Space group | $P2_1$ | $P2_1$ |
| Cell parameters (Å) | $a$ = 41.89, $b$ = 41.10, $c$ =71.67 | $a$ = 42.32, $b$ = 41.77, $c$ =71.73 |
| $\alpha, \beta, \gamma$ (°) | 90.00, 104.33, 90.00 | 90.00, 104.17, 90.00 |
| No. of observations (outer shell) | 26787 (3273) | 17313 (2431) |
| No. of unique reflections (outer shell) | 7293 (877) | 5191 (819) |
| $R_{merge}$[a] (outer shell) | 0.223 (0.539) | 0.344 (0.429) |
| $R_{meas}$ (outer shell) | 0.260 (0.628) | 0.411 (0.524) |
| Completeness (%) (outer shell) | 98.22 (98.5) | 99.6 (100.0) |
| Average I/σ(I) (outer shell) | 11.8 (3.3) | 18.2 (7.8) |
| Multiplicity (outer shell) | 3.7 (3.7) | 3.3 (3.0) |
| $CC_{1/2}$ | 0.948 (0.663) | 0.740 (0.707) |
| **Data Refinement Parameters** | | |
| $R_{work}/R_{free}$ | 0.177 / 0.259 | 0.218 / 0.248 |
| No. of atoms | | |
| Protein | 2049 | 2049 |
| Ions | 1 | 1 |
| Ligands | 27 | 23 |
| $H_2O$ | 85 | 45 |
| B factors (Å$^2$) | | |
| Protein | 13.5 | 5.27 |
| Ligands | 34.1 | 10.29 |
| Ions | 7.55 | 2.28 |
| $H_2O$ | 7.46 | 1.72 |
| For all atoms | 15.65 | 4.89 |
| RMSD from ideal bond length (Å) | 0.0076 | 0.006 |
| RMSD from ideal bond angles (°) | 1.5 | 1.54 |
| Ramachandran plot, % residues in regions: | | |
| Favored | 92.0 | 90.91 |
| Allowed | 7.9 | 8.30 |
| Outliers | 0 | 0.79 |
| PDB ID | 8J2O | 8JEE |

[a] $R_{merge} = \Sigma_{hkl} \Sigma_i |I_i(hkl) - \langle I(hkl) \rangle| / \Sigma_{hkl} \Sigma_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ are the observed individual and mean intensities of a reflection with the indices $hkl$, respectively, $\Sigma_i$ is the sum over i measurements of a reflection with the indices $hkl$, and $\Sigma_{hkl}$ is the sum over all reflections.

## 3.5. *In vivo* carbonic anhydrase inhibition studies:

*In vivo* results are crucial to recognize the physiological effects of the CAIs. Outcomes of *in vivo* studies are also helpful in analyzing the competency of test compounds/ CAIs before planning for *in vivo* glaucoma studies, a disease characterized by elevated intraocular pressure (IOP), affecting a large number of people worldwide.

Based on the interesting results obtained from *in vitro* and X-ray crystallographic investigations, we have decided to conduct *in vivo* carbonic anhydrase inhibition studies. Since acetohexamide (**1**) is no longer available due to its association with disulfiram-like reactions and hepatotoxicity [32], therefore, we have opted levosulpiride (**54**) for *in vivo* carbonic anhydrase inhibition studies. In our experiments, we determined the effect of levosulpiride (**54**) on the activity of mouse erythrocytes CA at the 1$^{st}$, 3$^{rd}$, and 5$^{th}$ hour of intraperitoneal administration and then compared it with the control group of the respective hour. The results of the *in vivo* effects of levosulpiride (**54**) are presented in Figure 9. Results showed that levosulpiride (**54**) significantly inhibited the mouse erythrocytes' CA activity at the 1$^{st}$, 3$^{rd}$ and 5$^{th}$ hour, and activities were observed as 10472.841 ± 799.78 ($p$<0.001), 8238.864 ± 14.604 ($p$<0.001), and 7311.668 ± 1294.896 EU/g Hb ($p$<0.001), respectively, when compared with the control of the respective hour. It was observed that levosulpiride showed the strongest inhibition at the 5$^{th}$ hour after administration (Figure 9).

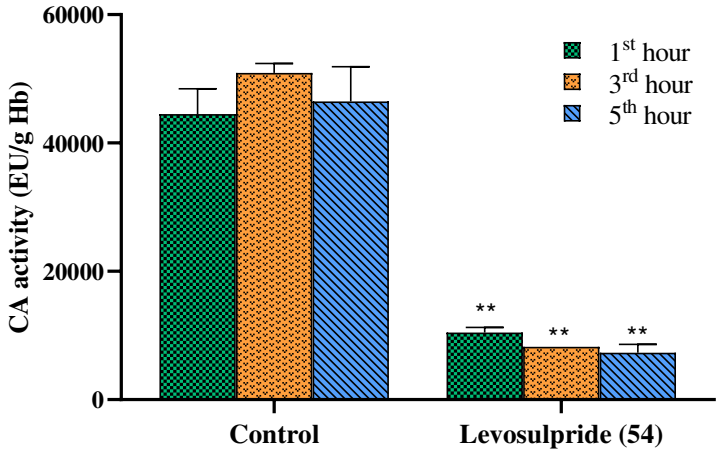



**Figure 9:** Effect of levosulpiride (**54**) (100 mg/kg of body weight) on mice erythrocytes carbonic anhydrase activity. Values are expressed as mean ± SD. ** values regarded as highly significant ($p$<0.001) when compared with the control groups at the respective hour of treatment.

Although extensive literature is reported describing the effects of several FDA approved drugs against hCA II isoform (Figure 10), but there is no report describing the detailed studies on these two drugs, acetohexamide and levosulpiride. Türkes reported five antiviral drugs, abacavir, emtricitabine, lamivudine, ribavirin, and ritonavir against hCA I and hCA II isoforms. He reported that hCA II was inhibited by these drugs with Ki values in the range of 0.64 – 5.80 $\mu$M [33].

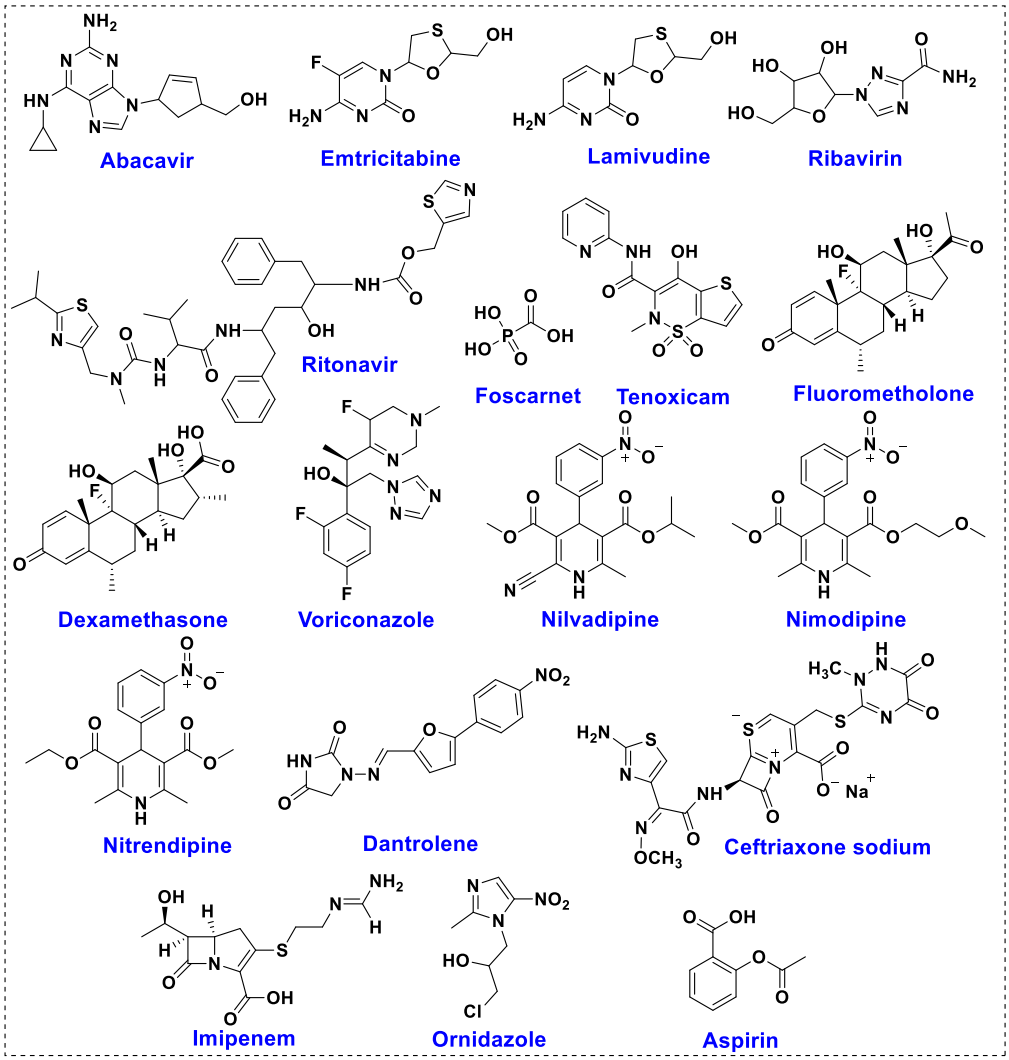



**Figure 10:** Some of the FDA approved drugs reported as inhibitors of hCA II isoform.

Temperini and cowokers reported the interaction of foscarnet, an antiviral drug, with 11 CA isozymes including hCA II (Ki = 14.2 mM) [34]. In another study, Alım *et al.* reported the anti-inflammatory drugs tenoxicam, fluorometholone acetate, and dexamethasone as inhibitors of hCA II with $IC_{50}$ values of 0.11, 17.5, and 14 $\mu$M, respectively [35]. Topal reported the hCA II

inhibitory activity of antifungal drug, voriconazole (Ki = 36.76 ± 2.57 *n*M) [36]. Türkes and coworkers reported calcium channel blockers, *i.e.* nilvadipine, nimodipine, and nitrendipine as inhibitor of hCA II isoform with $IC_{50}$ ranging between 70-139 $\mu$M [37]. Gülçin *et al*., reported the *in vitro* hCA II inhibitory activity of muscle relaxant, dantrolene ($IC_{50}$ = 3.24 x $10^{-5}$ M) as well as *in vivo* CA inhibitory activity [38]. Ekinci and coworkers reported the *in vitro* hCA II inhibitory effects of ceftriaxone sodium, imipenem and ornidazole with $IC_{50}$ vales of 2.542, 0.0258, 0.343 mM, respectively [39]. In another study, Andring *et al*., reported aspirin as suicide inhibitor of hCA II enzyme ($IC_{50}$ = 6.6 ± 0.5 mM) and reported its binding mechanism *via* crystallographic studies [40].

## Conclusion:

The newly identified inhibitory activities of acetohexamide (**1**) and levosulpiride (**54**) against the CA II enzyme are in line with the drug repurposing approach. Based on the results presented here, it might be concluded that both drugs were found to be weak inhibitors of the CA II enzyme. Inhibitory activity of acetohexamide (**1**) against CA II could be due to the presence of *p*-acetyl group, while levosulpiride (**54**) inhibited the activity of hCA II enzyme by interacting through its sulfonamide group. Structural analysis revealed that these groups showed multiple interactions with the $Zn^{+2}$ ion and also with the active site's amino acid residues of the hCA II enzyme. From complementary *in vivo* studies, we found that the intraperitoneal administration of levosulpiride (**54**) significantly inhibited CA activity for up to 5 h. Although both drugs showed weak inhibitory potential (*in vitro*) than the standard CAI, acetazolamide, but our findings will provide comprehensive insights for the optimization of the pharmacological profile of these drugs, and provide a platform for the exploration of different derivatives of these drugs. Additionally, as part of our ongoing project, we have begun synthesizing different derivatives of both drugs. In essence, the exploration of acetohexamide and levosulpiride as inhibitors of carbonic anhydrase II underscores the importance of interdisciplinary approaches in modern drug discovery.

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## Authors' contributions:

**Saima Rasheed** designed and supervised the study; she was responsible for funding acquisition, project administration, data collection, data analysis, and writing original draft of the manuscript and its finalization. **Noor ul Huda:** (an M. Phil. scholar worked under the supervision of Saima Rasheed) performed *in vitro* laboratory experimentations. Saima Rasheed & Noor ul Huda: wrote the manuscript. **Zoha Warsi** and **Syeda Sarah Tahir**: (M. Phil. scholars working under the supervision of Saima Rasheed) performed *in vivo* experiments. **Sadaf Gul:** (Ph.D. scholar under the supervision of Saima Rasheed) helped in hCA II expression and purification. **Sven Falke:** Data analysis, manuscript review, and valuable inputs. All authors have read and approved the final manuscript.

## Competing interest:

The authors declare that they have no competing interests.

## Availability of data and materials:

All data generated or analyzed during this study are included in this article. Data is deposited to the Protein Data Bank (PDB) under accession IDs 8J2O and 8JEE.

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