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STRUCTURE-BASED DESIGN OF NON-COVALENT CARBONIC ANHYDRASE IX/XII INHIBITORS IN CANCER THERAPEUTICS

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ABSTRACT

Abstract Carbonic anhydrases IX and XII (CA-IX/XII) are Tran's membrane metalloenzymes that display overexpression in hypoxic tumor microenvironments; both have been well established as clinically relevant targetable molecules for anticancer drug discovery. Here we describe the computational design, synthesis and biological evaluation of a series of eight novel sulfonamide-based non-covalent inhibitors (CAI−01 to CAI−08) targeting with selectivity against CA-XII over the off-target isoform CA-II. Ligand-based and structure-based drug design methods together with 100 ns molecular dynamics (MD) simulation were used to assess binding stability of Auto Dock Vine 1.2 and GLIDE XP docking approaches, respectively. Swiss ADME and pk CSM-based in silicon ADMET profiling predicted good oral bioavailability (58.2–78.6%), low her cardio toxicity risk, and compliance with Lipinski's Rule of Five for six of the eight compounds. Development of the synthesized compounds was achieved via experimental validation through enzyme inhibition assays, which showed IC50 values at NM (7.1 to 21.3 for CA-IX and 9.2 to 28.6 for CA-XII) based competitive aspect obtained from selectivity indices above 20 with respect to CA-II. The lead compound CAI-06 displayed the greatest inhibition (CA-IX IC50 = 7.1 NM; CA-XII IC50 = 9.2 nM), high selectivity between CA-IX and CA-II with a ratio of activity estimated to be 44.0, and was further identified as one of the most potent inhibitors with respect to MCF-7 cell viability across all preforming compounds at 10 uM (72.4%). Pearson correlation analysis indicated that docking calculated binding free energies highly correlated with experimental IC50 values ($r = 0.912$, $p < 0.01$). Our results therefore identify the sulfonamide-triazole scaffold as a novel chemo type for pH-selective tumor -targeting CA inhibition and therefore further pre-clinical drug development.

Keywords: *Carbonic anhydrase IX[1]; carbonic anhydrase XII[2]; non-covalent inhibitors[3]; Sulfonamide scaffold[4]; Molecular docking[5], ADMET profiling[6]; Hypoxic tumor microenvironment[7].*

I. INTRODUCTION

1.1 CARBONIC ANHYDRASES AS VALIDATED ONCOLOGICAL TARGETS

Carbonic anhydrases (CAs) are a class of zinc dependent metalloenzymes that catalyze the reversible hydration of carbon dioxide in bicarbonate and a proton ($CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$). Of the sixteen genetically distinct isoforms that have been identified in humans, CA-IX and CA-XII are two very attractive targets in oncology as they are selectively and highly overexpressed in hypoxic solid tumors compared to normal tissues [1]. CA-IX is a well-established endogenous marker of tumors hypoxia in various carcinomas, such as breast, colorectal, and cervical carcinomas as well as non-small cell lung cancer [2], where its expression is directly controlled by the transcriptional axis hypoxia-inducible factor-1α (HIF-1α). CA-XII is expressed in a much wider range of tissues but has also been found to be over-expressed in renal cell carcinoma and hormone-receptor-positive breast cancers. Both isoforms play crucial roles in maintaining intracellular pH homeostasis in the acidic tumors microenvironment that assists tumors invasion, metabolic adaptation, and sustained survival by exporting protons [3]. Pharmacological blockade of these isoforms, therefore, has therapeutic promise not only as a cytostatic but also to reverse tumors acidification, to tumors hypoxic cells to chemo- and radiotherapy and thus impair metastatic dissemination [4].

1.2 ADVANCES IN NON-COVALENT INHIBITOR DESIGN STRATEGIES

The most common CA inhibitors (CAIs) are dependent on a core sulfonamide or sulfonic function that coordinates the active-site zinc ion, displacing the Zn-coordinated hydroxide and forming an ultra-high-affinity non-covalent interaction network with comparatively conserved active residues such as His94, His96, His119, and Thr200 [5]. The lack of isoform selectivity in early pan-CAI agents, e.g. acetazolamide and methazolamide, limits therapeutic use because systemic side effects emerge from inhibition of cytosolic CA-I or CA-II leading to diuretic action, metabolic acidosis, and neurological adverse effects [6]. The main structural differences between tumors -associated and cytosolic isoforms are the arrangement of hydrophilic and hydrophobic sub packets surrounding the catalytic zinc [7], as well as in vestibular and rim regions accessible to extended pharmacophoric tails. Recent medicinal chemistry efforts have thus shifted toward inhibitors designed to bind the tails, whereby a ZBG is connected to a hydrophobic scaffold via a linker that probes these paralog-variable binding sub-regions [8]. Among these are coumarone, saccharin's, benzoxazoles, carbohydrate-based sulfonamides, and photo-switchable azo-derivatives; however, none of these agents have yet found the optimal fulcrum between potency, selectivity between protease isoforms and pharmacokinetic suitability for clinical deployment [9]. As opposed to irreversible covalent warheads, the non-covalent mode of inhibition is favorable in this context as it provides limited off-target toxicity, tunable duration of action and more manageable structure–activity relationships.

1.3 RATIONALE AND OBJECTIVES OF THE PRESENT STUDY

Notably, all advances mentioned above collectively represent a large gap; the systematic combination of multi-parametric computational screening with isoform-selective experimental validation across a structurally diverse compound library targeting concurrent inhibition of both CA-IX and CA-XII has not been effectively achieved to date. The majority of studies in the literature focus on a single-isoform selectivity, where dual CA-IX/XII inhibition strategy remains to be explored [10]. In addition, the activity profiles for candidate CAIs with respect to pH which is particularly relevant in the context of the acidic tumor's microenvironment are seldom reported, reducing their translational relevance from in vitro enzyme inhibition data [11]. We addressed these gaps in the present study with an integrated workflow comprising four stages: (i) rational pharmacophore-guided design of

eight sulfonamide-triazole hybrids, (ii) multi-conformational molecular docking against CA-IX and CA-XII crystal structures available from the Protein Data Bank (PDB: 3IAI; PDB: 1JD0), using inhibition data for acetazolamide as a benchmark, (iii) 100 ns all-atom MD simulations to evaluate binding mode stability, and finally (iv) experimental validation including enzyme inhibition kinetics, in vitro cytotoxicity assays in MCF-7 breast cancer cells, and pH-activity profiling. The goals were to identify Nano molar dual CA-IX/XII lead compounds, to perform quantitative structure activity relationship studies, to conduct statistical correlation and comparative analysis against published clinical trial data, and to outline a medicinal chemistry path toward preclinical optimization.

II. LITERATURE SURVEY

Inhibitors designed to target CA-IX/XII have been the focus of nearly thirty years of work incorporating aspects of enzymology and medicinal chemistry, resulting in a transition from scaffold-nonspecific acetazolamide-like compounds to precision-engineered isoform-selective small-molecules. The structural work of described the underlying catalytic mechanism, active-site architecture and zinc coordination geometry that provides the foundation for all modern CAI design. The availability of the first crystal structures deposited in the Protein Data Bank (PDB) for CA-IX and CA-XII, including 3IAI and 1JD0 entries which describe the structure of CA-IX: acetazolamide complex [12], provided unprecedented opportunities for development of structure-based drug design at atomic-resolution level. Additional Identifying Characteristics: CA-IX is associated with a proteoglycan-like (PG) domain, has a relatively larger hydrophobic pocket at the active site compared to the CA-XII is enzyme, and displays distinct glycosylation patterns that affect inhibitor binding kinetics [13]. Prior to the year 2010, early classes of inhibitors reported, still using primary sulfonamide ZBGs with simple scaffolds such as benzene or pyridine systems only achieved sub micro molar albeit non-selective decrements. Small molecular saccharin-based inhibitors were synthesized by Carta et al. The authors showed that five-membered ring heterocyclic ZBGs can bind the zinc ion in a slightly different geometry, improving target selectivity for CA-IX versus CA-II only to a modest level [14]. The first class of mechanism-based inhibitors that do not directly coordinate to zinc were discovered by Maresca et al. 2010, 2022) as hydrolysis-dependent pro drug CAIs targeting the hydrophobic patch of the active site [15]. In addition, reported CA-XII IC50 values were sub-micro molar with >40-fold selectivity over CA-II, which rivalled the most potent compounds within this study as they are based on urea-sulfonamides also developed by the same group. Angeli et al. 2021 used CA-IX as a test case in to explore a series of N-substituted benzamine sulfonamides, finding that the ortho-chloro-substituted analogue had an IC50 against CA-IX of 11.2 nM and a remarkable degree of agreement between docking binding free energies determined using Auto Dock-GPU (TMP) and experimental Kis from established approaches, justifying the approach taken in this current study.

However, the compound SLC-0111 (WBI-5111) which is a fluorobenzyl ureido sulfonamide developed by Winum, Supuran and co-workers [20], has emerged as the most advanced CA-XII-selective inhibitor entering Phase I/II clinical trials in patients with solid tumors in combination with gemcitabine with strong selectivity for CA-XII (CA-XII IC50 = 4.5 nM; >10-fold selectivity CA-XI vs. CA-XII; >70-fold selectivity versus all other classes of CAs). Nonetheless, its substantial sparing of CA-IX raises the question of whether a combined dual inhibition could provide greater antitumor efficacy in hypoxic and/or CA-IX-overexpressing tumors. Ronca et al. (2022) reported a series of indole-fused sulfonamides that were nanomolar inhibitors against both isoforms,

IC50 8.4 nM (CA-IX), 11.3 nM (CA-XII), and useful in MCF-7 xenograft models which resemble the desired pharmacological profile for the present work most closely [18]. Systematic stability analyses based on MD simulations have been performed (simulating complexes using GROMACS 2020) and reported RMSD values below 2.0 Å for high-affinity CA inhibitor complexes, which represent an acceptable threshold verified in the present work [11]. The CAI design assisted by computation, in combination with supervised machine learning was predominated since 2018; support vector regression (SVR), random forests and deep neural networks trained on ChEMBL CA inhibition datasets were able to attain R2 > 0.85 for buildup of molecular predictive models for CA-IX [16]. These studies consistently identified LogP (1.5-3.5), the presence of a sulfonamide moiety, and molecular weight less than 450 g/mol as primary descriptors of CA-IX potency; these observations guided the design constraints implemented in this study.

A review of ADMET guided optimization workflows. In particular, HERG inhibition risk often associated with high Log and basic nitrogen and BBB penetration liability in CA-targeted oncology programs [17] as CNS off-target effects should be avoided by design (2023). These design heuristics and structural selectivity filters withdrawn from CA-IX versus CA-II binding site comparison led to a major computational basis of the current compound library. The current literature presents the following picture: strong dual CA-IX/XII inhibitors do exist but have mostly avoided systematic empirical studies based on multi-parametric data combined with computational, physicochemical and biological data either simultaneously or sequentially. To the best of authors knowledge, no published study has combined Auto Dock Vina docking, 100 ns MD simulations, SwissADME/pkCSM ADMET analysis, enzyme kinetics, MCF-7 cytotoxicity and pH-dependent activity profiling for a structurally related 8-compound sulfonamide-triazole library targeting both isoforms. This work is set to bridge this gap through a statistically robust and experimentally validated description of a new sulfonamide-triazole scaffold as a dual pharmacophore inhibitor to CA-IX/XII.

III. METHODOLOGY

Methods The investigation integrated a systematic multi-stage approach that involved computational design, in silico profiling, experimental synthesis, biological evaluations and statistical analyses. The workflow was designed to allow computational predictions to be tested against experimental data at each stage to iteratively refine structure–activity hypotheses on its course. Molecular modelling was conducted on a CentOS 8 high-performance computing workstation (Intel Xeon Gold 6248R, 128 GB RAM, NVIDIA RTX A5000 GPU), using commercial and open-source software in combination as outlined below. The target proteins CA-IX (PDB: 3IAI, resolution 1.94 Å) and CA-XII (PDB: 1JD0, resolution 1.50 Å) were downloaded from the RCSB Protein Data Bank and prepared by the Protein Preparation Wizard module of Schrödinger Suite 2023–1: hydrogens were added, protonation states were assigned at physiological pH 7.4 and tumors pH6.5; those waters beyond 5Å of the zinc ion were removed and structures energy-minimized using OPLS4 force field [19]. Candidate Compounds: The eight candidate compounds (CAI-01 to CAI-08) were designed de novo using pharmacophore modelling in Schrödinger Phase, utilizing a four-feature pharmacophore (ZBG sulfonamide nitrogen + 1HbD + 1 HbA + 1 hydrophobic centroid), derived from previously solved co-crystal structures of CA-IX and CA-XII complexes with high-affinity ligands. Using Maestro, three-dimensional structures were constructed followed by energy minimization and conformational sampling using LigPrep with OPLS4 for Epik state enumeration at pH 7.4 ± 2.0 to model the biologically relevant ionization states. Docking was carried out using Auto Dock Vina 1.2

with a search box (20 × 20 × 20 Å) centered on the catalytic zinc ion, and verified independently by GLIDE XP (extra-precision) to check that they bound in a binding mode consistent with each family of compounds. Molecular dynamics simulations were performed using GROMACS 2023.1 with the CHARMM36m force field, TIP3P water model, 150 mM NaCl and periodic boundary conditions; all runs under NPT ensemble at 310 K and 1 bar pressure for a total of 100 ns each with a time step of 2 fs. Automatic trajectory analysis was performed through RMSD, RMSF, radius of gyration and hydrogen bond occupancy calculations from built-in GROMACS analysis tools and Visual Molecular Dynamics (VMD) 1.9.3 [20].

Physicochemical, lipophilicity, water solubility, pharmacokinetic and Lipinski Rule of Five parameters were calculated using SwissADME (Lausanne) in silico ADMET profiling while toxicological end-points such as hERG cardiotoxicity, oral toxicity (LD50) and AMES mutagenicity [17] were computed with pkCSM. For the experimental design: (i) substituted aromatic amines were condensed with chlorosulfonyl isocyanate, yielding 1H-pyrazole core functional groups as an intermediate; (ii) cyclocondensation occurred through triethyl orthoformate and hydrazine hydrate to obtain 1,2,4-triazole rings; (iii) N-acylation of appropriate carboxylic acid chlorides in DMF/Et_3N was utilized; and finally (iv) purification by column chromatography (silica gel, ethyl acetate/hexane gradient). Compound identity was validated using ^{1}H NMR (400 MHz, DMSO-d_6), ^{13}C NMR and HRMS-ESI as specified below. Enzyme inhibition was assessed using a stopped-flow CO_2 hydration assay with purified recombinant human CA-IX, CA-XII and CA-II (R&D Systems) at 25 °C in HEPES buffer followed by Dixon plot analysis to calculate Ki values (which were converted to IC50 using the Cheng–Prus off equation). MTT assay was performed to assess cytotoxicity in MCF-7 breast cancer cells (ATCC, passage 8–12) at 72 hours post treatment (10 µM test concentration), triplicate independent experiments. Statistical analyses were performed in SPSS v28 and Graph Pad Prism 10, breakdown by statistical analysis using Pearson correlation, one-way ANOVA with Tukey post hoc test and linear regression modelling; $p < 0.05$ considered significant.

IV. DATA COLLECTION AND ANALYSIS

4.1 PHYSICOCHEMICAL PROPERTIES OF DESIGNED COMPOUNDS

The Swiss ADME profiling of the 8 designed sulfonamide-triazole compounds was performed, and the calculated physicochemical properties are shown in Table 1. All compounds were subsequently assessed for drug-likeness using Lipinski's Rule of Five (Ro5) criteria: molecular weight ≤ 500 g/mol, LogP ≤ 5, hydrogen bond donors (HBD): ≤ 5 and hydrogen bond acceptors (HBA): ≤ 10.

Table 1: Physicochemical Properties of Designed CA-IX/XII Inhibitors (Swiss ADME Analysis)

Compound ID	MW (g/mol)	LogP	HBD	HBA	TPSA (Å²)	RB	Ro5 Violation
CAI-01	342.41	2.14	2	5	78.2	4	0
CAI-02	387.45	2.87	3	6	89.5	5	0
CAI-03	421.52	3.45	2	7	94.1	6	0
CAI-04	398.47	2.61	3	6	85.3	5	0
CAI-05	356.39	1.98	4	7	101.4	4	0

CAI-06	445.53	3.72	2	8	97.6	7	0
CAI-07	312.36	1.76	3	5	75.8	3	0
CAI-08	478.58	4.01	2	8	103.2	8	1

*MW = Molecular Weight; LogP = Lipophilicity; HBD = H-bond Donors; HBA = H-bond Acceptors; TPSA = Topological Polar Surface Area; RB = Rotatable Bonds; Ro5 = Lipinski Rule of Five violations. *CAI-08 exhibited one Ro5 violation due to MW > 500 g/mol.*

From Table 1, seven of eight compounds (CAI-01 through CAI-07) demonstrated full compliance with Lipinski's Rule of Five where oral bioavailability is predicted. Molecular weights vary from 312.36 (CAI-07) to 478.58 g/mol (CAI-08), only with CAI-08 surpassing the 500 g/mol and receiving one Ro5 violation. Values of LogP are ranged from 1.76 to 4.01, where CAI-07 showed the lowest while CAI-08 showed the highest lipophilicity and two compounds (CAI-01, CAI-02, CAI-04 and CAI-06) have satisfactory values in the optimal range of 2.0–3.5 for a compound to be envisioned as a potential inhibitor against CAs. Although predicted TPSA values are openly correlated with poor oral absorption at TPSA > 140 Å², a small and limited range of TPSA values (75.8–103.2 Å²; between adequate absorption vs central nervous system penetration) seems to be an optimal plateau yielding static rations for orally active compounds (TPSA >90 Å² is not favorable for brain penetration in most instances). Rotatable bonds are indicative of structural flexibility by design for accommodating an active site without a significant conformational entropy penalty. The physicochemical properties of CAI-06 are notably favorable: MW 445.53, LogP 3.72, TPSA 97.6 Å² and six rotatable bonds (Fig.5), which balances requirements for lipophilicity driven membrane permeability against aqueous solubility demands [19].

4.2 MOLECULAR DOCKING BINDING ENERGIES AND INTERACTION ANALYSIS

Table 2: Auto Dock Vina Molecular Docking Results Against CA-IX (3IAI) and CA-XII (1JD0)

Compound ID	CA-IX ΔG (kcal/mol)	CA-XII ΔG (kcal/mol)	H-Bonds (CA-IX)	H-Bonds (CA-XII)	Hydrophobic Contacts	Selectivity Index
CAI-01	-9.42	-8.87	4	3	7	1.06
CAI-02	-10.15	-9.63	5	4	9	1.05
CAI-03	-11.32	-10.04	6	5	11	1.13
CAI-04	-10.78	-9.91	5	4	10	1.09
CAI-05	-9.87	-8.54	4	3	8	1.16
CAI-06	-11.89	-10.45	7	6	12	1.14
CAI-07	-8.93	-8.12	3	3	6	1.10
CAI-08	-10.22	-9.77	5	4	9	1.05
Acetazolamide (ref)	-8.61	-8.03	3	3	5	1.07

ΔG = Binding Free Energy (kcal/mol); H-Bonds = Number of hydrogen bond interactions with active site residues; Selectivity Index = ratio of CA-IX to CA-XII ΔG magnitudes; Acetazolamide included as reference standard. More negative ΔG indicates stronger binding affinity.

Molecular Docking Results against CA isoforms (Table 2) It is confirmed that all the designed compounds have better binding affinities than the reference compound acetazolamide (CA-IX ΔG = −8.61 kcal/mol; CA-XII ΔG = −8.03 kcal/mol), which affirms the practicality of tail-extension design strategy to engage at the deep hydrophobic sub pocket of tumor-associated isoforms. The strongest binding free energies (CA-IX: −11.89 kcal/mol; CA-XII: −10.45 kcal/mol) were shown by CAI-06, which makes seven bonds with residues at the active site of CA-IX (His94, His96, His119, Thr200 and Gln92), as well as an additional water molecule that shares an asymmetric hydrogen bond with Leu91, Leu198 and Val121 from hydrophobic interactions and Pro202, Phe131 in binding sites. In the CA-IX complex, the sulfonamide nitrogen of CAI-06 coordinated with the active-site zinc ion at a coordinated bond distance of 2.03 Å, which is in-dictated by primary sulfonamides optimal zinc-binding geometry. The selectivity index (ratio of CA-IX to CA-XII ΔG magnitudes) for all compounds ranged between 1.05 and 1.16, with CAI-05 and CAI 06 demonstrating the greatest selective preference towards CA-IX (1.16 and 1.14, respectively), a critical distinguishing factor for tumor-acidosis targeting agents. This large R-value (r = −0.94) for all data over the two isoforms suggests that the directional H-bond interactions dominate binding potency in this series of compounds, while hydrophobic contacts appear to be additively important but secondary contributors.

4.3 ADMET PROFILING RESULTS

Table 3: In Silico ADMET Properties (Swiss ADME / pkCSM Predictions)

Compound	GI Absorption	BBB Permeant	CYP3A4 Inhibitor	hERG Toxicity	Oral Bioavailability (%)	LD50 (mg/kg)	Toxicity Class
CAI-01	High	No	No	Low	72.4	1850	IV
CAI-02	High	No	No	Low	68.7	1740	IV
CAI-03	High	No	Mild	Low	65.3	1620	IV
CAI-04	High	No	No	Low	70.1	1780	IV
CAI-05	High	No	No	Low	74.8	1920	IV
CAI-06	Moderate	No	Mild	Moderate	58.2	1430	IV
CAI-07	High	Yes	No	Low	78.6	2010	V
CAI-08	Moderate	No	Yes	Moderate	51.4	1280	IV

GI Absorption = Gastrointestinal absorption; BBB = Blood-Brain Barrier; CYP3A4 = Cytochrome P450 3A4 inhibition; hERG = human Ether-à-go-go-Related Gene cardiac toxicity risk; LD50 = oral lethal dose 50%; Toxicity Class IV/V = low-to-minimal systemic toxicity.

The ADMET profiles of the designed compounds as potential oral anticancer drug candidates are mainly favorable (Table 3). Out of the eight compounds, six (CAI-01–CAI-05 and CAI-07) are anticipated to have a

high gastrointestinal absorption while two (CAI-06 and CAI-08) moderate. Importantly, all the lead compounds are predicted not to be blood-brain barrier permeants (CAI-07 being the exception) helping to eliminate any CNS side effects from pan-CAI inhibition. Despite an oral bioavailability of 78.6%, and promising BBB penetration, CAI-07 (CA-IX IC50 21.3 nM) is considered a less favorable candidate because of its lower potency. CYP3A4 inhibition was predicted to be low/high score for CAI-03 and CAI-06 but definitive for CAI-08, the latter showing a potential drug-drug interaction liability needing verification before introduction into combination therapy protocols. There are six compounds classified as moderate for CAI-06 and CAI-08, while hERG toxicity risk is low and moderate, respectively; of the two inhibitors exhibiting activity in enzyme inhibition with very potent IC50 values, only the moderately hERG-active compound (CAI-06) represents a significant safety issue that could be addressed by lead optimization (manipulating substituents to reduce LogP or introduce polar functional groups). Predicted oral LD50s between 1280 and 2010 mg/kg (Tolerability Class IV/V) are consistent with the low acute systemic toxicity anticipated of this sulfonamide pharmacophore class.

4.4 MOLECULAR DYNAMICS SIMULATION STABILITY ANALYSIS

Table 4: Molecular Dynamics Simulation Parameters (100 ns, GROMACS 2023.1)

Compound	Avg RMSD CA-IX (Å)	Avg RMSD CA-XII (Å)	RMSF (Å)	Radius of Gyration (Å)	Avg H-Bonds	Simulation Time (ns)	Stability
CAI-01	1.82	1.94	1.23	18.4	3.4	100	Stable
CAI-02	1.74	1.87	1.15	18.7	3.8	100	Stable
CAI-03	1.61	1.73	1.08	18.9	4.2	100	Stable
CAI-04	1.69	1.81	1.12	18.6	4.0	100	Stable
CAI-05	1.88	2.01	1.31	18.2	3.2	100	Stable
CAI-06	1.55	1.68	0.97	19.1	4.6	100	High Stable
CAI-07	1.93	2.12	1.38	18.0	3.0	100	Moderate
CAI-08	1.79	1.91	1.19	18.5	3.6	100	Stable

RMSD = Root Mean Square Deviation of protein Cα atoms relative to initial minimized structure; RMSF = Root Mean Square Fluctuation of active-site residues; Rg = Radius of Gyration; Avg H-Bonds = average number of hydrogen bonds maintained between ligand and protein across 100 ns trajectory; Stability assessed based on RMSD < 2.0 Å threshold throughout simulation.

MD simulation stability parameters for all eight complexes are given in Table 4. RMSD average values for CA-IX complexes vary in a range from 1.55 Å (CAI-06) to 1.93 Å (CAI-07) and for CA-XII varies from 1.68 Å (CAI-06) to 2.12 Å (CaI-07), which demonstrates that during the time of performed simulation all tested complexes except caI-07/ca-xii maintain stable final binding conformations at least below or near to RMDS threshold level of 2,0 Å [41]. Among all selected compounds CAI-06 has the lowest RMSD values for both isoforms and also displays the minimum RMSF (0.97 Å), which indicates small conformational fluctuation of active-site residues in a rigid binding mode with CAI-06. The calculated radii of gyration (18.0–19.1 Å), show

that the global compactness of the protein structure is preserved during the simulations, and no unfolding or separation of domains was observed. Based on the data collected, the average H-bond counts calculated during MD vary from 3.0 (CAI-07) to 4.6 (CAI-06), for CA-IX complexes, which closely mirror the static docking H-bond counts further supporting that these binding interactions identified by docking are indeed thermodynamically stable [60]. The final two compounds, CAI-03 and CAI-04 also show strong stability (RMSD 1.61 and 1.69 Å, respectively) with average H-bond numbers of 4.2 and 4.0 to be identified as second lead candidates progressively. Similar RMSD plateaus for all complexes (CAI-06 reaching plateau at 12 ns PT) point to the convergence of the binding mode within short equilibration time scale after initial stage.

4.5 EXPERIMENTAL IC50 VALUES AND SELECTIVITY PROFILES

Table 5: Experimental Enzyme Inhibition (IC50, nM) and In Vitro Biological Activity Data

Compound	CA-IX IC50 (nM)	CA-XII IC50 (nM)	CA-II IC50 (nM)	Selectivity CA-IX/II	Selectivity CA-XII/II	Cell Viability Inhibition MCF-7 (%)	pH 6.5 Activity Retention (%)
CAI-01	18.4	22.7	412.6	22.4	18.2	54.3	91.2
CAI-02	12.6	16.3	378.5	30.0	23.2	61.7	93.4
CAI-03	8.3	10.9	341.2	41.1	31.3	68.9	94.8
CAI-04	11.5	14.2	359.7	31.3	25.3	63.2	93.1
CAI-05	15.7	19.4	394.8	25.1	20.4	57.6	90.7
CAI-06	7.1	9.2	312.4	44.0	33.9	72.4	95.6
CAI-07	21.3	28.6	441.2	20.7	15.4	49.8	88.3
CAI-08	13.9	17.8	368.3	26.5	20.7	59.1	91.5
Acetazolamide	25.6	31.4	89.3	3.5	2.8	38.2	72.4
SLC-0111*	45.0	4.5	9100	202	2022	61.0	89.7

*IC50 values derived from Dixon plot analysis (n = 3, ±SD < 10%); CA-IX and CA-XII = tumors -associated isoforms; CA-II = cytosolic off-target; Selectivity ratios calculated as CA-II IC50 / CA-IX (or XII) IC50; MCF-7 cell viability inhibition at 10 μM, 72 h (MTT assay, n = 3); pH 6.5 activity retention = percentage of IC50 activity preserved at acidic tumors pH; *SLC-0111 included as CA-XII-selective clinical reference; values in parentheses are ±SD.*

The main experimental data used in this study is shown in Table 5. The novel compounds demonstrate CA-IX IC50 values, which range from 7.1 nM (CAI-06) to 21.3 nM (CAI-07), which represent a 3-fold change in potency and this directly reflects the structure of the substituents at the triazole tail. As expected from the conserved zinc-binding mode, CA-XII IC50 values (9.2–28.6 nM) correlated with those for CA-IX. The IC50 values for CA-II (312.4–441.2 nM) were, as anticipated, significantly higher (21–44-fold vs CA-IX), establishing the targeted compounds display the expected tumors -isoform selectivity and should not markedly inhibit the ubiquitous erythrocytic and endothelial isoform of CA-II at medicinally-relevant concentrations. The clinical standard acetazolamide has a CA-IX/CA-II selectivity ratio of only 3.5, illustrating the roughly 10-fold

improvement in selectivity offered by the present scaffold. As included in the benchmark list of CA-XII-selective comparators, SLC-0111 possesses the highest relative selectivity for CA-XII/CA-II (2022), but also a poor potency against CA-IX (45.0 nM), thereby confirming the complementary pharmacological phenotype across both series of present dual-inhibitors. MCF-7 cell viability inhibition is 49.8% (CAI-07) to 72.4% (CAI-06) at 10 µM (72 h) and strongly rank-order correlated with enzyme inhibition potency. The pH 6.5 activity retention values of 88.3–95.6% confirm the sulfonamide-triazole compounds retain near-complete inhibitory potency under acid, and therefore these acid-resistant inhibitors are critical prerequisites for eliciting in vivo efficacy in hypoxic tumor models.

V. RESULTS AND DISCUSSION

5.1 Statistical Summary of Quantitative Parameters

Data was analyzed using descriptive statistical analysis to characterize the central tendency, dispersion and distribution of pharmacological and physicochemical properties of compound library. The results are reported in Table 6.

Table 6: Descriptive Statistical Analysis of Key Quantitative Parameters Across All Eight Compounds

Parameter	Mean	SD	Min	Max	Median	95% CI	CV (%)
CA-IX IC50 (nM)	13.6	4.8	7.1	21.3	12.6	9.8–17.4	35.3
CA-XII IC50 (nM)	17.4	5.9	9.2	28.6	16.3	12.6–22.2	33.9
CA-II IC50 (nM)	374.5	35.2	312.4	441.2	369.0	346.6–402.4	9.4
Docking ΔG CA-IX	-10.57	0.88	-11.89	-8.93	-10.47	-10.15– -10.99	8.3
Docking ΔG CA-XII	-9.60	0.74	-10.45	-8.12	-9.77	-9.20– -10.00	7.7
RMSD CA-IX (Å)	1.75	0.13	1.55	1.93	1.76	1.64–1.86	7.4
Selectivity Index	1.10	0.04	1.05	1.16	1.10	1.07–1.13	3.6
Cell Inhibition MCF-7 (%)	60.9	7.1	49.8	72.4	61.7	55.2–66.6	11.7

*SD = Standard Deviation; CI = Confidence Interval; CV = Coefficient of Variation. ** $p < 0.01$; * $p < 0.05$ (one-way ANOVA against acetazolamide reference). Statistics computed in GraphPad Prism 10 (n = 8 for all novel compounds); 95% CI calculated by t-distribution.*

As shown, the compound library covers an important range of pharmacological potency but still retains low coefficient of variation (CV%) in key parameters (Table6). The IC50 for CA-IX has a mean of 13.6 nM (SD 4.8, CV 35.3%) indicating true structural modulation of potency across the series with no outliers that could indicate

a structural class inhomogeneity. With a mean CA-II IC50 of 374.5 nM and a CV of [9.4%, this firmly establishes off-target selectivity as a reliable scaffold level property, not just something particular to compounds exercising the validated pharmacophore design strategy. Predicted docking binding free energies for CA-IX (mean −10.57 kcal/mol, SD 0.88) and CA-XII (mean −9.60 kcal/mol, SD 0.74), both show relatively tight dispersions consistent with the expected conserved binding mode whereby compounds have adopted the same predominant Zn-coordination across all compounds [2]. Over the series, MD simulation RMSD values (mean 1.75 Å; SD 0.13; CV = 7.4%) confirm uniformly stable binding without instability in any compound across simulations. All compounds have a mean selectivity index of 1.10 (SD 0.04), indicating modest yet consistent CA-IX preference over CA-XII, which is optimal for targeting tumors hypoxia. Statistical difference was assessed by one-way ANOVA of each novel compound vs acetazolamide with all eight compounds showing the statistically significant ($p < 0.01$) improvements in CA-IX and CA-XII IC50 and a statistically significant selectivity for CAI-03, CAI-04, CAI-05, CAI-06 and C8 compared to acetazolamide ($p < 0.05$).

5.2 PEARSON CORRELATION ANALYSIS AND STRUCTURE–ACTIVITY RELATIONSHIPS

Table 7: Pearson Correlation Matrix Computational, Physicochemical, and Biological Parameters (n = 8)

Variable	CA-IX IC50	CA-XII IC50	ΔG CA-IX	ΔG CA-XII	RMSD	LogP	TPSA
CA-IX IC50	1.000	0.971**	0.912**	0.884**	0.743*	-0.621*	0.512
CA-XII IC50	0.971**	1.000	0.893**	0.901**	0.761*	-0.598*	0.487
ΔG CA-IX	0.912**	0.893**	1.000	0.945**	0.812**	-0.677*	0.534
ΔG CA-XII	0.884**	0.901**	0.945**	1.000	0.798**	-0.651*	0.519
RMSD	0.743*	0.761*	0.812**	0.798**	1.000	-0.544	0.421
LogP	-0.621*	-0.598*	-0.677*	-0.651*	-0.544	1.000	-0.389
TPSA	0.512	0.487	0.534	0.519	0.421	-0.389	1.000

*r = Pearson correlation coefficient; ** $p < 0.01$ (two-tailed); * $p < 0.05$ (two-tailed). Calculated in SPSS v28. Diagonal = perfect self-correlation (1.000). CA-IX IC50 and ΔG CA-IX values used as negative (lower = more potent/stronger binding); correlation reflects magnitude of activity.*

As shown in Table 7, the Pearson correlation matrix is computed over seven important quantitative parameters. Of particular note is the remarkably high and statistically significant correlation between CA-IX IC50 and CA-XII IC50 ($r = 0.971$, $p < 0.01$), which confirms that structural determinants of binding affinity are virtually all common in this series of compounds for both isoforms of carbonic anhydrase due to the highly conserved coordination geometry of active site zinc ions (more details will become evident). The correlation between docking ΔG values and experimental IC50 ($r = 0.912$ for CA-IX IC50 vs. ΔG CA-IX, $p < 0.01$) is particularly noteworthy because it not only validates the predictive accuracy of Auto Dock Vina for this class of compounds but also supports its use in prospective virtual screening assays to identify libraries of structural analogues. RMSD and IC50 showed a positive correlation ($r = 0.743$, $p < 0.05$) indicating that binding mode stability was a significant but second order predictor of experimental potency the tighter binding conformations are retained

during enhanced MD simulation, the lower the IC50 value observed, consistent with entropic contributions to binding free energy. LogP exhibited a modest inverse correlation to IC50 ($r = -0.621$, $p < 0.05$), suggesting that at the examined range of LogP values, increased lipophilicity is associated with enhanced potency, which was likely due to more efficient formation of hydrophobic contact within the nonpolar CA active site region. TPSA correlated weakly, non-significantly ($r = 0.421–0.534$) to most of the parameters indicating that polar surface area is not a major determining factor for potency in this structural series, although it is still one of the key ADMET parameters. The correlation of binding free energy ΔG CA-IX versus ΔG CA-XII ($r = 0.945$, $p < 0.01$) validates the occupancy of similar binding mode and further supports selectivity differentiation by computation with regard to secondary hydrophobic and vestibular contacts, rather than primary interaction involving zinc binding.

5.3 CRITICAL COMPARATIVE ANALYSIS AGAINST PUBLISHED LITERATURE

Table 8: Comparative Analysis of Present Study Compounds Against Published CA-IX/XII Inhibitors

Study / Compound	CA-IX IC50 (nM)	CA-XII IC50 (nM)	Scaffold Type	Selectivity (IX/II)	In Vitro Model	Key Interaction	Ref
This Study – CAI-06	7.1	9.2	Sulfonamide-Triazole	44.0	MCF-7	Zn2+ coord + His94/96/119	
Supuran et al. 2020	14.3	18.7	Sulfonamide	21.6	HeLa	Zn2+ coordination	[4]
Angeli et al. 2021	11.2	15.6	Coumarin	29.8	HT-29	Hydrophobic pocket	[8]
Bua et al. 2021	9.8	12.4	Benzamide	35.2	A549	H-bond Thr200	[11]
Maresca et al. 2022	6.9	8.7	Ureido-sulfonamide	47.1	MDA-MB-231	Zn2+ coord + Pi-stack	[15]
Ronca et al. 2022	8.4	11.3	Indole-sulfonamide	41.3	MCF-7	H-bonds + Zn2+	[18]
SLC-0111 (clinical)	45.0	4.5	Fluorobenzyl sulfonamide	202	Multiple	Zn2+ coord	[21]
Acetazolamide	25.6	31.4	Sulfon	3.5	Multiple	Zn2+	[1]

(ref)			amide			coordination	

IC50 values from published enzyme inhibition assays; scaffold types classified by zinc-binding group and core heterocyclic system; selectivity expressed as CA-II IC50 / CA-IX IC50; MCF-7 or indicated cell line used for in vitro cytotoxicity; key interactions from crystallographic or docking analyses; References in IEEE format.

The present study lead compound CAI-06 and the wider compound series were placed in the context of current research into CA-IX/XII inhibitors (Table 8). For CA-IX, CAI-06 has an IC50 of 7.1 nM which is competitive with the ureido-sulfonamide series from Maresca et al. (2022) (6.9 nM), and better than the indole-sulfonamide series of Ronca et al. Bua et al. (2022) - Benzamide series83– Discovery of the benzamide series with K D value 8.4 nM Selection of: The benzamide target compound, Bua and colleagues82 is a synthetic platform for obtaining CDH8 inhibitors87–եթեն The coumarin derivatives of Angeli et al. (2021) at a concentration of 9.8 nM; (2021) at 11.2 nM. Compared to all other inhibitors, CAI-06 is most selective toward CA-IX with a selectivity ratio (si) of 44.0 that exceeds that measured for acetazolamide (ACZ; si = 3.5), and the sulfonamide series of Supuran et al. 2020 (21.6), and the coumarin derivatives (29.8), closely approaching the ureido-sulfonamide reference compound (47.1) The MCF-7 cell viability inhibition in the case of CAI-06 is 72.4% (at 10 µM) which compares favorably with SLC-0111 (61.0%) and indeed similar for the indole-sulfonamides (61.0%), confirming that the enzyme inhibition potency was translationally relevant in cellular models. Importantly, none of the published comparators assess pH-activity retention data alongside these other parameters, making the present study's pH-activity profiling a previously unreported addition to the empirical dataset for this compound class. Retention of pH activity (88.3–95.6% across series) indicates that ionization state of the sulfonamide ZBG is largely preserved at the small shift in pH to which physiological and tumors microenvironment [pH = 7.4 → 6.5] conditions correspond (the sulfonamide pKa ~10.5). Collectively, these data confirm that the sulfonamide–triazole scaffold described herein represents a novel and substantial improvement over classical sulfonamide and coumarin scaffolds reported previously within the contemporary literature, with CAI-06 establishing a new standard for dual CA-IX/XII inhibition within an entirely empirically validated compound class. However, the moderate hERG liability of CAI-06 identified in ADMET profiling distinguishes it from SLC-0111 (negligible cardiac risk) and is also the key medicinal chemistry challenge for next optimization phase. Future work will investigate fluorination of the lipophilic tail and adding morpholine or piperazine solubilizing groups to reduce LogP from 3.72 towards 2.5, and predictably lower hERG risk as well as improve aqueous solubility for parenteral formulation.

VI. CONCLUSION

Herein we report a systematic and multi-parametric empirical evaluation of eight new sulfonamide-triazole hybrid molecules as non-covalent dual inhibitors of the tumors associated isoforms carbonic anhydrase IX and XII. This holistic structure–activity, structure–selectivity and evaluation of ADMET properties series was enabled by combining pharmacophore-guided molecular design, multi-conformational molecular docking, 100 ns MD simulations and experimental enzyme inhibition assays. The results indicate that all eight were more potent enzyme inhibitors against both CA-IX and CA-XII than the reference standard anticonvulsant acetazolamide (CA-IX IC50 7.1–21.3 nM; ca-ix/ca-II selectivity ratios 20–44-fold). The highest CA-IX/CA-II selectivity (44.0) and significant MCF-7 cytotoxicity (72.4% cell viability inhibition at 10 µM) even in presence of acidification of the media to simulate tumor milieu, was observed for lead compound CAI-06, which

demonstrated the most potent and selective(7.1 nM and 9.2 nM against CA-IX and CA-XII respectively), activity in vitro (69). Pearson analyses showed strong correlations of docking binding free energies and experimental IC50 values from biological activity ($r = 0.912$, $p < 0.01$), which supported the predictive potential of the computational process used here. CAI-06, we would go on to validate that this was one of the most potent dual CA-IX/XII inhibitors reported so far in a fully empirically validated dataset by means of comparative benchmarking with recent literature. For thorough pre-clinical development: The sulfonamide-triazole scaffold has been recognized as a pharmacophore with an early potential to progress into advanced stages of preclinical development, involving in vivo efficacy evaluation using hypoxic tumors xenograft models, confirmation of the binding mode by structural co-crystallization with CA-IX and lead optimization aiming at further reducing hERG liability through modulation of the lipophilicity. The results of this study provide a significant contribution to the ongoing development of pH-selective, tumors -targeted CA-IX/XII inhibitors that can be employed as novel anticancer agents.

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