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Design and synthesis of a novel class of carbonic anhydrase-IX inhibitor 1-(3-(phenyl/4-fluorophenyl)-7-imino-3H-[1,2,3]triazolo[4,5d]pyrimidin 6(7H)yl)urea

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ABSTRACT

Carbonic anhydrase IX (CAIX) is a promising target in cancer therapy especially in the case of hypoxia-induced tumors. The selective inhibition of CA isozymes is a challenging task in drug design and discovery process. Here, we performed fluorescence-binding studies and inhibition assay combined with molecular docking and molecular dynamics (MD) simulation analyses to determine the binding affinity of two synthesized triazolo-pyrimidine urea derived (TPUI and TPUII) compounds with CAIX and CAII. Fluorescence binding results are showing that molecule TPUI has an excellent binding-affinity for CAIX (k_D = 0.048 μM). The TPUII also exhibits an appreciable binding affinity (k_D = 7.52 μM) for CAIX. TPUI selectively inhibits CAIX as compared to TPUII in the 4-NPA assay. Docking studies show that TPUI is spatially well-fitted in the active site cavity of CAIX, and is involve in H-bond interactions with His94, His96, His119, Thr199 and Thr200. MD simulation studies revealed that TPUI efficiently binds to CAIX and essential active site residual interaction is consistent during the entire simulation of 40 ns. These studies suggest that TPUI appeared as novel class of CAIX inhibitor, and may be used as a lead molecule for the development of potent and selective CAIX inhibitor for the hypoxia-induced cancer therapy.

1. Introduction

Carbonic anhydrase (CAs) are zinc containing metalloenzymes which primarily regulate physiological pH in human body [1]. CA catalyzes a rapid reversible conversion of carbon dioxide (CO_2) and water (H_2O) into a proton (H^+) and bicarbonate ion (HCO_3^-) which regulates the physiological processes connected with the transport of CO_2/HCO_3^-, homeostasis, electrolyte secretion in a varieties of tissues/organs, biosynthetic reactions, respiration, calcification, tumorigenicity and bone reabsorption [2,3]. In human there are 16 αCA isozymes have been reported which exhibit variable tissue distribution and functions [4].

CAIX is an attractive target for cancer treatment because of its limited expression in the normal tissues and predominant expression in varieties of tumour cells [5,6]. CAIX is a hypoxia-inducible protein that regulates cellular pH to encourage cancer cell survival and invasion in the hypoxic microenvironments and serve as a potential biomarker in the poor prognosis of breast cancer metastasis and survival [7–9]. Furthermore, extracellular location of this isozyme is favorable for designing of a selective inhibitor which inhibits the membrane associated CAs without interacting to other cytosolic and mitochondrial CAs [10–12]. Expression of CAIX is induced under hypoxic conditions and their activity leads to pH imbalance in tumor tissues and thus provides favorable environment for the survival of cancer cells [13,14]. Moreover, it contributes to cancer progression by stimulating cancer cell migration, adhesion and invasion. Now a day's CAIX has been identified as an important biomarker of hypoxia and its over-expression is often associated with a poor responsiveness to the classical radio and chemotherapies [15,16]. Therapeutic inhibition of CAIX has been

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shown to decrease primary tumor growth and metastasis in various tumor models [17].

Inorganic anions, sulfonamides, sulfamates, sulfamides, phenols are important class of pharmacophores that inhibit CAIX very efficiently by participating in Zn binding [18–20]. Acetazolamide **1** (Fig. S1) is a potent CA inhibitor which inhibits CA isoform in a nonselective manner through participating in Zn-binding [21]. Recently, Korkmaz et al. [22] reported a series of phenyl thiourea derivatives as novel class of CA inhibitors. In this series derivative **2** (Fig. S1) inhibit CA with micro molar range activities. Coumarins appeared as new class of CAIs that binds (in hydrolyzed form) at the entrance of the CA active site and does not interact with the Zn metal. Thiocoumarine derivatives **3** (Fig. S1) exhibits an excellent inhibitory activity for CAIX without showing Zn metal binding in active site of CA [23]. Moreover, coumarin derivative **4** (Fig. S1) was also successfully inhibits CA isoform without displaying Zn-binding [24]. Thus, coumarins show entirely new category of mechanism to inhibit CA enzyme. Numerous, potent sulfonamide based CAIX inhibitors have already been identified in the last years that can efficiently inhibit CAIX activity [22]. However, most of the clinically used CA-inhibitors exhibit broad CA-inhibition and possesses poor isozyme subtype selectivity [25,26]. Therefore, a selective CAIX inhibitor is essentially required in cancer therapy without side effects related to the inhibition of other CA isozymes [2,27]. Novel chemical scaffolds which endowed with improved activity as well as selectivity for CAIX are essentially required for the development of successful anticancer agents.

Triazoles have emerged as an important class of heterocyclic compound to design potent therapeutic drug molecules [28]. Numerous triazole derivatives have already showed promising anticancer activity in various *in vitro* as well as *in vivo* models [29–31]. Recently, triazole-linked O-glycosides (compound **5**, Fig. S1) have been used as a selective and potent CAIX inhibitor [27]. Moreover, pyrimidine derivatives (compound **6**, Fig. S1) also displayed potent CA inhibitory activity. [32,33]. Hence, triazole coupled pyrimidine molecules would be a promising scaffold for the development of potent and selective CAIX inhibitors toward the cancer therapy.

In this view, we have design and synthesized urea pendant triazolo-pyrimidine derivatives (TPUI and TPUII) as a novel class of CAIX inhibitors (Fig. S1, compound **7**). The designed molecules are endowed with essential pharmacophoric requirement such as hydrophobic domain (triazolo-pyrimidine) as well as hydrogen bond donor (HBD)/hydrogen bond acceptor (HBA) (Urea fragment) domain (Fig. S1). The designed compounds have a urea functional group instead of the traditional sulfonamide group. This alteration might open a new door for a novel class of pharmacophore as selective CAIX inhibitors. Binding affinities of TPUI and TPUII with CAIX as well as CAII were assessed by spectrofluorimeter. *In vitro* CA inhibition studies of TPUI and TPUII against CAIX as well as CAII were studied by the 4-NPA spectrophotometric assay. Moreover, CAIX selective derivative TPUI was docked with human CAIX to evaluate its interaction potential and sitting pattern in active site CAIX isoforms. Furthermore, MD simulation studies were also performed to evaluate the extensive binding prototype of synthesized triazolo-pyrimidine urea derivatives with CAIX.

2. Materials and methods

2.1. Synthesis of TPUI and TPUII

All chemicals, reagents and solvents were procured from Sigma–Aldrich (St. Louis, MO, USA), S.D Fine Chemicals (India) and Merck (Darmstadt, Germany). The homogeneity and purity of the compounds were checked by thin layer chromatography (TLC), performed on commercially available silica gel (Kieselgel 60, F_{254}) coated aluminium sheets (Merck) by using methanol: chloroform (5:95) as solvent system. The visualization on TLC was done by both ultra-violet (UV) light ($\lambda = 254$ nm) and iodine indicator. All compounds were purified by column chromatography by using silica gel 100–200 mesh (Merck). Melting points were determined in open capillary tubes in a Hicon melting point apparatus (Hicon, India). The nuclear magnetic resonance (NMR) spectra were obtained on high resolution Jeol-400 MHz NMR spectrophotometer (USA) in $CDCl_3$ and DMSO-d_6 using Tetramethylsilane (TMS) as the internal reference. Chemical shifts (δ) were expressed in parts per million relative to TMS and the following abbreviations were used to describe the peak patterns when appropriate: s, (singlet); d, (doublet); t, (triplet); m, (multiplet); brs, (broad singlet); dd, (double doublet). The coupling constant (J) values are given in hertz (Hz). Mass spectra (LRMS) were recorded on an Agilent 6310 Ion trap LC/MS and elemental analysis (C, H and N) was carried on Elementar analysensysteme GmbH. A detail of chemical analysis is provided in the Supplementary Text for Chemistry Section.

2.2. Cloning, expression and purification of CAIX and CAII

The 1131-bp coding region (38-414aa) of catalytic domain of CAIX gene was sub-cloned into pET21c (Novagen) vector with the C-terminal His6 tag. Similarly, CAII gene of 801-bp was sub-cloned into pET15d vector (Novagen) with N-terminal His6Xtag. Both proteins were expressed in *Escherichia coli* BL21 (DE3) cells. CAIX was expressed at 16 °C for 14 h after induction with 0.5 mM IPTG (Sigma). CAIX was expressed as insoluble protein and form inclusion bodies (IBs) and pellets were dissolved in the suspension buffer (50 mM phosphate, pH 7.4, 300 mM NaCl, 1% Triton-X and 1% *N*-lauroylsarcosine) to solubilize IBs. These solubilized IBs were sonicated and centrifuged for 30 min at 12,000 rpm. This step was repeated thrice and finally supernatant was applied on a Ni-NTA Sepharose column (GE Health-care). CAIX was eluted from the column with elution buffer (50 mM phosphate, pH 7.4, 300 mM NaCl, 1% *N*-lauroylsarcosine and 350 mM imidazole). CAIX was refolded by dialyzing against refolding buffer (50 mM phosphate buffer pH 7.4 and 150 mM NaCl) for 36 h at 4 °C with five successive buffer changes. We also performed enzyme assay of refolded CAIX (*34*). Fig. S2 A and B are showing the SDS-PAGE and Western blot, respectively of purified CAIX.

CAII was expressed at 37 °C for 3 h after induction with 0.25 mM IPTG (Sigma) as soluble protein, harvested cells were suspended in buffer (50 mM phosphate, pH 7.4, 300 mM NaCl, 1% Triton-X and 0.1 mg/ml lysozyme). Cell lysate was sonicated and centrifuged to remove cell debris. Supernatant was applied to Ni-NTA Sepharose column (GE Health-care) and eluted with elution buffer (50 mM phosphate, pH 7.4, 300 mM NaCl, and 300 mM imidazole). The eluent was collected, concentrated and loaded to gel-filtration chromatography (superdex200, GE Healthcare Bio-Sciences) in 50 mM phosphate, pH 7.4 and 150 mM NaCl buffer (*35*). Both proteins were analyzed by SDS-PAGE and identified by Western blotting with anti-histidine antibodies. Fig. S2 C and D are showing the SDS-PAGE and Western blot, respectively of purified CAII.

2.3. Fluorescence spectra measurements

Fluorescence measurement was performed in the Jasco spectrofluorimeter (Model FP-6200) using 5 mm quartz cuvette. Protein concentration of 3.4 μM and 2.8 μM were used for CAII and CAIX, respectively. Compounds (TPUI and TPUII) were used in a concentration 1–10 μM. The protein solution was excited at 292 nm, and emission spectra were recorded between 300–400 nm. Both compounds were dissolved in DMSO and then diluted to 1 mg/ml in the 50 mM phosphate buffer pH 7.4 and 150 mM NaCl. The