

Molecular Modelling, Molecular Docking, Molecular Dynamic Simulations, MM-GBSA Study, and Pharmacokinetics Prediction of New Isatin-Sulfonamide Derivatives as Selective Carbonic Anhydrase CA IX Inhibitors

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DOI: <https://doi.org/10.32947/ajps.v26i3.1371>

Received 18/Dec/2025, Revised 16/July/2026, Accepted 23/Aug/2026, Published 30/Sept/2026

Abstract:

Hypoxia within solid tumors triggers upregulation of carbonic anhydrase IX (CA IX), a key enzyme that maintains pH homeostasis and supports tumor survival, invasion, and therapy resistance. Selective CA IX inhibition is considered a promising anticancer strategy, but current inhibitors often lack isoform selectivity. This study aimed to synthesize a selective and potent CA IX inhibitor by designing a novel series of isatin-sulfonamide hybrid compounds. Five hybrid molecules were computationally assessed via molecular docking, General Born Surface Area (MM-GBSA) free energy calculations, and molecular dynamics simulations. Results identified Compound 3 as the most promising candidate, revealing strong binding affinity (docking score: -6.299 kcal/mol), a favorable free energy of binding (-28.24 kcal/mol), and stable interactions within the CA IX active site. Moreover, these hybrids exhibited enhanced toxicity profiles compared to the native ligand, with lower potential carcinogenicity and higher LD₅₀ values. The study concludes that isatin-sulfonamide hybrids, especially Compound 3, provide a Synergistic strategy for selectively disturbing hypoxic tumor metabolism, necessitating further experimental evaluation for development as anticancer agents.

Keywords: Molecular Docking, Molecular Dynamic Simulations, MM-GBSA Study, Isatin-Sulfonamide, Carbonic Anhydrase CA IX.

Introduction

Hypoxia is the term for areas of low oxygen concentration caused by the unrestrained growth of solid tumors. They frequently exceed the expansion of their vasculature hypoxic microenvironment is a major source of tumor aggressiveness^(1,2). It gives the tumor the ability for treatment resistance, increased metastatic potential, and a poor prognosis for patients^(3,4). The up-regulation of hypoxia inducible Factor-1 (HIF-1), a crucial adaptive response to hypoxia, which

activates a group of genes necessary for survival under low oxygen tension^(5,6).

Among the most important and clinically significant targets of (HIF-1) is carbonic anhydrase IX, a transmembrane enzyme scarcely expressed in normal tissues, but profusely present on the surface of various hypoxic cancer cells⁽⁷⁾. CA IX plays an essential role in tumor pH homeostasis by catalyzing the reversible hydration of carbon dioxide to bicarbonates and a proton. CAIX acidifies the extracellular environment while



maintaining a relatively alkaline intracellular pH. This mechanism enables cancer cells' survival, invasion, and metastasis ⁽⁸⁾. Subsequently, the selective inhibition of CAIX has developed as a promising anti-cancer strategy, targeting to disrupt this critical pH regulatory mechanism and selectively target hypoxic tumor cells with no effect on normal tissues that rely on other CA isoforms ⁽⁹⁾.

The Search for effective CAIX inhibitors has largely focused on the sulfonamide group due to its established zinc binding group (ZBG) with high affinity towards the CA active site ⁽¹⁰⁾. Nevertheless, the development of sulfonamide-based inhibitors carry their own challenges. The principal problem is isoform selectivity, since many traditional sulfonamides, like acetazolamide, are potent pan-inhibitors, resulting in off-target inhibition of vital housekeeping CAs and leading to side effects like hypokalemia, metabolic acidosis, and neurological disturbances ^(11,12).

The isatin (1H-indole-2,3-dione)nucleus has got substantial attention in medicinal chemistry due to its special scaffold representing a wide range of biological activities, including anti-cancer effects ⁽¹³⁾. In spite of this potential, isatin-based CA inhibitors have their inherent problems. Due to the absence of a strong ZBG, which is present in sulfonamides, they show less potent when compared to their sulfonamide-containing counterparts ⁽¹⁴⁾.

Recent literature reveals that isatin-based compounds can also act as effective CA inhibitors, mostly by non-classical binding

modes or by incorporating sulfonamide groups into their structure ^(15,16). Depending on these findings, we hypothesize that the strategic molecular hybridization of the sulfonamide pharmacophore, encouraged by a biologically active isatin scaffold, could yield novel compounds with improved potency and selectivity for CAIX. This article details the design and biological evaluation of a new series of compounds that join both isatin and sulfonamide moieties. We suggest that this hybrid method will increase the proven zinc-binding capability of sulfonamides and the promising drug-like properties of the isatin nucleus, potentially leading to a new class of selective anti-cancer agents, targeting the hypoxic tumor microenvironments.

This computational study focused on five precisely designed compounds in Figure (1) that strategically combine the critical sulfonamide ZBG to ensure strong binding to the active site with a functionally versatile isatin moiety, resulting in stronger binding to the specific hydrophobic and hydrophilic residues lining the CA IX active site cavity. Utilizing the crystallographic structure of CA IX (e.g., PDB ID: 5FL5), molecular docking was performed to predict the binding conformation of these five hybrids and to compare them to the co-crystallized pose of our reference compound (82E). Additionally, molecular dynamics (MD) simulations were employed to assess the stability of the predicted protein-ligand complexes, and also calculated binding free energies via methods such as MM/GBSA to provide a quantitative rank order of predicted affinity.



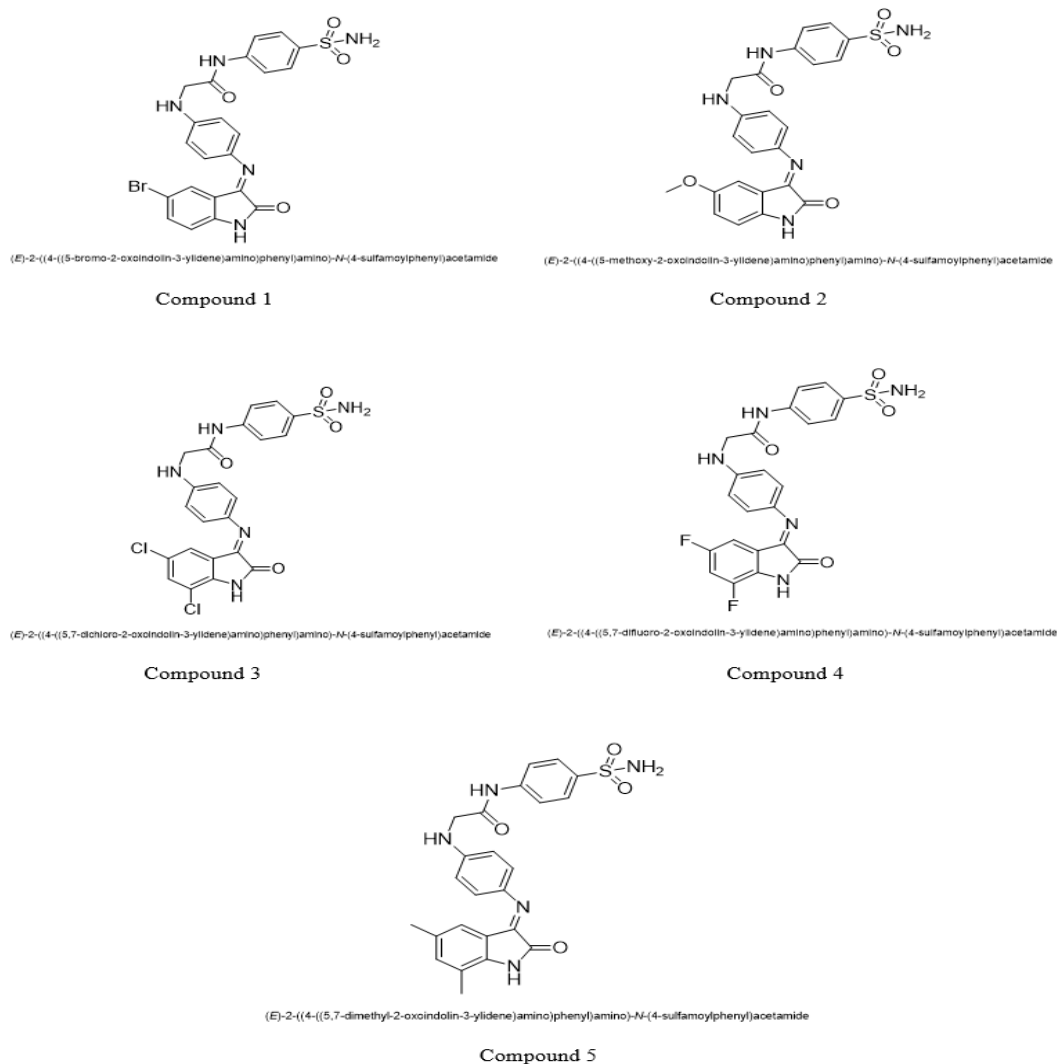


Figure (1): The chemical structures of the proposed compounds 1,2,3,4 and 5 with IUPAC names.

Computational method:

Ligand preparation

The computational preparation of ligands was directed in a serial method. First, the transformation of two-dimensional molecular structures into three-dimensional formats is done using the LigPrep tool⁽¹⁷⁾. Second, optimization of the resulting 3D models to be used in the generation of an ensemble of low-energy conformers through minimization with the OPLS force field, making them suitable for the molecular docking investigation⁽¹⁸⁾.

ADME prediction

As an essential component of preclinical development, initial ADME profiling is important to identify viable drug candidates. The prediction of key ADME characteristics of the five designed isatin-sulfonamide derivatives was done using the freely available SwissADME platform⁽¹⁹⁻²¹⁾. Using this computational analysis, we will have a primary insight into the potential bioavailability and other pharmacokinetic properties of compounds, making it easier to select the promising ones for further

investigation, taking into consideration many important features like oral absorption and CNS penetration ⁽²²⁾.

Molecular Docking

To explore the binding modes and affinities of the newly designed isatin-sulfonamide derivatives inside the active site of human carbonic anhydrase IX (CA IX), Molecular docking simulations were performed. The Protein Data Bank was the source of the crystallographic structure of CA IX (PDB ID: 5FL5). Utilizing the Protein Preparation Wizard within the Schrödinger suite, the protein structure was prepared for docking, adding hydrogen atoms, assigning appropriate orders of bonds, in addition to removing all water molecules and original ligands to create a clean system ⁽²³⁾.

The grid for docking was created centered on the co-crystallized native ligand's binding site to express the specific catalytic region for exploration. The ligands were prepared, and using the Glide module (Schrödinger LLC), their conformations were docked flexibly ^(23,24). This method enabled a comparative analysis of the binding interactions and poses of the novel compounds in relation to the native ligand, giving insights into the structural determining factors of binding affinity and selectivity for CA IX ⁽²⁴⁾.

MM-GBSA Binding Free Energy Calculations

The binding free energies of the designed isatin-sulfonamide compounds, in addition to the native ligand, against the carbonic anhydrase IX (CA IX) enzyme (PDB: 5FL5) were predicted using the MM-GBSA (Molecular Mechanics/Generalized Born Surface Area) method. These calculations were accomplished with the Prime module of the Schrödinger suite ⁽²⁵⁾. The OPLS3e force field, providing a comprehensive framework for modeling molecular energetics and interatomic forces, was applied to define the potential energy of the system, thereby

allowing a reliable estimation of binding affinity ⁽²⁶⁾.

For accurate modeling of the role of the solvent, the VSGB 2.0 solvation model was applied. By effectively accounting for solvation forces and the energetic contributions of the solvent environment, this model was able to predict realistic binding free energy ⁽²⁷⁾. The computations were directed at the optimized ligand-enzyme complexes to clarify the thermodynamics of the binding interaction. The resulting binding free energy (ΔG_{bind}) values offer a quantitative basis for evaluating and ranking the comparative binding strengths of the compounds to the CA IX active site ⁽²⁸⁾.

Molecular Dynamics (MD) Simulation

A simulation was performed to explore the stability as well as the binding characteristics of the protein-ligand complex. The primary system was set by solvating the enzyme-ligand complex within an orthorhombic box using the TIP3P water model. System neutrality was achieved by adding sodium and chloride ions ⁽²⁹⁾.

Using the Desmond module within the Schrödinger software suite on a Linux platform, and a duration of 150 nanoseconds, the simulation was performed ⁽³⁰⁾. The calculation employed the OPLS4e force field under the NPT ensemble, with temperature maintained constant at 300 K and pressure of 1.01325 bar.

After the simulation, the trajectories were analyzed to assess system behavior. The stability and interactions of the complex were interpreted by the use of Simulation Interaction Diagram, which provided data on the root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), and specific protein-ligand contacts ⁽³¹⁾.

Toxicological Profiles

The toxicological properties of the lead compounds were predicted *in silico* using the ProTox 3.0 platform. Machine-learning models were employed to estimate LD₅₀



values and GHS toxicity classes, supporting early identification of potential safety concerns.

Results and Discussion

Molecular Docking Analysis for CA IX Inhibitors

Molecular docking studies were conducted to assess the binding mode and predicted affinity of the designed compounds toward carbonic anhydrase IX (CA IX). All designed ligands showed promising binding affinities, with docking scores ranging from -4.151 to -6.299 kcal/mol, in comparison with the reference inhibitor 5-(1-(4-methoxyphenyl)-1H-1,2,3-triazol-4-yl) thiophene-2-sulfonamide (82E), which showed a docking score of -2.90 kcal/mol, Table (1). The more negative docking scores of the designed compounds suggest stronger interactions and better stabilization within the CA IX active site relative to the reference molecule.

Hydrogen Bonding Interactions

A central role is played by Hydrogen bonding in ligand binding and stabilization within the CA IX catalytic pocket. Most studied compounds exhibited interactions with key residues such as Thr200, Pro201, Gln71, and Asp131, in addition to water-mediated hydrogen bonds, an important factor in the placement of ligands close to the catalytic zinc ion.

Compound 3, with the highest docking score (-6.299 kcal/mol), formed several strong hydrogen bonds: Thr200 (1.82 Å), Pro201–H₂O (2.59 Å), Gln71 (2.24 Å), and Asp131–H₂O (1.84 Å). The short hydrogen-bond distances are an indication of strong polar interactions that significantly enhance stability within the active site. Figure 2 (A and B). Compound 2 (-6.078 kcal/mol) also showed high affinity due to strong hydrogen bonds with Thr200 (1.91 Å) and Arg129 (2.69 Å). The weaker binding energy of the

reference ligand compared to the designed ligands was explained by its single hydrogen bond with Thr201 (2.04 Å).

Metal Coordination with Zinc (Zn^{2+})

An important requirement for potent CA IX inhibition is the direct and stable coordination with the catalytic Zn^{2+} ion (Zn1260); this target was achieved by all designed compounds, indicating their proper chemical scaffold. Coordination to Zn^{2+} anchors the ligand inside the catalytic site, leading to enzyme inhibition and blocking CO₂ hydration.

Hydrophobic Interactions

Hydrophobic contacts constitute a considerable contribution to ligand stabilization within the relatively hydrophobic CA IX active site. Frequent residues that contribute in hydrophobic contacts include: Pro202, Pro203, Leu134, Val130, Val121, Cys204, Leu199, Val208, Ala22, Trp210, Leu91, Val142, Pro76, etc.

Of all studied ligands, Compound 3 and Compound 2 coordinated the largest hydrophobic networks, explaining their higher docking scores. In addition, compound 5 showed several interactions with key hydrophobic residues like Pro202, Pro203, Val130, and Leu199, producing moderately high affinity.

Hydrophobic residues adjacent to the catalytic cavity, mainly Pro202/203, Val130, and Leu199, are crucial in CA IX selectivity, and their interaction creates a stronger binding of the designed ligands.

On the other hand, the reference inhibitor exhibited fewer hydrophobic interactions in general, and despite its interaction with residues such as Val130, Val142, and Leu199, the hydrophobic profile is weaker, supporting its lower docking score, Figure 3 (A and B).



Table (1): Docking scores and interaction type for different isatin-sulfonamide derivatives inside CA IX (PDB code: 5FL5) active site.

Compounds	Docking score Kcal/mol	Interaction		
		H-bonding with distance	Metal coordination	Hydrophobic
1	-4.151	THR 200(1.74), PRO+H ₂ O (2.59) GLN 71+ H ₂ O (2.35), GLN 92 (2.59)	Zn 1260	PRO 202, PRO 203, LEU 134, VAL 130, VAL 121, CYS 204, LEU 199, VAL 208, PRO 23, ALA 22, TRP 210,
2	-6.078	THR 200(1.91), ARG 129 (2.69)	Zn 1260	PRO 202, PRO 203, LEU 134, VAL 130, VAL 121, CYS 204, LEU 199, LEU91,
3	-6.299	THR 200(1.82), PRO+H ₂ O (2.59) GLN 71(2.24), ASP 131+H ₂ O (1.84)	Zn 1260	PRO 202, PRO 203, LEU 134, VAL 130, VAL 121, CYS 204, LEU 91, LEU 199, LEU 140, VAL 142
4	-5.247	THR 200(1.90), PRO+H ₂ O (2.24) GLN71+ H ₂ O (2.26) VAL130 (2.58),	Zn 1260	PRO 202, PRO 203, LEU 134, VAL 130, VAL 121, CYS 204, LEU 199, VAL 208, ALA 128, LEU 74, PRO 75, PRO 76
5	-5.94	THR 200(1.82), PRO+H ₂ O (1.92)	Zn 1260	PRO 202, PRO 203, LEU 134, VAL 130, VAL 121, CYS 204, LEU 199, LEU91,
Reference 82E	-2.90	THR 201 (2.04)	Zn 1260	PRO 202, PRO 203, LEU 134, VAL 130, VAL 142, LEU91, LEU 199, LEU 144, VAL 121



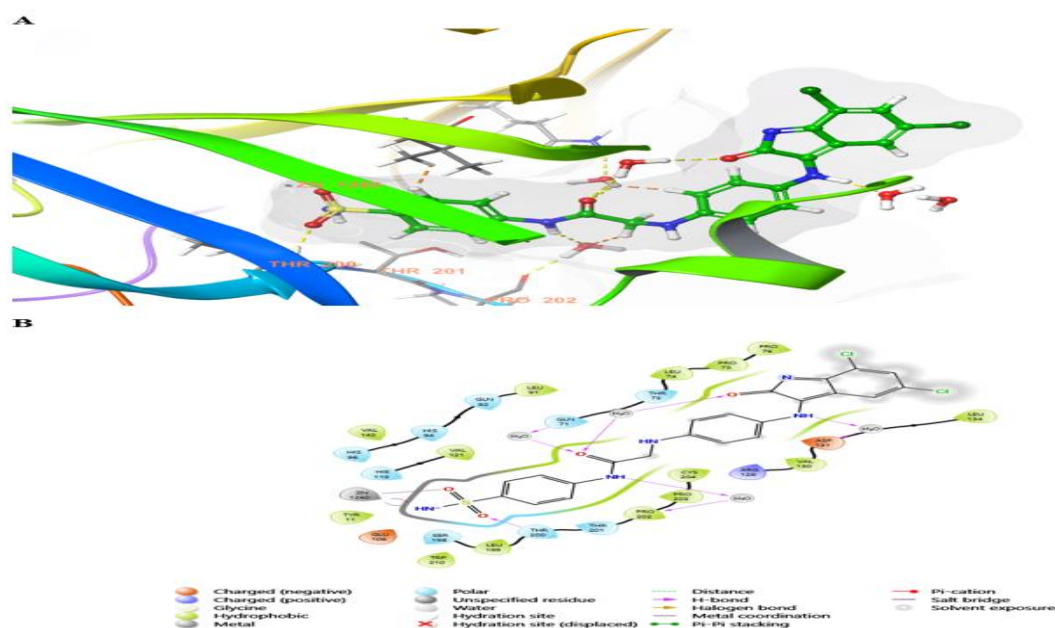


Figure 2: The interaction of compound 3 with the active binding site of CA IX in 3D (A) and 2D (B) structures.

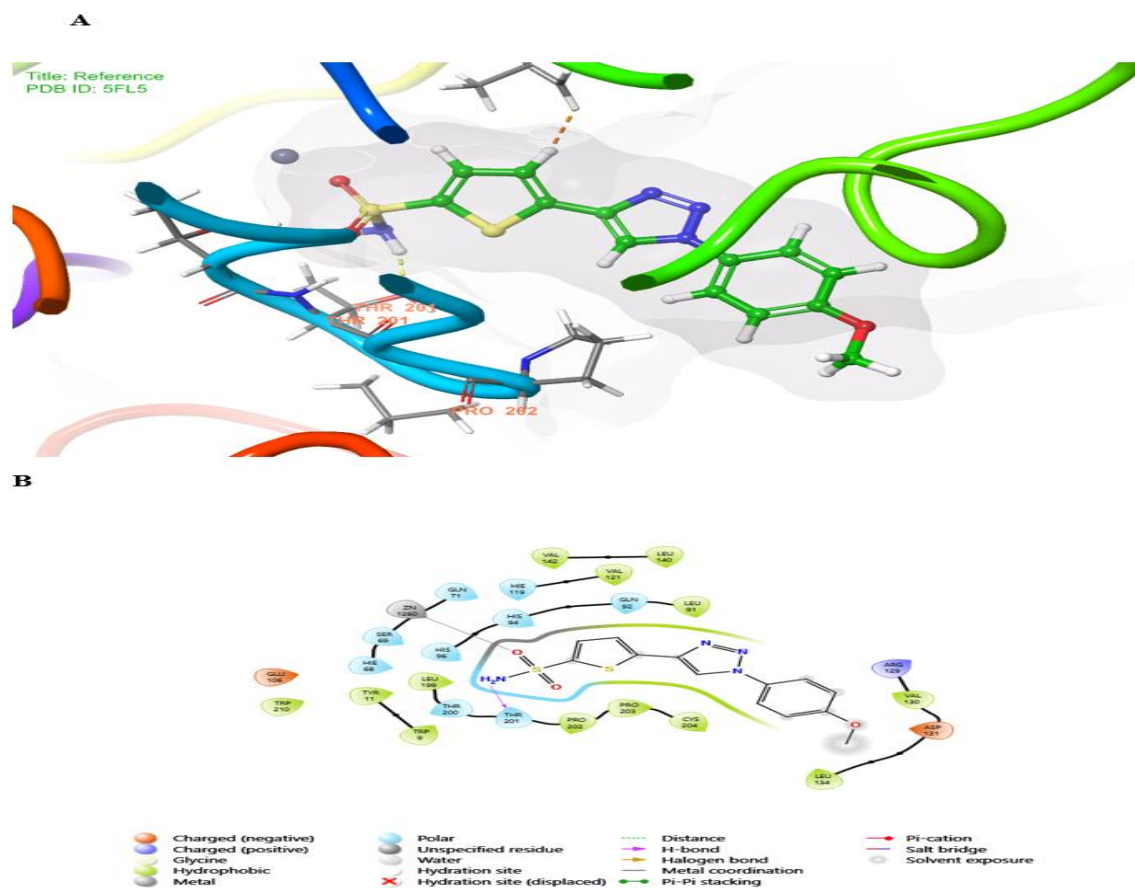


Figure 3: The interaction of native ligand (82E) with the active site of CA IX enzyme in 3D (A) and 2D (B) structures.

MM-GBSA Binding Free Energy

Analysis

MM-GBSA binding free energies calculations for the native ligand 8E2 and proposed compounds revealed that Compound 3 exhibited a favorable binding affinity toward CA IX, with a binding energy ΔG of -28.24 kcal/mol, this is significantly more favorable than that of the native ligand (-18.86 kcal/mol) Table (2). This represents an improvement in the interaction profile. As shown by the decomposed energy terms, van der Waals component (-37.53 kcal/mol) as well as lipophilic contacts (-11.81 kcal/mol) are the chief contributors to the stability of the compound 3-CA IX complex. A

moderate hydrogen bonding (-3.37 kcal/mol) is consistent with compact fitting inside the binding pocket. A comparison across the series showed that compound 3 had the third strongest overall binding energy, exceeded by compounds 1 (-33.39 kcal/mol) and 4 (-29.50 kcal/mol). Despite this, Compound 3 was selected for emphasis due to its superior stability profile in subsequent MD simulations. These MM-GBSA results are consistent with the MD observations, where Compound 3 maintained stable interactions throughout the 150 ns trajectory, supporting its further investigation as a CA IX inhibitor candidate.

Table (2): MM-GBSA values of different isatin-sulfonamide derivatives and native ligand in CA IX enzyme (PDB code:5FL5)

compounds	ΔG bind (Kcal/mol)	ΔG bind Vander	ΔG binds lipophilic	ΔG binds H-bond
1	-33.39	-44.14	-14.28	-2.22
2	-24.68	-36.21	-9.03	-3.18
3	-28.24	37.53	-11.81	-3.37
4	-29.50	-40.89	-10.36	-3.14
5	-24.38	-36.26	-9.04	-3.15
82E	-18.86	-31.05	-10.60	-2.41

Molecular Dynamics Simulation

To estimate the stability, binding mode, and dynamic interactions of compound 3 within the active site of carbonic anhydrase IX (CA IX), a 150 ns molecular dynamics simulation was performed (PDB: 5FL5). The simulation offers many insights into the conformational stability of the protein, the ligand mobility, in addition to the main interactions maintaining the complex.

Throughout the 140 ns MD simulation, the RMSD profile of the CA IX-compound 3 complex showed a stable protein backbone, while C α RMSD values changed from 1.0 to 1.8 Å, representing structural stability and the system equilibration. The ligand exhibited moderate fluctuations in RMSD (2–6 Å),

indicating conformational flexibility within the binding pocket with no evidence of dissociation. The detected fluctuations are consistent with induced-fit adjustments typical of CA IX inhibitors, indicating that compound 3 remains steadily accommodated within the active site throughout the entire simulation, Figure (4).

The C α backbone RMSD fluctuated within 1.0–1.8 Å throughout the 150 ns trajectory. This narrow range indicates that the protein remained structurally stable and did not undergo global unfolding. The system reached equilibration, as no continuous drift or increasing RMSD was observed. The dynamics represent realistic thermal motions rather than structural instability.



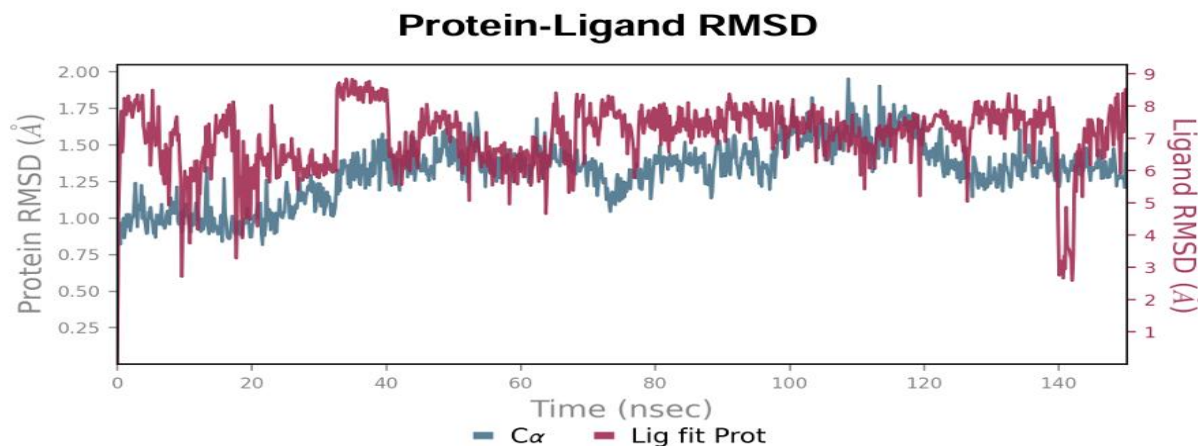


Figure 4: Compound 3 RMSD plot.

Secondary Structure

Approximately ~37.7% secondary structure elements of the protein were preserved, mostly β -strands (~30%) and a less helical portion (~7%), with slight changes over time. This approves that CA IX conserved its native fold, and the simulation conditions did not disturb its architecture.

According to RMSF values, the terminal regions show the major fluctuations (typical

for most proteins). While the catalytic core remained rigid, Figure (5).

Numerous residues adjacent to the ligand-binding pocket showed mild but constant fluctuations. Residues with green vertical bars in the RMSF plot point to persistent ligand contacts, which confirms their structural contribution to ligand stabilization.

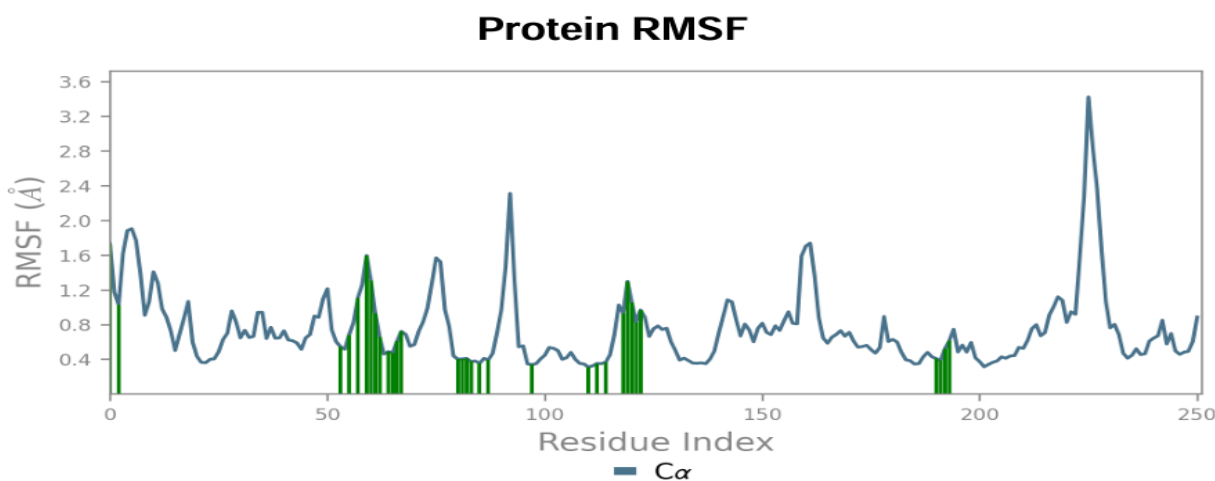


Figure 5: Compound 3 RMSF.

Hydrogen Bonds

There were persistent H-bond interactions with key residues. The simulation interaction diagram demonstrates H-bonds continued for significant portions of the trajectory (>30%). Alongside the essential zinc coordination, these hydrogen bonds contribute to the precise orientation and stabilization of the ligand.

Hydrophobic Interactions

Hydrophobic contacts showed dominance throughout the simulation. The aromatic

rings of the ligand consistently formed π - π stacking and van der Waals interactions with hydrophobic residues in the CA IX pocket. These interactions are crucial for CA IX-sulfonamide inhibitors stabilization.

Ionic & Water Bridges

Occasionally, ionic contacts complemented the hydrogen bond network, in addition to the water bridges, which formed dynamically and participated in the flexibility and entropic stabilization of the ligand, Figure 6 (A and B).

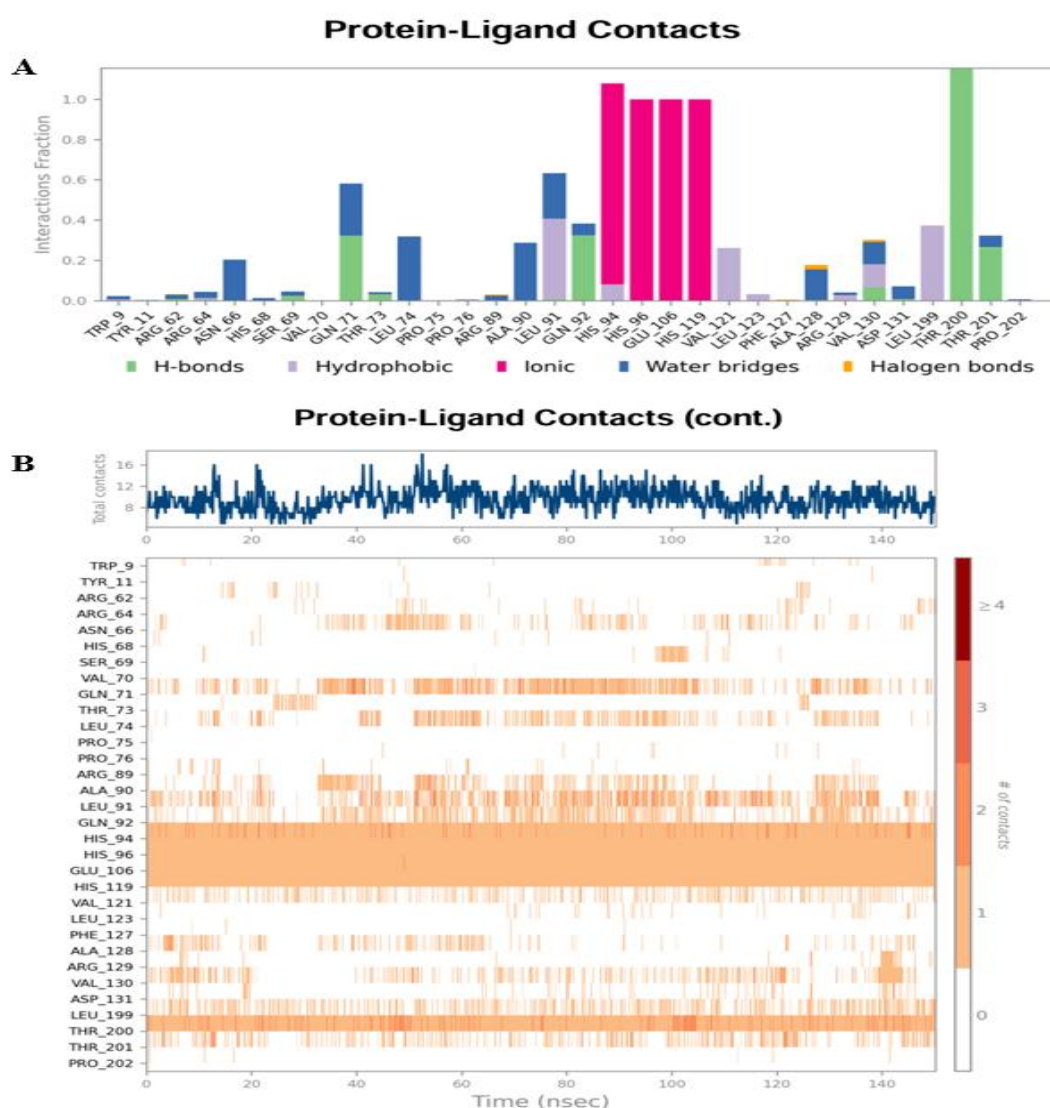


Figure 6: CA IX - compound 3 contacts. (A, B) explain the proportion of binding interactions through 150 ns of MDS.



Physicochemical properties

For a comparison study, important physicochemical properties like Molecular Mass (MW), topological polar surface area (TPSA), hydrogen bond donors (HBD), and hydrogen bond acceptors (HBA) were calculated for all candidate compounds, in addition to the native ligand (Table 3). These features characterize key molecular aspects and general structural similarity between molecules.

1. Molecular Mass

Molecular Mass (g/mol) of compounds fell below and above 500 g/mol. It is known that higher molecular Mass (>500 g/mol) often reduce oral absorption and permeability. Here, all compounds had higher MWT than the native ligand, rendering them less absorbable.

2. LogP (lipophilicity)

For native ligand: 1.56 (moderately hydrophilic), while for other compounds: 1.66–2.63. Although the native ligand has a lower LogP value than other compounds, all compounds fall within an acceptable range, leading to balanced solubility and permeability, since the ideal LogP for oral drugs lies between 1 and 3.

3. Hydrogen Bond Donors (HBD)

It is a measurement of the number of hydrogen atoms in molecules that can contribute to H-bonding. While for the Native ligand, it is 1 other candidate have 4. More donors in a molecule create stronger interactions with their targets, as is the case with candidates. Nevertheless, more HBD decreases drug permeability, and if HBD exceeds 5, it generally violates Lipinski's rules. here the maximum HBD = 4, which lies in the acceptable limit.

4. Hydrogen Bond Acceptors (HBA):

Represents the number of atoms that accept hydrogen bonds. For native ligand= 6, while the candidates range from 6 to 8. Higher HBA may strengthen binding interactions but may decrease membrane permeability. In spite of higher HBA in candidates, it is still in the acceptable range (ideal < 10).

5. TPSA (Topological Polar Surface Area)

Represents overall polarity, influencing absorption as well as blood–brain barrier permeability. The native ligand had the least value: 136.72 Å², while others: ~151 Å², except compound 2 (160.36 Å²). Ideally, a TPSA value under 140 Å² permits better intestinal absorption. Since most compounds (151–160 Å²) slightly exceed this limit, they demonstrate somewhat less oral absorption.

Table (3): Physicochemical properties of native ligand and isatin-sulfonamide derivatives

Compound	Molecular weight(g/mol)	LogP	H-bond donors	H-Bond acceptors	TPSA
native ligand	336.39	1.56	1	6	136.72
1	528.38	2.21	4	6	151.13
2	479.51	1.66	4	7	160.36
3	518.37	2.63	4	6	151.13
4	485.46	2.18	4	8	151.13
5	477.54	2.33	4	6	151.13



Toxicity profile:

The toxicity profiles of the candidate compounds were estimated, utilizing an in-silico toxicity model (software that predicts toxicological properties).

Regarding Hepatotoxicity, neurotoxicity, Immunogenicity, and Mutagenicity, all the candidate compounds and the reference drug were found inactive. Concerning carcinogenicity, all candidate compounds showed inactive results, unlike the native ligand, which was active with probability 0.54. While the native ligand and all compounds were found Respiratory toxic, only compound 9 was inactive in terms of Respiratory toxicity. Thus, compound 2 may have promising safety for further development as a drug.

According to GHS (Globally Harmonized System) classification, compounds are

classified into Class 1–3: Highly toxic, Class 4: Moderately toxic, Class 5: Low toxicity, and Class 6+: Relatively safe. Since all the studied compounds fell in class 5, they have low acute toxicity.

The LD50 values for the candidate compounds ranged from 4000mg/kg to 5000mg/kg. Compounds 3,4, and 5 had the highest LD50 of 5000mg/kg, indicating a relatively higher Safety margin when compared to other compounds. Overall, the candidate compounds exhibited favorable toxicity profiles, Table (4). The native ligand compound is considered moderately toxic, with an LD50 equal to 400, making it less favorable than the newly tested molecules. This highlights the importance of constant investigation of the new chemical series.

Table (4): Toxicity profile of the native ligand and isatin-sulfonamide derivatives.

Toxicity	Native ligand	1	2	3	4	5
Hepatotoxicity	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Neurotoxicity	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Immunogenicity	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Carcinogenicity	Active 0.54	Inactive	Inactive	Inactive	Inactive	Inactive
Mutagenicity	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Respiratory toxicity	Active 0.58	Active 0.79	Inactive	Active 0.8	Active 0.79	Active 0.79
Toxicity class	4	5	5	5	5	5
LD50	400mg/kg	4000mg/kg	4000mg/kg	5000mg/kg	5000mg/kg	5000mg/kg

Conclusion

This computational study revealed that the strategic hybridization of isatin and sulfonamide pharmacophores has generated a series of novel compounds possessing favorable potential as selective carbonic anhydrase IX (CA IX) inhibitors. Namely, Compound 3 demonstrated higher binding affinity as well as stable interactions within

the CA IX active site. In addition, it is considered safer when compared to the native ligand. Evaluation of stability and selectivity of these compounds was accomplished via a combination of computational methods, such as molecular dynamics simulations, molecular docking, and MM-GBSA free energy calculations.



These findings propose that the designed isatin-sulfonamide hybrids may have a promising dual-targeted approach for selective. Thus, preventing the hypoxic tumor cells from pH regulation, leading to their death. In addition to the advantageous safety and efficacy profiles. It is advised to apply further experimental evaluations prior to involvement in preclinical development as potential anti-cancer agents.

Acknowledgement

The authors gratefully acknowledge the College of Pharmacy, Mustansiriyah University (www.uomustansiriyah.edu.iq), Baghdad, Iraq, for providing the necessary facilities and support to successfully carry out this work.

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