

Drug Design, In Silico ADME, Drug-Likeness, Molecular Docking and In Vitro Evaluation of Mannich-Modified Dihydropyrimidinone Derivatives Targeting HPV16 E6

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Abstract: Persistent infection with high-risk human papillomavirus (HPV) is a necessary cause of cervical cancer, and the viral E6 oncoprotein is a candidate target for small-molecule design. Five 3,4-dihydropyrimidinone derivatives (C1–C5) were designed on the Biginelli scaffold and modified by a Mannich-type reaction with benzamide, aniline, sulphadiazine, para-aminobenzoic acid (PABA) and 2-aminobenzothiazole, respectively. Drug-likeness and ADME properties were predicted with SwissADME, toxicity risks with OSIRIS Property Explorer, and binding to HPV16 E6 (PDB ID: 4GIZ) by molecular docking with PyRx/AutoDock. The top-ranked compound was tested against HeLa cells by the MTT assay. All five compounds showed no Lipinski violations, acceptable predicted ADME characteristics and no major predicted toxicity alerts. Docking scores were –7.3, –7.2, –7.8, –8.1 and –8.8 kcal/mol for C1–C5, respectively. The 2-aminobenzothiazole derivative C5 gave the most favorable score (–8.8 kcal/mol) and reduced HeLa cell viability in a concentration-dependent manner (IC₅₀ 36.01 µg/mL). As HeLa cells are HPV18-positive, this cytotoxicity cannot be attributed to inhibition of HPV16 E6; the docking result is predictive, and C5 is regarded as a candidate for further structural optimization and mechanistic testing in HPV16-positive cells.

Keywords: 3,4-dihydropyrimidinone; Mannich base; HPV16 E6; molecular docking; SwissADME; drug-likeness; toxicity prediction; PyRx; Auto Dock; in silico drug design.

INTRODUCTION

Cervical cancer remains a major cause of cancer morbidity and mortality worldwide [1], and persistent infection with high-risk human papillomavirus (HPV) is a necessary cause of the disease [2]. The E6 oncoprotein of HPV16 and HPV18 promotes degradation of the tumor suppressor p53 [3], a process that requires the cellular ubiquitin ligase E6AP [4,5]. E6 binds E6AP through a helical LxxLL motif. Crystal structures of full-length HPV16 E6 bound to an E6AP LxxLL peptide (PDB 4GIZ) and of the E6/E6AP/p53 ternary complex (PDB 4XR8) have defined a basic-hydrophobic pocket that is a candidate site for small-molecule ligands [6–8].

Dihydropyrimidinones, accessible through the Biginelli reaction [9,10], and Mannich bases [11,12] are scaffolds of long-standing interest in medicinal chemistry. In this work, five Mannich-modified dihydropyrimidinone derivatives were designed and prepared, their drug-likeness, ADME properties and toxicity were predicted in silico, and their binding to HPV16 E6 was evaluated by molecular docking. The highest-ranked compound was then examined in a preliminary cytotoxicity assay.

2. Materials and Methods

2.1 Molecular Design

Five candidate molecules were designed as substituted 3,4-dihydropyrimidinone derivatives and coded C1–C5. Structures were drawn with ACD/ChemSketch (v2020/v2021). The derivatives carry an amine-derived substituent introduced through a Mannich-type reaction: benzamide (C1), aniline (C2), sulphadiazine (C3), PABA (C4) and 2-aminobenzothiazole (C5). The molecular formulas were C₂₃H₂₅N₃O₄ (C1), C₂₂H₂₅N₃O₃ (C2), C₂₂H₂₆N₄O₅S (C3), C₂₃H₂₅N₃O₅ (C4) and C₂₃H₂₄N₄O₃S (C5).

2.2 Chemistry

Step 1: Biginelli synthesis of the 3,4-dihydropyrimidin-2(1H)-one intermediate. Urea, benzaldehyde and ethyl acetoacetate (1:1:1 molar ratio) were placed in a round-bottom flask with a few drops of concentrated hydrochloric acid as catalyst. The reaction was carried out solvent-free or in ethanol. The mixture was refluxed for 1–2 h at 80–90 °C, and completion was monitored by colour change (colorless to yellowish orange) and thin-layer chromatography (TLC). The reaction mixture was filtered, the filtrate was evaporated, and the product was recrystallized from a mixture of DMF and ethanol to give the intermediate, following the Biginelli reaction [9,10] (Figure 1).

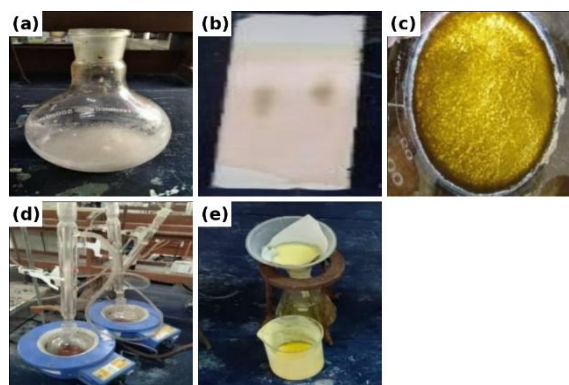


Figure 1. Step 1: Biginelli reaction – synthesis of the 3,4-dihydropyrimidinone intermediate (photographs (a)–(e) recorded during the synthesis).

Step 2: Mannich-type reaction. The intermediate was stirred with acetaldehyde for 2 h on a magnetic stirrer. The appropriate primary or secondary amine (benzamide, aniline, sulphadiazine, PABA or 2-aminobenzothiazole) was then added in equimolar proportion to the intermediate and acetaldehyde, and the mixture was refluxed for 2 h at 80–90 °C in ethanol (with hydrochloric acid for C2 and C4) [11,12].

Reaction progress was followed by color change and TLC. The mixture was poured into cold ice water, and the precipitated product was filtered and recrystallized from DMF and ethanol. The compounds were named as follows: C1, ethyl 1-[1-(benzoylamino)ethyl]-6-methyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate; C2, ethyl 6-methyl-2-oxo-4-phenyl-1-[1-(phenylamino)ethyl]-1,2,3,4-tetrahydropyrimidine-5-carboxylate; C3, ethyl 1-[1-(4-aminobenzenesulfonamido)ethyl]-6-methyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate; C4, ethyl 1-[1-[(4-carboxyphenyl)amino]ethyl]-6-methyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate; and C5, ethyl 1-[1-(1,3-benzothiazol-2-ylamino)ethyl]-6-methyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate.

2.3 Ligand and Target Preparation

The molecular target was the HPV16 E6 oncoprotein. The crystal structure with PDB ID 4GIZ (full-length HPV16 E6 in complex with an LxxLL peptide of E6AP, X-ray diffraction, 2.55 Å resolution) was obtained from the RCSB Protein Data Bank [6,8] and cleaned in UCSF ChimeraX [13]: water molecules and the original ligand were removed, the E6AP peptide and chains A, B and C were retained, and hydrogens and charges were added.

2.4 Drug-Likeness, ADME and Toxicity Prediction

Physicochemical, lipophilicity, solubility, pharmacokinetic and drug-likeness parameters were predicted with SwissADME [17]. Drug-likeness was assessed with Lipinski's rule of five [18]: molecular weight below 500 Da, no more than five hydrogen-bond donors, no more than ten hydrogen-bond acceptors and a calculated logP not greater than 5 (or MlogP not greater than 4.15). Rotatable-bond (10 or fewer) and polar surface area (140 Å² or less) limits belong to Veber's rules [19] and are reported separately. Toxicity-related risks (mutagenicity, tumorigenicity, irritant and reproductive effects) were predicted with OSIRIS Property Explorer (version 2.16), a rule-based tool.

2.5 Molecular Docking

Docking against HPV16 E6 (PDB 4GIZ) was performed with PyRx [14] using AutoDock [15,16] and Auto Dock Tools version 1.5.6, following protein preparation, ligand preparation, docking and visualization of results. Binding affinity was

expressed in kcal/mol, with more negative values interpreted as more favorable predicted binding [20]. The grid was centered at X = 1.95, Y = 58.69, Z = 25.68, with grid-box dimensions of 26.8 Å (X), 29.9 Å (Y) and 32.7 Å (Z); the box was confirmed to cover the E6AP LxxLL-binding pocket.

2.6 In Vitro Cytotoxicity (MTT Assay)

The cytotoxicity of C5 was examined in the HeLa cell line, which is HPV18-positive [21,22], using the MTT assay [23,24]. Cells were treated with C5 at 0 (control), 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/mL, and absorbance was read in triplicate wells alongside blank wells. Percentage viability was calculated relative to the untreated control, and the IC50 was derived from the concentration–viability data.

3. Results

3.1 Chemical Series

The five derivatives retain the dihydropyrimidinone core and differ in the amine-derived substituent (Figure 2, Table 1), allowing a simple comparison of how aromatic and heteroaromatic substituents influence predicted physicochemical suitability and docking affinity.

C	MOL.FORMULA	DESCRIPTION	MELTING POINT	% YEILD
C1	C ₂₃ H ₂₅ N ₃ O ₄	White crystal	175°C-179°C	78.5%
C2	C ₂₂ H ₂₅ N ₃ O ₃	light yellow crystal	170°C-175°C	84.3%
C3	C ₂₂ H ₂₆ N ₄ O ₅ S	yellow crystal	170°C-173°C	85.8%
C4	C ₂₃ H ₂₅ N ₃ O ₅	white colour crystal	180°C-184°C	80.4%
C5	C ₂₃ H ₂₄ N ₄ O ₃ S	Yellow crystal	160°C-163°C	85.2%

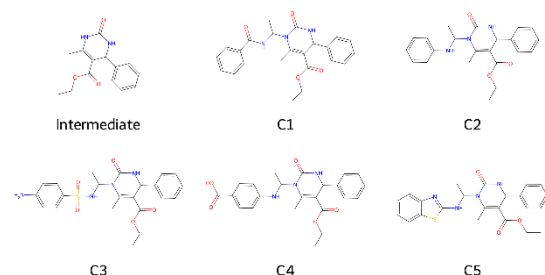


Figure 2. Structures of the dihydropyrimidinone intermediate and the Mannich-modified derivatives C1–C5 (N1-substituted with a 1-(amino)ethyl side chain).

Table 1. Compounds C1–C5, molecular formulas, molecular weights calculated from the formulas, and docking scores against HPV16 E6 (PDB ID: 4GIZ).

Code	Amine component	Molecular formula	Mol. weight (g/mol)	Docking score (kcal/mol)
C1	Benzamide	C ₂₃ H ₂₅ N ₃ O ₄	407.47	−7.3
C2	Aniline	C ₂₂ H ₂₅ N ₃ O ₃	379.46	−7.2
C3	Sulphanilamide	C ₂₂ H ₂₆ N ₄ O ₅ S	458.54	−7.8
C4	PABA	C ₂₃ H ₂₅ N ₃ O ₅	423.47	−8.1
C5	2-Aminobenzothiazole	C ₂₃ H ₂₆ N ₄ O ₃ S	436.54	−8.8

3.2 Drug-Likeness, ADME and Toxicity Prediction

All five compounds complied with Lipinski's rule of five without any violation and showed acceptable predicted ADME properties (Table 2). No major toxicity alerts were predicted for any compound by OSIRIS Property Explorer. These are rule-based predictions and do not replace experimental ADME and toxicity testing.

Table 2. Lipinski drug-likeness parameters of C1–C5 predicted with SwissADME. MW, molecular weight; HBD, hydrogen-bond donors; HBA, hydrogen-bond acceptors.

Code	MW (g/mol)	HBD	HBA	Log P	Lipinski violations
C1	407.47	2	4	3.37	0
C2	379.46	2	4	4.05	0
C3	458.54	3	6	2.50	0
C4	423.47	3	5	3.75	0
C5	436.54	2	6	3.49	0

3.3 Molecular Docking against HPV16 E6

The principal computational result was the docking of C1–C5 against HPV16 E6 (Figure 3). Binding affinities were −7.3 kcal/mol for C1, −7.2 kcal/mol for C2, −7.8 kcal/mol for C3, −8.1 kcal/mol for C4 and −8.8 kcal/mol for C5. C5 had the most negative score and was therefore the computationally prioritized compound under the stated scoring protocol. The binding mode of C5 within HPV16 E6 is shown in Figure 4; the interacting residues were Arg131, Ser74, Gln107, Leu50, Val62 and Tyr32.

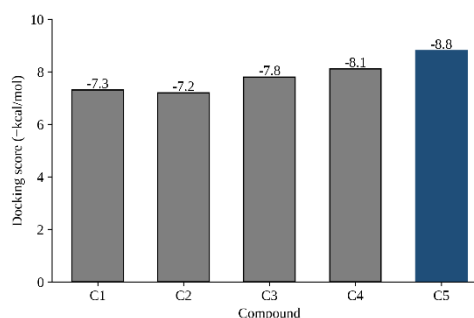


Figure 3. Docking scores of C1–C5 against HPV16 E6 (PDB ID: 4GIZ). Bars show the magnitude of the predicted binding affinity (kcal/mol); C5 (dark bar) had the most favorable score.

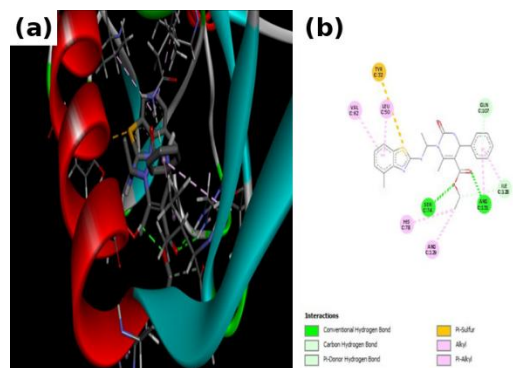


Figure 4. Binding mode of C5 within HPV16 E6 (PDB ID: 4GIZ): (a) three-dimensional view and (b) two-dimensional interaction diagram. Interacting residues: Arg131, Ser74, Gln107, Leu50, Val62 and Tyr32.

3.4 In Vitro Cytotoxicity of C5 against HeLa Cells

C5 reduced HeLa cell viability in a concentration-dependent manner (Table 3, Figure 5). Viability fell from 90% at 10 $\mu\text{g/mL}$ to 4% at 70 $\mu\text{g/mL}$, and the IC_{50} was 36.01 $\mu\text{g/mL}$ (approximately 82.5 μM , using the molecular weight of 436.54 g/mol).

Table 3. MTT absorbance and viability of HeLa cells treated with C5. Mean blank absorbance was 0.040 ± 0.004 . Viability is expressed relative to the untreated control (0 $\mu\text{g/mL}$). $\text{IC}_{50} = 36.01 \mu\text{g/mL}$.

Concentration ($\mu\text{g/mL}$)	Absorbance (mean $\pm$ SD, n = 3)	Viability (%)
0	0.790 ± 0.002	100
10	0.709 ± 0.002	90
20	0.588 ± 0.001	74
30	0.469 ± 0.002	59
40	0.345 ± 0.004	44
50	0.229 ± 0.002	29
60	0.103 ± 0.001	13
70	0.031 ± 0.002	4
80	0.001 ± 0.000	0
90	0.001 ± 0.000	0
100	0.001 ± 0.000	0

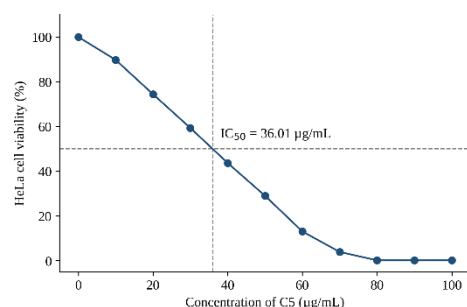


Figure 5. Concentration-dependent reduction in HeLa cell viability by C5 in the MTT assay (mean $\pm$ SD, n = 3). Dashed lines mark 50% viability and the IC_{50} (36.01 $\mu\text{g/mL}$).

4. Discussion

The target structure, 4GIZ, is a crystallographic structure of HPV16 E6 complexed with an LxxLL peptide from E6AP [6,8]. The associated structural study established the molecular basis of LxxLL recognition by HPV16 E6 and identified a basic-hydrophobic pocket involved in recognition of host-protein motifs. This context supports the use of HPV16 E6 as a target for structure-based ligand exploration. The present docking results alone, however, do not establish that the compounds inhibit the E6–E6AP interaction or disrupt E6 biological function. HeLa cells carry HPV18 rather than HPV16 [21,22]. The cytotoxicity of C5 therefore indicates general cytotoxicity and cannot be attributed to inhibition of HPV16 E6. All five candidates satisfied the applied drug-likeness criteria, showed acceptable predicted ADME characteristics and had no major predicted toxicity alerts, so docking affinity was the principal differentiating result. C5, the 2-aminobenzothiazole derivative, produced the most favorable score (–8.8 kcal/mol), followed by C4 (–8.1), C3 (–7.8), C1 (–7.3) and C2 (–7.2 kcal/mol). C5 was selected for experimental evaluation on this basis and is regarded as a putative lead for optimization rather than a confirmed antiviral or anticancer agent. The main limitations are as follows. Docking scores come from a single run per compound, and the 0.7 kcal/mol gap between C5 and C4 is small relative to the error usually attributed to docking scoring functions. The interacting residues shown in Figure 4 come from a single docked pose, which was not validated by re-docking or molecular dynamics. ADME and toxicity data are predictions only. The cytotoxicity assay tested a single compound in a single, HPV16-negative cell line without controls for selectivity. Molecular dynamics simulations, binding free-energy estimation, residue-level interaction analysis and target-engagement assays (for example E6–E6AP binding or p53 stabilization in HPV16-positive cells) would be needed to test the proposed mechanism.

5. Conclusions

Five Mannich-modified dihydropyrimidinone derivatives were designed and evaluated in silico. All satisfied Lipinski's rule of five and showed no major predicted toxicity alerts. Compound C5 (2-aminobenzothiazole) had the most favorable docking score against HPV16 E6 (−8.8 kcal/mol) and reduced HeLa cell viability with an IC₅₀ of 36.01 µg/mL. The docking result is predictive. C5 is a candidate for further structural optimization and mechanistic testing in HPV16-positive cells.

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Author Contributions: Rekha S: Conceptualization, drug design, synthesis of dihydropyrimidinone derivatives, in silico ADME and toxicity analysis, biological data analysis, visualization and writing—original draft. Ramesh Kumar S: Supervision, project administration, validation, critical review. Iswarya M, Vigneshwaren K, Vishnu V: editing of the manuscript. All authors contributed to the interpretation of the results, reviewed the manuscript critically, and approved the final version.

Conflicts of Interest: The authors declare no conflict of interest.

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