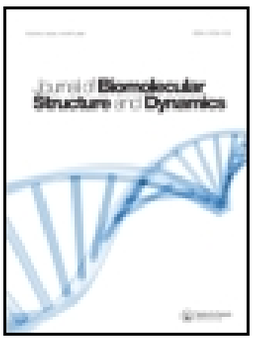

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Design and development of 5-(4H)-oxazolones as potential inhibitors of human carbonic anhydrase VA: towards therapeutic management of diabetes and obesity

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Design and development of 5-(4H)-oxazolones as potential inhibitors of human carbonic anhydrase VA: towards therapeutic management of diabetes and obesity

Amarjyoti Das Mahapatra[a#], Aarfa Queen[b#], Mohd Yousuf[c], Parvez Khan[c], Afzal Hussain[d], Md. Tabish Rehman[d], Mohamed F. Alajmi[d], Bhaskar Datta[a] and Md. Imtaiyaz Hassan[c]

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ABSTRACT

Inhibitors of carbonic anhydrase (CAIs) hold promise for addressing various diseases, including cancer, diabetes, and other metabolic syndromes. CAV is the only isoform present in the mitochondria and is considered a potential drug target for obesity. In this work, we have developed C2, and C4 substituted oxazole-5(4H)-one derivatives as a new scaffold for the selective inhibition of human carbonic anhydrase VA (hCAVA). Synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, and LC-MS mass spectrometry and subsequently evaluated for *in vitro* hCAVA inhibition. Two compounds, 4 and 5 showed a considerably higher binding affinity for hCAVA in comparison to the hCAII. Further, cell-based studies showed that these compounds decrease the expression of CAVA and GLUT4 in adipocytes and non-toxic to HEK293 cells. The present work opens a platform for the use of oxazole-5(4H)-ones and holds promise for further refinement of potent and selective hCAVA inhibitors.

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KEYWORDS
Carbonic anhydrase inhibitors; carbonic anhydrase VA; oxazolone derivatives; obesity; drug discovery

1. Introduction

Oxazolones are widely studied five-membered heterocyclic molecules as part of pharmacologically relevant scaffolds (Cannella et al., 1996; Joshi et al., 2003; Keni & Tepe, 2005). Highly substituted oxazolones derivatives can be easily synthesized by straight-forward modification at different active sites. Diverse pharmacological activities of oxazolone derivatives can be accessed by modification at C2 and C4 positions. For example, the presence of a nitro group containing exocyclic phenyl group (-Ph-NO2) at the C4 position of oxazolone contributes to immunosuppressive activity (Mesaik et al., 2004). An extended conjugation through an aliphatic double bond at C4 position and a phenyl ring at C2 position plays an important role in tyrosinase inhibitory activity (Khan et al., 2006). Electron donating and electron-withdrawing effect of substituents at C2 and C4 positions of oxazolone moieties have a crucial effect on chymotrypsin inhibitory activities (Khan et al., 2008). Substituted oxazolone derivatives have been reported towards numerous pharmacological applications such as antimicrobial (Pasha et al., 2007; Tandon et al., 2004), DAPK inhibitors (Okamoto et al., 2010), urease inhibition (Fareed et al., 2013), and potential anti-obesity or antidiabetic agents (Carpene et al., 2019; Kakkar & Narasimhan, 2019; Soldavini & Kaunitz, 2013) (Figures 1 and 2).

The different isoforms of carbonic anhydrase (CA, EC4.2.1.1) catalyzes the simplest and most fundamental reaction in living systems, namely the interconversion of carbon dioxide and bicarbonate ion (Sly & Hu, 1995; Tashian et al., 1990). The α-CA family of isoforms is present in vertebrates, where the dominant discourse surrounding CAs has focused around the cytosolic isoform CAII. However, several other isoforms have garnered attention in recent years (Lehtonen et al., 2004; Supuran, 2004). For example, the membrane-bound isoforms CAIX and CAXII are correlated with tumors, thereby rendering them important as anti-cancer therapeutic targets (Nocentini & Supuran, 2018; Pastorekova et al., 2005; Salaroglio et al., 2018). The development of isoform-selective CA inhibitors has been a useful strategy for gaining insight into the role of tissue or organelle localized isoforms (Pastorekova et al., 2004; Supuran & Scozzafava, 2002). The cytosolic and membrane-associated α-CA isoforms have been implicated in the maintenance of acid-base balance and transport of carbon dioxide and bicarbonate across membranes that ultimately underlie distinctive tissue-specific physiological events (Gilmour & Perry, 2009; McConnell et al., 1961; Minkin & Jennings, 1972; Postel & Sonnenberg, 2012; Supuran, 2004, 2018).

CA isoforms VA and VB are present in mitochondria. CAVA is mainly expressed in the liver, skeletal muscle, and kidney

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#These authors contributed equally to this manuscript.

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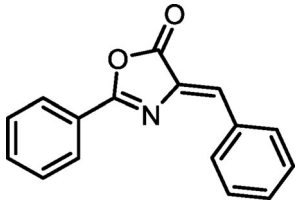


Figure 1. General chemical structure of (Z)-4-benzylidene-2-phenyloxazol-5(4H)-one.

while CAVB has a wider tissue distribution (Shah et al., 2000). Both CAVA and CAVB have been suggested as contributing to biosynthetic reactions involving bicarbonate as a reactant (Chegwidden & Spencer, 1996; Forster & Dodgson, 2000). The localization of the CAVA and CAVB isoforms in the mitochondrial matrix, and presence of highly active CAII in cytosol have been linked to pyruvate carboxylase functioning, and efflux of acetyl groups during fatty acid biosynthesis (Hazen et al., 1996; Winum et al., 2005). The mitochondrial CAV isoforms are implicated in lipogenesis (Chegwidden & Spencer, 1996), gluconeogenesis (Dodgson & Cherian, 1989), ureagenesis (Dodgson, 1987), and all processes that require access to free bicarbonate or on a related note to the tricarboxylic acid transporter system (Imtaiyaz Hassan et al., 2013). Interestingly, structural analysis of CAVA has sought to support the lower activity of the isoforms when compared to CAII (Boriack-Sjodin et al., 1995; Heck et al., 1994). Nevertheless, such analysis is complicated by the higher than cytosolic pH in mitochondria. Also, the broader distribution of CAVB compared to CAVA indicates distinctive functional contexts for the two isoforms (Fujikawa-Adachi et al., 1999).

While the druggable target properties of both CAVA and CAVB have been reported, but relatively few reports for the isoform-selective inhibitors of these enzymes are available (Nishimori et al., 2005; Vullo et al., 2004). Mitochondrial CAVA could serve as an interesting therapeutic target for modulating ureagenesis and gluconeogenesis, both of which are unregulated in renal failure and obesity. The case of Topiramate (TPM) is relevant in this regard (Gordon & Price, 1999; Supuran, 2003). The use of TPM resulted in a significant loss in the weight of obese patients and this was attributed to the inhibitory action against CAV and CA II isoforms that actively participate in the lipogenesis of adipocytes (Dodgson et al., 2000). Sulfonamides and their isosteres form the dominant class of CA inhibitory drugs (Supuran, 2010). Sulfonamides and sulfamides have also been evaluated for their CAV inhibitory activities (Nishimori et al., 2005; Smaine et al., 2008; Vullo et al., 2004). Due to the well-known cross-reactivity of the pharmacophore, the identification of CAV isoform-selective inhibitors within sulfonamides is non-trivial (Supuran, 2010). This points to the need for identifying scaffolds that have superior isoform-selective character.

In the present work, we have explored C2, and C4 substituted oxazole-5(4H)-one derivatives as a new scaffold for developing hCAVA inhibitors. The synthesized inhibitors could be implicated in the therapeutic targeting of CAVA to manage obesity and other metabolic disorders.

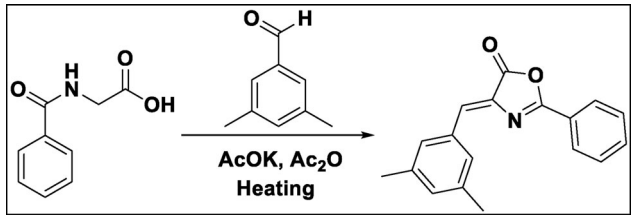


Figure 2. General reaction scheme for the synthesis of (Z)-4-benzylidene-2-phenyloxazol-5(4H)-one (Wang, 2010).

2. Materials and methods

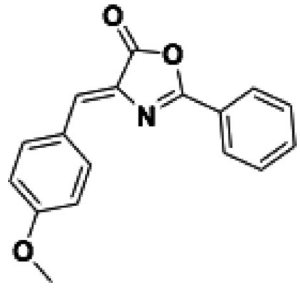
2.1. General chemical procedures

^{1}H NMR spectra were recorded on a 500 MHz Bruker Instrument. ^{13}C NMR spectra (125.8 MHz) were recorded on a 500 MHz Bruker Instrument. Tetramethyl silane (TMS) was used as an internal standard for NMR studies. Chemical shifts were measured in ppm (δ). Coupling constants (*J*) are reported in Hertz (Hz). Mass spectra were measured by LC-MS on a Waters SYNAPT-G2S-S using the electrospray ionization technique. Silica gel plates were used for TLC (Thin-layer chromatography) analysis.

2.2. General procedure for the synthesis of oxazol-5(4H)-ones

Oxazol-5(4H)-ones were synthesized according to the reported method (Beloglazkina et al., 2016). A mixture of aromatic aldehydes (1 equiv.), hippuric acid (1 equiv.) in acetic anhydride (3 equiv.) was refluxed at 100 °C for 3–4 h in a nitrogen environment in the presence of potassium acetate (1 equiv.) to obtain the desired compounds with moderate yields. After synthesis, we focused on the most intense spot on TLC (thermodynamically more stable Z-isomer) which was successfully purified and characterized. While we did not observe the formation of E-isomers, we cannot exclude their formation from the reaction *per se*, albeit in a significantly smaller proportion. After cooling, the precipitate was filtered and washed with distilled water and ether to obtain the desired compound. Pure Oxazol-5(4H)-one derivatives were obtained either after washing with solvents or after column chromatography or finally characterized by ^{1}H NMR, ^{13}C NMR, and LC-MS mass spectrometry.

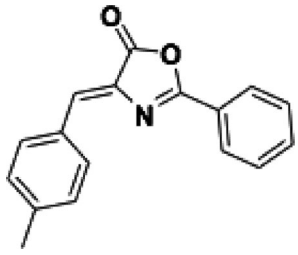
2.2.1. (Z)-4-(4-methoxybenzylidene)-2-phenyloxazol-5(4H)-one (1)



The title compound was synthesized as described above and obtained as solid, yield 57%, ^{1}H NMR (DMSO-d_6, 500 MHz):δ 8.31 (d, 2H, J_{obs} = 8.5), 8.12 (d, 2H, J_{obs} = 8), 7.74 – 7.71 (m, 1H), 7.66 – 7.63 (m, 2H), 7.34 (s, 1H), 7.12 (d,

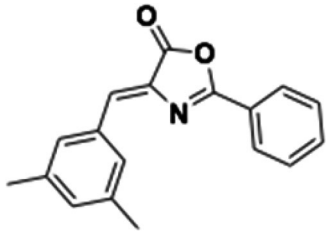
2H, J_{obs} = 9), 3.88 (s, 3H); ^{13}C NMR (DMSO-d_6, 125.8 MHz):δ167.5, 162.4, 162.4, 135.0, 133.8, 131.8, 131.1, 129.8, 128.2, 126.7, 125.8, 115.2, 56.0; MS (ESI): *m/z*: calculated $[M+H]^+$: 280.0968; found $[M+H]^+$: 280.0947.

2.2.2. (Z)-4-(4-methylbenzylidene)-2-phenyloxazol-5(4H)-one (2)



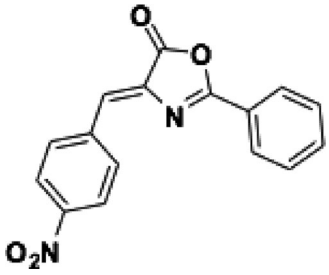
The title compound was synthesized as described above and obtained as solid, yield 60%, ^{1}H NMR (DMSO-d_6, 500 MHz):δ8.22 (d, 2H, J_{obs} = 8.5), 8.13 (d, 2H, J_{obs}s = 7), 7.75 – 7.72 (m, 1H), 7.67 – 7.64 (m, 2H), 7.37 (d, 2H, J_{obs} = 8), 7.33 (s, 1H), 2.40 (s, 3H); ^{13}C NMR (DMSO-d_6, 125.8 MHz):δ167.4, 163.1, 142.3, 134.0, 132.9, 132.7, 131.6, 131.2, 130.2, 129.8, 128.4, 125.7, 21.8; MS (ESI): *m/z*: calculated $[M+H]^+$: 264.1019; found $[M+H]^+$: 264.1089.

2.2.3. (Z)-4-(3,5-dimethylbenzylidene)-2-phenyloxazol-5(4H)-one (3)



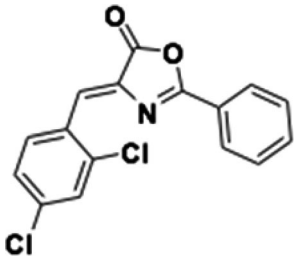
The title compound was synthesized as described above and obtained as solid, yield 52%, ^{1}H NMR (DMSO-d6, 500 MHz): δ 8.14 (d, 2H, J_{obs} = 7), 7.93 (s, 2H), 7.76 – 7.73 (m, 1H), 7.68 – 7.65 (m, 2H), 7.26 (s, 1H) , 7.18 (s, 1H) , 2.37 (s, 6H); ^{13}C NMR (DMSO-d6, 125.8 MHz): δ 167.4, 163.3, 138.5, 134.1, 133.7, 133.5, 133.2, 131.7, 130.5, 129.8, 128.5, 125.6, 21.4; MS (ESI): *m/z*: calculated $[M+H]^+$: 278.1176; found $[M+H]^+$: 278.1183.

2.2.4. (Z)-4-(4-nitrobenzylidene)-2-phenyloxazol-5(4H)-one (4)



The title compound was synthesized as described above and obtained as solid, yield 49%, ^{1}H NMR (DMSO-d_6, 500 MHz):δ8.57 (d, 2H, J_{obs} = 9), 8.37 (d, 2H, J_{obs} = 9), 8.20 (d, 2H, J_{obs} = 7), 7.81 – 7.78 (m, 1H), 7.70 – 7.67 (m, 2H), 7.49 (s, 1H); ^{13}C NMR (DMSO-d_6, 125.8 MHz):δ166.9, 165.4, 148.3, 140.1, 136.7, 134.8, 133.4, 129.9, 128.9, 127.5, 125.3, 124.4; MS (ESI): *m/z*: calculated $[M+H]^+$: 295.0713; found $[M+H]^+$: 295.0774.

2.2.5. (Z)-4-(2,4-dichlorobenzylidene)-2-phenyloxazol-5(4H)-one (5)



The title compound was synthesized as described above and obtained as solid, yield 54%, ^{1}H NMR (DMSO-d_6, 500 MHz):δ8.92 (d, 1H, J = 8.5), 8.17 (d, 2H, J_{obs} = 7), 7.87 (d, 1H, J_{obs} = 2), 7.80 – 7.77 (m, 1H), 7.70 – 7.66 (m, 3H), 7.41 (s, 1H); ^{13}C NMR (DMSO-d_6, 125.8 MHz):δ167.0, 165.2, 136.6, 136.4, 136.0, 134.7, 134.3, 130.2, 130.1, 129.9, 128.8, 128.7, 125.3, 122.7; MS (ESI): *m/z*: calculated $[M+H]^+$: 318.0083; found $[M+H]^+$: 318.0088.

2.3. Molecular docking

Molecular docking was carried out using Autodock Vina and AutoDock 4 (Zn) with an improved force field package (Morris et al., 2009; Santos-Martins et al., 2014; Trott & Olson, 2010). The atomic coordinates of previously reported modeled structures of CAVA were taken from the Protein Model Database (PMDB ID: PM0080287), while for CAIX and CAII the structure coordinates were taken from PDB ID: 3IAI, PDB ID: 2AW1, respectively (Idrees et al., 2016, 2017). The 2D and 3D structures of all the synthesized molecules were drawn in ChemBio3D Ultra 12.0. Molecular docking studies were performed to see the binding conformations and types of protein-ligand interactions (Thakur & Hassan, 2011). In short, the torsion angles were identified using Autodock Tools, and Kollman atomic charges were applied to the protein molecule. Grid spacing was defined, and the selected synthesized molecules were subjected to robust molecular docking procedures as described previously (Queen et al., 2018; Shamsi et al., 2020). The selected docking poses were analyzed, refined, and visualization/structure analysis of each docked complex was performed using PyMOL viewer (Schrödinger, LLC) and "Receptor-ligand Interactions" modules of BIOVIA/Discovery Studio 2017R2 were used (Biovia, 2013).

2.4. Protein expression and purification

The catalytic domain of human CAVA (39-305aa), CAIX (38-414aa), and full-length CAII were cloned, expressed, and purified using a prokaryotic expression system (*E. coli*, BL21 strain) as described previously (Aneja et al., 2020; Idrees et al., 2016). In brief, CAVA and CAII were expressed as soluble proteins using a

prokaryotic expression system. The expression constructs of CAVA and CAII were separately transformed in the BL21 strain of *E. coli,* and individual colonies were picked for establishing the primary and secondary expression cultures. The secondary cultures were induced for protein expression with isopropyl β-D-1-thiogalactopyranoside, and cultures were pelleted down after 5–6 h of induction. The pelleted cultures of CAVA and CAII were lysed using lysis buffer (50 mM Tris, 250 mM NaCl, 0.1 mM PMSF and 1% Triton-100), and filtered supernatant obtained after cell lysis was directly loaded on pre-equilibrated Ni-NTA affinity column. The bound protein was eluted using imidazole gradients, and each elution was analyzed for the presence of protein. The protein-containing fractions were pooled, concentrated, and further purified using gel-filtration chromatography (Superdex 200 pg connected to the Akta purifier, GE Healthcare). The CAIX was purified from inclusion bodies using sarcosine buffer (50 mM Tris, 1.5% N-lauroyl sarcosine, pH 8.0). The purified CAIX was refolded by dialyzing extensively in refolding buffer (50 mM Tris pH 8.0 and 150 mM NaCl) for 30–36 h, at 4 °C with 5–6 successive buffer exchanges.

2.5. CA enzyme inhibition assay

Enzyme inhibition assay of CA was carried out using our earlier reported method (Peerzada et al., 2018). This assay spectrophotometrically measured the *p*-nitrophenol, a yellow-colored product which is formed by the hydrolysis of *p*-nitrophenyl acetate (4-NPA) catalyzed by CA. The absorbance of the hydrolyzed reaction product (yellow color) was measured at 400 nm with the help of a UV/visible spectrophotometer (Jasco V-660, Model B028661152) equipped with a Peltier-type temperature regulator. The synthesized compounds are DMSO soluble, so we prepared the stock solutions in DMSO, and working solutions were prepared by making the subsequent dilutions from the stock solutions of respective compound in the assay buffer (50 mM Tris buffer, pH 8.0). For each measurement of enzyme inhibition studies, nearly 5 μM CAVA/CAII/CAIX protein was used, and protein solution without compound (with DMSO only, as vehicle control) was taken as control, whereas, acetazolamide (a non-specific inhibitor of CAs) was used as a positive control. The IC_{50} values for each synthesized compound were determined by analyzing the absorption data using Graph Pad Prism (Version 6.0) software.

2.6. Fluorescence measurements

Binding affinities of CAVA and selected synthesized molecules (compounds 4 and 5) were performed using fluorescence emission studies as described previously (Khan et al., 2018, 2019). The stock solutions of each compound were prepared in DMSO and working solutions (1 μM/μl concentration) were diluted in Tris buffer (50 mM, pH 8.0). The CAVA, CAIX, and CAII consist of tryptophan residues, which absorb at 280 nm and bear characteristic emission maxima at 346 nm. Thus, we excited protein solution at 280 nm and fluorescence emission was recorded in the 300–400 nm range. A significant decrease in the fluorescence intensity of CAVA with the increasing concentration of compound 4 or 5 was used to calculate the binding constant (K_a) and the number of binding sites (n) present on the protein molecule using the modified Stern-Volmer equation (Boaz & Rollefson, 1950):

$$\log\ (F_o - F)/F = \log K_a + n \log [L] \quad (1)$$

where, F_o = Fluorescence intensity of native protein, F = Fluorescence intensity of protein in the presence of ligand, K_a = Binding constant, n = number of binding sites, L = concentration of ligand. The binding constant (K_a) and the number of binding sites (n) were obtained from the intercept and slope, respectively.

2.7. Protein isolation and western blot

The 3T3-L1 cells were treated with the IC_{50} dose of selected compounds and were lysed in RIPA cell lysis buffer (Thermo Fisher Scientific (USA). The protein concentrations were measured using a Bicinchoninic Acid Assay (BCA-protein estimation kit). Approximately 40–50 μg of whole-cell lysate was diluted with 6X Laemmli's buffer, boiled for 3–5 min and resolved using 12% SDS-polyacrylamide electrophoresis under reducing conditions. The resultant polypeptides were transferred to polyvinylidene fluoride (PVDF) membrane using blotting. The protein of interest was identified with the help of peptide-specific primary antibodies (Carbonic Anhydrase VA Polyclonal Antibody, Catalog # PA5-36931, GLUT4 Monoclonal Antibody, Catalog # MA5-17176, from Thermo Fisher Scientific) and horseradish peroxidase coupled secondary conjugates using luminol as a chemiluminescent substrate for HRP (Khan et al., 2014).

2.8. Cell viability studies

The cell viability studies of HEK293 cells with selected compounds was analyzed using MTT assay (Khan et al., 2017, 2018). The percentage of cell viability was estimated and plotted as a function of ligand concentration.

3. Results and discussion

3.1. Chemical synthesis

In the present work, we have investigated C2 and C4 substituted oxazol-5(4H)-one derivatives as a new scaffold for the development of hCAVA inhibitors. Five oxazole-5(4H)-one derivatives with varying substitutions at C4 substituted phenyl ring were synthesized from aromatic aldehydes, hippuric acid, acetic anhydride, and potassium acetate according to literature reported procedure (Beloglazkina et al., 2016). A mixture of aromatic aldehydes (1 equiv.), hippuric acid (1 equiv.) in acetic anhydride was refluxed at 100 °C for 3–4 h under the nitrogen environment in the presence of potassium acetate (1 equiv.) to obtain desired compounds with moderate yields. Scrutiny of literature towards the synthesis of similar 5-(4H)-oxazolones derivatives revealed that the thermodynamically stable Z isomer is usually formed and in the case of certain specific aldehydes, a mixture of both isomers might be formed (de Castro et al., 2016; Rao, 1976;

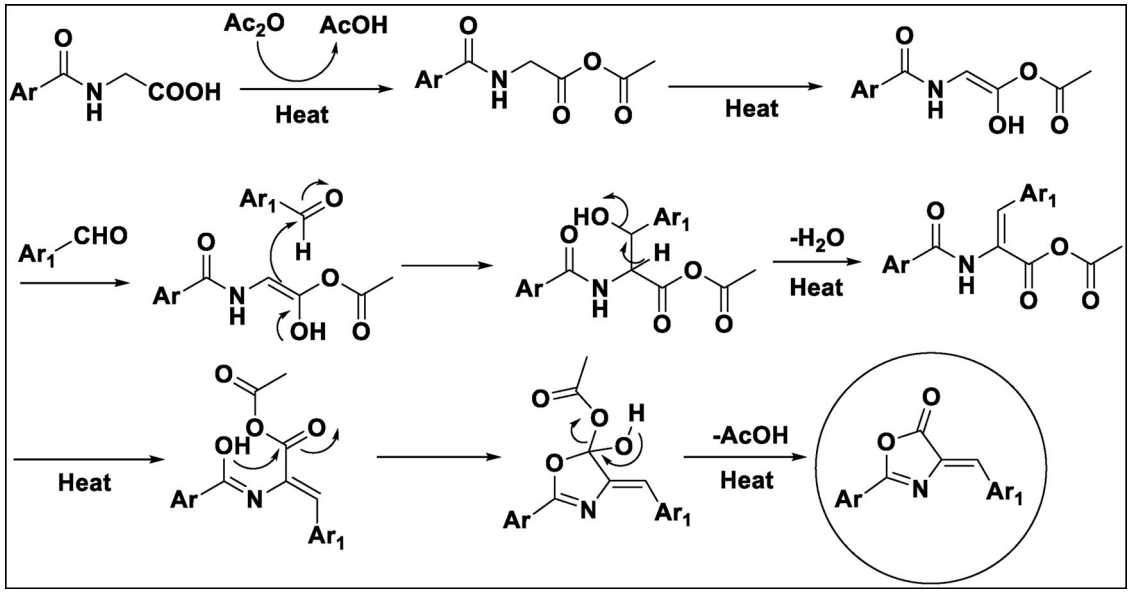


Figure 3. Proposed mechanism for the synthesis of (Z)-4-benzylidene-2-phenyloxazol-5(4H)-one.

Table 1. Enzyme activity and binding study outcomes of compounds 4 and 5 with CAVA.

	Esterase assay IC_{50}, (μM)				Molecular Docking	
Compound No.	hCAVA	hCAII	hCAIX	Binding constant (*Ka*), M^{-1}	Binding energy (kcal/mol)	No. of Hydrogen Bonds and other binding residues
4	0.224 ± 0.36	11.41 ± 0.36	2.44 ± 0.012	1.9×10^7	−8.5	8 (Zn, Trp96, Leu101, Gln103, Gln128, His130, His132, His155, Val157, Tyr167, Lys168, Val171, Thr235, Thr236, Pro238)
5	2.75 ± 0.22	9.11 ± 0.36	5.33 ± 0.01	4.9×10^4	−8.1	4 (Zn, Trp96, Thr98, Leu101, Gln103, His130, His155, Gln128, Val157, Tyr167, Leu177, Val179, Thr235, Thr236, Val243, Trp245)
AZM	0.067 ± 0.004	0.015±.001	0.036 ± 0.002	Not determined	−6.6	5 (Zn, Gln103, Gln128, His130, His132, His155, Val157, Tyr167, Val171, Val179, Leu234, Thr235, Thr236, Val243, Trp245)

Rao & Filler, 1975). Finally, Oxazole-5(4H)-one derivatives were characterized by different spectroscopic techniques including ^{1}H NMR, ^{13}C NMR, and LC-MS mass spectrometry. All the compounds displayed only one ^{1}H NMR signal for the vinyl proton in the region δ 7.1- 7.4, which confirmed the presence of only one geometric isomer, which is the thermodynamically more stable Z isomer with established literature. Mechanism for the synthesis of (Z)-4-benzylidene-2-phenyloxazol-5(4H)-one is shown in Figure 3 (Wang, 2010).

3.2. Enzyme inhibition studies

The enzymatic activity of human CAVA, CAIX, and CAII was evaluated with increasing concentration of each synthesized compound while acetazolamide was used as a standard positive control. We have found that, out of five synthesized molecules, only two molecules (compound 4 and 5) show higher inhibition activity towards hCAVA. Inhibitory results were quite riveting as demonstrated by the significant inhibitory activity of compounds 4 and 5 for the hCAVA with IC_{50} values of 0.224 ± 0.36 μM and 2.75 ± 0.22 μM, respectively. The corresponding IC_{50} values of compound 4 and 5 for hCAIX were 2.44 ± 0.012 μM and 5.33 ± 0.01 μM, respectively. Whereas for hCAII, the IC_{50} values of compound 4 and 5 were 11.41 ± 0.36 μM and 9.11 ± 0.36 μM, respectively (Table 1). These results indicate that compound 4 and 5 have a greater affinity towards hCAVA compared to hCAIX and hCAII. The similar architecture of the binding cavity may be responsible for modest activity towards hCAIX and hCAII.

3.3. Molecular docking

Molecular docking has been extensively used to investigate the mode of interactions of designed inhibitors to their receptors (Naqvi et al., 2018, 2019; Naz et al., 2015, 2016, 2017, 2018). Docking results showed that compound 4 and 5 binds to the active site cavity of CAVA with affinities of −8.5 kcal/mol and −8.1 kcal/mol, respectively. Docking studies of compound 4 and 5 into the active site of CAVA showed that these molecules offer several interactions to the binding residues (Figure S1). In particular, the compound 4 forms eight hydrogen bonds with Gln103, Hius130, His132, Thr235, Thr236, and zinc ion, and interacts with Trp96, Leu101, Gln128, His155, Val157, Tyr167, Lys168, Val171 and

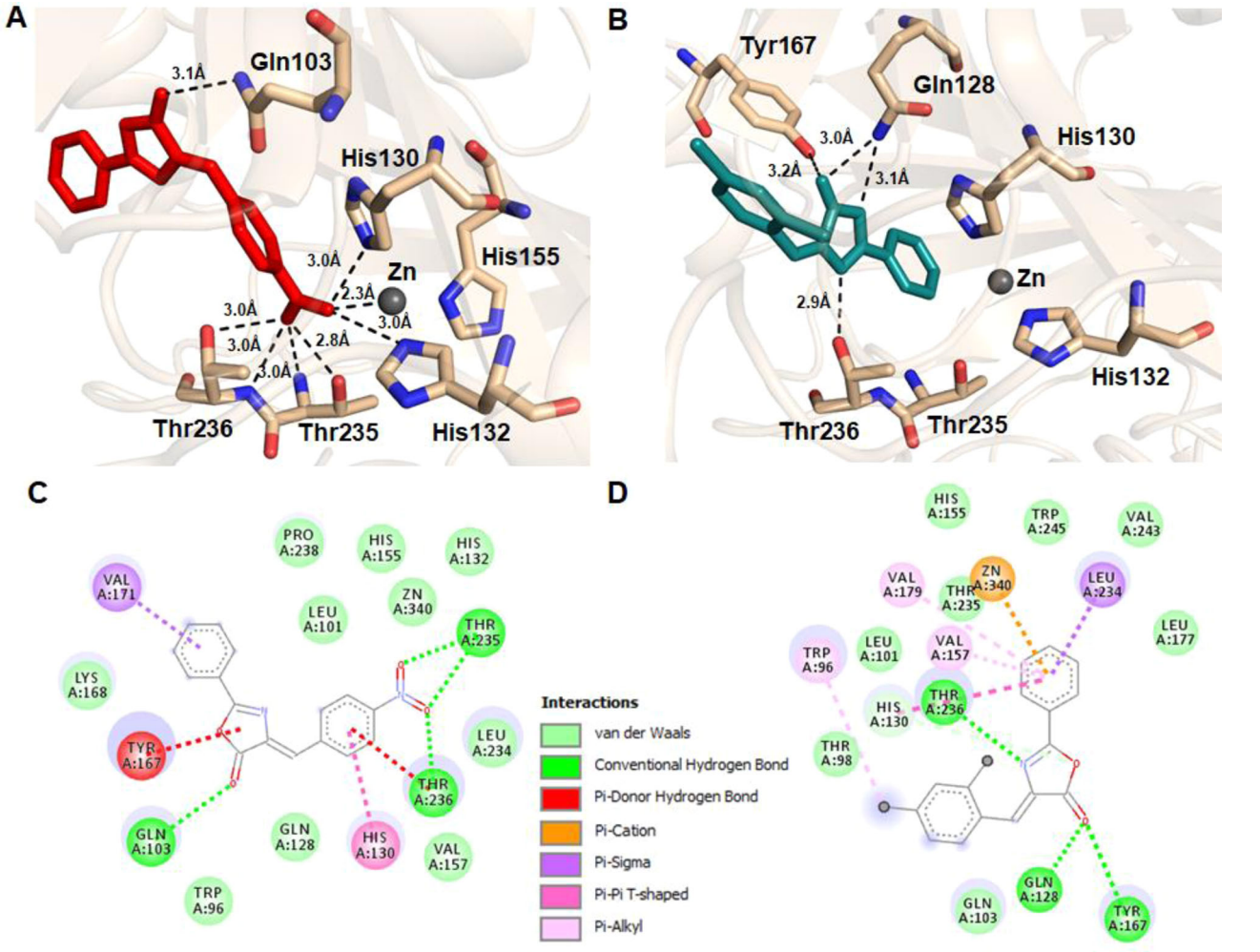


Figure 4. Molecular docking studies of compound 4 and 5 with CAVA: Three-dimensional focused view of binding pocket residues showing the hydrogen bond-forming residues of CAVA docked with **(A)** compound 4 **(B)** compound 5 (compound 4: red color stick model, compound 5: deep teal color stick model, residues of CAVA participating in H-bonding interactions are shown connected in dotted black lines). The grey color sphere represents the zinc atom. 2D representation of residues of CAVA docked complex involved in different interactions like van der Waals interactions, hydrogen bonding, charge or polar interactions with, **(C)** compound 4, **(D)** compound 5 (see inset for each type of interaction and respective color).

Pro238 (Figure 4(A, C)). Compound 5 also forms four hydrogen bonds with Gln128, Tyr167, and Thr236. It also interacts with zinc ion, Trp96, Thr98, Leu101, Gln103, His130, His155, Val157, Leu177, Val179, Thr235, Val243, Trp245 (Figure 4(B, D)). The binding poses of compound 4 show that the para-substituted nitro group offers hydrogen bonds to Thr235 and Thr236, the important residues of CAVA active site. O-atom of oxazol-5(4H)-one ring of compound 4 stabilizes the complex by forming a hydrogen bond with Gln103 and, forms π-alkyl, π-donor, and π-sigma interactions with His130, Tyr167, and Val171, respectively (Figure 4(C)). Interestingly, compound 4 directly interacts with the zinc metal ion present in the cavity of CAVA. These interactions hinder the involvement of zinc atom with the active site residues of CAVA and thus decreasing the activity.

In the case of compound 5, binding analysis shows that chlorine atoms form π-alkyl bonds with Trp96. N-atom of oxazol-5(4H)-one ring of compound 5 stabilizes the complex by forming a hydrogen bond with Thr236 and O-atom form two hydrogen bonds with Gln128 and Tyr167 (Figure 4(B, D)). Phenyl ring of compound 5 forms π-alkyl and π-sigma interactions with Val157, Val179, and Leu234, respectively (Figure 4(D)). The reported crystal structure and inhibition studies of other CA isoforms suggested that these residues are mainly responsible for the activity and interaction of known inhibitor of CAs, and these interactions stabilizes the protein-ligand complex (Mujumdar et al., 2019; Sjoblom et al., 2009). The binding of these compounds also involves similar types of interactions with the CAVA catalytic domain, which might be responsible for the reduced catalytic activity. The surface representations also showed that these compounds occupy the internal cavity of CAVA (Figures 4 and S1).

Interestingly, when compared the binding mode of these compounds with acetazolamide (AZM), a non-specific inhibitor of CAs, it was found that these compounds bind to the same binding site of CAVA where AZM binds (Figure 5). These compounds shared the identical residues of the binding cavity for hydrogen bonding as AZM (Figure 5(A–C)). These molecules share the critical important binding residues with AZM, and the results of our study corroborate previous reports, which suggested that CAVA inhibitors significantly bind with these residues (Capkauskaite et al., 2018; Costa et al., 2019; Poli et al., 2020). It means that these compounds

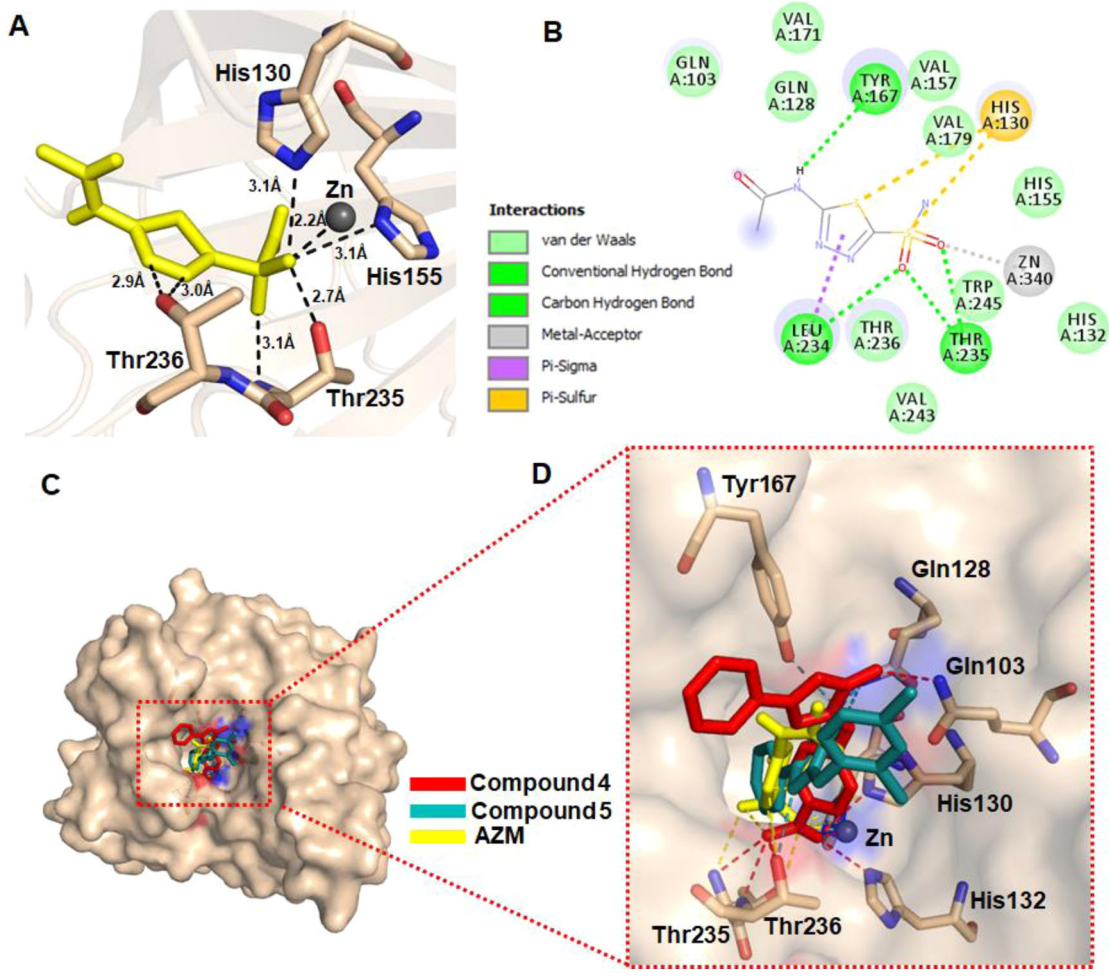


Figure 5. Comparative molecular docking studies of compound 4, 5, and acetazolamide with CAVA: **(A)** Three-dimensional focused view of binding pocket residues of CAVA showing the hydrogen bond-forming residues with acetazolamide (AZM). The yellow color stick model represents AZM, H-bonding interactions are shown in dotted black lines. The grey color sphere represents the zinc atom. **(B)** 2D representation of residues of CAVA involved in different interactions like van der Waals interactions, hydrogen bonding, charge, or π-interactions with AZM (see inset for each type of interaction and respective color). AZM: acetazolamide. The sphere represents a zinc atom. **(C)** Three-dimensional surface view of the docked complex of compounds 4, 5, and AZM in the binding pocket of CAVA: **(D)** Intensive view of binding pocket residues of CAVA that commonly participated in the hydrogen bonding with compound 4, 5, and AZM. The residues of CAVA participating in bonding are shown connected through H-bonding interactions with each ligand as presented in respective colored dotted lines.

might follow the similar mechanism of CA inhibition that AZM follows. Overall, all these observations established that the synthesized molecules interacted with catalytically essential residues of CAVA.

3.4. Fluorescence binding studies

Enzyme inhibition assay and *in silico* studies suggested that compounds 4 and 5 binds with CAVA and inhibited the activity, thus the actual binding affinity of these compounds with CAVA was carried out using fluorescence binding assay. The considerable decrease in the fluorescence emission of CAVA with the increasing concentrations of compounds 4 and 5 (Figure 6) suggested that these compounds bind with CAVA. The reduction of fluorescence intensity was analyzed using the modified Stern-Volmer equation and the binding constant (*Ka*) was calculated (Figure 6). The value of *Ka* for the binding of compound 4 and 5 with CAVA was found to be 1.9×10^7 M^{-1} and 4.9×10^4 M^{-1}, respectively. These results supported the *in-silico* results and further suggested that compound 4 and 5 physically interacted with CAVA and form a stable complex with it. The higher binding affinity of compound 4 towards CAVA compared to compound 5 also supports the strong inhibition potential of the former that had been observed. The obtained stoichiometry of each protein-ligand complex is 1:1.

3.5. Protein expression

To observe the effect of compound 4 and 5 on CAVA expression, 3T3-L1 cells were incubated with IC_{50} concentration of compounds for 24–48 h, and protein expression was analyzed using immunoblotting. It was found that the treatment of compounds decreases the expression of CAVA in 3T3-L1 cells (Figure 7(A)). These results suggested that the selected compound reduces the expression of CAVA. The high expression of CAVA directly correlated with obesity and thus to further validate the effect of CAVA inhibition, the expression of GLUT4 (transporter overexpressed in obesity) was also evaluated. Expression results showed that compound 4 and 5 also decreases the expression of GLUT4 (Figure 7(A)). The 3T3-L1 cells were used as an established model for obesity

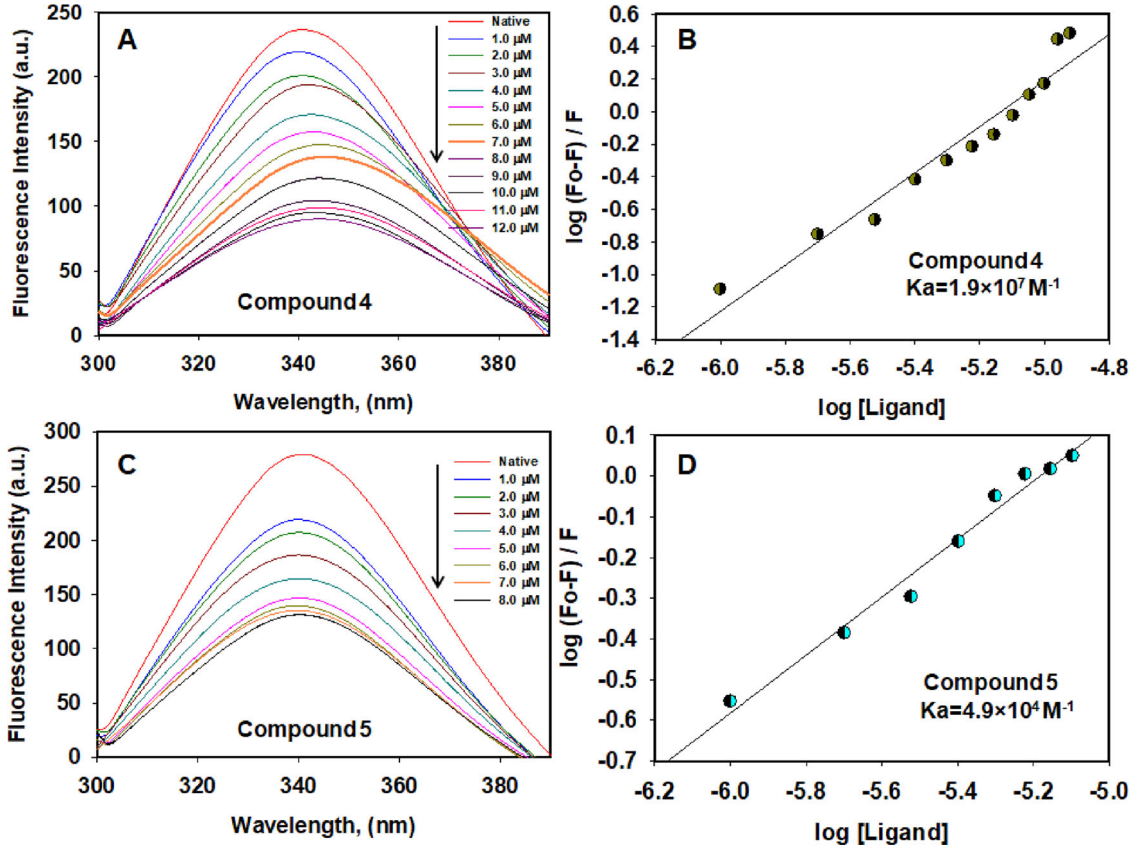


Figure 6. Fluorescence binding of compound 4 and 5 with CAVA. **(A)** The fluorescence emission spectrum of CAVA (5–10 µM) with increasing concentration of compound 4. **(B)** Stern-Volmer plot obtained from the fluorescence quenching data of CAVA with compound 4. **(C)** Fluorescence emission spectra of CAVA with increasing concentration of compound 5. **(D)** Stern-Volmer plot obtained from the fluorescence quenching data of CAVA with compound 5. For fluorescence studies protein sample was excited at 280 nm and emission of each titration was recorded in 300–400 nm range. The value of binding constant/number of binding sites was estimated using the Stern-Volmer plot.

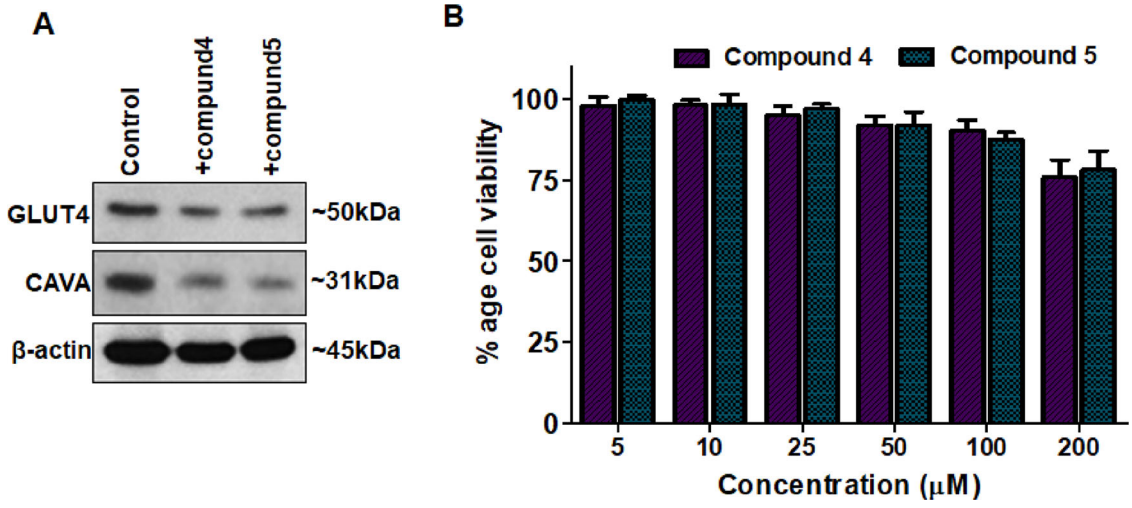


Figure 7. Protein expression and cytotoxicity studies of selected compounds. **(A)** Representative expression profile of CAVA and GLUT4 in compound 4 or 5 treated adipocytes (3T3-L1) cells w.r.t. control. Immunoblotting studies were performed after stipulated time treatment of compound 4 and 5, the expression studies showing that the treatment of cells with compound 4/5 decreases the protein level expression of CAVA and GLUT4. β-actin is taken as a loading control. **(B)** Cell viability studies of HEK293 cells in the presence of compound 4 and 5 as measured by MTT assay. Cells were treated with increasing concentration of compound 4/5 and the percentage cell viability was estimated with respect to the control cells (cells treated with vehicle control/DMSO) and plotted as a function of concentration.

or related disorders. Further, the expression of GLUT4 is very high in these cells (Dissanayake et al., 2018). In other words, the overexpression of CAVA and GLUT4 has been directly associated with obesity (Price et al., 2017; Shah et al., 2013). Therefore, the targeting of CAVA or GLUT4 is of great importance, particularly for obesity. Expression studies suggested that compound 4 and 5 decreases the expression of CAVA/GLUT4. These results support our rationale of CAVA

targeting molecule synthesis and advocate that these molecules have the potential to be evaluated for CAVA oriented obesity.

3.6. Cell cytotoxicity

The cytotoxicity studies of compound 4 and 5 were carried out on HEK293 cells. The cells were treated with increasing concentration (0–200 μM) of each compound and cell viability was accessed using MTT assay. Results showed that in the studied concentration range these compounds do not inhibit the growth of HEK293 cells (Figure 7(B)). These results suggested that the studied compounds does not show toxicity to HEK293 cells in the studied concentration range.

4. Conclusions

We have synthesized a series of molecules and evaluated them for *in vitro* hCAVA and hCAII inhibition activity. The biological assays reveal that electron-donating and electron-withdrawing functional groups at C4 substituted phenyl ring and a phenyl ring at C2 position of oxazolone moiety contribute towards inhibition of hCAVA more significantly. The presence of electron-withdrawing groups, 4-nitro and 2,4-dichloro, on the oxazole-5(4H)-one showed significant inhibitory activity of the hCAVA enzyme. On the other hand, the electron-donating substituent such as 4-methoxy, 4-methyl, and 3,5-dimethyl have negligible inhibitory activity towards hCAVA. Our studies suggest that the distinct architecture of C2 and C4 substituted oxazole-5(4H)-one is responsible for significant inhibitory activity hCAVA. The strong inhibition profile, high binding affinity, and potential to decrease the protein level expression suggested that these compounds target CAVA-related transporters and might be considered prospective anti-obesity lead molecules.

Disclosure statement

The authors declare no conflict of interest.

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References

Aneja, B., Queen, A., Khan, P., Shamsi, F., Hussain, A., Hasan, P., Rizvi, M. M. A., Daniliuc, C. G., Alajmi, M. F., Mohsin, M., Hassan, M. I., & Abid, M. (2020). Design, synthesis & biological evaluation of ferulic acid-based small molecule inhibitors against tumor-associated carbonic anhydrase IX. *Bioorganic & Medicinal Chemistry*, *28*(9), 115424. https://doi.org/10.1016/j.bmc.2020.115424

Beloglazkina, A. A., Wobith, B., Barskaia, E. S., Zefirov, N. A., Majouga, A. G., Beloglazkina, E. K., Zyk, N. V., Kuznetsov, S. A., & Zefirova, O. N. (2016). Synthesis and biological testing of (5Z)-2-aryl-5-arylmethylidene-3,5-dihydro-4H-imidazol-4-ones as antimitotic agents. *Medicinal Chemistry Research*, *25*(6), 1239–1249.

Biovia, D. S. (2013). *Discovery studio modeling environment*. Dassault Systemes.

Boaz, H., & Rollefson, G. (1950). The quenching of fluorescence. Deviations from the Stern-Volmer law. *Journal of the American Chemical Society*, *72*(8), 3435–3443.

Boriack-Sjodin, P. A., Heck, R. W., Laipis, P. J., Silverman, D. N., & Christianson, D. W. (1995). Structure determination of murine mitochondrial carbonic anhydrase V at 2.45-A resolution: Implications for catalytic proton transfer and inhibitor design. *Proceedings of the National Academy of Sciences*, *92*(24), 10949–10953.

Cannella, R., Clerici, F., Gelmi, M. L., Penso, M., & Pocar, D. (1996). A new synthesis of functionalized 2-alkylidenetetrahydro-5-furanones by tandem alkylation and translactonization reactions of 5(4H)-oxazolones. *The Journal of Organic Chemistry*, *61*(5), 1854–1856. https://doi.org/10.1021/jo951213n

Capkauskaite, E., Zaksauskas, A., Ruibys, V., Linkuviene, V., Paketuryte, V., Gedgaudas, M., Kairys, V., & Matulis, D. (2018). Benzimidazole design, synthesis, and docking to build selective carbonic anhydrase VA inhibitors. Bioorganic *and Medicinal Chemistry*, *26*, 675–687. https://doi.org/10.1016/j.bmc.2017.12.035

Carpene, C., Boulet, N., Chaplin, A., & Mercader, J. (2019). Past, present and future anti-obesity effects of flavin-containing and/or copper-containing amine oxidase inhibitors. *Medicines (Basel)*, *6*, E9. https://doi.org/10.3390/medicines6010009.

Chegwidden, W. R., & Spencer, I. M. (1996). Carbonic anhydrase provides bicarbonate for de novo lipogenesis in the locust. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *115*(2), 247–254.

Costa, G., Carta, F., Ambrosio, F. A., Artese, A., Ortuso, F., Moraca, F., Rocca, R., Romeo, I., Lupia, A., Maruca, A., Bagetta, D., Catalano, R., Vullo, D., Alcaro, S., & Supuran, C. T. (2019). A computer-assisted discovery of novel potential anti-obesity compounds as selective carbonic anhydrase VA inhibitors. *European Journal of Medicinal Chemistry*, *181*, 111565. https://doi.org/10.1016/j.ejmech.2019.111565

de Castro, P. P., Carpanez, A. G., & Amarante, G. W. (2016). Azlactone reaction developments. *Chemistry - A European Journal*, *22*(30), 10294–10318.

Dissanayake, W. C., Sorrenson, B., Cognard, E., Hughes, W. E., & Shepherd, P. R. (2018). beta-catenin is important for the development of an insulin responsive pool of GLUT4 glucose transporters in 3T3-L1 adipocytes. *Experimental Cell Research*, *366*(1), 49–54.

Dodgson, S. J. (1987). Inhibition of mitochondrial carbonic anhydrase and ureagenesis: A discrepancy examined. *Journal of Applied Physiology (1985)*, *63*(5), 2134–2141. https://doi.org/10.1152/jappl.1987.63.5.2134

Dodgson, S. J., & Cherian, K. (1989). Mitochondrial carbonic anhydrase is involved in rat renal glucose synthesis. *American Journal of Physiology-Endocrinology and Metabolism*, *257*(6), E791–E796.

Dodgson, S. J., Shank, R. P., & Maryanoff, B. E. (2000). Topiramate as an inhibitor of carbonic anhydrase isoenzymes. *Epilepsia*, *41*(s5), S35–S39.

Fareed, G., Afza, N., Versiani, M. A., Fareed, N., Mughal, U. R., Kalhoro, M. A., Iqbal, L., & Lateef, M. (2013). Synthesis, spectroscopic characterization and pharmacological evaluation of oxazolone derivatives. *Journal of the Serbian Chemical Society*, *78*(8), 1127–1134.

Forster, R. E., & Dodgson, S. J. (2000). Membrane transport and provision of substrates for carbonic anhydrase in vertebrates. In W. R.

Chegwidden, Y. Edwards & N. Carter (Eds.), *The carbonic anhydrases - New horizons* (pp. 263–280). Birkhauser Verlag.

Fujikawa-Adachi, K., Nishimori, I., Taguchi, T., & Onishi, S. (1999). Human mitochondrial carbonic anhydrase VB. cDNA cloning, mRNA expression, subcellular localization, and mapping to chromosome x. *The Journal of Biological Chemistry*, *274*(30), 21228–21233.

Gilmour, K. M., & Perry, S. F. (2009). Carbonic anhydrase and acid-base regulation in fish. *The Journal of Experimental Biology*, *212*(Pt 11), 1647–1661. https://doi.org/10.1242/jeb.029181

Gordon, A., & Price, L. H. (1999). Mood stabilization and weight loss with topiramate. *The American Journal of Psychiatry*, *156*(6), 968–969. https://doi.org/10.1176/ajp.156.6.968a

Hazen, S. A., Waheed, A., Sly, W. S., LaNoue, K. F., & Lynch, C. J. (1996). Differentiation-dependent expression of CA V and the role of carbonic anhydrase isozymes in pyruvate carboxylation in adipocytes. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *10*(4), 481–490. https://doi.org/10.1096/fasebj.10.4.8647347

Heck, R. W., Tanhauser, S. M., Manda, R., Tu, C., Laipis, P. J., & Silverman, D. N. (1994). Catalytic properties of mouse carbonic anhydrase V. *The Journal of Biological Chemistry*, *269*(40), 24742–24746.

Idrees, D., Kumar, S., Rehman, S. A. A., Gourinath, S., Islam, A., Ahmad, F., & Imtaiyaz Hassan, M. (2016). Cloning, expression, purification and characterization of human mitochondrial carbonic anhydrase VA. *3 Biotech*, *6*(1), 16. https://doi.org/10.1007/s13205-015-0334-1

Idrees, D., Prakash, A., Haque, M. A., Islam, A., Ahmad, F., & Hassan, M. I. (2016). Spectroscopic and MD simulation studies on unfolding processes of mitochondrial carbonic anhydrase VA induced by urea. *Journal of Biomolecular Structure and Dynamics*, *34*(9), 1987–1997. https://doi.org/10.1080/07391102.2015.1100552

Idrees, D., Shahbaaz, M., Bisetty, K., Islam, A., Ahmad, F., & Hassan, M. I. (2017). Effect of pH on structure, function, and stability of mitochondrial carbonic anhydrase VA. Journal *of Biomolecular Structure and Dynamics*, *35*(2), 449–461. https://doi.org/10.1080/07391102.2016.1149097

Imtaiyaz Hassan, M., Shajee, B., Waheed, A., Ahmad, F., & Sly, W. S. (2013). Structure, function and applications of carbonic anhydrase isozymes. *Bioorganic & Medicinal Chemistry*, *21*(6), 1570–1582. https://doi.org/10.1016/j.bmc.2012.04.044

Joshi, H., Upadhyay, P., Karia, D., & Baxi, A. J. (2003). Synthesis of some novel imidazolinones as potent anticonvulsant agents. *European Journal of Medicinal Chemistry*, *38*(9), 837–840.

Kakkar, S., & Narasimhan, B. (2019). A comprehensive review on biological activities of oxazole derivatives. *BMC Chemistry*, *13*(1), 16. https://doi.org/10.1186/s13065-019-0531-9531.

Keni, M., & Tepe, J. J. (2005). One-pot Friedel-Crafts/Robinson-Gabriel synthesis of oxazoles using oxazolone templates. *The Journal of Organic Chemi*stry, *70*(10), 4211–4213. https://doi.org/10.1021/jo0501590

Khan, K. M., Mughal, U. R., Khan, M. T. H., Perveen, S., & Choudhary, M. I. (2006). Oxazolones: New tyrosinase inhibitors; synthesis and their structure-activity relationships. *Bioorganic & Medicinal Chemistry*, *14*, 6027–6033. https://doi.org/10.1016/j.bmc.2006.05.014

Khan, K. M., Mughal, U. R., Lodhi, M. A., & Choudhary, M. I. (2008). Synthesis and chymotrypsin inhibitory activity of substituted oxazolones. *Letters in Drug Design & Discovery*, *5*(1), 52–56.

Khan, N. S., Khan, P., Ansari, M. F., Srivastava, S., Hasan, G. M., Husain, M., & Hassan, M. I. (2018). Thienopyrimidine-chalcone hybrid molecules inhibit fas-activated serine/threonine kinase: An approach to ameliorate antiproliferation in human breast cancer cells. *Molecular Pharmaceutics*, *15*(9), 4173–4189. https://doi.org/10.1021/acs.molpharmaceut.8b00566

Khan, P., Idrees, D., Moxley, M. A., Corbett, J. A., Ahmad, F., von Figura, G., Sly, W. S., Waheed, A., & Hassan, M. I. (2014). Luminol-based chemiluminescent signals: Clinical and non-clinical application and future uses. *Applied Biochemistry and Biotechnology*, *173*(2), 333–355. https://doi.org/10.1007/s12010-014-0850-1

Khan, P., Queen, A., Mohammad, T., Smita Khan, N. S., Hafeez, Z. B., Hassan, M. I., & Ali, S. (2019). Identification of alpha-mangostin as a potential inhibitor of microtubule affinity regulating kinase 4. *Journal of Natural Products*, *82*, 2252–2261. https://doi.org/10.1021/acs.jnatprod.9b00372

Khan, P., Rahman, S., Queen, A., Manzoor, S., Naz, F., Hasan, G. M., Luqman, S., Kim, J., Islam, A., Ahmad, F., & Hassan, M. I. (2017). Elucidation of dietary polyphenolics as potential inhibitor of microtubule affinity regulating kinase 4: In silico and and in vitro studies. *Scientific Reports*, *7*(1), 1–15. 10.1038/s41598-017-09941-4.

Lehtonen, J., Shen, B., Vihinen, M., Casini, A., Scozzafava, A., Supuran, C. T., Parkkila, A. K., Saarnio, J., Kivela, A. J., Waheed, A., Sly, W. S., & Parkkila, S. (2004). Characterization of CA XIII, a novel member of the carbonic anhydrase isozyme family. *The Journal of Biological Chemistry*, *279*(4), 2719–2727. https://doi.org/10.1074/jbc.M308984200

McConnell, D., Frajola, W. J., & Deamer, D. W. (1961). Relation between the inorganic chemistry and biochemistry of bone mineralization. *Science (New York, N.Y.)*, *133*(3448), 281–282. https://doi.org/10.1126/science.133.3448.281

Mesaik, M. A., Rahat, S., Khan, K. M., Choudhary, M. I., Murad, S. Z. I., & Ahmad, A. (2004). Synthesis and immunomodulatory properties of selected oxazolone derivatives. *Bioorganic & Medicinal Chemistry*, *12*, 2049–2057. https://doi.org/10.1016/j.bmc.2004.02.034

Minkin, C., & Jennings, J. M. (1972). Carbonic anhydrase and bone remodeling: sulfonamide inhibition of bone resorption in organ culture. *Science (New York, N.Y.)*, *176*(4038), 1031–1033. https://doi.org/10.1126/science.176.4038.1031

Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., & Olson, A. J. (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of Computational Chemistry*, *30*(16), 2785–2791.10.1002. https://doi.org/10.1002/jcc.21256

Mujumdar, P., Kopecka, J., Bua, S., Supuran, C. T., Riganti, C., & Poulsen, S. A. (2019). Carbonic anhydrase XII inhibitors overcome temozolomide resistance in glioblastoma. *Journal of Medicinal Chemistry*, *62*(8), 4174–4192. https://doi.org/10.1021/acs.jmedchem.9b00282

Naqvi, A. A. T., Jairajpuri, D. S., Noman, O. M. A., Hussain, A., Islam, A., Ahmad, F., Alajmi, M. F., & Hassan, M. I. (2019). Evaluation of pyrazolopyrimidine derivatives as microtubule affinity regulating kinase 4 inhibitors: Towards therapeutic management of Alzheimer's disease. *Journal of Biomolecular Structure and Dynamics*, *38*(13), 3892–3907. https://doi.org/10.1080/07391102.2019.1666745

Naqvi, A. A. T., Mohammad, T., Hasan, G. M., & Hassan, M. I. (2018). Advancements in docking and molecular dynamics simulations towards ligand-receptor interactions and structure-function relationships. *Current Topics in Medicinal Chemistry*, *18*(20), 1755–1768. https://doi.org/10.2174/1568026618666181025114157.

Naz, F., Khan, F. I., Mohammad, T., Khan, P., Manzoor, S., Hasan, G. M., Lobb, K. A., Luqman, S., Islam, A., Ahmad, F., & Hassan, M. I. (2018). Investigation of molecular mechanism of recognition between citral and MARK4: A newer therapeutic approach to attenuate cancer cell progression. *International Journal of Biological Macromolecules*, *107*(Pt B), 2580–2589. https://doi.org/10.1016/j.ijbiomac.2017.10.143

Naz, F., Shahbaaz, M., Bisetty, K., Islam, A., Ahmad, F., & Hassan, M. I. (2015). Designing new kinase inhibitor derivatives as therapeutics against common complex diseases: Structural basis of microtubule affinity-regulating kinase 4 (MARK4) inhibition. *Omics*: A *Journal of Integrative Biology*, *19*(11), 700–711. https://doi.org/10.1089/omi.2015.0111

Naz, H., Islam, A., Ahmad, F., & Hassan, M. I. (2016). Calcium/calmodulin-dependent protein kinase IV: A multifunctional enzyme and potential therapeutic target. *Progress in Biophysics and Molecular Biology*, *121*(1), 54–65. https://doi.org/10.1016/j.pbiomolbio.2015.12.016.

Naz, H., Khan, P., Tarique, M., Rahman, S., Meena, A., Ahamad, S., Luqman, S., Islam, A., Ahmad, F., & Hassan, M. I. (2017). Binding studies and biological evaluation of β-carotene as a potential inhibitor of human calcium/calmodulin-dependent protein kinase IV. *International Journal of Biological Macromolecules*, *96*, 161–170. https://doi.org/10.1016/j.ijbiomac.2016.12.024

Nishimori, I., Vullo, D., Innocenti, A., Scozzafava, A., Mastrolorenzo, A., & Supuran, C. T. (2005). Carbonic anhydrase inhibitors. The mitochondrial isozyme VB as a new target for sulfonamide and sulfamate inhibitors. *Journal of Medicinal Chemistry*, *48*(24), 7860–7866. https://doi.org/10.1021/jm050483n

Nocentini, A., & Supuran, C. T. (2018). Carbonic anhydrase inhibitors as antitumor/antimetastatic agents: A patent review (2008–2018). Expert

Opinion on Therapeutic Patents, *28*(10), 729–740. https://doi.org/10.1080/13543776.2018.1508453

Okamoto, M., Takayama, K., Shimizu, T., Muroya, A., & Furuya, T. (2010). Structure-activity relationship of novel DAPK inhibitors identified by structure-based virtual screening. *Bioorganic & Medicinal Chemistry*, *18*(7), 2728–2734. https://doi.org/10.1016/j.bmc.2010.02.018

Pasha, M. A., Jayashankara, V. P., Venugopala, K. N., & Rao, G. K. (2007). Zinc Oxide (ZnO): An efficient catalyst for the synthesis of 4-arylmethylidene-2-phenyl 5(4H)-oxazolones having antimicrobial activity. *Journal of Pharmacology and Toxicology* , *2*(3), 264–270.

Pastorekova, S., Parkkila, S., Pastorek, J., & Supuran, C. T. (2004). Carbonic anhydrases: Current state of the art, therapeutic applications and future prospects. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *19*(3), 199–299.

Pastorekova, S., Vullo, D., Casini, A., Scozzafava, A., Pastorek, J., Nishimori, I., & Supuran, C. T. (2005). Carbonic anhydrase inhibitors: Inhibition of the tumor-associated isozymes IX and XII with polyfluorinated aromatic/heterocyclic sulfonamides. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *20*(3), 211–217.

Peerzada, M. N., Khan, P., Ahmad, K., Hassan, M. I., & Azam, A. (2018). Synthesis, characterization and biological evaluation of tertiary sulfonamide derivatives of pyridyl-indole based heteroaryl chalcone as potential carbonic anhydrase IX inhibitors and anticancer agents. *European Journal of Medicinal Chemistry*, *155*, 13–23. https://doi.org/10.1016/j.ejmech.2018.05.034

Poli, G., Bozdag, M., Berrino, E., Angeli, A., Tuccinardi, T., Carta, F., & Supuran, C. T. (2020). N-aryl-N′-ureido-O-sulfamates as potent and selective inhibitors of hCA VB over hCA VA: Deciphering the binding mode of new potential agents in mitochondrial dysfunctions. *Bioorganic Chemistry*, *100*, 103896. https://doi.org/10.1016/j.bioorg.2020.103896

Postel, R., & Sonnenberg, A. (2012). Carbonic anhydrase 5 regulates acid-base homeostasis in Zebrafish. *PLoS ONE*, *7*(6), e39881.

Price, T. O., Sheibani, N., & Shah, G. N. (2017). Regulation of high glucose-induced apoptosis of brain pericytes by mitochondrial CA VA: A specific target for prevention of diabetic cerebrovascular pathology. *Biochimica et Biophysica Acta. Molecular Basis of Disease*, *1863*(4), 929–935.

Queen, A., Khan, P., Idrees, D., Azam, A., & Hassan, M. I. (2018). Biological evaluation of p-toluene sulphonylhydrazone as carbonic anhydrase IX inhibitors: An approach to fight hypoxia-induced tumors. *International Journal of Biological Macromolecules*, *106*, 840–850. https://doi.org/10.1016/j.ijbiomac.2017.08.082

Rao, Y. S. (1976). Reactions in polyphosphoric acid. I. New stereospecific synthesis of the E isomers of 2-phenyl-4-arylmethylene-2-oxazolin-5-ones. *The Journal of Organic Chemistry*, *41*(4), 722–725.

Rao, Y. S., & Filler, R. (1975). Geometric isomers of 2-aryl(aralkyl)-4-arylidene(alkylidene)-5-(4H)-oxazolones. *Synthesis*, *1975*(12), 749–764.

Salaroglio, I. C., Mujumdar, P., Annovazzi, L., Kopecka, J., Mellai, M., Schiffer, D., Poulsen, S.-A., & Riganti, C. (2018). Carbonic anhydrase XII inhibitors overcome P-glycoprotein-mediated resistance to temozolomide in glioblastoma. *Molecular Cancer Therapeutics*, *17*(12), 2598–2609.

Santos-Martins, D., Forli, S., Ramos, M. J., & Olson, A. J. (2014). AutoDock4(Zn): an improved AutoDock force field for small-molecule docking to zinc metalloproteins. *Journal of Chemical Information and Modeling*, *54*(8), 2371–2379.10.1021. https://doi.org/10.1021/ci500209e

Shah, G. N., Hewett-Emmett, D., Grubb, J. H., Migas, M. C., Fleming, R. E., Waheed, A., & Sly, W. S. (2000). Mitochondrial carbonic anhydrase CA VB: Differences in tissue distribution and pattern of evolution from those of CA VA suggest distinct physiological roles. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(4), 1677–1682. https://doi.org/10.1073/pnas.97.4.1677

Shah, G. N., Rubbelke, T. S., Hendin, J., Nguyen, H., Waheed, A., Shoemaker, J. D., & Sly, W. S. (2013). Targeted mutagenesis of mitochondrial carbonic anhydrases VA and VB implicates both enzymes in ammonia detoxification and glucose metabolism. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(18), 7423–7428. https://doi.org/10.1073/pnas.1305805110

Shamsi, F., Hasan, P., Queen, A., Hussain, A., Khan, P., Zeya, B., King, H. M., Rana, S., Garrison, J., Alajmi, M. F., Rizvi, M. M. A., Zahid, M., Imtaiyaz Hassan, M., & Abid, M. (2020). Synthesis and SAR studies of novel 1,2,4-oxadiazole-sulfonamide based compounds as potential anticancer agents for colorectal cancer therapy. *Bioorganic Chemistry*, *98*, 103754. https://doi.org/10.1016/j.bioorg.2020.103754

Sjoblom, B., Polentarutti, M., & Djinovic-Carugo, K. (2009). Structural study of X-ray induced activation of carbonic anhydrase. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(26), 10609–10613. https://doi.org/10.1073/pnas.0904184106

Sly, W. S., & Hu, P. Y. (1995). Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annual Review of Biochemistry*, *64*, 375–401. https://doi.org/10.1146/annurev.bi.64.070195.002111

Smaine, F.-Z., Pacchiano, F., Rami, M., Barragan-Montero, V., Vullo, D., Scozzafava, A., Winum, J.-Y., & Supuran, C. T. (2008). Carbonic anhydrase inhibitors: 2-substituted-1,3,4-thiadiazole-5-sulfamides act as powerful and selective inhibitors of the mitochondrial isozymes VA and VB over the cytosolic and membrane-associated carbonic anhydrases I, II and IV. *Bioorganic & Medicinal Chemistry Letters*, *18*(24), 6332–6335. https://doi.org/10.1016/j.bmcl.2008.10.093

Soldavini, J., & Kaunitz, J. D. (2013). Pathobiology and potential therapeutic value of intestinal short-chain fatty acids in gut inflammation and obesity. *Digestive Diseases and Sciences*, *58*(10), 2756–2766. https://doi.org/10.1007/s10620-013-2744-4

Supuran, C. T. (2003). Carbonic anhydrase inhibitors in the treatment and propylaxis of obesity. *Expert Opinion on Therapeutic Patents*, *13*(10), 1545–1550.

Supuran, C. T. (2004). Carbonic anhydrases: Catalytic and inhibition mechanisms, distribution and physiological roles. In C. T. Supuran, A. Scozzafava & J. Conway (Eds.), *Carbonic anhydrase* (pp. 1–23). CRC Press.

Supuran, C. T. (2010). Carbonic anhydrase inhibitors. *Bioorganic & Medicinal Chemistry Letters*, *20*(12), 3467–3474. https://doi.org/10.1016/j.bmcl.2010.05.009

Supuran, C. T. (2018). Carbonic anhydrase inhibitors as emerging agents for the treatment and imaging of hypoxic tumors. *Expert Opinion on Investigational Drugs*, *27*(12), 963–970. https://doi.org/10.1080/13543784.2018.1548608

Supuran, C. T., & Scozzafava, A. (2002). Applications of carbonic anhydrase inhibitors and activators in therapy. *Expert Opinion on Therapeutic Patents*, *12*(2), 217–242.

Tandon, M., Coffen, D. L., Gallant, P., Keith, D., & Ashwell, M. A. (2004). Potent and selective inhibitors of bacterial methionyl tRNA synthetase derived from an oxazolone-dipeptide scaffold. *Bioorganic & Medicinal Chemistry Letters*, *14*(8), 1909–1911. https://doi.org/10.1016/j.bmcl.2004.01.094

Tashian, R. E., Venta, P. J., Nicewander, P. H., & Hewett-Emmett, D. (1990). Evolution, structure and expression of the carbonic anhydrase multigene family. *Progress in Clinical and Biological Research*, *344*, 159–175.

Thakur, P. K., & Hassan, M. I. (2011). Discovering a potent small molecule inhibitor for gankyrin using de novo drug design approach. *International Journal of Computational Biology and Drug Design*, *4*(4), 373–386. https://doi.org/10.1504/IJCBDD.2011.044404

Trott, O., & Olson, A. J. (2010). AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, *31*(2), 455–461.10.1002. https://doi.org/10.1002/jcc.21334

Vullo, D., Franchi, M., Gallori, E., Antel, J., Scozzafava, A., & Supuran, C. T. (2004). Carbonic anhydrase inhibitors. Inhibition of mitochondrial isozyme V with aromatic and heterocyclic sulfonamides. *Journal of Medicinal Chemistry*, *47*(5), 1272–1279. https://doi.org/10.1021/jm031057+

Wang, Z. (2010). Erlenmeyer-Plochl Azlactone Synthesis, Chapter 217. In Comprehensive Organic Name Reactions and Reagents (pp. 997–1000). John Wiley & Sons.

Winum, J. Y., Scozzafava, A., Montero, J. L., & Supuran, C. T. (2005). Sulfamates and their therapeutic potential. *Medicinal Research Reviews*, *25*(2), 186–228. https://doi.org/10.1002/med.20021