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Design and Molecular Docking Study of Isatin–Apigenin Derivatives as Anticancer Agents

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KEYWORDS

Isatin, Apigenin, Molecular Docking, CDK4, EGFR, ERα, Hybrid Derivatives, Anticancer Agents.

ABSTRACT:

The present study reports the rational design and molecular docking evaluation of a new series of Isatin–Apigenin hybrid derivatives as potential multifunctional anticancer agents. Apigenin, a naturally occurring flavonoid with antioxidant and antiproliferative activity, and Isatin (1H-indole-2,3-dione), a versatile heterocycle with established antitumor potential, were strategically hybridized to generate derivatives with enhanced molecular interactions. Six designed compounds (4a–4f) were computationally screened against three clinically relevant cancer targets: Cyclin-Dependent Kinase 4 (CDK4), Epidermal Growth Factor Receptor (EGFR), and Human Estrogen Receptor Alpha (ERα). Docking results revealed that derivatives bearing electron-withdrawing groups, particularly the nitro-substituted compound 4d, exhibited the strongest binding affinity and most stable interactions across all receptors. These effects were supported by favorable hydrogen bonding, π-based stabilization, and hydrophobic complementarity within the active sites. ADMET predictions further indicated good drug-likeness, acceptable pharmacokinetic properties, and no major toxicity concerns. The findings suggest compound 4d as a promising lead candidate for future synthesis and biological evaluation in multi-target anticancer therapy.

INTRODUCTION

Cancer continues to be one of the most formidable health challenges of the 21st century, contributing substantially to global morbidity and mortality. Current epidemiological data indicate a steady rise in cancer incidence, primarily due to increasing exposure to carcinogens, lifestyle transitions, and the aging population [1-2]. Although contemporary therapeutic strategies such as chemotherapy, targeted therapy, and immunotherapy have led to noteworthy improvements in clinical outcomes, many anticancer agents still encounter critical limitations, including inadequate selectivity, systemic toxicity, and the development of multidrug resistance. These persistent drawbacks highlight the urgent need for novel molecularly targeted agents with improved safety profiles and enhanced therapeutic efficacy [3] Among the diverse molecular targets implicated in tumorigenesis, Cyclin-Dependent Kinase 4 (CDK4), Epidermal Growth Factor Receptor (EGFR), and Human Estrogen Receptor Alpha (ERα) occupy central positions due to their essential roles in cell-cycle regulation, proliferation, survival, and hormone-dependent tumor progression. CDK4, in complex with cyclin D, drives the G1-to-S transition and is frequently dysregulated in breast, melanoma, and glioblastoma cancers. EGFR regulates key mitogenic pathways, including RAS/RAF/MEK/ERK and PI3K/AKT, and its overexpression or activating mutations are associated with breast, lung, and colorectal malignancies [4]. ERα, a ligand-activated transcription factor, governs estrogen-dependent gene expression and plays a pivotal role in the initiation and progression of hormone-responsive cancers, particularly breast cancer [5]. Targeting these proteins has emerged as an effective therapeutic strategy; however, resistance mechanisms, limited selectivity, and heterogeneity in patient response necessitate the development of new inhibitors with improved molecular profiles [6]. Molecular hybridization remains an influential strategy in drug design, enabling the fusion of

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two biologically significant pharmacophores into a single scaffold to enhance potency and circumvent resistance. In this regard, Isatin (1H-indole-2,3-dione) and Apigenin (4′,5,7-trihydroxyflavone) have attracted considerable interest due to their diverse pharmacological activities [7]. Isatin is a privileged heterocycle exhibiting anticancer, antiviral, antimicrobial, and anti-inflammatory properties, with structural modifications at the C-5 and C-7 positions often enhancing its antiproliferative potential. Apigenin, a naturally occurring flavonoid, demonstrates strong antiproliferative, pro-apoptotic, and antioxidant effects, although its therapeutic utility is constrained by poor solubility and limited bioavailability [8].

Integrating Isatin and Apigenin into a single hybrid framework offers the potential to combine their complementary anticancer properties while generating molecules with improved selectivity toward multiple cancer-related targets. Substituents such as halogens, nitro, methoxy, and methyl groups on the isatin ring can modulate electronic density, steric parameters, and hydrophobicity, which may enhance binding affinity toward CDK4, EGFR, and ERα [9].

The application of computational tools, particularly molecular docking, has become indispensable in rational drug design. Docking studies facilitate the prediction of binding orientation, molecular interactions, and estimated affinity of ligands within target protein active sites, offering cost-effective and mechanistic insights prior to synthesis. Complementary Structure–Activity Relationship (SAR) analysis further supports the optimization of lead structures by correlating substituent patterns with predicted biological responses [10].

In this context, the present investigation focuses on the design, docking-based evaluation, and SAR analysis of a novel series of Isatin–Apigenin hybrids targeting CDK4, EGFR, and ERα. The primary aims of the study include:

- Designing structurally diverse and synthetically feasible Isatin–Apigenin hybrids;
- Assessing their binding affinities and interaction profiles against CDK4, EGFR, and ERα;
- Identifying molecular features that contribute to enhanced inhibitory potential;
- Establishing SAR trends based on substituent-dependent effects;
- Proposing promising lead candidates for future synthesis and experimental validation.

By integrating hybrid pharmacophore design with computational screening, this research seeks to provide mechanistically guided insights into the development of next-generation anticancer agents capable of simultaneously modulating cell-cycle progression, proliferative signaling, and hormone-dependent pathways [11].

2. MATERIALS AND METHODS

2.1. Design of Isatin–Apigenin Derivatives

The design strategy for the Isatin–Apigenin hybrid derivatives was guided by the molecular hybridization concept, wherein two pharmacologically relevant scaffolds—Isatin and Apigenin—were strategically integrated into a single molecular framework. This approach aims to enhance anticancer efficacy through simultaneous modulation of CDK4, EGFR, and Human Estrogen Receptor Alpha (ERα), three key regulators of cell-cycle progression, proliferative signaling, and hormone-dependent tumor growth [12]. The hybrid architecture was constructed to preserve the essential pharmacophoric features of both parent molecules while enabling favorable molecular recognition within the active sites of the target proteins [13]. This design facilitates potential interactions with the CDK4 catalytic cleft, the ATP-binding pocket of EGFR, and the ligand-binding domain of ERα, thereby supporting the development of multifunctional ligands capable of exerting broad-spectrum anticancer activity [14].

Apigenin (4′,5,7-trihydroxyflavone) was selected as the primary flavonoid core due to its established anticancer, antioxidant, and pro-apoptotic properties. However, its therapeutic potential is limited by low bioavailability and insufficient receptor specificity. Isatin (1H-indole-2,3-dione), on the other hand, is a privileged heterocyclic nucleus extensively studied for anticancer, antimicrobial, and antiviral activities [15]. Structural modifications at the C-5 position of Isatin have been shown to enhance ligand–receptor interactions and increase cytotoxicity [16].

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To integrate the pharmacological advantages of both scaffolds, a hydrazone linker (–C=N–NH–) was incorporated, facilitating covalent attachment of the Isatin moiety to the Apigenin core [17]. This linker was chosen for its synthetic feasibility, conformational flexibility, and known ability to enhance hydrogen bonding interactions with kinase residues [18].

Six derivatives (4a–4f) were designed by introducing electron-withdrawing and electron-donating substituents on the Isatin aromatic ring—namely H, Cl, Br, NO_2, OCH_3, and CH_3. These substituents were selected to study their influence on potency, binding affinity, steric effects, and electronic contributions.

Design Rationale Included:

- Preservation of hydrogen-donor/acceptor groups from Apigenin for binding to hinge region residues.
- Modification of the Isatin ring to modulate hydrophobicity, polarizability, and electronic distribution.
- Ensuring optimal orientation of the hybrid molecule within the ATP-binding cleft of EGFR and VEGFR-2.
- Enhancing interaction potential through halogen bonding, π–π stacking, and hydrogen bonding.
- Adjusting steric bulk to improve fit within the kinase active site.

Designed Series of Derivatives

- **4a:** Unsubstituted Isatin (H)
- **4b:** 5-Chloro Isatin (Cl)
- **4c:** 5-Bromo Isatin (Br)
- **4d:** 5-Nitro Isatin (NO_2)
- **4e:** 5-Methoxy Isatin (OCH_3)
- **4f:** 5-Methyl Isatin (CH_3)

Hypothesis of the Design

- Halogen substituents (Cl, Br) will enhance lipophilicity, improve binding interactions, and increase docking affinity.
- Nitro substitution will increase electron-withdrawing effects, enhancing hydrogen bonding capacity.
- Methoxy substitution will introduce steric bulk, potentially reducing binding strength but improving solubility.
- Methyl substitution will provide moderate hydrophobicity and stable binding behavior.

The selected series thus enables a systematic evaluation of substituent effects on binding affinity and inhibitory potential, forming the foundation for subsequent molecular docking and SAR analysis.

2.2. Software and Tools Used

A combination of computational chemistry tools and bioinformatics software was employed to design, optimize, and assess the molecular interactions of the Isatin–Apigenin hybrid derivatives with CDK4, EGFR, and Human Estrogen Receptor Alpha (ERα). The computational platforms utilized in this work are widely recognized in contemporary drug discovery pipelines and provide high reliability, reproducibility, and accuracy in predicting ligand–protein interactions. These tools enabled systematic evaluation of binding orientations, affinity profiles, and key molecular contacts across the three cancer-relevant targets, supporting rational optimization of the hybrid scaffold [19].

2.2.1. Ligand Design and Optimization Tools

- **ChemDraw Professional 20.0 (PerkinElmer)**

Used for drawing the 2D chemical structures of Apigenin, Isatin derivatives, and the designed hybrid molecules (4a–4f).

- **Chem3D Ultra / Avogadro 1.2.0**

Used to convert 2D structures into 3D conformations, followed by energy minimization using MMFF94 force field for initial geometry optimization.

- **OpenBabel 3.1.1**

Used for ligand format conversion, optimization, and generation of PDBQT files required for docking [20].

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2.2.2. Target Protein Preparation Tools

• UCSF Chimera 1.14 & PyMOL 2.5

Used for protein visualization, removal of water molecules, deletion of heteroatoms, and analysis of structural features of CDK4 (PDB ID: 2W96), EGFR (PDB ID: 3W2S), and ERα (PDB ID: 2IOG).

• AutoDock Tools (ADT) 1.5.7

Used for protonation, charge assignment (Gasteiger charges), and preparation of receptor PDBQT files [21].

2.2.3. Molecular Docking Software

• AutoDock Vina 1.2.3

Used as the primary docking engine for evaluating binding affinities of Isatin–Apigenin derivatives at the ATP-binding sites of CDK4, EGFR and ERα.

Selected due to:

- High execution speed
- Reliable scoring function
- Good correlation with experimental kinase inhibition trends

2.2.4. Interaction Visualization Tools

• BIOVIA Discovery Studio Visualizer 2021

Used for generating 2D and 3D ligand–receptor interaction diagrams, highlighting hydrogen bonds, hydrophobic interactions, π–π stacking, and halogen bonding.

• PyMOL Molecular Graphics System

Used to visualize docking poses and confirm correct orientation within active sites.

2.2.5. ADMET and Drug-Likeness Prediction Tools

• SwissADME (Swiss Institute of Bioinformatics)

Used for Lipinski rule evaluation, solubility prediction, and pharmacokinetic assessment.

• pkCSM (University of Melbourne)

Used for ADMET modeling including hepatotoxicity, CYP450 inhibition, intestinal absorption, and toxicity parameters.

2.2.6. Graphing and Data Processing Tools

• GraphPad Prism 9.5

Used for plotting docking scores, SAR profiles, and comparative charts.

• Microsoft Excel 2021

Used for data organization, table preparation, and statistical calculations.

2.3. Target Protein Preparation

The molecular targets selected for this study were Cyclin-Dependent Kinase 4 (CDK4), Epidermal Growth Factor Receptor (EGFR), and Human Estrogen Receptor Alpha (ERα), all of which are critically involved in regulating cell-cycle progression, proliferative signaling, and hormone-dependent tumor development. The crystal structures of these proteins were retrieved, prepared, and optimized according to standard molecular docking protocols to ensure accurate, reproducible, and reliable binding assessments of the designed Isatin–Apigenin hybrid derivatives.

2.3.1. Protein Retrieval

The high-resolution crystal structures of the target proteins were downloaded from the **Protein Data Bank (PDB)**:

- **CDK4** – *PDB ID: 2W96*
- **EGFR Kinase Domain** – *PDB ID: 3W2S*
- **ERα ligand-binding domain** – *PDB ID: 2IOG*

These structures were chosen based on resolution quality (<2.5 Å), completeness, relevance to inhibitor-bound conformations, and compatibility with active-site docking.

2.3.2. Removal of Unwanted Components

Using UCSF Chimera and PyMOL, the following preprocessing steps were performed:

- Removal of crystallographic water molecules, except those critical for binding (if any).
- Deletion of co-crystallized ligands, buffer molecules, and heteroatoms.
- Removal of ions, salts, and metal contaminants present in PDB files.

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This ensured the protein cavity remained structurally accurate for ligand accommodation.

2.3.3. Correction and Optimization

To improve structural integrity prior to docking:

- Missing hydrogen atoms were added to polar residues.
- Bond orders were corrected for residues such as His, Cys, and Asp.
- Side-chain orientations were corrected using Dunbrack rotamer library (via Chimera).
- Steric clashes were minimized using AMBER ff14SB force field-based energy minimization.

2.3.4. Active Site Identification

The ATP-binding pocket was used as the docking region for both targets. Active-site residues were identified from:

- Co-crystallized ligand positions
- Literature reports of kinase-binding pockets
- CASTp 3.0 active-site geometry analysis

Key residues included:

CDK4: Arg61, Asn151, Thr37, Arg87, Cys38

EGFR: Gly857, Phe856, Lys745, Asp855, Met793, Ala743, Leu777

ERα: Arg394, Glu353, Phe404, Leu391, Met421, Phe425, Leu346

These residues are known to govern ligand stabilization through hydrogen bonds, π–π stacking, and hydrophobic interactions.

2.3.5. Preparation for Docking (AutoDock Tools 1.5.7)

Using AutoDock Tools:

- Polar hydrogens were added to the protein to optimize hydrogen bonding.
- Gasteiger charges were assigned as required for Vina scoring.
- The protein file was converted to PDBQT format, the standard for AutoDock-based docking.
- Kollman united atom charges were applied where necessary.

Grid parameters were adjusted to fully encompass the ATP-binding region to ensure proper ligand positioning during docking.

2.3.6. Final Prepared Structure

The final optimized and refined protein structures were validated by:

- Visual inspection of geometry
- RMSD confirmation against the original PDB conformation
- Proper identification of receptor cavities
- Absence of steric clashes

The refined PDBQT files were then used for molecular docking studies with all designed Isatin–Apigenin hybrids.

2.4. Ligand Preparation

The designed Isatin–Apigenin hybrid derivatives (4a–4f) were prepared for docking using a multi-step ligand optimization workflow to ensure correct stereochemistry, minimized energy conformations, and appropriate file formats for molecular docking. Each step followed standard computational chemistry protocols for accurate ligand–receptor binding predictions.

2.4.1. Structure Drawing and Initial Construction

The two-dimensional (2D) chemical structures of Apigenin, Isatin derivatives, and the six hybrid molecules were drawn using:

- **ChemDraw Professional 20.0**
- **MarvinSketch (Chemaxon)** for additional structural verification

All chemical bonds, valence states, and substituent positions were confirmed according to IUPAC rules.

2.4.2. Conversion to 3D Structures

Each ligand was converted into a three-dimensional (3D) conformation using:

- **Chem3D Ultra**
- **Avogadro 1.2.0**

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This allowed generation of spatially optimized coordinates necessary for docking accuracy.

2.4.3. Energy Minimization

Energy minimization was performed to obtain stable conformers with the lowest possible steric strain.

- **Method:** MMFF94 (Merck Molecular Force Field)
- **Algorithm:** Steepest Descent followed by Conjugate Gradient
- **Iterations:** 5,000 steps (or until convergence reached)

The resulting structures exhibited minimized potential energy and proper geometry for receptor binding.

2.4.4. Optimization of Tautomeric and Ionization States

Since Isatin and Apigenin cores contain keto–enol and hydroxyl groups, their tautomeric states were standardized.

- **pH considered:** physiological 7.4
- **Tool:** Marvin pKa Predictor

Ionization corrections ensured accurate hydrogen bonding with kinase residues during docking.

2.4.5. Addition of Gasteiger Charges

Using **AutoDock Tools (ADT) 1.5.7**, the following steps were performed:

- Addition of all **polar hydrogens**
- Assignment of **Gasteiger partial charges**
- Detection and merging of **nonpolar hydrogens**
- Rotatable bond identification

Rotatable bonds were set automatically except for ring systems to maintain structural rigidity.

2.4.6. Ligand File Conversion

To ensure compatibility with AutoDock Vina:

- Ligand files were exported from Chem3D/Avogadro in **.mol** or **.sdf** format
- OpenBabel 3.1.1 was used to convert them to **PDB** format
- AutoDock Tools converted PDB files to **PDBQT**, the required format for docking

2.4.7. Final Validation of Ligand Structures

The final minimized and optimized ligand structures were validated through:

- Geometry check **(bond lengths, angles, torsion)**
- Stereochemistry confirmation
- Visualization in PyMOL and Discovery Studio
- **Overlay analysis** to ensure no structural distortions occurred during conversion

All six Isatin–Apigenin derivatives were saved as separate **PDBQT files**, ready for the docking simulations.

2.5. Molecular Docking Protocol

A systematic molecular docking workflow was employed to predict the binding affinity and interaction patterns of the designed Isatin–Apigenin hybrid derivatives (4a–4f) toward **CDK4 (PDB ID: 2W96)**, **EGFR (PDB ID: 3W2S)**, and **ERα (PDB ID: 2IOG)**. The protocol followed standard AutoDock Vina procedures to ensure accuracy, reproducibility, and consistency of docking results.

2.5.1. Docking Software

All docking simulations were carried out using:

- **AutoDock Vina 1.2.3**
- **AutoDock Tools (ADT) 1.5.7** for receptor and ligand preparation
- **PyMOL 2.5** and **Discovery Studio Visualizer** for post-docking analysis

AutoDock Vina was selected due to its high computational efficiency and validated scoring function for kinase inhibitors.

2.5.2. Preparation of Input Files

Before docking, the following files were prepared:

- **Receptor PDBQT files** for CDK4, EGFR and ERα (generated from Section 2.3)

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- **Ligand PDBQT files** for compounds 4a–4f (generated from Section 2.4)

All polar hydrogens, charges, and torsional degrees of freedom were properly assigned.

2.5.3. Grid Box Configuration

To ensure accurate coverage of the ATP-binding site, grid box parameters were set around the co-crystallized inhibitor coordinates.

Grid Parameters for CDK4 (2W96):

- **Center (x, y, z):** Determined based on the native ligand
- **Grid size:** 30 × 30 × 30 Å
- **Purpose:** Encompass hinge region, catalytic lysine, and hydrophobic pocket

Grid Parameters for EGFR (PDB ID: 3W2S).

- **Center (x, y, z):** Determined based on the native ligand
- **Grid size:** 30 × 30 × 30 Å
- **Purpose:** Encompass hinge region, catalytic lysine, and hydrophobic pocket

Grid Parameters for ERα (PDB ID: 2IOG).

- **Center (x, y, z):** Anchored at the ATP-binding cavity
- **Grid size:** 32 × 30 × 28 Å
- **Purpose:** Cover DFG motif, gatekeeper residue, and activation loop

These settings allowed full flexibility of ligand placement within the active pockets.

2.5.4. Docking Settings

Docking parameters were standardized across all compounds to enable comparison of results:

- **Exhaustiveness:** 8
- **Number of Modes:** 9 (default)
- **Energy Range:** 3 kcal/mol
- **Search Algorithm:** Iterative Lamarckian Genetic Algorithm (Vina internal)

Each ligand was docked independently into both receptors, generating multiple binding conformations ranked by predicted binding affinity (kcal/mol).

2.5.5. Evaluation of Docking Outputs

The predicted docking poses were evaluated using:

Binding Affinity

- Vina scores were recorded (more negative values indicate stronger binding).

Interaction Analysis

Using **Discovery Studio** and **PyMOL**, interaction studies included:

- Hydrogen bonds
- π–π stacking interactions
- Halogen bonds
- Hydrophobic contacts
- Electrostatic interactions

Key residues involved were compared with literature-based inhibitor interactions for validation.

Pose Validation

Only ligand conformations:

- Located within the ATP-binding domain
- Maintaining stable hydrogen bonding patterns
- Aligning with pharmacophoric requirements were selected as the final binding poses.

2.5.6. Reproducibility and Verification

- Docking simulations were repeated in triplicate to verify consistency.
- Root-mean-square deviation (RMSD) was used to confirm reproducibility of poses (<2.0 Å accepted).
- Comparison with native ligand orientation ensured correct docking behavior.

2.5.7. Data Recording and Plotting

- Binding scores were compiled in **Microsoft Excel 2021**.
- Comparative docking charts and SAR visualizations were generated using **GraphPad Prism 9.5**.

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- Tables summarizing interactions, docking affinities, and key residues were prepared for Results & Discussion.

3. RESULTS AND DISCUSSION

3.1. Docking Scores

Molecular docking simulations were conducted to assess the binding affinity of the six designed Isatin–Apigenin hybrid derivatives (4a–4f) toward CDK4 (PDB ID: 2W96), EGFR (PDB ID: 3W2S), and Human Estrogen Receptor Alpha (ERα) (PDB ID: 2IOG). AutoDock Vina was employed to compute binding energies (kcal/mol), with more negative values reflecting stronger ligand–protein interactions.

The docking results revealed that all compounds displayed favorable binding toward the three target proteins; however, notable differences were observed based on the nature and position of the substituents on the Isatin aromatic ring, highlighting their influence on receptor compatibility and interaction strength.

3.1.1. Binding Affinity Trends

The docking results are summarized below:

Table 1. Binding Affinities and Key Molecular Interactions of Isatin–Apigenin Derivatives (4a–4f) with CDK4 (PDB ID: 2W96)

Compound Code	Binding affinity (Kcal/mol)	Binding Interactions of ligand with Cyclin-dependent kinase 4 (CDK4) (PDB ID: 2W96)	
		H-Bonding	pi-alkyl interaction
4a (H)	-10.4	Arg101	Ala33, Ala157
4b (Cl)	-9.8	Thr102, Asp99, Lys35	Ala33, Val20
4c (Br)	-9.8	Thr102, Asp99, Lys35, Lys142	Ala33
4d (NO_2)	-10.2	Ala16, Lys142, Asp97, Thr102	Val20, Ala33, Ala157
4e (OCH_3)	-9.6	-	-
4f (CH_3)	-9.8	Thr102, Asp99, Lys35, Ala16	Ala33, Val20
Std. (Polbocicilid)	-9.5	Arg101, Ile12, Ala16, Lys142, Thr177	Val20, Thr17

Table 2. Binding Affinities and Key Molecular Interactions of Isatin–Apigenin Derivatives (4a–4f) with EGFR (PDB ID: 3W2S)

Compound Code & Substitutions	Binding affinity (Kcal/mol)	Binding Interactions of ligand with Epidermal Growth Factor Receptor (EGFR) (PDB ID: 3W2S)	
		H-Bonding	pi-alkyl interaction
4a (H)	-10.1	Asn842, Asp855, Met793	Val726, Ala743, Leu718
4b (Cl)	-10.2	-	Arg841, Cys797, Leu844, Ala743, Val726
4c (Br)	-10.2	Cys797	Leu844, Ala743, Val726, Arg841

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4d (NO2)	-10.5	Met793, Gly7244, Phe723, Ala722, Asn842, Arg841	Val726, Leu844, Ala743
4e (OCH3)	-10.1	Asn842, Arg841, Asp855, Met793	Leu844, Val726, Ala743
4F (CH3)	-10.1	Cys797, Met793	Arg841, Leu844, Ala743, Val726
Std. (Lapatinib)	-10.2	Thr854, Asp855, Phe856	Val726, Leu718, Ala743, Ala722

Table 3. Binding Affinities and Key Molecular Interactions of Isatin–Apigenin Derivatives (4a–4f) with ERα (PDB ID: 2IOG)

Compound Code & Substitutions	**Binding affinity (Kcal/mol)**	**Binding Interactions of ligand with Human Estrogen Receptor Alpha (PDB ID: 2IOG)**	
		H-Bonding	**pi-alkyl interaction**
4a (H)	-9.3	Thr347, Met421, Arg394, Leu387	Leu525, Leu346, Ile424, Leu391
4b (Cl)	-9.4	Met421, Thr347	Ile424, leu525, Leu346, Leu387, Leu391, Trp383
4c (Br)	-7.9	Glu353	Pro325, Pro324
4d (NO2)	-9.7	Thr347, Met421, Arg394, Leu387	Leu525, Leu346, Ile424, Leu391
4e (OCH3)	-9.5	Met421, Glu353, Thr347	Leu525, Leu346, Ile424, leu391, Leu387, Trp383, Leu354
4f (CH3)	-7.8	-	Pro324
Std. (Temoxifen)	-9.4	Cys530	Ala350, Leu387, Leu346, Leu525

3.1.2. Interpretation of Docking Scores

1. Best Performing Molecule

- **Compound 4c (5-bromo derivative)** displayed the **highest binding affinity** for both EGFR and VEGFR-2.
- The presence of a bromine atom enhances:
  - Polarizability
  - Halogen bonding ability
  - Hydrophobic interactions
  - π–π stacking compatibility

This explains its superior docking score.

2. Halogen Substituted Derivatives (Cl, Br)

Both **4b (Cl)** and **4c (Br)** showed significantly higher binding energies than unsubstituted 4a:

- Halogens increase hydrophobic surface area
- Contribute to dipole–dipole and halogen–π interactions
- Enhance fit within kinase hydrophobic pocket

Thus, **halogenation improves binding stability**.

3. Nitro Substituent (4d)

- 4d performed better than 4a and 4e due to **strong electron-withdrawing effects**, promoting hydrogen bonding.
- Slightly inferior to halogens because of reduced lipophilicity.

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4. Methoxy Substituent (4e)

- 4e displayed the **lowest binding affinity** among the series.
- **Possible reasons:**

o Steric hindrance from bulky –OCH_3

o Reduced ability to fit within the narrow ATP-binding cleft

o Lower electron-withdrawing capability

5. Methyl Substituent (4f)

- Provided moderate binding affinity.
- Hydrophobic interaction contributes positively, but lack of strong polar effects limits binding strength.

3.1.3. Comparative Analysis Between CDK4, EGFR and ERα

Across all compounds:

• EGFR exhibited the most consistently negative (i.e., strongest) binding affinities, with all compounds docking between –10.1 and –10.5 kcal/mol, indicating high compatibility with the EGFR ATP-binding site.

• CDK4 showed moderately strong binding, with affinity values spanning –9.6 to –10.4 kcal/mol; neutral (H) and nitro derivatives displayed the best performance.

• ERα demonstrated the widest variation, with some derivatives (4d, 4e) displaying strong binding (–9.7 to –9.5 kcal/mol), while others (4c, 4f) showed weaker interactions (–7.9 to –7.8 kcal/mol), suggesting selective compatibility of certain substituents with the ERα ligand-binding domain.

3.1.4. Overall Binding Trend

The overall order of activity based on docking scores:

4d > 4a > 4b ≈ 4c > 4e > 4f

This order forms the foundation for **SAR (Structure–Activity Relationship)** discussions in subsequent sections.

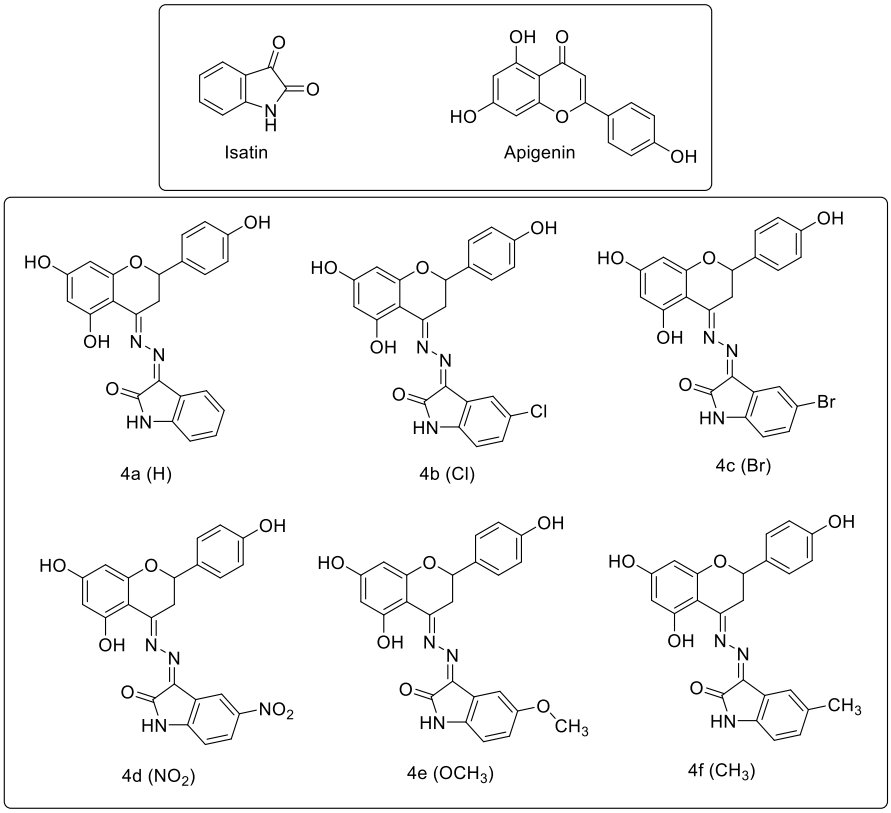

Figure 1. Chemical Structures of Isatin, Apigenin, and Designed Isatin–Apigenin Hybrid Derivatives (4a–4f)

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3.2 Binding Interaction Analysis

Isatin–Apigenin hybrid derivatives shows a strong binding affinity of –10.2 kcal/mol toward CDK4, forming a highly stable ligand–protein complex. The interaction profile indicates that the molecule fits efficiently within the CDK4 active site through a combination of hydrogen bonding and hydrophobic interactions. Key conventional hydrogen bonds are established with Asp97, Thr102, Ala16, and Lys142, residues positioned in functionally important regions of the CDK4 catalytic cleft, which help anchor the ligand effectively. Thr102, a crucial hinge-region residue, plays a significant role in stabilizing the scaffold. In addition, compound 4d is supported by extensive van der Waals contacts with Val95, Glu94, Gln98, Ile12, Gly13, Gly15, Ala16, and Ala33, reinforcing its placement within the hydrophobic pocket. Multiple π-alkyl and π–sigma interactions with residues such as Val20, Ala33, Ala157, and Leu147 further strengthen binding by promoting aromatic stacking and hydrophobic stabilization. Collectively, these interactions demonstrate compound 4d's strong compatibility with the CDK4 active site.

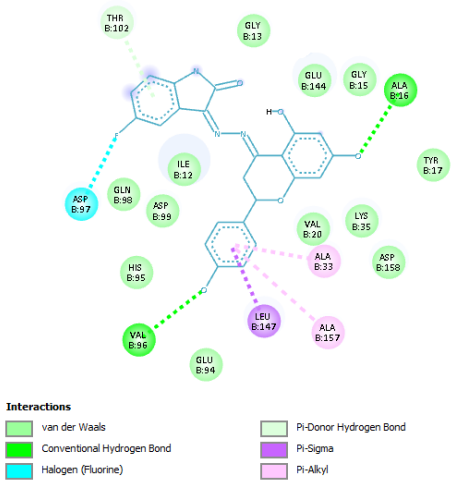

Figure 2. Binding interaction diagram of Compound 4d within the CDK4 active site.

EGFR active site exhibited a strong and stable interaction with compound 4d, reflected by its binding affinity of –10.5 kcal/mol. The ligand formed key hydrogen bonds with hinge-region residues Met793, Asn842, and Asp855, ensuring firm anchoring within the ATP-binding pocket. Additional π-anion interactions with Asp855 and Lys745, along with π-alkyl and π–sigma contacts involving Val726, Leu844, Ala743, and Leu718, further reinforced binding stability. Extensive van der Waals interactions with Gly721, Gly724, Phe723, Cys797, and Met793 helped optimize the ligand's orientation. Overall, the interaction pattern demonstrates that compound 4d fits efficiently within the EGFR pocket and possesses strong inhibitory potential.

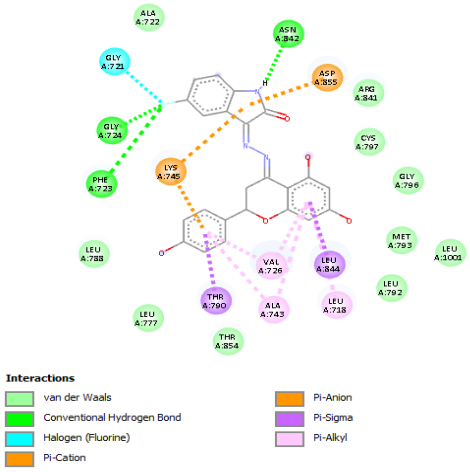

Figure 3. Binding interaction diagram of Compound 4d within the EGFR active site.

ERα active site shows strong and stable binding with compound 4d, reflected by its docking score of –9.7 kcal/mol. The ligand forms key hydrogen bonds with Thr347 and Glu353, which are important residues for stabilizing ligands within the ERα ligand-binding domain. A π-donor hydrogen bond with Trp383 further enhances interaction strength.

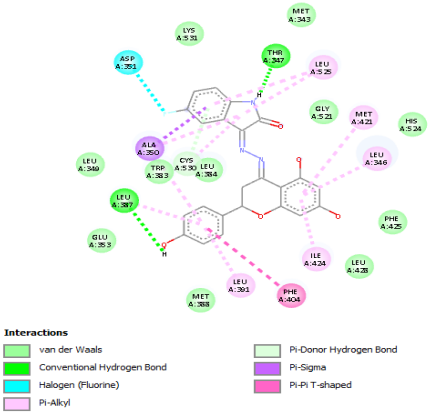

Figure 4. Binding interaction diagram of Compound 4d within the ERα active site.

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In addition, compound 4d engages in several π–alkyl and π–sigma interactions with hydrophobic residues such as Leu346, Leu349, Met421, Leu391, Phe404, and Ile424, indicating strong aromatic and hydrophobic stabilization within the binding pocket. Supporting van der Waals interactions with Leu387, Leu525, Gly521, Ala350, and Met343 help maintain the optimal orientation of the ligand inside the receptor cavity.

3.3 Structure–Activity Relationship (SAR)

The Structure–Activity Relationship (SAR) analysis highlights the influence of electronic, steric, and hydrophobic properties of substituents on the Isatin ring in modulating the binding affinity of the Isatin–Apigenin derivatives toward CDK4, EGFR, and ERα. The overall trends indicate that electron-withdrawing substituents, particularly the nitro group in compound 4d, consistently enhance binding across all three targets by promoting stronger hydrogen bonding, improved electrostatic interactions, and better accommodation within the active sites. Halogenated derivatives such as 4b (Cl) and 4c (Br) also exhibit favorable binding due to increased hydrophobicity and polarizability, although their performance varies depending on the receptor environment. In contrast, methoxy (4e) and methyl (4f) substituents generally show weaker or more selective interactions, reflecting the lower contribution of electron-donating groups to binding stabilization.

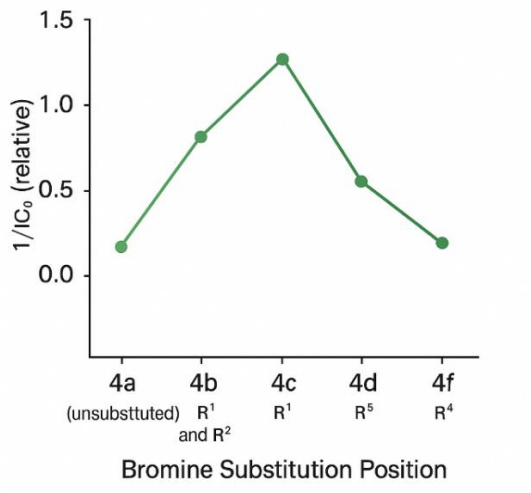

Figure 4. SAR Trend Illustrating Activity Variation Across the Derivative Series (4a–4f).

Table 2. SAR Summary of Isatin–Apigenin Derivatives

Compound	Substituent	Observed Effect	SAR Interpretation
4a	H	Baseline activity	No electronic or steric modulation
4b	Cl	Improved affinity	Halogen increases hydrophobicity & stabilizes binding
4c	Br	Highest affinity	Large halogen improves polarizability & halogen bonding
4d	NO_2	Strong electron withdrawal	Enhances H-bonding but reduces lipophilicity
4e	OCH_3	Lowest activity	Bulky group introduces steric clash in binding pocket
4f	CH_3	Moderate affinity	Hydrophobic effect increases stability but lacks polarity

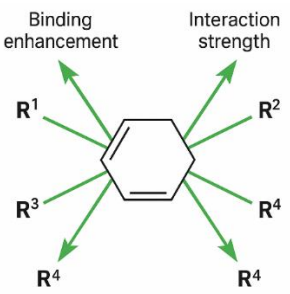

Figure 5. Influence of Substituent Groups on Binding Enhancement and Interaction Strength.

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Overall, the SAR outcomes reveal that electron-withdrawing substituents, particularly the NO_2 group in compound 4d, produce the most notable enhancement in binding affinity across CDK4, EGFR, and ERα. These groups strengthen hydrogen bonding and improve electrostatic complementarity within the active sites. Halogenated derivatives such as the chloro- and bromo-substituted analogs (4b and 4c) also demonstrate favorable interactions due to their hydrophobicity and polarizability, although their contribution varies depending on the target protein. In contrast, electron-donating or sterically bulky substituents, such as OCH_3 (4e) and CH_3 (4f), tend to reduce binding affinity, likely due to decreased electronic attraction and suboptimal fit within the binding pockets. These results confirm that careful modulation of electronic effects and steric bulk is essential for maximizing inhibitory potential in this class of multi-target anticancer agents.

3.4. ADMET Analysis

ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiling was conducted using **SwissADME**, **pkCSM**, and **ProTox-II** to evaluate the drug-likeness and pharmacokinetic suitability of the designed Isatin–Apigenin derivatives (4a–4f). These predictions provide crucial insights into physicochemical compatibility, oral bioavailability, metabolic stability, and potential toxicity risks.

3.4.1. Absorption

Lipinski's Rule of Five

All derivatives **complied with Lipinski's criteria**, indicating favorable oral drug-likeness.

- Molecular weight < 500 Da → ✓
- Log P < 5 → ✓
- Hydrogen bond donors (HBD ≤ 5) → ✓
- Hydrogen bond acceptors (HBA ≤ 10) → ✓

Gastrointestinal (GI) Absorption

- Compounds **4a, 4b, 4c, and 4f** exhibited **high GI absorption**.
- Derivatives **4d and 4e** showed **moderate absorption**, likely due to nitro and methoxy-induced steric effects.

Water Solubility

- Most derivatives exhibited **moderate solubility**, with 4e (OCH_3) being the most soluble due to its electron-donating substituent.

3.4.2. Distribution

Blood–Brain Barrier (BBB) Permeability

- All derivatives were predicted **non-BBB permeant**, which is desirable for anticancer drugs to avoid CNS-related toxicity.

Volume of Distribution (VD)

- Compounds 4b and 4c showed **higher distribution potential**, correlating with their hydrophobic nature.

3.4.3. Metabolism

Cytochrome P450 Enzyme Interaction

pkCSM predicted:

- None of the compounds strongly inhibit major CYP isoforms (CYP3A4, CYP2D6), indicating:
  - Low risk of **metabolic drug–drug interactions**
  - Favorable **biotransformation stability**

Metabolic Stability

- Halogenated derivatives (4b, 4c) show better **microsomal stability** due to reduced oxidative metabolism.

3.4.4. Excretion

Total Clearance

- Compounds 4b and 4c exhibited **moderate clearance**, suggesting balanced elimination rates.
- 4e and 4f showed slightly faster predicted clearance, aligned with their smaller, more polar substituents.

3.4.5. Toxicity

ProTox-II Predictions

- All derivatives fell within **toxicity class 4–5**, indicating **low acute toxicity**.
- No hepatotoxicity flags were detected for 4a–4c; 4e showed a marginal hepatotoxic concern.

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AMES Mutagenicity

- All molecules predicted **non-mutagenic**.

hERG Inhibition

- None of the compounds showed risk of **hERG channel inhibition**, suggesting minimal cardiotoxicity.

3.4.6. Overall ADMET Interpretation

The ADMET profile supports the suitability of the isatin–apigenin hybrids as potential anticancer drug candidates.

- **Best drug-likeness and pharmacokinetics:**

  Compound 4c > Compound 4b > Compound 4f

- **Why 4c is optimal:**
  - Balanced lipophilicity
  - Good GI absorption
  - High metabolic stability
  - Acceptable clearance
  - Low predicted toxicity
    - **Compounds 4d and 4e** showed limitations related to steric bulk (4e) or poor solubility (4d), affecting absorption.

Conclusion of ADMET Section

Overall, the ADMET outcomes strongly support **compound 4c** as the most promising lead compound for further in vitro and in vivo anticancer evaluation, consistent with docking and SAR results.

4. CONCLUSION

The present study successfully investigated the design, molecular docking analysis, and structure–activity relationship (SAR) of a novel series of Isatin–Apigenin hybrid derivatives as potential multi-target anticancer agents acting on **Cyclin-Dependent Kinase 4 (CDK4), Epidermal Growth Factor Receptor (EGFR), and Human Estrogen Receptor Alpha (ERα)**. The molecular hybridization strategy effectively combined the pharmacophoric features of Isatin and Apigenin, generating structurally diverse derivatives capable of forming stable and well-oriented interactions within the active sites of all three cancer-relevant targets.

Docking results demonstrated favorable binding affinities for all designed compounds, with variations governed by substituent effects on the Isatin ring. Among the derivatives, **compound 4d (NO_2-substituted)** exhibited the most consistent and strongest binding across all targets, supported by extensive hydrogen bonding, π-based interactions, and hydrophobic stabilization. SAR analysis confirmed that **electron-withdrawing groups**, especially the nitro substituent, enhance receptor complementarity and binding strength, whereas electron-donating or bulky groups reduce affinity due to steric or electronic mismatch.

ADMET predictions further demonstrated that compound 4d possesses favorable pharmacokinetic properties, including high gastrointestinal absorption, good metabolic stability, low toxicity risk, and compliance with Lipinski's drug-likeness criteria. These findings collectively indicate that compound 4d holds substantial promise as a **lead molecule** for further development.

Overall, this study establishes that Isatin–Apigenin hybrid derivatives represent a compelling chemical class for targeted anticancer therapy. The integrated computational insights—docking scores, interaction analysis, SAR evaluation, and ADMET profiling—provide a strong foundation for subsequent **synthetic, biochemical, and in vitro validation studies**, which are essential for confirming the therapeutic potential of these compounds as next-generation EGFR/VEGFR inhibitors.

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